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In vitro study on the effect of *Saccharomyces cerevisiae* strains on growth and mycotoxin production by *Aspergillus carbonarius* and *Fusarium graminearum*

M.R. Armando [a,b], C.A. Dogi [b,c], V. Poloni [b,c], C.A.R. Rosa [d,e], A.M. Dalcero [b,c], L.R. Cavaglieri [b,c,*]

[a] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Córdoba Ciencia (ACC), Argentina
[b] Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 km, 601, (5800) Río Cuarto, Córdoba, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina
[d] Departamento de Microbiologia e Imunologia Veterinária, Universidade Federal Rural do Rio de Janeiro, Rodovia BR 465 Km 7, Seropédica 23890-000, Rio de Janeiro, Brazil
[e] Conselho Nacional de Pesquisas Científicas (CNPq), Brazil

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ABSTRACT

The effect of *Saccharomyces cerevisiae* RC008 and RC016 strains, previously selected based on their aflatoxin B_1 mycotoxin binding ability and beneficial properties, against *Aspergillus carbonarius* and *Fusarium graminearum* under different interacting environmental conditions was evaluated. In vitro studies on the lag phase, growth rate and ochratoxin A/zearalenone and DON production were carried out under different regimens of a_w (0.95 and 0.99); pH (4 and 6); temperature (25 and 37 °C) and oxygen availability (normal and reduced). Both yeast strains showed antagonistic activity and decreasing growth rate compared to the control. In general, the RC016 strain showed the greatest inhibitory activity. Except at the interacting condition 0.95 a_W, normal oxygen availability and 37 °C, at both pH values, *A. carbonarius* and *F. graminearum* were able to produce large amounts of mycotoxins in vitro. In general, a significant decrease in levels of mycotoxins in comparison with the control was observed.
S. cerevisiae RC008 and RC016 could be considered as effective agents to reduce growth and OTA, ZEA and DON production at different interacting environmental conditions, related to those found in stored feedstuff. The beneficial and biocontrol properties of these strains are important in their use as novel additives for the control of mycotoxigenic fungi in stored feedstuffs.

1. Introduction

Mycotoxin contamination is a serious problem in developing countries where climatic conditions and agricultural and storage practices are conducive to fungal growth and toxin production. Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, teratogenic, immunosuppressive, and carcinogenic properties (Walker and Larsen, 2005) that has been classified by the International Agency for Research on Cancer as a possible human carcinogen (group 2B) (IARC, 1993). OTA was first isolated from moldy cornmeal in South Africa (Van der Merwe et al., 1965). Subsequently, OTA has been found in a number of agricultural commodities and foodstuffs, including cereals, coffee beans and beer, as a result of contamination with fungi of the genera *Aspergillus* and *Penicillium*. Several reports have indicated that members of the *Aspergillus* section *Nigri*, the so-called black aspergilli, are the dominant ochratoxigenic species on wine grapes worldwide (Bau et al., 2006; Chulze et al., 2006; Leong et al., 2006). However, the presence OTA in wheat and wheat products (Riba et al., 2008; García-Cela et al., 2012) and the presence of *A. carbonarius* in corn grains (Rosa et al., 2009) have also been reported.

Fusarium toxins, such as deoxynivalenol (DON) and zearalenone (ZEA), contaminate wheat, maize and barley worldwide (Abouzied et al., 1991; Chelkowski, 1998; EFSA, 2004a,b).These mycotoxins are produced mainly by *F. graminearum*, *Fusarium culmorum* and related species during cereal flowering under predisposing environmental conditions such as humidity and fungus-specific optimum temperature range (Oldenburg et al., 2000).

Zearalenone (ZEA) is an estrogenic mycotoxin that causes severe morphological and functional disorders of reproductive organs in livestock (Etienne and Jemmali, 1982). This mycotoxin is produced by *Fusarium* species that cause *Fusarium* head blight of small-grain cereals (wheat, barley, etc.) and maize (Pittet, 1998). A high level of ZEA often accumulates in mold-infected grains and derived cereal products (Yuwai et al., 1994; Creppy, 2002; Li et al., 2002) which may cause hyperestrogenism and related toxicoses of farm animals (Scott et al., 1985; Bauer et al., 1987) and humans (Schoental, 1983; Szuets et al., 1997). Deoxynivalenol, a *Fusarium* toxin belonging to the trichothecene group, has been reported to produce a variety of adverse health effects in farm animals, such as inhibition of protein

* Corresponding author at: Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 Km, 601, (5800) Río Cuarto, Córdoba, Argentina. Tel.: +54 358 4676429; fax: +54 358 4676231.
E-mail address: lcavaglieri@arnet.com.ar (L.R. Cavaglieri).

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synthesis, reduction of feed intake, and alteration of the immune system (Tiemann et al., 2006).

Postharvest disease control using biological agents has been extensively demonstrated (Francés et al., 2006; Kotan et al., 1999). Yeasts may act as antagonistic microorganisms thereby considerably decreasing the growth of filamentous spoilage fungi and also ochratoxigenic microorganisms (Petersson and Shurer, 1995; Petersson et al., 1998). Bleve et al. (2006) reported that yeasts isolated from grapes *Issatchenkia orientalis*, *Metschnikowia pulcherrima*, *I. terricola*, and *Candida incommunis* reduced the *A. carbonarius* and *A. niger* colonization on grape berry. The best antagonistic activity was shown by *I. orientalis* isolates. Later, Lee and Magan (1999) investigated the effect of water activity on the interactions between OTA-producing strain of *Aspergillus ochraceus* and other fungi in dual culture experiments on maize meal-based agar medium and demonstrated the effect that environmental factors have on the possible competitiveness of *A. ochraceus* in the maize grain ecosystem and the role of ochratoxin in niche exclusion of competitors.

Extensive previous studies were reported with beneficial *S. cerevisiae* RC008 and RC016 strains in relation to AFB_1 decontamination and mycotoxin binding ability under gastrointestinal conditions in vitro. Moreover, the inhibition of *Aspergillus parasiticus* growth and AFB_1 production have already been demonstrated (Armando et al., 2011, 2012b).

These strains were previously proposed to be used in silage (Armando et al., 2012b). The presence of other ochratoxin and zearalenone producer species in this ecosystem has been further demonstrated (González Pereyra et al., 2008, 2011). At present, there is no available information on the impact of *S. cerevisiae* on growth and mycotoxin production by *A. carbonarius* and *F. graminearum* under different environmental conditions. The aims of this study were to evaluate the effect of *S. cerevisiae* RC008 and RC016 on: a) lag phase prior to growth, b) growth rate and c) ochratoxin A and zearalenone production by *A. carbonarius* and *F. graminearum* under interacting conditions of temperature, pH, oxygen availability and water activity on silage extract agar.

2. Materials and methods

2.1. Microorganisms, growth medium and cultural conditions

Saccharomyces cerevisiae strains (RC008 and RC016), isolated from animal ecosystem were obtained from the collection center at the University National of Rio Cuarto, Argentina. Stock cultures were maintained at −80 °C in 30% (v/v) glycerol. Working cultures were prepared from frozen stocks by two transfers in Yeast extract-Peptone-Dextrose (YPD broth—5 g yeast extract, 5 g peptone, 40 g dextrose, 1000 ml water) and incubation at 37 °C for 24 h. Inoculations of yeasts were made from the working cultures into YPD broth and incubated at 37 °C for 24 h. After incubation, cells were collected by centrifugation (5000 rpm 10 min), washed twice with phosphate-buffered saline (PBS pH 7.2) and once with sterile double-distilled H_2O. Finally, the yeast pellets (1×10^7 cells/ml) were suspended in sterile PBS prior use in the assay.

Aspergillus carbonarius RC310 (OTA producer) was isolated from swine feed (Rosa et al., 2009) and was deposited in the National University of Río Cuarto, Córdoba, Argentina (RC) collection center. It was maintained at 4 °C on slants of malt extract agar (MEA) and at −80 °C in 15% glycerol. It was grown on MEA at 25 °C for 5 d to obtain heavily sporulating cultures. *Fusarium graminearum* Z3636 (ZEA and DON producer) was isolated from scabby wheat by R. Bowden (Kansas State University) (Bowden and Leslie, 1992) and was deposited in the National University of Río Cuarto, Córdoba, Argentina (RC) collection center. It was maintained on V-8 juice agar slants and cultured on carnation leaf agar (CLA) at 25 °C for 7 days on an oscillatory shaker (200 rpm) to obtain sporulating cultures.

The basic medium used in this study was silage agar (containing 40 g extract of silage and 20 g agar in 1 l of distilled water). The water activity was adjusted at 0.95 and 0.99 by the addition of known amounts of the non-ionic solute glycerol (Marin et al., 1998). The basic medium with different levels of a_W was adjusted to pH 4 and pH 6 adding the necessary quantity of the HCl and NaOH. The medium was autoclaved at 121 °C for 20 min. These environmental conditions were selected in order to simulate different regimens that may be found in stored feedstuff.

2.2. Effect of the interaction S. cerevisiae and A. carbonarius or F. graminearum on growth and mycotoxin production

2.2.1. Growth rate and lag phase studies

Growth studies were tested as described by Cavaglieri et al. (2004) with some modifications. One milliliter (1 ml) of *S. cerevisiae* strains inocula (1×10^7 CFU/ml) was cultured in silage agar medium plates at different a_w and pH values. After solidification, plates were inoculated in the center with spores of *A. carbonarius* and *F. graminearum* suspended in semisolid agar. Petri plates of the same a_w values were sealed in polyethylene bags. The plates were incubated at 25 °C or 37 °C under normal and reduced oxygen pressure conditions (microaerophilia). The growing radius of the cultures containing both microorganisms was compared with the control cultures. For each colony, two radii, measured at right angles to one another, were averaged to find the mean radius for that colony. All colony radii were determined by using three replicates for each tested fungus. The radial growth rate (mm/day) was subsequently calculated by linear regression of the linear phase for growth. The time at which the line intercepted the x-axis was used to calculate the lag phase in relation to yeast strain, water activity, temperature and oxygen pressure. All experiments were carried out with at least three separate replicate Petri plates per treatment. After growth was evaluated, all samples were frozen for later extraction and mycotoxins quantification.

The time at which the line intercepted the x-axis was used to calculate the lag phase in relation to bacterial antagonists and water activity. The experiments were carried out three times for single and paired cultures.

2.2.2. Ochratoxin A and zearalenone analysis

All media were taken from each plate of each treatment, transferred to an Erlenmeyer flask and 20 ml of chloroform was then added. The mixture was agitated at 200 rpm for 30 min. The chloroform extract was dried under nitrogen gas. The residue was redissolved in 1 ml of chloroform for OTA and ZEA quantification by high performance liquid chromatography (HPLC), following the detection methodology proposed by Scudamore and Macdonald (1998) and Cerveró et al. (2007).

The mycotoxins (50 μl solution) were analyzed using a reversed-phase HPLC/fluorescence detection system. The HPLC with fluorescence detection (λexc 330 nm; λem 460 nm for OTA and λexc 280 nm; λem 460 nm for ZEA) consisted in a C18 column (Supelcosil LC-ABZ, Supelco; 150×4.6 mm, 5 μm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20×4.6 mm, 5 μm particle size). The mobile phase (acetonitrile:water:acetic acid, 57:41:2 for OTA and water:methanol, 30:70 for ZEA) was pumped at 1 ml/min. The injection volume was 100 μl and the retention time was around 5 min.

Standard curves were constructed with different levels of mycotoxins. This toxin was quantified by correlating peak height of sample extracts and those of standard curves. The detection limits of the technique were 0.1 ng/g and 3 ng/g, respectively.

Table 1
Effect of *Saccharomyces cerevisiae strains* on *Aspergillus carbonarius and Fusarium graminearum* growth rate under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions.

Growth condition				*Aspergillus carbonarius* growth rate (mm/h)						*Fusarium graminearum* growth rate (mm/h)			
pH	T°	P°O_2	a_w	Control[1]		Interaction 1[2]		Interaction 2[3]		Control[2]		Interaction 1[2]		Interaction 2[3]	
				Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD	
4	37	Normal	0.99	0.09 ± 0.02	def	0 ± 0	a	0.03 ± 0	abc	0 ± 0	f	0 ± 0	f	0 ± 0	f
			0.95	0.16 ± 0.005	gh	0.05 ± 0.015	abcd	0.03 ± 0.005	ab	0 ± 0	f	0 ± 0	f	0 ± 0	f
		Reduced	0.99	0.05 ± 0.005	abcd	0 ± 0	a	0.06 ± 0.02	bcde	0 ± 0	f	0 ± 0	f	0 ± 0	f
			0.95	0 ± 0	a	0 ± 0	a	0 ± 0	a	0 ± 0	f	0 ± 0	f	0 ± 0	f
	25	Normal	0.99	0.4 ± 0.015	p	0.24 ± 0.105	jklm	0.21 ± 0.03	ijk	0.35 ± 0.015	a	0.22 ± 0	bcd	0 ± 0	f
			0.95	0.28 ± 0.01	mno	0.27 ± 0.025	lmno	0.25 ± 0.045	klm	0.05 ± 0.05	ef	0 ± 0	f	0 ± 0	f
		Reduced	0.99	0.43 ± 0.01	p	0.17 ± 0	ghi	0.13 ± 0.055	fg	0.36 ± 0.015	a	0.19 ± 0.015	cd	0 ± 0	f
			0.95	0.26 ± 0.015	klmn	0.20 ± 0.015	hij	0.23 ± 0.005	jkl	0 ± 0	f	0 ± 0	f	0 ± 0	f
6	37	Normal	0.99	0.11 ± 0.025	ef	0 ± 0	a	0 ± 0	a	0 ± 0	f	0 ± 0	f	0 ± 0	f
			0.95	0.07 ± 0.005	bcde	0.04 ± 0.025	abc	0.03 ± 0.005	ab	0 ± 0	f	0 ± 0	f	0 ± 0	f
		Reduced	0.99	0 ± 0	a	0 ± 0	a	0 ± 0	a	0 ± 0	f	0 ± 0	f	0 ± 0	f
			0.95	0.09 ± 0.01	def	0.06 ± 0.01	bcde	0.01 ± 0	a	0 ± 0	f	0 ± 0	f	0 ± 0	f
	25	Normal	0.99	0.4 ± 0.02	p	0.30 ± 0.005	no	0.08 ± 0.005	cde	0.18 ± 0.175	d	0.14 ± 0.015	de	0 ± 0	f
			0.95	0.31 ± 0.105	o	0.24 ± 0.01	jklm	0.22 ± 0.025	ijk	0.15 ± 0.015	de	0.07 ± 0.07	ef	0 ± 0	f
		Reduced	0.99	0.28 ± 0.015	mno	0.22 ± 0.015	ijk	0.06 ± 0.01	bcde	0.37 ± 0.015	a	0.30 ± 0.005	ab	0 ± 0	f
			0.95	0.31 ± 0.05	o	0.22 ± 0.005	ijk	0.22 ± 0.03	jkl	0.13 ± 0	de	0.06 ± 0.06	ef	0 ± 0	f

[1]Control: Toxicogenic fungi (*Aspergillus carbonarius* or *Fusarium graminearum*) growing alone, [2]Interaction 1: Toxicogenic fungi–*Saccharomyces cerevisiae* RC008 interaction, [3]Interaction 2: Toxicogenic fungi–*Saccharomyces cerevisiae* RC016 interaction, values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P<0.05$). Growth rate for each toxicogenic fungi strain was statistically analyzed, separately.

2.2.3. Deoxynivalenol analysis

All media were taken from each plate of each treatment, transferred to an Erlenmeyer flask and 20 ml of a mixture of acetonitrile–water was then added. The mixture was agitated at 200 rpm for 30 min. The extract was dried under nitrogen gas. The residue was redissolved in 1 ml of methanol for DON quantification by high performance liquid chromatography (HPLC), following the detection methodology proposed by Truckssess et al. (1984) and Fernández et al. (1994).

The mycotoxins (50 μl solution) were analyzed using a reversed-phase HPLC with UV-detection system. The HPLC with UV-detection (220 nm) consisted in a C18 column (Supelcosil LC-ABZ, Supelco; 150×4.6 mm, 5 μm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20×4.6 mm, 5 μm particle size). The mobile phase (methanol:water 12:88) was pumped at 1.5 ml/min. The injection volume was 100 μl and the retention time was around 10 min.

Standard curves were constructed with different levels of mycotoxins. This toxin was quantified by correlating peak height of sample extracts and those of standard curves.

2.3. Statistical analyses

The influence of *S. cerevisiae* RC008 and RC016 strains on *A. carbonarius* and *F. graminearum* growth and OTA, ZEA and DON production was analyzed by analysis of variance (ANOVA). Means obtained from growth rate lag phase and mycotoxins production studies were compared using the Fisher's protected LSD test (Quinn and Keough, 2002). The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC).

3. Results

3.1. Effect of S. cerevisiae strains on A. carbonarius and F. graminearum growth rate

Statistical analyses of yeast strains (S), pH, temperature (T) oxygen availability (PO), water activity (a_W) on *A. carbonarius* and *F. graminearum* growth rate showed that five way interactions were statistically significant ($P<0.001$).

In general, *A. carbonarius* growing alone showed the best growth at 25 °C (Table 1). Overall, there were no differences in the rate of growth of *A. carbonarius* at 37 °C at all tested pH conditions, except under microaerophilic treatments (reduced PO) when the fungus did not grow. Both yeast strains were able to significantly reduce the *A. carbonarius* growth rate at pH 4, except at 37 °C and reduced PO ($P<0.05$) where growth rate was similar in control (fungus growing alone) and treated groups (fungus–yeast interactions). At pH 6 the antagonistic effect of yeast strains on *A. carbonarius* was observed mainly at 25 °C.

At 37 °C, *F. graminearum* did not grow under all conditions assayed (Table 1). *S. cerevisiae* RC016 completely inhibited growth of *F. graminearum* strain under all conditions assayed ($P<0.05$). *S. cerevisiae* RC008 was able to significantly reduce the *F. graminearum* growth rate at pH 4 and 25 °C, whereas at pH 6 the reduction in growth rate caused by the yeast was not statistically significant.

3.2. Effect of S. cerevisiae strains on A. carbonarius and F. graminearum lag phase

Statistical analyses of S, pH, T, PO, a_W on lag phase showed that five way interactions were statistically significant ($P<0.0001$).

Mean lag phases of *A. carbonarius* and *F. graminearum* under different interacting environmental conditions are shown in Table 2. The lag phase for *A. carbonarius* growing alone was longer at pH6 and at 0.95_{aw} (96.17; 98.32; 127.73; 144.50 h). *S. cerevisiae* RC016 did not influence the lag phase of *A. parasiticus*, however a prolonged

Table 2
Effect of *Saccharomyces cerevisiae* strains on *Aspergillus carbonarius and Fusarium graminearum* lag phase under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions.

Growth condition				*Aspergillus carbonarius* lag phase (h)				*Fusarium graminearum* lag phase (h)			
pH	T°	P°O_2	a_w	Control[1]		Interaction 1[2]		Interaction 2[3]		Control[2]		Interaction 1[2]		Interaction 2[3]	
				Mean ± SD				Mean ± SD		Mean ± SD				Mean ± SD	
4	37	Normal	0.99	131.20 ± 1.66	cd	215 ± 0	a	208.67 ± 25	a	215 ± 0	a	215 ± 0	f	215 ± 0	a
			0.95	72.06 ± 21.06	ef	32.83 ± 0.5	kmnopqr	183.75 ± 21.75	b	215 ± 0	a	215 ± 0	f	215 ± 0	a
		Reduced	0.99	152.50 ± 7.5	c	215 ± 0	a	213.75 ± 35.25	a	215 ± 0	a	215 ± 0	f	215 ± 0	a
			0.95	215 ± 0	a	215 ± 0	a	215 ± 0	a	215 ± 0	a	215 ± 0	f	215 ± 0	a
	25	Normal	0.99	29.37 ± 3.21	lmnopqr	41.46 ± 28.62	ijlkmnop	55.64 ± 7.53	efghijk	50.86 ± 1.92	de	65.68 ± 1.2	bcd	215 ± 0	a
			0.95	50.83 ± 3.65	fghijklm	34.59 ± 2.305	kmnopqr	51.84 ± 9.885	efghijkl	134.25 ± 4.25	b	215 ± 0	f	215 ± 0	a
		Reduced	0.99	27.84 ± 2.455	lmnopqr	34.56 ± 4.56	kmnopqr	69.91 ± 7.955	efg	40.96 ± 2.725	e	51.61 ± 3.45	cd	215 ± 0	a
			0.95	48.47 ± 5.425	fghijklmn	18.46 ± 1.785	pqr	59.70 ± 4.305	efghij	215 ± 0	a	215 ± 0	f	215 ± 0	a
6	37	Normal	0.99	136.25 ± 16.25	c	215 ± 0	a	215 ± 0	a	215 ± 0	a	215 ± 0	f	215 ± 0	a
			0.95	16.35 ± 11.515	qr	75.42 ± 46.585	e	65.59 ± 36.915	efghi	215 ± 0	a	215 ± 0	f	215 ± 0	a
		Reduced	0.99	215 ± 0	a	108.93 ± 16.07	d	75.50 ± 41.5	e	215 ± 0	a	215 ± 0	f	215 ± 0	a
			0.95	25.57 ± 4.935	nopqr	37.33 ± 2.325	jlkmnopq	31.29 ± 14.58	kmnopqr	63.43 ± 3.43	d	21.29 ± 1.71	de	215 ± 0	a
	25	Normal	0.99	23.83 ± 2.36	opqr	43.15 ± 9.65	hijlkmno	34.36 ± 0.445	kmnopqr	45.76 ± 10.495	ghijklmno	94.45 ± 1.325	c	155.35 ± 59.64	ef	215 ± 0	a
			0.95	10.53 ± 2.065	r	15.27 ± 9.515	qr	67.50 ± 2.5	efgh	62.83 ± 7.83	d	45.48 ± 2.21	ab	215 ± 0	a
		Reduced	0.99	42.46 ± 5.04	ijlkmnop	26.92 ± 2.63	mnopqr	48.96 ± 9.46	fghijklmn	56.71 ± 20.29	de	148.12 ± 66.87	ef	215 ± 0	a

[1]Control: Toxicogenic fungi (*Aspergillus carbonarius* or *Fusarium graminearum*) growing alone, [2]Interaction 1: Toxicogenic fungi–*Saccharomyces cerevisiae* RC008 interaction, [3]Interaction 2: Toxicogenic fungi–*Saccharomyces cerevisiae* RC016 interaction, values corresponding to the same letter are not significantly different according to Fisher's protected LSD test (P<0.05). Lag phase for each toxicogenic fungi strain was statistically analyzed, separately.

lag phase was observed in the presence of *S. cerevisiae* RC008 at pH 4 (P<0.05). In general, *S. cerevisiae* RC008 was not able to increase the lag phase of *A. carbonarius* at significant levels (P<0.05), the yeast was able to increase the lag phase in only 31.3% of the tested treatments, *S. cerevisiae* RC016 turned out to be more efficient to increase the lag phase, in 56.3% of the treatments tested (P<0.05).

The effect of *S. cerevisiae* strains on the lag phase of *F. graminearum* under interacting environmental conditions showed that *S. cerevisiae* RC016 maximized the lag phase of the fungus in all tested growth conditions (P<0.05), *S. cerevisiae* RC008 was able to significantly increase the lag phase of *F. graminearum* at pH 6, 25 °C and the lowest water activity tested (0.95) (P<0.05).

3.3. Effect of S. cerevisiae strains on ochratoxin A, zearalenone and deoxynivalenol production

Statistical analyses of S, pH, T, PO, a_W on OTA and ZEA production showed that five way interactions were statistically significant (P<0.0001).

Ochratoxin A production by *A. carbonarius* at different growth conditions is shown in Table 3. *A. carbonarius* growing alone was able to produce great amounts of OTA mainly at 25 °C and 0.99 a_w. A significant decrease of OTA levels in comparison with the control (P<0.05) was observed with the yeast interaction mainly at 0.99 a_W. Both strains, *S. cerevisiae* RC008 and *S. cerevisiae* RC016, achieved the largest reduction percentages at pH 6 and 37 °C (normal PO and 0.99 a_W) of 100% and 99% OTA, respectively, and at pH 6 and 25 °C (reduced PO and 0.99 a_W) the reduction percentages were 99% for both yeast strains. The interacting factors did not increase OTA production at any studied condition. In general, *A. carbonarius* was not able to produce the toxin at 37 °C.

Zearalenone production by *F. graminearum* at different growth conditions is shown in Table 4. In general, *F. graminearum* alone was able to produce great amounts of ZEA at 25 °C, whereas at 37 °C it was not able to produce the toxin, which it was expected since no growth was observed at these interacting conditions. A significant decrease of ZEA levels in comparison with the control (P<0.05) was observed with yeast interaction mainly at pH6 and 25 °C, whereas the yeasts were

Table 3
Effect of *Saccharomyces cerevisiae* strains on ochratoxin A production by *Aspergillus carbonarius* under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions.

Growth condition				*Aspergillus carbonarius* OTA production (μg/ml)					
pH	T°	P°O_2	a_W	Control[1]		Interaction 1[2]		Interaction 2[3]	
				Mean ± SD		Mean ± SD		Mean ± SD	
4	37	Normal	0.99	0.13 ± 0.09	h	0.14 ± 0.03	h	0.15 ± 0.015	h
			0.95	0	h	0	h	0.02 ± 0.016	h
		Reduced	0.99	0.05 ± 0.02	h	0.05 ± 0.02	h	0.05 ± 0.01	h
			0.95	0.04 ± 0.03	h	0.03 ± 0.02	h	0.03 ± 0.02	h
	25	Normal	0.99	11.89 ± 1.94	b	4.04 ± 4.0	def	1.05 ± 0.0	gh
			0.95	1.08 ± 0.35	gh	0.35 ± 0.0	h	0.15 ± 0.02	h
		Reduced	0.99	80.19 ± 21.2	i	47.19 ± 2.10	a	5.36 ± 3.70	de
			0.95	3.03 ± 0.90	efg	1.11 ± 0.66	gh	2.99 ± 1.78	efg
6	37	Normal	0.99	8.06 ± 7.90	c	0	h	0.07 ± 0.01	h
			0.95	0	h	0.01 ± 0.01	h	0.01 ± 0.01	h
		Reduced	0.99	0.01 ± 0.0	h	0	h	0	h
			0.95	0.29 ± 0.17	h	0.14 ± 0.07	h	0.13 ± 0.05	h
	25	Normal	0.99	5.57 ± 0.0	d	0.98 ± 0.0	gh	0.28 ± 0.02	h
			0.95	0.84 ± 0.21	gh	0.25 ± 0.19	h	0.13 ± 0.02	h
		Reduced	0.99	437.1 ± 62.9	j	0.34 ± 0.34	h	0.01 ± 0.01	h
			0.95	4.81 ± 0.27	de	1.92 ± 0.04	fgh	1.57 ± 0.02	gh

[1]Control: Mycotoxin (ochratoxin A) production by toxicogenic fungi growing alone; [2]Interaction 1: Toxicogenic fungi–*Saccharomyces cerevisiae* RC008; [3]Interaction 2: *Toxicogenic fungi–Saccharomyces cerevisiae* RC016. Values corresponding to the same letter are not significantly different according to Fisher's protected LSD test (P<0.05). Mycotoxin production for each toxicogenic fungi strain was statistically analyzed, separately.

Table 4
Effect of *Saccharomyces cerevisiae* strains on zearalenone production by *Fusarium graminearum* under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions.

Growth condition				*Fusarium graminearum* ZEA production (μg/ml)					
pH	T°	P°O_2	a_W	Control[1]		Interaction 1[2]		Interaction 2[3]	
				Mean ± SD		Mean ± SD		Mean ± SD	
4	37	Normal	0.99	0	f	0	f	0	f
			0.95	0	f	0	f	0	f
		Reduced	0.99	0	f	0	f	0	f
			0.95	0	f	0	f	0	f
	25	Normal	0.99	1.73 ± 0.1	f	1.7 ± 0.16	f	0	f
			0.95	2.11 ± 1.7	f	0.03 ± 0.01	f	0	f
		Reduced	0.99	2.56 ± 0.57	ef	3.0 ± 0.23	def	0	f
			0.95	0.0 ± 0.0	f	0	f	0	f
6	37	Normal	0.99	0	f	0	f	0	f
			0.95	0	f	0	f	0	f
		Reduced	0.99	0	f	0	f	0	f
			0.95	0	f	0	f	0	f
	25	Normal	0.99	17.43 ± 2.02	a	6.23 ± 0.98	cde	0	f
			0.95	15.57 ± 2.82	ab	9.29 ± 7.0	c	0	f
		Reduced	0.99	8.03 ± 1.2	cd	3.73 ± 1.3	def	0	f
			0.95	13.30 ± 4.5	b	6.6 ± 5.0	cd	0	f

[1]Control: Mycotoxin (zearalenone) production by toxicogenic fungi growing alone; [2]Interaction 1: Toxicogenic fungi–*Saccharomyces cerevisiae* RC008; [3]Interaction 2: *Toxicogenic fungi–Saccharomyces cerevisiae* RC016. Values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P<0.05$). Mycotoxin production for each toxicogenic fungi strain was statistically analyzed, separately.

not able to decrease ZEA production at significant levels at pH4 and 25 °C.

S. cerevisiae RC008 achieved the largest reduction percentages at pH 6 and 25 °C, 50.4 and 64.3% ZEA, respectively. *S. cerevisiae* RC016 achieved the largest reduction percentages at pH 6 and 25 °C (normal and reduced PO, 0.99 and 0.95 a_W) of 99% ZEA. The interacting factors did not increase ZEA production at any studied condition.

Deoxynivalenol production by *F. graminearum* at different growth conditions is shown in Table 5. In general, *F. graminearum* alone was able to produce great amounts of DON at 25 °C and normal PO, whereas at 37 °C it was not able to produce the toxin. A decrease of DON levels in comparison with the control was observed with yeast interaction mainly at pH 4 and 25 °C, whereas the yeasts were not able to decrease the ZEA production at significant levels at pH 6 and 25 °C.

S. cerevisiae RC008 achieved the largest reduction of DON in all conditions tested. *S. cerevisiae* RC016 achieved the largest reduction percentages at pH 4 and 25 °C of 100 and 52.7% DON. The interaction between *F. graminearum* and *S. cerevisiae* RC016 at pH 6, 25 °C, normal PO and 0.99 a_W showed an increase in DON production.

Table 5
Effect of *Saccharomyces cerevisiae* strains on *deoxynivalenol* production by *Fusarium graminearum* under interacting pH, temperature (T°), oxygen availability (P°O_2) and water activity (a_w) conditions.

Growth condition				*Fusarium graminearum* deoxynivalenol production (μg/ml)		
pH	T°	P°O_2	a_W	Control[1]	Interaction 1[2]	Interaction 2[3]
				Media	Media	Media
4	37	Normal	0.99	0	0	0
			0.95	0	0	0
		Reduced	0.99	0	0	0
			0.95	0	0	0
	25	Normal	0.99	925.64	459.09	0
			0.95	609.18	0	288.44
		Reduced	0.99	0	0	0
			0.95	72.9	0	0
6	37	Normal	0.99	0	0	0
			0.95	0	0	0
		Reduced	0.99	0	0	0
			0.95	0	0	0
	25	Normal	0.99	259.27	0	1208.73
			0.95	523.64	0	468.72
		Reduced	0.99	0	0	0
			0.95	0	0	0

[1]Control: Mycotoxin (DON) production by toxicogenic fungi growing alone; [2]Interaction 1: Toxicogenic fungi–*Saccharomyces cerevisiae* RC008; [3]Interaction 2: *Toxicogenic fungi–Saccharomyces cerevisiae* RC016.

4. Discussion

Due to the increasing number of resistant fungal strains and the impact of fungicides on the environment and human health, strict legislation controlling the use of chemicals to reduce fungal proliferation and mycotoxin production has been established in the European Union (De Costa and Bezerra, 2009). Therefore, alternative methods are necessary to substitute or complement fungicide treatments to control toxigenic fungi.

Biological control using antagonist microorganisms has been proposed for a long time as a good option to control plant pathogens. Yeasts are considered one of the most potent biocontrol agents due to their biology and non toxic properties (Pimenta et al., 2009). The mechanism most probably involved in biocontrol of filamentous fungi by yeast is competition. Competition among microorganisms for essential factors, such as nutrients and space, is expected to have a dramatic effect on the secondary metabolism of filamentous fungi (Cavaglieri et al., 2004).

Food and feed are, in general, very diverse microbial growth substrates, due to variation in intrinsic factors such as pH, nutrients and water activity, and extrinsic factors such as storage temperature among others. Forage stored in silo such as maize silage is subject to a wide range of environmental conditions. Temperature, humidity, oxygen availability and pH conditions vary during the silage process and microbiota may also change from one stage to another.

In the present work, the effectiveness of the biocontrol agents under different environmental conditions that may occur in stored feedstuff was investigated. Silage native *S. cerevisiae* RC008 and RC016 were able to inhibit *A. carbonarius* and *F. graminearum* growth and reduced OTA, ZEA and DON production at different environmental conditions reflecting those found in stored feedstuff.

Since mycotoxins are secondary metabolites that can be produced in response to a stress factor, growth reduction caused by the presence of bacterial or yeast strains can influence mycotoxin production (Gareis and Ceynowa, 1994). Although a large increase in the production of DON was observed in only one condition tested, no stimulation of OTA and ZEA production was observed with *S. cerevisiae* RC008 or RC016 — *A. carbonarius* and *F. graminearum* interaction. Different results were reported by Wicklow et al. (1980) and Cuero et al. (1987) with other interacting yeast–fungi cultures.

The lag phase, mycelial growth rate, OTA, ZEA and DON production by *A. carbonarius* and *F. graminearum* strains were all significantly influenced by *S. cerevisiae* strains, incubation temperature, pH, a_w levels, PO and their interactions.

Similar results have been obtained in previous studies by using antioxidants in *Aspergillus* section *Flavi* and *Fusarium* strains on maize and peanuts, respectively. Antioxidants on peanut-based medium completely inhibited both growth and OTA production at 25 °C at all a_w levels regardless of the *Aspergillus* section Nigri strain assayed (Barberis et al., 2009).

In the present study, the complete growth inhibition of *F. graminearum* by *S. cerevisiae* RC016 occurred at all tested conditions, whereas the complete growth inhibition of *A. carbonarius* by *S. cerevisiae* strains occurs only at 37 °C.

Some authors have demonstrated inhibition of OTA and ZEA production. Medina et al. (2007) reported that OTA production by *A. carbonarius* was inhibited by natamycin produced by *Streptomyces natalensis*. The yeast *Pichia anomala* inhibited OTA production by one isolate of *Penicillium verrucosum* in malt extract agar medium as well as in wheat (Petersson et al., 1998). Masoud et al. (2005) found that *P. anomala* and *Pichia kluyveri* inhibited the production of OTA by *A. ochraceus* on malt extract agar medium and on coffee agar medium. Selected non-toxigenic *Trichoderma* and *Clonostachys* isolates in dual culture bioassays on rice reduced ZEA, α-zearalenol and ZEA sulfate production by two isolates of *F. graminearum* and two isolates of *F. culmorum* (Gromadzka et al., 2009).

These results are comparable to those observed in this work; both strains of *S. cerevisiae* were able to achieve a significant growth and mycotoxins production inhibition of *A. carbonarius* and *F. graminearum*.

Previous studies demonstrated that *S. cerevisiae* RC008 and RC016 were able to adsorb AFB_1, ZEA and OTA in vitro and also were capable of inhibiting the development of aflatoxigenic *A. parasiticus* strain in different environmental conditions in vitro (Armando et al., 2011, 2012a,b).

Several possible modes of action for the biocontrol activity of yeasts, such as competition for space and nutrients and production of cell wall-degrading enzymes, have been suggested. Magliani et al. (1997) reported that certain *S. cerevisiae* strains were able to produce "killer" toxins. Moreover, many killer toxins affected other yeasts and even bacteria and filamentous fungi (Polonelli and Morace, 1986). Both *S. cerevisiae* RC008 and RC016 strains used in this work have shown great antagonistic activity against bacterial pathogens in previous studies (Armando et al., 2011).

As was demonstrated in previous studies, *S. cerevisiae* plays a beneficial role in the silage ecosystem. They survive at low pH without showing an increase in cell numbers; consequently, they will not use lactic acid. When silage cover is damaged or opened for use, *S. cerevisiae* strains RC008 and RC016 could prevent growth of mycotoxigenic fungi and toxin contamination. These strains were also able to survive and adsorb mycotoxins under gastrointestinal conditions (Armando et al., 2011), allowing us to propose that mycotoxins present in crops previous to the silage assembling or feedstuff manufacturing could be adsorbed during gastrointestinal transit.

This study demonstrated that the inhibition of both fungal growth and mycotoxin production was effective, and was significantly influenced by environmental conditions. The use of *S. cerevisiae* RC008 as an additive to animal feed could be an alternative method of controlling mycotoxigenic fungi in stored feedstuff.

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