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Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species

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Abstract

The effect of water activity (a_w) (0.82–0.99) on growth and ochratoxin A (OTA) production by twelve *Aspergillus niger* aggregate strains, cultured in Czapek Yeast Autolysate agar (CYA) and Yeast Extract Sucrose agar (YES), was studied for an incubation period of 30 days. The strains were selected to include diverse sources, different reported abilities to produce OTA and different ITS-5.8 S rDNA Restriction Fragment Length Polymorphism (RFLP) pattern. They were characterized by Random Amplification of Polymorphic DNA (RAPD) and ITS-5.8 S rDNA and 28 S rDNA (D1/D2) sequencing. Regardless of the a_w value tested, YES was a better culture medium than CYA for OTA production. The a_w range for OTA production was narrower than that for growth. OTA was produced from 0.90, 0.92, 0.94 or 0.96 to 0.99 a_w depending on the strain and the culture medium. The molecular study differentiated strains into two groups which corresponded to the RFLP types N and T although it did not distinguish them by their source of isolation or OTA producing abilities. Our results show that *A. niger* aggregate strains are able to grow and produce OTA over a wide a_w range. These results will lead to a better understanding of the contribution of *A. niger* aggregate in OTA contamination of food and feed.

Keywords: *Aspergillus niger*; Black aspergilli; Ochratoxin A; Water activity

1. Introduction

Mycotoxin contamination of food and feed represents a high risk for human and animal health. Ochratoxin A (OTA) is a mycotoxin which contaminates foods such as cereals, coffee, grapes, cocoa, wine, beer and spices. It commands attention as it has been implicated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity and immunotoxicity in both animals and humans (O'Brien and Dietrich, 2005). It has been classified as a possible human carcinogen (group 2B) by the IARC (IARC, 1993). The European Commission has established maximum limits for OTA in cereals, dried vine fruits, wine, coffee and infant foods (Commission of the European Communities, 2002, 2004, 2005).

OTA has been mainly associated with *Aspergillus ochraceus* and other species included in section *Circumdati* and to *Penicillium verrucosum*. Nevertheless, since the first description of OTA production by *Aspergillus niger* var. *niger* (Abarca et al., 1994) and by *A. carbonarius* (Horie, 1995) members of *Aspergillus* section *Nigri* are achieving a greater significance regarding OTA content in some food commodities. The source of OTA contamination in grapes, raisins and wine is due mainly to *A. carbonarius* and the species included in the *A. niger* aggregate (Abarca et al., 2004). It is worth noting that *Aspergillus niger* products hold the GRAS (Generally Regarded as Safe) status from the Food and Drug Administration and is a widely applied industrial species for large-scale biotechnological production of organic acids and enzymes (Bigelis and Lausure, 1987) in the food industry.

The taxa included in the *A. niger* aggregate are very difficult to distinguish by morphological means. Hence, many attempts have been made to divide these taxa into two or more species

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(Abarca et al., 2004). On the basis of their ITS-5.8 S rDNA Restriction Fragment Length Polymorphism (RFLP) patterns, two groups can be observed, designated as N and T (Accensi et al., 1999). To date, all OTA-positive isolates belonging to the *A. niger* aggregate were classified in type N, whereas type T strains were not able to produce OTA (Accensi et al., 2001; Cabañes et al., 2002; Abarca et al., 2003; Leong et al., 2004).

Knowledge of the influence of environmental parameters on OTA production may contribute to prevention of OTA contamination in food commodities. Nevertheless, very little is known about optimal conditions for OTA production by *A. niger* aggregate strains. In a previous study focused on the influence of temperature, maximum OTA levels were achieved at 20–25 °C in YES medium (Esteban et al., 2004). In synthetic grape juice medium, maximum OTA production of two *A. niger* aggregate strains has been recently reported at 0.98–0.995 a_w (Bellí et al., 2004).

The aim of the current work is to study the influence of a_w on growth and OTA production by different species belonging to the *A. niger* aggregate. The strains were selected to include different sources, different reported ochratoxigenic ability and different ITS-5.8 S rDNA RFLP patterns. Analysis of genetic relationships between the strains included in the study was performed using several molecular techniques.

2. Materials and methods

2.1. Fungal strains and culture conditions

The reported OTA production, RFLP pattern and source of isolation of the strains used in this study are shown in Table 1. The strains were grown on malt extract agar at 25 °C for 7 days. Conidia suspensions of each isolate were prepared in an aqueous solution of 0.05% Tween 80. The inocula were adjusted to approximately 10^6–10^7 conidia per milliliter, determined by a counting chamber. The physiological study was carried out on two culture media: Czapek Yeast Extract (CYA) agar and Yeast Extract Sucrose (YES) agar (Pitt and Hocking, 1997) with a final pH of 6.9–7. Both media were adjusted to different water activity values (0.82, 0.86, 0.90, 0.92, 0.94, 0.96, 0.98, 0.99) by addition of appropriate quantities of glycerol determined by the construction of a calibration curve. The final a_w of the culture media was determined with Novasina Thermoconstanter TH 200 (Novasina, Zurich, Switzerland). Plates were centrally point inoculated with 1 μl of the adjusted suspension and incubated at 25 °C. Each assay was performed in duplicate. For each experiment, the a_w values of control plates were determined after 30 days of incubation and not significant variation was detected.

2.2. Determination and quantification of OTA

OTA production was determined after 5, 10, 15, 20 and 30 days of incubation at each a_w value assayed following a previously described high-pressure liquid chromatography (HPLC) screening method (Bragulat et al., 2001). Three agar plugs were removed from different points of the colony and these were extracted with 0.5 ml of methanol. The extracts were filtered and 20 μl was injected into the HPLC. OTA detection and quantification was made by a Waters LCM1 chromatograph with a fluorescence detector Waters 2475 (excitation wavelength: 330 nm/emission wavelength: 460 nm), and with a column C18 Spherisorb S5 ODS2, 250×4.6 mm. The mobile phase, with a flow rate of 1 ml/min, consisted of the following isocratic program: acetonitrile, 57%; water, 41% and acetic acid, 2% (Bauer and Gareis, 1987). The extracts with a peak at the same retention time as OTA (around 6.8 min) were considered positive. Confirmation was made through derivatization of OTA to its methylester (Hunt et al., 1980). The detection limit of the extraction procedure and the HPLC technique was 0.02 ng OTA and the quantification limit of HPLC technique with the extraction procedure was 0.05 μg g^{-1} for this mycotoxin.

Data obtained from the different a_w conditions tested were statistically analysed by means of one-way analysis of variance test and Student's test. All statistical analyses were performed using SPSS software (Version 12.0).

2.3. DNA extraction

Fungal DNA was extracted as described by Accensi et al. (1999). The strains were inoculated in 1.5 ml Eppendorf tubes

Table 1
Aspergillus niger aggregate strains used in this study

Species	Strain (source)[a]	OTA production reported (Ref.)	RFLP pattern (Ref.)
A. niger var. *niger*	A-75 (feedstuffs, CCFVB)	+ (Abarca et al., 1994; Esteban et al., 2004)	N (Accensi et al., 2001)
A. niger var. *niger*	A-136 (soy, CCFVB)	+ (Abarca et al., 1994; Esteban et al., 2004)	N (Accensi et al., 2001)
A. niger var. *niger*	A-942 (raisins, CCFVB)	+ (Abarca et al., 2003; Esteban et al., 2004)	N (Abarca et al., 2003)
A. niger var. *niger*	A-943 (grapes, CCFVB)	+ (Esteban et al., 2004)	N (Esteban et al., 2004)
A. awamori	CBS 139.52 (kuro-koji)	+ (Ono et al., 1995; Esteban et al., 2004)	N (Accensi et al., 2001)
A. foetidus	CBS 618.78 (unknown)	+ (Téren et al., 1996; Esteban et al., 2004)	N (Accensi et al., 2001)
A. niger	CECT 2088 (unknown)	− (Varga et al., 2000; Esteban et al., 2004)	N (Esteban et al., 2004)
A. niger	CBS 554.65 (tannin–gallic acid fermentation)	− (Accensi et al., 2001; Esteban et al., 2004)	N (Accensi et al., 2001)
A. niger var. *niger*	A-946 (coffee, CCFVB)	− (Esteban et al., 2004)	N (Esteban et al., 2004)
A. niger	CBS 121.55 (otomycosis)	− (Esteban et al., 2004)	N (Esteban et al., 2004)
A. tubingensis	CBS 134.48 (unknown)	− (Accensi et al., 2001; Esteban et al., 2004)	T (Accensi et al., 2001)
A. niger var. *niger*	A-947 (grapes, CCFVB)	− (Cabañes et al., 2002; Esteban et al., 2004)	T (Cabañes et al., 2002)

[a]Abbreviations: CCFVB, Culture Collection of Veterinary Faculty of Barcelona, Bellaterra (Barcelona), Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CECT, Colección Española de Cultivos Tipo, Valencia, Spain.

containing 500 μl of Sabouraud broth (2% glucose, w/v; 1% peptone w/v) supplemented with chloramphenicol (1 mg l^{-1}) and incubated overnight in an orbital shaker at 300 rpm at 30 °C. Mycelium was recovered after centrifugation and washed with NaCl 0.9% (w/v), frozen in liquid nitrogen and ground to a fine powder with a pipette tip. The powder was incubated for 1 h at 65 °C in 500 μl extraction buffer (Tris–HCl 50 mM, EDTA 50 mM, SDS 3% and 2-mercaptoethanol 1%). The lysate was extracted with phenol : chloroform (1 : 1, v/v), 3 M NaOAc and 1 M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 8). DNA was cleaned with 'Geneclean kit II' (BIO 101, La Jolla, CA, USA), according to the manufacturer's instructions.

2.4. Sequencing of the ITS1-5.8 S rDNA-ITS2 and D1/D2 of 28 S rDNA regions

The sequencing reaction was carried out using the sequencing commercial system 'ABI Prism Big Dye Terminator v 3.0 Ready Reaction Cycle Sequencing Kit' (Applied Biosystems, Gouda, The Netherlands). The sequence of both chains was obtained with the primers ITS5 (5′-GGAAGTAAAAG TCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′), described by White et al. (1990) for the ITS1-5.8 S-ITS2 region and NL1 (5′-GCATATCAATAAGCGGAGGAA AAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (O'Donnell, 1993) for the D1/D2 region of 28 S rDNA, following the indications of the manufacturer. An Applied Biosystems 'ABI Prism 310 Genetic Analyzer' sequencer was used to obtain the DNA sequences. The sequences were aligned by using the software program Clustal X (1.8) (Thompson et al., 1997). Once aligned, they were analysed using the Neighbor-Joining method (Saitou and Nei, 1987) with the program 'Mega' (2.1) (Kumar et al., 2001).

2.5. RAPD analysis

PCR reaction was carried out in a 20 μl reaction volume using a Perkin-Elmer 2400 thermocycler. The mixture for the reaction contained: 10× PCR buffer (10 mM); 3 mM of $MgCl_2$; 280 μM of each dNTP (dATP, dCTP, dTTP and dGTP); 0.55 μM of primer ari1 (5′-TGCTTGGCACAGTTGGCTTC-3′) (Castellá et al., 2002); 1 U of Tag DNA polymerase and between 0.1 and 10 ng of DNA. The reaction started with an initial denaturing step of 5 min at 95 °C, 44 cycles of 1 min at 95 °C, 1 min at 36 °C and 4 min at 72 °C, followed by a final elongation step of 7 min at 72 °C.

Electrophoresis was conducted on a 2% (w/v) agarose gel in Tris–boric acid–EDTA (TBE). The molecular weights of the amplified products were obtained in comparison to the '100 bp molecular ruler' (Bio-Rad Laboratories S.A., Barcelona). The profiles of bands obtained were analysed using the UPGMA

Table 2
OTA concentration (mean and mean standard error) produced by the five OTA-producing strains of *A. niger* aggregate in CYA medium at 25 °C at each a_w value tested

Strain	Days	OTA concentration(μg/g)						
		0.86	0.90	0.92	0.94	0.96	0.98	0.99
A-75	5	NG	nd	nd	5.86±1.87^{a,b}	10.17±3.53^{b}	4.87±2.49^{a,b}	3.98±0.63^{a,b}
	10	nd	nd	0.40±0.25^{a}	6.74±2.37^{b}	6.63±3.42^{b}	2.46±0.43^{a,b}	1.47±0.20^{a}
	15	nd	nd	0.04±0.05^{a}	4.59±1.26^{b}	3.85±0.95^{b}	1.67±1.00^{a}	1.82±0.10^{a}
	20	nd	nd	0.10±0.06^{a}	3.72±0.95^{a}	2.55±0.92^{a}	6.91±8.02^{a}	1.72±0.62^{a}
	30	nd	nd	nd	1.91±0.91^{b}	1.72±0.08^{b}	1.78±0.03^{b}	1.03±0.21^{a,b}
A-136	5	NG	nd	nd	12.95±4.82^{b}	12.7±1.56^{b}	13.36±0.90^{b}	4.08±0^{a}
	10	nd	nd	nd	2.06±0.28^{b}	3.65±0.12^{c}	3.05±1.17^{b,c}	1.89±0.39^{b}
	15	nd	nd	nd	1.38±0.25^{a,b}	2.55±0.34^{b}	1.96±1.20^{b}	1.73±0.45^{a,b}
	20	nd	nd	nd	1.50±0.52^{b}	2.84±0.49^{c}	4.75±0.30^{a,d}	2.54±0.28^{c}
	30	nd	nd	nd	1.00±0.09^{a}	1.92±0.45^{a}	6.76±1.56^{b}	1.63±0.43^{a}
A-942	5	NG	nd	nd	1.50±0.66^{a,b}	3.35±1.51^{b}	10.28±0.01^{c}	1.37±0.04^{a,b}
	10	nd	1.27±1.51^{a}	6.47±0.18^{b}	11.15±1.29^{c}	4.17±1.32^{a,b}	2.50±1.96^{a}	0.75±0.20^{a}
	15	nd	1.86±0.66^{a,b}	2.22±0.51^{a,b}	9.31±0.21^{c}	3.42±1.27	3.92±1.14^{b}	0.94±0.04^{a}
	20	nd	1.47±0.76^{a}	1.88±0.13^{a}	8.21±2.74^{b}	2.54±0.86	3.94±0.26^{a}	0.98±0.23^{a}
	30	nd	0.45±0.42^{a}	1.48±0.37^{b}	4.91±0.19^{c}	3.44±0.47	4.89±0.04^{c}	0.66±0.13^{a}
A-943	5	NG	nd	nd	nd	0.06±0^{b}	0.07±0.01^{b}	0.12±0.03^{c}
	10	nd	nd	0.34±0.08^{a}	0.24±0.21^{a}	0.22±0.08^{a}	nd	nd
	15	nd	nd	0.12±0.04^{a}	0.19±0.11^{a}	0.31±0.20^{a}	nd	0.16±0.04^{a}
	20	nd	nd	nd	0.29±0.03^{b}	0.73±0.13^{c}	nd	0.07±0.01^{a}
	30	nd	nd	nd	0.25±0.08^{b}	0.51±0.14^{c}	nd	nd
CBS 139.52	5	NG	nd	nd	nd	0.49±0.11^{a}	12.6±7.86^{a}	3.05±1.22^{a}
	10	nd	nd	nd	nd	8.79±2.47^{b}	5.49±1.33^{c}	2.22±0.17^{a}
	15	nd	nd	nd	nd	1.94±1.22^{b}	5.09±0.41^{c}	2.35±0.28^{b}
	20	nd	nd	nd	nd	0.83±0.35^{a}	4.23±1.18^{b}	2.18±0.35^{c}
	30	nd	nd	nd	nd	1.05±0.28^{b}	6.04±0.09^{c}	0.29±0.04^{a}

^{a,b,c,d}Values with the same superscript within each strain and incubation time are not significantly different ($p<0.05$); nd: not detected (limit of detection 0.05 μg g^{-1}).; NG: no growth.

Table 3
OTA concentration (mean and mean standard error) produced by the five OTA-producing strains of *A. niger* aggregate in YES medium at 25 °C at each a_w value tested

Strain	Days	OTA concentration (μg/g)						
		0.86	0.90	0.92	0.94	0.96	0.98	0.99
A-75	5	NG	nd	nd	3.31±0.57[a]	9.99±14.13[a]	23.86±4.70[a]	54.12±16.88[b]
	10	nd	nd	nd	6.99±3.08[a,b]	13.04±1.97[b,c]	18.50±6.36[c]	20.54±1.57[c]
	15	nd	nd	nd	1.19±0.13[a]	3.06±1.85[a]	2.92±1.61[a]	9.91±2.79[b]
	20	nd	nd	nd	0.45±0.04[a]	3.82±4.50[a]	1.39±0.86[a]	15.32±2.28[b]
	30	nd	nd	nd	0.96±0.27[a]	0.65±0.72[a]	2.91±2.32[a,b]	5.02±0.86[b]
A-136	5	NG	nd	nd	3.81±2.46[a]	32.96±0.97[b]	59.66±10.59[c]	49.40±14.92[b,c]
	10	nd	nd	nd	0.99±0.42[a]	15.61±3.71[b]	18.04±0.39 [b]	8.88±3.14[c]
	15	nd	nd	nd	0.81±0.10[a]	7.35±2.55[a]	3.89±3.92[a]	3.40±1.82[a]
	20	nd	nd	nd	0.28±0.13[a]	5.49±0.04[b]	6.72±0.81[b]	6.01±3.59[b]
	30	nd	nd	nd	0.23±0.06[a]	2.96±1.15[a]	8.07±7.21[a]	0.56±0.27[a]
A-942	5	NG	nd	nd	2.30±1.36[a]	16.78±3.54[b]	16.32±2.14[b]	16.50±3.79[b]
	10	nd	nd	0.56±0.52[a]	7.54±0.98[a]	18.23±4.82[b]	4.72±1.96[a]	1.08±0.04[a]
	15	nd	nd	0.19±0.10[a]	1.87±1.97[a]	9.69±4.99[b]	4.18±1.39[a]	1.58±0.16[a]
	20	nd	nd	0.09±0.01[a]	0.71±0.30[a]	6.39±4.22[a]	3.54±2.41[a]	1.66±0.04[a]
	30	nd	nd	nd	1.14±1.08[a,b]	3.22±0.17[c]	1.89±0.06[b]	0.64±0.27[a,b]
A-943	5	NG	nd	nd	nd	0.17±0.02[b]	0.21±0.11[b]	0.21±0.01[b]
	10	nd	nd	nd	nd	1.06±0.92[a]	0.06±0.08[a]	nd
	15	nd	nd	nd	nd	0.30±0.23[a]	nd	0.05±0[a]
	20	nd	nd	nd	nd	nd	nd	nd
	30	nd	nd	nd	nd	nd	nd	nd
CBS 139.52	5	NG	nd	nd	0.11±0.03[a]	8.90±2.79[a]	25.53±4.71[b]	41.07±3.14[c]
	10	nd	nd	nd	0.47±0.44[a]	11.46±2.09[a]	22.48±22.37[a]	31.69±5.26[a]
	15	nd	nd	nd	nd	0.37±0.04[a]	7.80±4.51[b]	17.99±0.51[c]
	20	nd	nd	nd	nd	0.55±0.55[a]	1.53±0.54[a]	8.88±2.19[b]
	30	nd	nd	nd	nd	0.35±0.22[a]	4,10±2.64[a,b]	4.83±1.12[b]

[a,b,c]Values with the same superscript within each strain and incubation time are not significantly different ($p<0.05$); nd: not detected (limit of detection 0.05 μg g^{-1}); NG: no growth.

method (Page and Holmes, 1998) of the 'Diversity Database' 2.2.0. software. The reactions were performed in triplicate.

3. Results and discussion

3.1. Water activity effect on growth and OTA production

All the strains grew in CYA and YES media from 0.86 to 0.99 a_w and three of them (CBS 554.65, A-946, CBS 121.55) also grew in YES medium adjusted at 0.82 a_w from 20 days of incubation. The germination of *A. niger* strains has been reported at 0.77 a_w (Pitt and Hocking, 1997) and 0.80 a_w (Marín et al., 1998). Slow growth of *A. niger* isolates was reported at 0.76 a_w on malt extract agar (MEA) (Vujanovic et al., 2001) whereas in another study the minimum water activity limit for growth was 0.82 using the same culture medium (Parra and Magan, 2004).

Five of the six reported ochratoxigenic strains produced quantifiable levels of OTA. The strain CBS 618.78 produced only trace amounts of OTA in this study. Tables 2 and 3 show the concentration detected at each a_w value and incubation time in CYA and YES medium, respectively. Mean OTA concentration produced by all the positive strains at all sampling times is shown in Fig. 1. The a_w range for OTA production was narrower than that for growth and each strain differed in its optimum conditions for OTA production. The a_w range in which OTA production occurred was wider for most of the strains in CYA than in YES, but the concentration achieved was higher in YES medium mainly at the 0.96–0.99 a_w range (Fig. 1). The mean OTA concentration produced in YES medium (4.38 μg g^{-1}) was significantly higher ($p<0.01$) than in CYA medium (1.88 μg g^{-1}). Regardless the initial a_w value, YES was more suitable for OTA production than CYA medium for the *A. niger* aggregate strains. These results were in accordance with two previous studies of *A. niger* aggregate strains

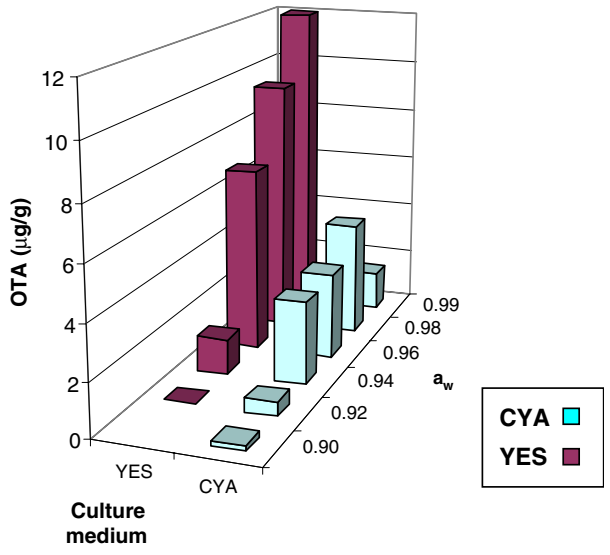

Fig. 1. Mean OTA concentration produced at all sampling times by all the OTA-producing strains at each a_w value and culture media tested.

(Bragulat et al., 2001; Esteban et al., 2004), however CYA medium was reported to be more suitable than YES medium for OTA production by *A. carbonarius* strains (Bragulat et al., 2001; Esteban et al., 2004, 2005).

All isolates produced OTA on both media after only 5–10 days of incubation. In CYA medium (Table 2) the maximum levels of OTA were detected within the range 0.94–0.98 a_w after 5–10 days of incubation. In YES medium the optimal conditions for OTA production were observed after 5–10 days of incubation at 0.96–0.99 a_w. Apart from CBS 618.78, which produced only trace amounts of OTA, A-943 produced in both media the lowest amounts of OTA in comparison with the rest of strains studied.

The amounts of OTA detected decreased when increasing incubation time. Some authors suggested that strains could remove and assimilate the phenylalanine moiety from the OTA molecule, as other nitrogen sources of the culture medium become exhausted (Téren et al., 1996).

The percentage of OTA producing strains in the *A. niger* aggregate is low (Abarca et al., 2004) although it is still unknown if this is due to a genetic based feature. It is necessary to test different environmental conditions to find out the optimal conditions for OTA production which may clarify this fact. In our study, the six strains initially considered as OTA-negative (Table 1) did not produce the mycotoxin at any of the a_w values tested for a period of 30 days. In a previous study, these six strains did not produce OTA at any of the temperatures tested (Esteban et al., 2004). Four of these strains showed N RFLP pattern whilst two of them showed T RFLP pattern. To date OTA production has never been demonstrated in type T strains (Accensi et al., 2001; Cabañes et al., 2002; Abarca et al., 2003; Leong et al., 2004).

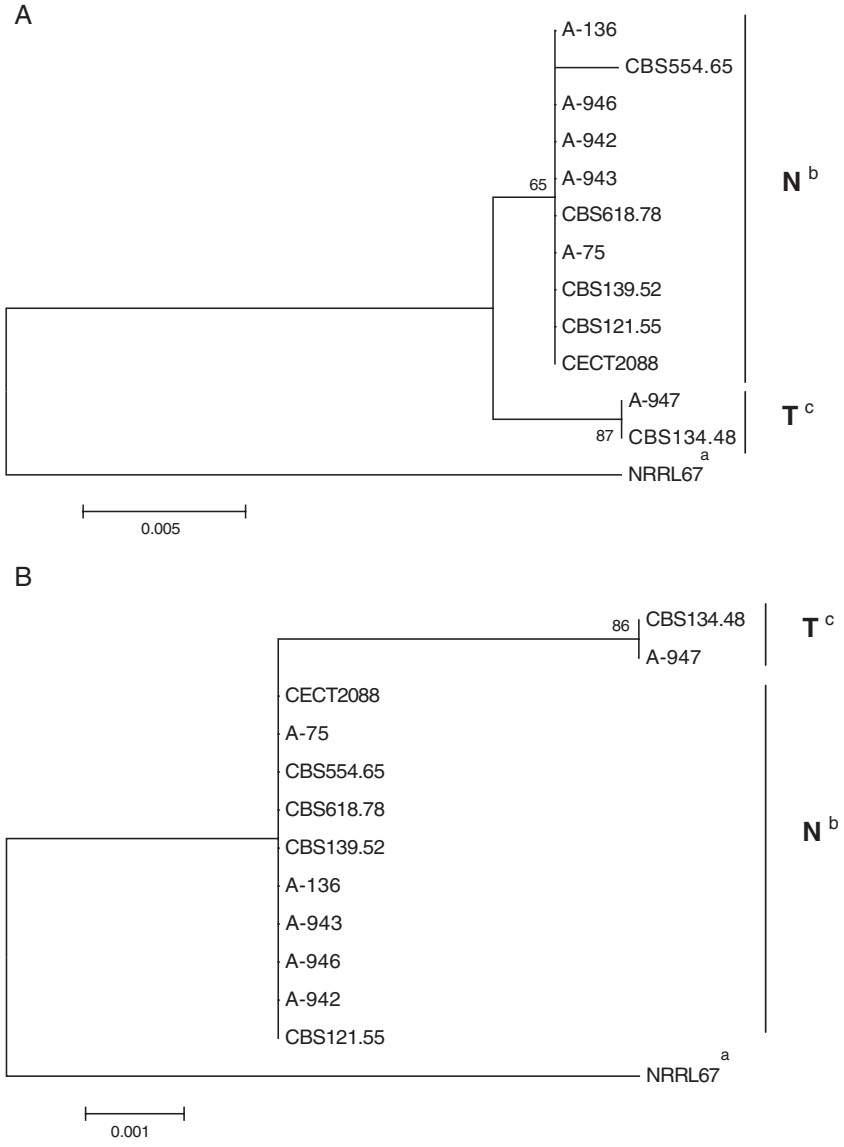

Fig. 2. Phylogenetic trees constructed from ITS-5.8S rDNA-ITS2 (A) and D1/D2 (B) sequences of the *A. niger* aggregate strains included in this study using neighbor-joining method. Bootstrap percentages calculated from 1000 resamplings are indicated at nodes. [a]*A. carbonarius* strain (NRRL67) was included as outgroup in the analysis. [b]N RFLP type; [c]T RFLP type (Accensi et al., 1999).

In synthetic grape juice medium the optimum a_w value for OTA production by two *A. niger* aggregate strains was recently reported to be 0.98–0.995 a_w, after 5 to 10 days of incubation (Bellí et al., 2004). Studies focused on *A. carbonarius* established the optimum a_w range for OTA production between 0.95 and 0.99 a_w in synthetic grape juice medium (Bellí et al., 2004, 2005; Mitchell et al., 2004), whereas maximum OTA accumulation was observed at 0.99 a_w when one isolate from green coffee was incubated on coffee cherries (Joosten et al., 2001).

3.2. Analysis of genetic relationships between the strains

Many attempts have been carried out to clarify the taxonomy within *A. niger* aggregate by using different molecular techniques and several authors have proposed division of the *A. niger* aggregate into two or more species (Abarca et al., 2004). The ITS1-5.8 S-ITS2 rDNA sequencing analysis of these strains showed very similar sequences which included 602 base pairs (bp). The strain CBS 554.65 showed a G insertion in the 5.8 S region. The two strains with T RFLP pattern (CBS 134.48 and A-947) differed from the type N strains at three substitutions in ITS1 and ITS2 regions. These differences were reflected in the phylogenetic tree assessed (Fig. 2A). Analysis of D1 and D2 regions has shown to be a suitable tool to differentiate species within *Aspergillus* section *Nigri* (Peterson, 2000). In our study, the sequences obtained by the analysis of 28 S rDNA (D1/D2 regions) included 586 bp. Strains with T RFLP pattern differed from N RFLP strains at two positions. Two different clusters were observed in the phylogenetic tree assessed (Fig. 2B). In both phylogenetic trees, one cluster contained the two strains with RFLP type T and the other one grouped the type N strains. This latter group included both OTA-positive and OTA-negative strains.

The differentiation between the two RFLP types was also reflected when observing the dendrogram obtained with the Random Amplification of Polymorphic DNA (RAPD) technique (Fig. 3). The two type T strains (CBS 134.48 and A-947) were also clustered separately from type N strains. Type N strains cluster showed a great variability and was divided in two main groups, both containing minor branches including producing and non-producing strains. On the basis of RAPD analysis, Megnegneau et al. (1993) proved the existence of a high level of intraspecific variability among strains of the *A. niger* aggregate, consistent with the RFLP analysis of rDNA which grouped the strains in two main groups.

In our study, no single molecular technique used could distinguish the strains by their source of isolation or their OTA producing abilities. For example, in the RAPD analysis, three OTA negative strains (CBS 554.65, A-946, CECT 2088) were clustered together with the producer strain CBS 618.78. The OTA negative strain CBS 121.55 was clustered jointly with the two OTA positive strains A-942 and A-943.

3.3. Implications on food sources

The strains studied have been able to grow at a wide range of a_w in both CYA and YES media. This would explain why *A. niger* is considered the most common *Aspergillus* species responsible for post-harvest decay of fresh fruit and is also among the most frequently fungi isolated from nuts and sun dried products, such as vine fruits (Pitt and Hocking, 1997; Heenan et al., 1988; Abarca et al., 2003). The black spores of this species apparently provide protection from sunlight and UV light, providing a competitive advantage in such habitats. *A. niger* aggregate species are isolated from grapes in the field, achieving the highest levels in late grape growth stages (Battilani et al., 2003; Serra et al., 2003; Bau et al., 2005). They are also isolated from coffee raw materials. *Aspergillus niger* has been frequently detected on OTA contaminated coffee cherries and green coffee (Bucheli and Taniwaki, 2002; Taniwaki et al., 2003). The isolation of these species from such products may represent one source of OTA contamination in the field.

Moreover, according to the results obtained, significant amounts of OTA can be achieved at only 5 days of incubation at 25 °C. This feature can also lead to the contribution of OTA contamination in those products, stored or manufactured after

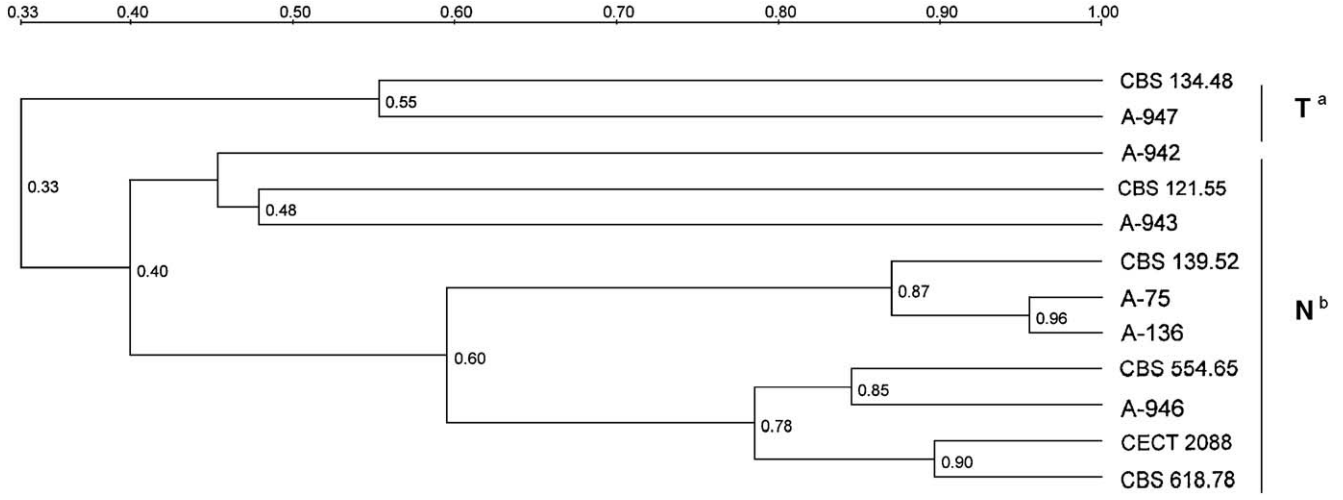

Fig. 3. UPGMA dendrogram of the *A. niger* aggregate strains used in this study assessed from the comparison of RAPD fingerprintings generated with primer ari1. The scale represents taxonomic distance. [a]T RFLP type; [b]N RFLP type (Accensi et al., 1999).

harvesting, where *A. niger* aggregate species are frequently isolated together with other ochratoxigenic black aspergilli. The drying process performed in some products such as coffee or raisins has not prevented OTA contamination (Bucheli and Taniwaki, 2002; Taniwaki et al., 2003). Leong et al. (2004) reported that black aspergilli can grow in the fruit during the initial stages of drying until the water activity falls below around 0.85 a_w. Coffee cherries contain still 25–50% of water after 5 days of drying, enough to support growth of black aspergilli (Bucheli and Taniwaki, 2002).

Our results show the ability of *A. niger* aggregate strains to grow and produce OTA in a wide a_w range. Moreover significant amounts of OTA can be produced after only five days of incubation since the a_w achieved by some substrates after the drying process could still allow the development of this species and the production of mycotoxin.

Further studies on the effect of physiological variables on growth and OTA production deserve a significant concern. It may lead us to better understanding of the role of *A. niger* aggregate in OTA contamination in food and feed where black aspergilli members are usually isolated.

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