Asian Journal of Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 3 (3): 176-181, 2008
ISSN 1815-9923

In vitro Antifungal Effect of EDTA Disodium Salt in Tested Black Aspergilli

Luís Abrunhosa and Armando Venâncio
IBB- Institute for Biotechnology and Bioengineering,
Centre for Biological Engineering, Universidade do Minho,
Campus de Gualtar 4710-057, Braga, Portugal

Abstract: The antifungal effect of Na_2EDTA on an *Aspergillus carbonarius*, an *A. ibericus*, an ochratoxigenic *A. niger* and a non-ochratoxigenic *A. niger* strain was studied. Also, the effect of Na_2EDTA on the production of ochratoxin A by *A. carbonarius* and the ochratoxigenic *A. niger* was evaluated. The poisoned food technique was used with CYA medium supplemented with 0, 1 and 10 mmol L^{-1} of Na_2EDTA. The colony diameters were recorded daily and the amount of ochratoxin A produced was quantified every two days. Significant reductions of growth rates were observed in the presence of Na_2EDTA being the calculated EC_{50} of 2.1 mmol L^{-1} for *A. carbonarius*, 0.9 mmol L^{-1} for *A. ibericus*, 2.0 mmol L^{-1} for the ochratoxigenic *A. niger* and 4.1 mmol L^{-1} for the non-ochratoxigenic *A. niger*. Furthermore, 10 mmol L^{-1} Na_2EDTA delayed the production of ochratoxin A and reduced the levels in approximately 99% during 8 days. Na_2EDTA is frequently used in the food industry and in agriculture agrochemicals and its effects on ochratoxigenic black aspergilli is not well known. This study showed that Na_2EDTA can significantly reduce the growth rates of tested fungi and its ochratoxin A production.

Key words: *Aspergillus carbonarius*, *Aspergillus ibericus*, *Aspergillus niger*, ochratoxin A, EDTA, fungicide

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by some *Aspergillus* and *Penicillium* species in several agricultural commodities. Therefore, OTA is found in food products such as breakfast cereals, coffee, cocoa products, dried vine fruits, dried figs, beer and wine. The presence of OTA in grapes is due to *A. carbonarius* and to some ochratoxigenic isolates of the *A. niger* aggregate (Serra *et al*., 2003; Gomez *et al*., 2006). *A. carbonarius* isolates are producers of higher amounts of OTA and are more frequent on grapes than the ochratoxigenic isolates of the *A. niger* aggregate (Serra *et al*., 2006; Esteban *et al*., 2004). *Aspergillus niger* aggregate strains are also able to degrade OTA into the less toxic ochratoxin α (Varga *et al*., 2000; Abrunhosa *et al*., 2002).

Ethylenediaminetetraacetic Acid (EDTA) is a strong chelating agent that forms several metal and salt complexes. EDTA and EDTA-complexes are widely used in cleaners and detergents, in agriculture or in food processing (Oviedo and Rodriguez, 2003). In the food industry, it is mostly used to remove metallic tastes by sequestering metal ions released during the processing or storage (e.g., in canned beans). In USA, Na_2EDTA concentrations of 36 to 500 ppm, which corresponds to 0.1-1.5 mmol L^{-1} in solution, are allowed in some food products (Heimbach *et al*., 2000). In agriculture, EDTA is used in agrochemical products to stabilize formulations or to provide micronutrients such as zinc, manganese, iron, copper, magnesium, calcium and potassium. EDTA is also recognized as an

Corresponding Author: A. Venâncio, Departamento de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
Tel: +351253604413 Fax: +351253678986

antibacterial agent which disrupts the membrane integrity and as a potentiator of other lethal agents (Oita, 2003). EDTA antifungal properties were mainly tested on yeasts (Siqueira and Sen, 2004; Kubo *et al*., 2005), being nevertheless reported its synergetic effect with another antifungal agent on the reduction of pulmonary aspergillosis (Hachem *et al*., 2006). Its effect in controlling mildew on tomato leaves was also reported (Ehret *et al*., 2002).

In this study, the *in vitro* effect of Na_2EDTA on the growth of an *A. carbonarius*, an *A. ibericus*, an ochratoxigenic *A. niger* and a non ochratoxigenic *A. niger* strains was assessed. All these strains were isolated from grapes.

MATERIALS AND METHODS

Chemical Material

Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) from Merck commercialized as Titriplex III.

Biological Material

Ochratoxigenic *Aspergillus carbonarius* strain MUM 03.59, non-ochratoxigenic *A. ibericus* strain MUM 03.49, ochratoxigenic *A. niger* strain MUM 03.57 and non-ochratoxigenic *A. niger* strain MUM 03.58.

Growth Conditions in Presence of Na_2EDTA

The antifungal activity of Na_2EDTA was evaluated by the agar dilution method using Czapek Yeast Extract Agar medium (CYA) (Mares *et al*., 2004). The medium was supplemented with 0, 1 or 10 mmol L^{-1} of Na_2EDTA (CYA, CYA+1 m and CYA+10 m, respectively). Strains were first grown in plates with MEA medium (Blakeslee formula), for 7 days, at 25°C, in the dark, for inoculum generation. For each strain, a spore suspension with 2.5×10^6 spores mL^{-1} was prepared in semi solid agar (0.2% agar and 0.05% Tween 80) using a NeuBauer chamber (Aberkane *et al*., 2002). Each strain was centrally inoculated in triplicate in each media with 10 µL of the respective spore suspension and incubated at 25°C, in the dark. The colony diameters were recorded daily and growth rates were calculated by linear regression of colony diameters against days. The concentration of Na_2EDTA at which survival was 50% (EC_{50}) was determined by fitting the experimental data to a four-parameter logistic model (Hill equation) using computer curve-fitting software (Prism 4, GraphPad Software, Inc, San Diego, CA, USA).

Ochratoxin A Analysis

Every two days, plates were extracted with methanol to quantify the amount of OTA produced. A modification of the method presented by Bragulat *et al*. (2001) was used as follows. The mycelia and media were cut and transferred to tubes with 20 mL of methanol. The tubes were vigorously vortexed and allowed to extract overnight. Methanol extracts were filtered with a 0.45 µm syringe filter of PTFE (Teknokroma) and 1 mL of the filtrate dried at 50°C with a gentle stream of nitrogen in a clean vial. Dried residues were resuspended in 1 mL of HPLC mobile phase and analyzed by high-performance liquid chromatography. The HPLC apparatus consisted of a Varian 9002 pump equipped with a Jasco FP-920 fluorescence detector (λ_{ex} = 333 nm; λ_{em} = 460 nm) and a Marathon Basic autosampler. The analytical column was a C_{18} reversed-phase YMC-Pack ODS-AQ (250×4.6 mm and 5 µm) fitted with a precolumn with the same stationary phase. The mobile phase was a mixture of acetonitrile/water/acetic acid (99/99/2, v/v/v) filtered and degassed. The flow rate was set to 0.8 mL min^{-1} and the column temperature to 30°C. The loop volume was 100 µL. Calibration curves were prepared with standards of OTA (Sigma).

Statistical Analysis

All statistic analyses were performed with the Statistic Package for Social Sciences (SPSS) version 15.0. Means were compared by analysis of variance followed by Duncan's post-test being the differences considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

The tested strains were found to be susceptible to the presence of Na_2EDTA in culture media. Na_2EDTA produced a significant increase in the colonies lag phase (Fig. 1) and a significant decrease on colonies growth rates (Table 1). In the presence of 1 mmol L^{-1} of Na_2EDTA, the growth rate of *A. carbonarius*, *A. ibericus*, *A. niger* MUM 03.57 and *A. niger* MUM 03.58 was reduced in 32, 56, 36 and 16%, respectively. When 10 mmol L^{-1} of Na_2EDTA was used, reductions in growth rate of 88, 89, 87 and 82% were obtained, respectively. The concentration of Na_2EDTA which reduces growth

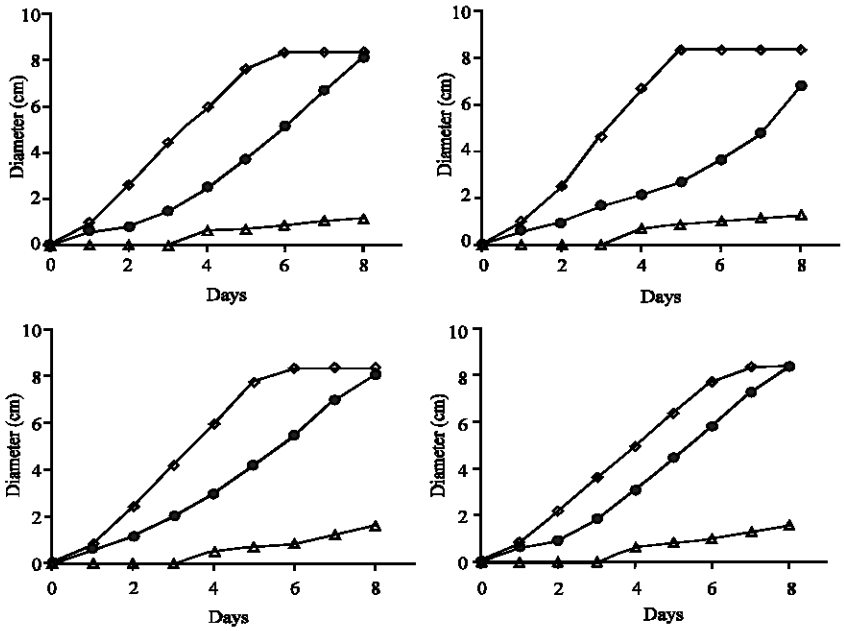

Fig. 1: Daily radial growth of A) *A. carbonarius* MUM 03.59, B) *A. ibericus* MUM 03.49, C) *A. niger* MUM 03.57 and D) *A. niger* MUM 03.58 cultivated in: (-◇-) CYA, (-●-) CYA+1 m and (-Δ-) CYA+10 m. Values presented are the mean of three replicates

Table 1: Radial growth rates (cm day^{-1}) of black aspergilli in CYA and in CYA supplemented with 1 and 10 mmol L^{-1} of Na_2EDTA (CYA+1 m and CYA+10 m, respectively)

	Radial growth rates (cm day^{-1})			
Medium	*A. carbonarius* MUM 03.59	*A. ibericus* MUM 03.49	*A. niger* MUM 03.57	*A. niger* MUM 03.58
CYA	1.66±0.02^a	1.88±0.01^a	1.72±0.01^a	1.38±0.01^a
CYA+1 m	1.25±0.01^b	0.90±0.01^b	1.18±0.01^b	1.27±0.01^b
CYA+10 m	0.15±0.02^c	0.15±0.01^c	0.26±0.02^c	0.24±0.02^c

Values are the mean of three replicates±Standard Deviation (SD). Data marked with different letter(s) in each column are significantly different at $p<0.05$ for the Ducan's post hoc test

Table 2: The 50% effective concentration (EC_{50}) of Na_2EDTA for the different black aspergilli tested

Strains	EC_{50} [a]	95% CI [b]	r^2 [c]
A. carbonarius (MUM 03.59)	2.1	1.99 to 2.23	0.9993
A. ibericus (MUM 03.49)	0.9	0.92 to 0.95	0.9999
A. niger (MUM 03.57)	2.0	1.98 to 2.10	0.9998
A. niger (MUM 03.58)	4.1	3.88 to 4.31	0.9995

[a]: EC_{50} = Concentration of Na_2EDTA (mmol L^{-1}) at which survival was 50% as determined by fitting the experimental data to a four-parameter logistic model (Hill equation) using computer curve-fitting software (Prism 4, GraphPad Software, Inc, San Diego, CA, USA), [b]: 95% Confidence Intervals of fitted EC_{50}, [c]: Correlation coefficient of fitted curves

Table 3: Production of ochratoxin A by *A. carbonarius* MUM 03.59 and *A. niger* MUM 03.57 in CYA and in CYA supplemented with 1 and 10 mmol L^{-1} of Na_2EDTA (CYA+1 m and CYA+10 m, respectively)

		OTA contents (μg $plate^{-1}$)		
		Medium		
Strain	Days	CYA	CYA+1 m	CYA+10 m
A. carbonarius	0	nd	nd	nd
MUM 03.59	2	1.49±0.29[a]	nd	nd
	4	14.59±3.39[b]	19.42±1.44[b]	nd
	6	48.35±4.83[d]	39.12±1.54[c]	nd
	8	68.61±5.62[e]	82.37±9.53[f]	0.08±0.11[a]
A. *niger*	0	nd	nd	nd
MUM 03.57	2	0.12±0.02[a]	nd	nd
	4	1.69±0.28[b]	0.30±0.04[a]	nd
	6	10.01±0.58[d]	1.68±0.06[b]	nd
	8	11.81±0.59[e]	7.50±0.39[c]	0.20±0.07[a]

Values are the mean of three replicates±Standard Deviation (SD). For each strain and column, data marked with different letter(s) are significantly different at $p<0.05$ for the Ducan's post hoc test, nd: not detected

in 50% (EC_{50}) is 2.1 mmol L^{-1} for *A. carbonarius*, 0.9 mmol L^{-1} for *A. ibericus*, 2.0 mmol L^{-1} for *A. niger* MUM 03.57 and 4.1 mmol L^{-1} for *A. niger* MUM 03.58 (Table 2).

The production of ochratoxin A was also affected by the presence of Na_2EDTA in culture media. When 1 mmol L^{-1} of Na_2EDTA was used, OTA was detected after 4 days of growth, while when 10 mmol L^{-1} were used, it was detected after 8 days (Table 3). The accumulation of OTA was significantly lower in most situations. In the presence of 1 mmol L^{-1} of Na_2EDTA and after 8 days of growth, *A. niger* MUM03.57 produced 7.5 μg OTA/plate. After the same period of incubation with 10 mmol L^{-1} of Na_2EDTA this strain produced only 0.2 μg OTA/plate, which is 98.3% less than the respective control. Under the same conditions, the *A. carbonarius* strain produced only 0.08 μg OTA/plate, 99.9% less than the control. The reduction in OTA production was not observed when *A. carbonarius* was grown with 1 mmol L^{-1} of Na_2EDTA (Table 3).

Several studies have previously reported the bactericidal proprieties of Na_2EDTA (Oita, 2003; Reidmiller *et al*., 2006). However, fungi tolerance to Na_2EDTA is not well documented despite its utilization by the food and agrochemical industry, being its antifungal effects mainly reported on clinical strains as *Candida albicans* (Sen *et al*., 2000) or *Aspergillus fumigatus* (Hachem *et al*., 2006).

In this study, it was demonstrated that Na_2EDTA can significantly inhibit the growth rate of black aspergilli isolated from grapes and significantly delay and reduce the ochratoxinA produced by the ochratoxigenic strains tested. Namely, 2 mmol L^{-1} of Na_2EDTA were sufficient to reduce the growth rate of ochratoxigenic strains in 50% and 10 mmol L^{-1} of Na_2EDTA to delay the production of OTA in 8 days and reduce the levels produced in approximately 99%. The effect of Na_2EDTA on strains growth is probably due to a defective cell wall construction mediated by its zinc binding capacity as presented by Brul *et al*. (1997) for yeasts. The reductions on OTA amounts produced are probably due to the inhibition of strains growth further than to the inhibition of the mycotoxin synthesis.

In agriculture, Na_2EDTA is commonly used on foliar products to supplement micronutrients to plants or to stabilize the formulation of several agrochemical products. It will be interesting to study, *in vivo*, if the application of products that contain EDTA can contribute to control the presence of black ochratoxigenic aspergilli and so the levels of ochratoxin A in agriculture commodities such as grapes.

SAFETY

Ochratoxin A is a toxic compound that needs to be manipulated with care and with appropriate safety precautions.

ACKNOWLEDGMENT

Luís Abrunhosa is grateful for grant SFRH/BD/11228/2002 from Fundação para a Ciência e Tecnologia-FCT, Portugal.

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