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Biocontrol of *Aspergillus ochraceus* by yeasts

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Aspergillus ochraceus is one of the main contaminant of products such as coffee, grapes, cereals and derivatives. This filamentous fungus can also produce ochratoxin A (OTA), a secondary metabolite with nephrotoxic and carcinogenic properties. The maximum OTA limits allowed in food and raw agroproducts are under legal regulation. Currently, biological control has been proposed as a useful strategy in integrated management to control these fungi. Yeasts would be suitable biocontrol agents because of their characteristics: capacity of growing in fermenters, few nutritional requests and inability to produce toxic metabolites.
In this study, we tested the antagonist ability of 16 yeast strains from seven different species against five *Aspergillus ochraceus* strains. Two strains of *Debaryomyces hansenii* (CYC 1021 and CYC 1244) showed inhibitory activity against these fungi when they both were grown in YMA-MB medium supplemented with sodium chloride (6%). Additional *in vitro* assays showed that salinity enhanced biocontrol activity of *Debaryomyces hansenii* CYC 1244. The effect of temperature on biocontrol activity was also studied. The highest reduction of fungal growth was achieved at 20ºC. OTA concentration in CYA medium was significantly lower at 28ºC compared with control when fungus and yeast were co-cultured.

Keywords *Aspergillus ochraceus*; biocontrol; *Debaryomyces hansenii*; ochratoxin A.

1. Introduction

Ochratoxin A (OTA) is a secondary metabolite produced by *Aspergillus* and *Penicillium* species. This mycotoxin has been shown to have nephrotoxic, inmunotoxic, genotoxic and teratogenic properties towards several animal species [1], and has been classified by International Agency for Research on Cancer as possible carcinogen to humans (group 2B) [2]. OTA occurs in various foodstuffs and beverages including a variety of cereals, beans, groundnuts, spices, dried fruits, grapes, coffee, milk, wine and beer [3, 4] and its maximum limits on several commodities for human consumption are under legal regulation. *Aspergillus ochraceus* is an important OTA producer specie and it is considered the main source in coffee [5].

Postharvest decay can be reduced by minimizing fruit injuries, by maintaining the natural resistance of the host and by delaying senescence. However, these beneficial practices are usually not sufficient to protect the product from fungal infection [6]. The use of fungicides immediately before or at postharvest to prevent rots is being increasingly limited by legislation, because of risks for consumers' health [7] environmental pollution, and the onset of resistant pathogen strains [8].

Biological control has been proposed as an alternative to the use of synthetic fungicides or in combination with them for reducing fungal growth and toxin biosynthesis [9, 10, 11]. Several yeast species have already been shown to be effective biological control agents in protecting plants against fungal diseases [12, 13]. Although the molecular basis of the natural process of biocontrol are still largely unknown; competition for nutrients [14], predation [15], secretion of cell wall degrading enzymes [16], killer toxins [17] or production of syringotoxins and syringomycins [18] are possible mechanisms involved in biological control.

The aim of the present work was to test the antagonistic ability of different yeast species against *A. ochraceus* strains and to determine the optimal conditions to reduce fungal growth and OTA concentration.

2. Materials and Methods

2.1. Organisms, media and culture conditions

All the isolates used in this study are given in Table 1. Yeast strains were maintained by regular subculturing on Yeast Morphology Agar (YMA) [19] of slopes at 25ºC for 48 h and subsequently stored at 4ºC until required. The *A. ochraceus* strains were maintained by regular subculturing on Potato Dextrose Agar (PDA) at 25ºC for 96 h and then stored at 4ºC until required and stored as spore suspension in 15% glycerol at –80ºC.

Table 1 Yeast and fungal strains used in biocontrol experiments.

Species	Strains			
Debaryomyces hansenii	CYC 1021	CYC 1244	CECT 10380	CECT 10386
Metschnikowia pulcherrima	L3	L4		L4.1
Pichia anomala	CECT 1114			
Pichia membranifaciens	CYC 1070			
Saccharomyces cerevisiae	CYC 1172		CYC 1174	
Torulaspora delbrueckii	CYC 1176	CYC 1177	CECT 10589	CECT 10676
Zygosaccharomyces rouxii	CYC 1150			
Aspergillus ochraceus	ALD*	ALF		AsO2*
	CECT 6795*		CECT 6825	

* OTA producers

2.2. Initial screening and salinity significance

The ability of 16 yeast strains to control *A. ochraceus* strains was tested. One ml of spore suspension (10^4 spores/ml) was cultured in YMA-MB medium either supplemented or not with sodium chloride (6%) at 20ºC. Plates were inoculated with a loopful of each yeast strain onto the surface of agar (4 yeasts/ plate). Positive biocontrol was considered when a clear zone of growth inhibition was visible after 7 days of incubation.

The effects of salinity on biocontrol of *D. hansenii* CYC 1244 against all five *A. ochraceus* strains were studied. One ml of a CYC 1244 cellular suspension (5 x 10^6 cells/ml) was mixed with 25 ml of melted YMA-MB medium with or without sodium chloride (6%). Spots of 1,5 µl of *A. ochraceus* spore suspension (10^7 spores/ml) were placed on each plate. Fungal growth was determined by measuring the fungal colony diameter at 4, 7 and 10 days. Plates were incubated at 20ºC.

2.3. Biocontrol activity: Effect of temperature and influence in OTA production

One ml of a yeast cellular suspension of *D. hansenii* (5 x 10^4 cells/ml) was mixed with 25 ml of melted CYA medium (Czapek Yeast Extract Agar). Spots of 2 µl of *A. ochraceus* spore suspension (10^6 spores/ml) were placed on each plate. Fungal growth was determined by measuring the colony diameter at 4, 7 and 10 days. This assay was carried out at 20ºC and 28ºC.

The influence of *D. hansenii* CYC 1244 in OTA concentration was analysed in the three *A. ochraceus* producers (ALD, CECT 6795 and AsO2). OTA was extracted by a method designed elsewhere [20] after 10 days of incubation in previously described conditions and measured by High Performance Liquid Chromatography (HPLC). Methanol – Monopotassium phosphate (2:1) was the mobile phase.

2.4. Statistical analysis

Statistical software SPSS 14.0 was used. Corresponding T-student test for independent or paired samples was applied. The level of significance was established as $p \leq 0.05$.

3. Results

3.1 Initial screening and salinity significance

In the initial screening we have used YMA-MB medium because it favours the growth of yeasts. Sodium chloride was added to enhance possible killer toxin production by yeasts [21]. Two out sixteen yeast strains tested in the screening, *D. hansenii* CYC 1021 and CYC 1244, showed biocontrol effect against all the *A. ochraceus* strains in assays performed in YMA-MB medium supplemented with sodium chloride (6%). Growth inhibition was not observed in experiments using YMA-MB medium without salt in these conditions. *D. hansenii* CYC 1244 was selected for additional *in vitro* assays because it produced a bigger zone of growth inhibition than CYC 1021.

Biocontrol efficiency of *D. hansenii* CYC 1244 was enhanced by high sodium chloride concentration (Table 2). Significant reduction of fungal growth (65%) by this yeast strain was observed in relation with control assays (fungi grown on free yeast plates) in YMA-MB with high salinity, while no significant reduction was observed

in experiments where the medium was not supplemented with salt. Figure 1 shows the evolution of fungal growth in YMA-MB with sodium chloride (6%).

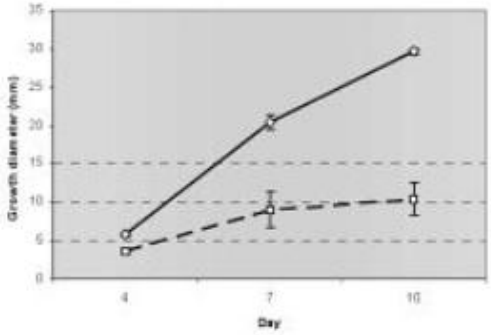

Fig. 1 Evolution of fungal growth in plates with YMA-MB supplemented with NaCl. Solid line represents control growth and discontinuous line indicates fungal growth with CYC 1244. The values are mean of five *A. ochraceus* strains. Bars indicate standard deviation.

Table 2. Effects of salinity and biocontrol activity on fungal growth. [a] Represents statistical signification ($p \leq 0.05$) when controls with or without sodium chloride were compared. [b] Represents statistical signification ($p \leq 0.001$) when control plates with fungi in YMA-MB medium were compared with YMA-MB plates where fungus and CYC 1244 were co-cultured. Values corresponded to 10-day-old cultures.

	Growth diameter without NaCl (mm)	**Growth diameter with NaCl (6%) (mm)**
Aspergillus ochraceus controls	42,40	29,60[a]
Aspergillus ochraceus + CYC 1244	36,80	10,40[b]

3.2 Biocontrol activity: Effect of temperature and influence in OTA production

This assay was carried out in CYA plates to test the effect of presence of *D. hansenii* CYC 1244 on OTA concentration. CYA is a permissive medium for OTA production [20]. The effect of temperature on both fungal growth and OTA concentration was studied at 20 and 28°C.

The temperature showed an effect on the biocontrol efficiency against *A. ochraceus* growth (Fig. 2). Biocontrol plates incubated at 28°C showed little reduction in growth (15.4%) compared to controls without yeast, while higher values (48.5%) were obtained at 20°C in the same experiments.

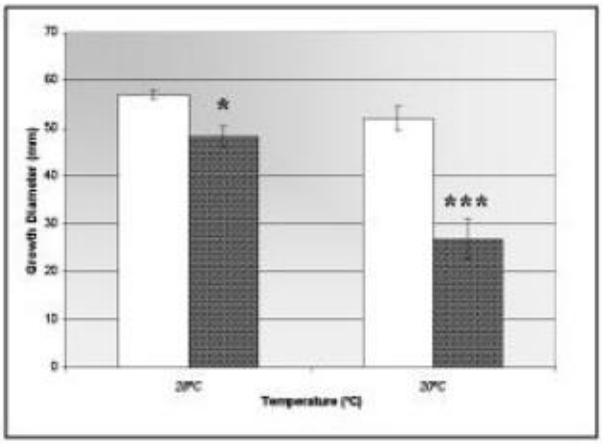

Fig. 2 Effect of temperature and presence of *D. hansenii* CYC 1244 on the reduction of fungal growth. White bars represent fungal growth in control plates without yeast and scratched bars in plates with CYC 1244. The values are mean of five *A. ochraceus* strains. Standard deviation of these values are indicated.
Statistical signification is indicated by asterisks: ***$p \leq 0.001$, **$0.001 < p \leq 0.01$, *$0.01 < p \leq 0.05$.

No OTA production by any of five *A. ochraceus* strains was detected in at 20°C. A high reduction of OTA concentration was observed at 28°C in plates of the three OTA-producing *A. ochraceus* strains co-cultured with *D. hansenii* CYC 1244 (Table 2).

Table 2 OTA concentration in plates at 28ºC after 10 days of incubation measured by HPLC.

A. ochraceus strain		[OTA] (µg/l)	Reduction (%)
AsO2	Control	10274	86,7
	+ CYC 1244	1371	
CECT 6795	Control	127,8	37,3
	+ CYC 1244	80,1	
ALD	Control	263	20,53
	+ CYC 1244	209	

4. Discussion

Yeasts would be suitable biocontrol agents because of their characteristics: capacity of growing in fermenters, few nutritional requests and inability to produce toxic metabolites [9]. Several yeast species seems to inhibit fungi [12, 13] and, specifically, species of *Pichia* and *Hanseniaspora* genera have been shown biocontrol against *Aspergillus ochraceus* [11]. In this work, two strains of *Debaryomyces hansenii* (CYC 1021 and CYC 1244) showed antagonistic activity against several strains of *A. ochraceus*, although the effect was more important in case of *D. hansenii* CYC 1244. This activity might be due to their ability to produce a killer toxin affecting fungal growth [17, 22]. Lethal activity of this toxin increases in mediums supplemented with sodium chloride [21]. The positive effect of high salinity found in the biocontrol assays might suggest an antagonist activity mediated by this mechanism.

Effects of temperature on growth and OTA production by *A. ochraceus* have been reported [23, 24]. In this work, we have observed antagonist activity at both temperatures tested, 28 ºC and at 20ºC. However, reduction of fungal growth was more drastic at 20ºC than at 28 ºC in all strains studied. This fact also supports the theory of a killer toxin mediated mechanism of biocontrol by *D. hansenii* CYC 1244 since it has been reported that toxin stability decreases at temperatures higher than 20ºC [17]. Additional experiments will be performed to rule out other possibilities such as competition.

The antagonist activity had also a remarkable effect on OTA reduction when *D. hansenii* CYC 1244 and *A. ochraceus* were co-cultured in the same plate, with values reaching 86% in AsO2 strain. In some studies, reduction of OTA concentration by yeast species might be achieved by the ability to adsorb or to retain mycotoxins [25]. Moreover, it has been described the capacity of several yeast species to produce volatile compounds that affect OTA production by *A. ochraceus* [26]. Both mechanisms could be possible in our case, although we do not have information yet about the mechanism underlying the effect of *D. hansenii* in OTA reduction.

The results obtained in the present work indicate a positive antagonistic effect by *D. hansenii* on all the *Aspergillus ochraceus* strains showed by reduction of fungal growth and OTA concentration in *in vitro* cultures. Further studies *in vivo* are needed to check if it is possible to apply this yeast in biological control of this fungus.

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