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ORIGINAL ARTICLE

Production of *Kluyveromyces* spp. and environmental tolerance induction against *Aspergillus flavus*

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Abstract The viability and biomass production of three isolates of *Kluyveromyces* spp. in six different growth media were studied. All yeast isolates showed good growth in all of the media tested, but nutrient yeast dextrose broth (NYDB 75 %) and molasses soy meal (MSB) media were selected for further analyses. The adaptive response of the yeasts to heat shock and water stress was studied, revealing that 60 min of incubation at 45 °C and a water activity value of 0.95 a_w were the appropriate conditions to adapt these yeasts for subsequent analyses. The physiological adaptation did not affect the ecological similarity between biocontrol agents and pathogen. The adapted yeasts also had a negative influence on the growth of *Aspergillus flavus* RCM89 mycelia and the accumulation of aflatoxin B_1 levels in vitro. These results have important implications for optimizing the formulation process of proven biocontrol agents against *A. flavus*. In addition, the applications of physiological methods are necessary for increasing the performance of biocontrol agents. Moreover, the physiological methods could enhance survival under environmental stress conditions of biological control agents.

Keywords *Kluyveromyces* spp. · *Aspergillus flavus* · Heat shock · Osmoadaptation

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Introduction

Maize (*Zea mays* L.) is a cereal crop and a staple food for a large proportion of the world population (von Braun 2007). About 60 % of the annual maize production in Argentina is exported as non-processed grain. In addition, Argentina exports about 500,000 tons of products derived from dry milling and 90,000 tons of products produced by wet milling (Lezcano 2012). Maize is used directly as a food source, but also as a forage crop for silage (Pimentel and Patzek 2005; ILSI 2006).

Components of the maize agroecosystem may be colonized by a variety of fungi (Cotty et al. 1994). A study conducted in Argentina found that potentially toxigenic *Aspergillus* section *Flavi* strains were extensively distributed at different levels in the soil, in debris and on insects in maize fields (Nesci and Etcheverry 2002). Several species of *Aspergillus* have the ability to invade agricultural commodities. *Aspergillus flavus* causes a variety of plant diseases because is a saprotrophic plant pathogen (Widstrom 1992), and its inocula may be transferred from the field to the storage ecosystem (Nesci and Etcheverry 2002). The conditions under which grain is stored can potentially provide a suitable environment for increased fungal growth. Maize is often harvested with a moisture content of 18–20 % and subsequently dried. An inefficient drying process and inappropriate environmental conditions often result in rapid spoilage and aflatoxin production (Etcheverry et al. 2012). Other factors that generate fungal contamination are probably reduced physiological activity of the grains and the inability of the grains to generate an active defense against the fungus. However, the increased fungal contamination may be due to an increase in the competitive ability of these fungi against the grain microbiota (Lacey and Magan 1991). The interaction between the substrate, biological factors and abiotic factors can cause a moldy substrate (Nesci et al. 2005, 2011). Aflatoxins are frequently

found in maize produced all over the world (Ghiasian et al. 2011). Aflatoxins are acutely and chronically toxic. The International Agency for Research on Cancer classified aflatoxin B_1 (AFB_1) as one of the more potent mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive natural substances (IARC 1993; JECFA 1998).

Control strategies are necessary to minimize the contamination in maize. The principles of storage for farmers, stockpiles and industry are the same: clean and sanitary facilities and good ventilation to provide cool and dry environment (Codex Alimentarius 2012). When such prevention measures are insufficient, other strategies must be applied. It is important to find a practical, cost-effective and non-toxic method to prevent fungal deterioration of stored grains. The use of microorganisms that antagonize cereals pathogens is free of health risks when it results in the enhancement of resident antagonist(s). These strategies of biological control using antagonistic microorganisms provide an environmentally friendly alternative to protect post-harvest fungal pathogens (Teixidó et al. 1998). Several species of yeasts have been shown to have an antagonistic effect against post-harvest fungal diseases (Petersson et al. 1999; Druvefors et al. 2002). Previous studies with yeasts have shown that *Kluyveromyces* spp. isolated from maize-based feedstuff significantly reduced *Aspergillus* section *Flavi* growth and AFB_1 accumulation in vitro (La Penna et al. 2004; La Penna and Etcheverry 2006).

Large-scale studies with mass-produced yeasts are needed to demonstrate that the antagonistic efficacy of yeasts is consistent. This scale-up process requires high densities of cells at a low cost, as well as the optimization of nutritional and environmental conditions (Hofstein et al. 1994; Wraight et al. 2001). However, growth media composed of waste and industrial products could provide a viable alternative (Costa et al. 2001; Wraight et al. 2001). The yeasts used for large-scale production must have certain characteristics, such as a fast growth rate, osmotolerant, not temperature-sensitive, among others (Wraight et al. 2001). This wide array of environmental conditions must not impact their effectiveness (Lui et al. 2011). If the antagonist is better adapted to a specific environment or have a rapid growth rate it could inhibit a pathogen by competition (Wilson et al. 1991). A current strategy available to enhance or maintain biocontrol efficiency includes enhancing stress tolerance (Edel-Hermann et al. 2009; Liu et al. 2011). Thermotolerance in yeasts to extreme heat treatment can enhance the ability of cells to withstand unfavorable environmental conditions, such as freeze drying (Diniz-Mendes et al. 1999), hydrostatic pressure (Iwahashi et al. 1991), high temperature (Biryukova et al. 2007) and oxidative stress (Deegenaars and Watson 1997). In addition, osmoadaptation has been found to improve the survival and biocontrol efficacy of different bacteria (Bonaterra et al. 2007; Sartori et al. 2012) and yeasts (Teixidó et al. 2005; 2006).

The aims of our study were: (1) to compare the viability and biomass production of three isolates of *Kluyveromyces* spp. in different growth media; (2) to study the effect of heat shock survival and water stress tolerance; (3) to establish the ecological similarity level between adapted yeasts and *Aspergillus flavus*; (4) to determine the impact of adapted yeasts on AFB_1 accumulation in vitro.

Materials and methods

Yeast isolates

The isolates used in this study were *Kluyveromyces* spp. Y16, Y22 and Y25 (La Penna et al. 2004). These yeasts were isolated from feedstuff. Stock cultures were maintained in 30 % v/v glycerol at −20 °C. These isolates were grown in malt extract broth (MEB) at 30 °C on a rotary shaker at 150 rpm for 16 h. At that time the cultures had reached the stationary phase with a concentration of 1×10^4 cell ml^{-1}.

Growth media and culture conditions

Three isolates of *Kluyveromyces* spp. (Y16, Y22, Y25) were grown in 100 ml of six different media: (1) MSB [molasses (20 g l^{-1})+soy meal (10 g l^{-1})] (Costa et al. 2001; Sartori et al. 2011); (2) MYE [molasses (20 g l^{-1})+yeast extract (1.2 g l^{-1})] (Kinay and Yildiz 2008); (3) NYDB 50 % [nutrient broth (4 g l^{-1})+dextrose (5 g l^{-1})+yeast extract (2.5 g l^{-1})]; (4) NYDB 75 % [nutrient broth (6 g l^{-1})+dextrose (7.5 g l^{-1})+yeast extract (3.75 g l^{-1})] (Teixidó et al. 1998); (5) GYP [glucose (10 g l^{-1})+yeast extract (5 g l^{-1})+peptone (10 g l^{-1})] and (6) YMB [peptone (5 g l^{-1})+glucose (10 g l^{-1})+yeast extract (3 g l^{-1})+malt extract (3 g l^{-1})] (Rodrigues et al. 2001).

The water activity (a_w) of the media was modified to 0.99, 0.97 and 0.95 a_w, respectively, using glucose (Dallyn and Fox 1980) and determined using an AquaLab water activity meter (model 4TE; AquaLab Technologies, Riverside, CA). Each treatment was inoculated with 1 % fresh culture of each *Kluyveromyces* spp. isolate, which was grown initially in MEB. Conical flasks were incubated on a rotary shaker (150 rpm) at 30 °C. The growth in each treatment was measured spectrometrically at 620 nm, and cell viability was estimated using the surface-plate method. Samples dilutions were made in sterile distilled water and spread-plated onto malt extract agar (MEA). Plates were incubated at 30 °C for 48 h, and the viable count was expressed as colony forming units per ml (CFU ml^{-1}). The experiments were conducted by triplicate for each treatment, and three independent assays. The growth parameters g (generation time) and k (constant growth rate) were calculated by linear regression of the exponential growth phase.

Survival and physiological adaptation to heat and water activity treatment

Based on the results obtained in preliminary assays on NYDB 75 % and MSB media, we selected isolates Y16 and Y25 for further study. The yeast cells were inoculated into 50 ml of media and the media osmotically modified by the addition of glucose (0.99, 0.97 and 0.95 a_w) and polyethylene glycol 300 (0.93 a_w) (Teixidó et al. 1998). The inoculated cultures were incubated on a rotary shaker at 150 rpm at 30 °C. In order to choose a suitable temperature treatment for heat shock induction of tolerance, when cultures reached the stationary phase of growth, the cells were harvested by centrifugation at 7000 *g* for 10 min at 10 °C. The cells were then resuspended in 5 ml of phosphate buffer (PBS) and the cell suspensions incubated in separate water baths at 35, 40 and 45 °C for 60 min. To determine the survival rates after heat shock, the cell paste was serially diluted in PBS. A new count of viable cells was done on MEA medium after 48 h of incubation at 30 °C. Three replicates per treatment were used. Survival at different temperatures was expressed as logarithmic values of N and N_0, where N refers to the yeast count following exposure to a particular holding time at the studied temperature, and N_0 represents the initial count prior to heat exposure.

Based on these results, a temperature of 45 °C for a duration of 60 min and a water activity of 0.95 were used in all subsequent experiments.

Influence of the physiological adaptation of yeasts on the interaction of these antagonistic agents with *Aspergillus flavus*

Determination of ecological similarity and co-existence between A. flavus RCM89 and adapted yeasts

Fungal strain The isolate used in this study was *Aspergillus flavus* (RCM89). This strain was isolated from stored maize, and it is an aflatoxin producer (Nesci 2005). The isolate was maintained at 4 °C on slants of MEA and in 15 % v/v glycerol at −80 °C.

Niche overlap index Experiments were carried out on agar plates to determine the carbon sources (C-sources) used by the filamentous fungi and the yeasts. The basic medium used was water agar containing 1.5 % agar supplemented with different C-compounds at a concentration of 10 mM. We assessed the following C-sources present in maize grains: D-fructose, D-galactose, α-D-glucose, D-rafinose, sucrose, aspartic L-acid, glutamic L-acid, L-histidine, L-phenylalanine, L-leucine, L-proline, L-treonine, dextrine, D-melobiose, L-alanine, D-serine, L-serine, L-arginine. The a_w of the basic medium was 0.99, and this was modified with glycerol to 0.97, 0.95 and 0.93, respectively (Dallyn and Fox 1980). *Aspergillus* spores obtained from MEA were suspended in 0.2 % water agar and inoculated onto each plate containing the different C-sources. Suspensions of yeasts were grown for 24 h in NYDB 75 % and MSB media. The water activity was modified to 0.99, 0.97 and 0.95 a_w, respectively, by adding different amounts of glucose (Dallyn and Fox 1980) and to 0.93 a_w by adding polyethylene glycol 300 (Teixidó et al. 1998). These cells were then heated to 45 °C, following which each isolate was inoculated onto each plate with the different C-source treatments. The plates were incubated at 25 °C for up to 10 days prior to examination for the utilization of the C-sources. Three replicates were used per treatment. The niche overlap index (NOI) was defined by Wilson and Lindow (1994a, b) as the number of C-sources utilized by the antagonist and pathogen as a proportion of the total number of C-sources utilized by the strains in question. This index was determined as the number of C-sources in common utilized by both strains (yeast-*Aspergillus*) as a proportion of total number of C-sources utilized by the aflatoxigenic strain. The NOIs were estimated for each strain in a pair. NOI values of >0.9 represent occupation or the same niche, while scores of <0.9 represent occupation of separate niches.

Effect of adapted yeasts on growth of A. flavus RCM89

The inhibitory activity on the growth rate of heat shock-adapted cells of yeasts against *A. flavus* was tested. Two media were used for this study: 3 % maize meal extract agar (MMEA) and MEA. The first medium was made by boiling 30 g dry maize meal in 1 l of water for 60 min and filtering the resultant mixture through a double layer of muslin. The volume was made up to 1 l with distilled water (Marín et al. 1998). This medium was specifically chosen because *A. flavus* RCM89 was isolated from stored maize (Nesci 2005). All experiments were carried out over the range of 0.99 to 0.93 a_w. The water activity was adjusted by the addition of known amounts of the non-ionic solute glycerol (Dallyn and Fox 1980). Yeast suspensions were prepared following the procedure used for the NOI studies. Each yeast suspension with 10^9 CFU ml^{-1} cells was pour-plated in 20 ml of MMEA and MEA. After solidification *A. flavus* RCM89 was spot inoculated with spores suspended in semi-solid agar (Pitt 1979). Petri plates with media of the same a_w were sealed in polyethylene bags, and the plates were incubated at 25 °C. The colony radius was measured daily. 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 test interaction. The radial growth rate (mm day^{-1}) was subsequently calculated by linear regression of the linear phase for growth. After the incubation period, controls and treatments were maintained at 4 °C for later extraction and AFB_1 quantification.

AFB_1 analysis

After 11 days of incubation at 25 °C, a plug of agar medium and biomass (1×1 cm) was taken from co-inoculated cultures (aflatoxin producer + yeast antagonist). The plug was transferred to an Eppendorf tube, and 500 μl of chloroform was added. The mixture was centrifuged at 850 *g* for 20 min. The solvent was removed from the chloroform extract under nitrogen gas. Samples were quantitatively determined by high-performance liquid chromatography (HPLC) following the methodology of detection of Trucksess et al. (1994). An aliquot (200 μl) was derivatized with 700 μl of trifluoroacetic acid:acetic acid:water (20:10:70). The aflatoxin-derivatized solution (50 μl sample) was analyzed using a reversed-phase HPLC/fluorescence detection system that consisted of an HP1100 pump (Hewlett Packard, Palo Alto, CA) connected to an HP1046A programmable fluorescence detector. The quantification was done using a Hewlett Packard workstation. Chromatographic separations were performed on a stainless steel, C18 reverse-phase column (150 ×4.6 mm ID; particle size 5 μm; Luna-Phenomenex, Torrance, CA). Water:methanol:acetonitrile (4:1:1 by volume) was used as the mobile phase at a flow rate of 1.5 ml min^{-1}. Aflatoxin derivative fluorescence was recorded at excitation and emission wavelengths of 360 and 440 nm, respectively. A standard curve was constructed at different levels of AFB_1. The limit of detection of the analytical method was 1 ng g^{-1}.

Statistical analysis

Analysis of variance was carried out on growth parameters and viable counts using a SAS program (SAS System for Windows 6.11; SAS Institute, Cary, NC). To establish significant differences, Duncan's multiple range test ($p<0.05$) was performed.

Results

Effect of growth media and water activity on yeast growth parameters

Statistical analyses on the constant growth rate (k) of isolates, water activity and their interaction were statistically significant (Table 1). The major effect was that of water activity. Statistical analyses on constant growth rate on different growth media were not significant ($F=1.31$, $p>0.2650$). Table 2 shows the significance of isolates, water activity, growth media and their interactions on the generation time (g) of yeast isolates Y16, Y22 and Y25. The factors isolates, a_w and growth media were statistically significant. The major effect was produced by a_w and the minor effect was produced by growth media.

Table 1 Significance of isolates, water activity, growth media and their interactions on constant growth rate (k) of yeast *Kluyveromyces* spp. isolates Y16, Y22 and Y25

Factor[a]	*df*	MS[b]	*F* value	*Pr*>Fr
I	2	0.202	70.17	0.0001
a_w	2	0.347	120.45	0.0001
GM	5	0.003	1.31	0.2650
I × a_w	4	0.057	19.88	0.0001
I × GM	10	0.004	1.43	0.1752
a_w × GM	10	0.003	1.21	0.2948
I × a_w × GM	20	0.006	2.23	0.0045

df Degrees of freedom, *MS* mean squares

[a] I, Isolate; a_w, water activity; GM, growth media

The growth parameters of the three *Kluyveromyces* spp. isolates Y16, Y22 and Y25 in all media plus the results of the a_w assay are shown in Tables 3, 4 and 5, respectively. We observed a decrease of k values and an increase of g values with decreasing water activity of the media.

The lowest g values were observed at 0.99 and 0.97 a_w for isolates Y16 and Y25 and at 0.97 a_w for isolate Y22. In general, the lowest g values were observed in medium NYDB 75 % for all three yeasts evaluated at all water activities.

The k values were similar at 0.99 and 0.97, but these values were observed to decrease at 0.95 a_w. The lowest number of generations per unit of time was observed at 0.95 a_w. Changes in a_w alone reduced the growth rate of *Kluyveromyces* spp. Y16 and Y22 by >30 % between 0.99 and 0.95 a_w. A reduction of 45 % was observed in the growth rate of *Kluyveromyces* spp. Y25.

In MSB, which is the most economical medium, the k values ranged from 0.218 to 0.5 h^{-1} for the three yeasts at all a_w evaluated. This range was similar that observed in the other media.

Based on these results, we chose NYDB 75 % and MSB as the media for subsequent assays. Moreover, since there was no

Table 2 Significance of isolates, water activity, growth media and their interactions on the generation time of yeast isolates Y16, Y22 and Y25

Factor[a]	*df*	MS	*F* value	*Pr*>Fr
I	2	4.348	101.28	0.0001
a_w	2	14.426	336.05	0.0001
GM	5	0.254	5.92	0.0001
I × a_w	4	0.134	3.13	0.0176
I × GM	10	0.007	0.18	0.9972
a_w × GM	10	0.048	1.13	0.3433
I × a_w × GM	20	0.041	0.98	0.4943

df Degrees of freedom, *MS* mean squares

[a] I, Isolate; a_w, water activity; GM, growth media

Table 3 Influence of growth media and water activities on growth parameters of *Kluyveromyces* spp. Y16

Growth media[a]	Water activity of media		
	0.99 a_w	0.97 a_w	0.95 a_w
GYP	*g*: 2.13 h b	*g*: 1.813 h b	*g*: 2.83 h a
	k: 0.326 h^{-1} A	*k*: 0.387 h^{-1} A	*k*: 0.237 h^{-1}B
MSB	*g*: 2.212 h b	*g*: 2.123 h b	*g*: 3.183 h a
	k: 0.313 h^{-1} A	*k*: 0.323 h^{-1} A	*k*: 0.218 h^{-1} B
MYE	*g*: 2.133 h b	*g*: 2.073 h b	*g*: 2.973 h a
	k: 0.325 h^{-1} A	*k*: 0.333 h^{-1} A	*k*: 0.233 h^{-1} B
NYDB 50 %	*g*: 2.007 h b	*g*: 1.897 h b	*g*: 3.07 h a
	*k*k 0.347 h^{-1} A	*k*: 0.363 h^{-1} A	*k*: 0.228 h–^{1} B
NYDB 75 %	*g*: 1.877 h b	*g*: 1.923 h b	*g*: 2.703 h a
	k: 0.37 h^{-1} A	*k*: 0.363 h^{-1} A	*k*: 0.257 h^{-1} B
YMC	*g*: 1.993 h b	*g*: 1.937 h b	*g*: 2.837 h a
	k: 0.35 h^{-1} A	*k*: 0.357 h^{-1} A	*k*: 0.247 h^{-1} B

g, Generation time; *k*, constant growth rate

For *g* values, values followed by the same lowercase letter for different water activities in the same medium are not significantly different according to Duncan's multiple range test ($p<0.05$). For k values, values followed by the same uppercase letter for different water activities in the same medium are not significantly different according to Duncan's multiple range test ($p<0.05$)

[a] For a detailed description of the various growth media, see text (Growth media and culture conditions)

Table 4 Influence of growth media and water activities on growth parameters of *Kluyveromyces* spp. Y22

Growth media[a]	Water activity of media		
	0.99 a_w	0.97 a_w	0.95 a_w
GYP	*g*: 1.95 h b	*g*: 1.89 h b	*g*: 2.18 h a
	k: 0.357 h^{-1} A	*k*: 0.46 h^{-1} A	*k*: 0.320 h^{-1} A
MSB	*g*: 1.79 h b	*g*: 1.69 h b	*g*: 2.270 h a
	k: 0.402 h^{-1} A	*k*: 0.445 h^{-1}A	*k*: 0.305 h^{-1} A
MYE	*g*: 1.77 h b	*g*: 1.86 h b	*g*: 2.05 h a
	k: 0.407 h^{-1}A	*k*: 0.435 h^{-1} A	*k*: 0.34 h^{-1} A
NYDB 50 %	*g*: 2.157 h a	g: 1.67 h b	*g*: 2.09 h a
	k: 0.327 h^{-1} B	*k*: 0.46 h^{-1} A	*k*: 0.33 h–^{1} AB
NYDB 75 %	*g*: 1.997 h a	*g*: 1.6 h b	*g*: 1.99 h a
	k: 0.347 h^{-1} B	*k*: 0.49 h^{-1} A	*k*: 0.35 h^{-1} B
YMC	*g*: 2.02 h a	*g*: 1.6 h b	*g*: 2.07 h a
	k: 0.343 h^{-1} B	*k*: 0.49 hs^{-1} A	*k*: 0.33 h^{-1} B

g, Generation time; *k*, constant growth rate

For *g* values, values followed by the same lowercase letter for different water activities in the same medium are not significantly different according to Duncan's multiple range test ($p<0.05$). For k values, values followed by the same uppercase letter for different water activities in the same medium are not significantly different according to Duncan's multiple range test ($p<0.05$)

[a] For a detailed description of the various growth media, see text (Growth media and culture conditions)

difference in *g* values between yeast isolates Y16 and Y22, we selected only Y16 from this pair of isolates for further testing. Isolate Y25 was also tested further because it presented different results.

Effect of heat shock and water activity treatment on yeast survival

In order to determine a suitable temperature–water activity combination to serve as a yeast treatment adaptation for inducing stress tolerance in *Kluyveromyces* spp. we evaluated the survival of heat shock-treated cells exposed to different temperatures and the water activities (Table 6). In general, a direct relationship was not observed between a decrease in viability and a decrease in water activity. Isolates Y16 and Y25 showed a low viability at 0.97 a_w and 35 °C in both media. At this temperature and in MSB medium, Y25 showed a high viability at 0.99 and 0.95 a_w, with values of 10.54 and 10.72 log, respectively. The temperature at which both isolates showed the highest viability at all a_w and media assayed was 40 °C. At 45 °C, the highest viability was observed at 0.95 a_w for isolate Y16 and at 0.97 and 0.95 a_w for isolate Y25 in NYDB 75% medium. In comparison, in MSB medium at the same temperature, the highest viability was observed at 0.97 a_w for both isolates. When we compared viability at 40 and 45 °C, we observed no difference at 0.97 a_w in both media. The same result was observed at 0.95 a_w. Moreover, cell viability at 45 °C was higher at 0.95 a_w than at 0.97 a_w for the isolate Y16 in NYDB 75% medium. No difference was observed between 0.97 and 0.95 a_w for isolate Y25 after heat treatment at 45 °C in both media.

Figure 1 shows the viability of isolates Y16 and Y25 before and after heat treatment at 45 °C at all water activities and in two media. In general, yeasts cells preserved their viability after heat treatment at 45 °C, with Y16 showing the highest viability after heat shock at 0.97, 0.95 and 0.93 a_w in both media. The same effect was observed for isolate Y25 at 0.97 and 0.95 a_w in NYDB 75% medium and at all a_w in MSB medium. Isolate Y16 showed the highest survival after heat shock at 0.95 and 0.97 a_w in NYDB 75% and MSB, respectively, and isolate Y25 showed the highest survival after heat shock at 0.97 a_w in both media.

Based on these results we chose the major stress situation to adapt yeasts cells in which a high viability was observed. Therefore, 45 °C and 0.95 a_w were chosen to be appropriate conditions to adapt these yeasts for subsequent analysis.

Table 5 Influence of growth media and water activities on growth parameters of *Kluyveromyces* spp. Y25

Growth media[a]	Water activity of media		
	0.99 a_w	0.97 a_w	0.95 a_w
GYP	*g*: 1.27 h b	*g*: 1.67 h b	*g*: 2.37 h a
	k: 0.54 h^{-1} A	*k*: 0.41 h^{-1} A	*k*: 0.29 h^{-1} B
MSB	*g*: 1.38 h b	*g*: 1.7 h b	*g*: 2.66 h a
	k: 0.5 h^{-1} A	*k*: 0.41 h^{-1} A	*k*: 0.26 h^{-1} B
MYE	*g*: 1.4 h b	*g*: 1.47 h c	*g*: 2.51 h a
	k: 0.49 h^{-1} B	*k*: 0.47 h^{-1} A	*k*: 0.28 h^{-1} B
NYDB 50 %	*g*: 1.37 h b	*g*: 1.43 h c	*g*: 2.36 h a
	k: 0.51 h^{-1} B	*k*: 0.48 h^{-1} A	*k*: 0.29 h^{-1} C
NYDB 75 %	*g*: 1.19 h b	*g*: 1.46 h b	*g*: 2.35 h a
	k: 0.58 h^{-1} A	*k*: 0.47 h^{-1} A	*k*: 0.29 h^{-1} B
YMC	*g*: 1.26 h b	*g*: 1.53 h b	*g*: 2.22 h a
	k: 0.55 h^{-1} A	*k*: 0.45 h^{-1} A	*k*: 0.31 h^{-1} B

g, Generation time; *k*, constant growth rate

For *g* values, values followed by the same lowercase letter for different water activities in the same medium are not significantly different according to Duncan's multiple range test ($p<0.05$). For k values, values followed by the same uppercase letter for different water activities in the same medium are not significantly different according to Duncan's multiple range test ($p<0.05$)

[a] For a detailed description of the various growth media, see text (Growth media and culture conditions)

Effect of yeasts adapted on interaction with *A. flavus*

Influence on NOI

Yeasts physiologically adapted were used in order to determine the level of niche overlap and co-existence between the yeasts cells and *A. flavus* RCM89. Table 7 shows the levels of ecological similarity and co-existence of paired cultures, based on the ability to utilize the C-sources tested. All three adapted*Kluyveromyces* isolates were able to use the 18 C-sources tested, showing a NOI >0.9 which is indicative of competence with the pathogen. This value demonstrates a 100 % of competence for the same C-sources used by *A. flavus*. Change of water activity had no influence on the number of carbon compounds utilized for the isolates examined.

Influence on A. flavus RCM89 growth rate

Control cultures of *A. flavus* RCM89 grew faster in MEA than in MMEA at all a_w. The growth rate decreased with decreasing a_w in both media, with growth rate values ranging from 5.41 to 4.24 mm day^{-1} in MEA and from 4.65 to 2.68 mm day^{-1} in MMEA (Table 8). The interaction of both yeasts adapted with *A. flavus* showed a decrease in growth rate in both media at all a_w assayed. The effect of this reduction was higher when paired cultures were grown in MEA. The range in the reduction was between 53.9 to 95.4 % and between 15.4 and 42.1 % in MEA and MMEA, respectively.

Influence on AFB_1 accumulation

Table 9 shows AFB_1 accumulation in paired cultures between *A. flavus* in interaction with isolates Y16 and Y25 adapted cells in MEA and MMEA at different a_w. At 0.99 a_w, *A. flavus* RCM89 accumulated AFB_1 in both MEA and MMEA media, with values of 1,642.3 and 53.23 ng g^{-1}, respectively. However, at 0.97 a_w *A. flavus* RCM89 accumulated AFB_1 only in MEA. At 0.95 and 0.93 a_w no AFB_1 accumulation was detected in the control and interaction cultures in both media. A total reduction of AFB_1 was achieved by the interaction of Y25 in MEA at 0.97 a_w. Under the same conditions, the highest percentage of reduction (98 %) was observed with isolate Y16.

Table 6 Viability of *Kluyveromyces* spp. Y16 and Y25 at high temperatures and different water activities

Temperature (°C)	Yeast	Water activity of growth media[a]						
		0.99		0.97		0.95		0.93
		NYDB 75%	MSB	NYDB 75%	MSB	NYDB 75%	MSB	NYDB 75%
35	Y16	10.48 a	10.27 ab	8.93 b	7.64 b	10.63 a	9.92 a	9.53 a
40	Y16	10.47 a	10.73 a	10.93 a	10.55 a	12.17 a	11.10 a	9.2 1ab
45	Y16	8.23 b	9.43 b	10.41 a	10.68 a	12.04 a	10.30 a	9.08 b
35	Y25	10.39 ab	10.54 a	8.77 b	9.34 b	10.47 a	10.72 a	9.07 b
40	Y25	11.03 a	10.46 a	10.83 a	11.09 a	11.02 a	10.59 a	9.77 a
45	Y25	9.96 b	9.88 a	10.75 a	10.71 a	10.64 a	10.33 a	8.32 c

Values followed by different lowercase letters for each water activity, each isolate and each medium are significantly different according to Duncan's multiple range test ($p<0.05$)

[a] For a detailed description of the various growth media, see text (Growth media and culture conditions)

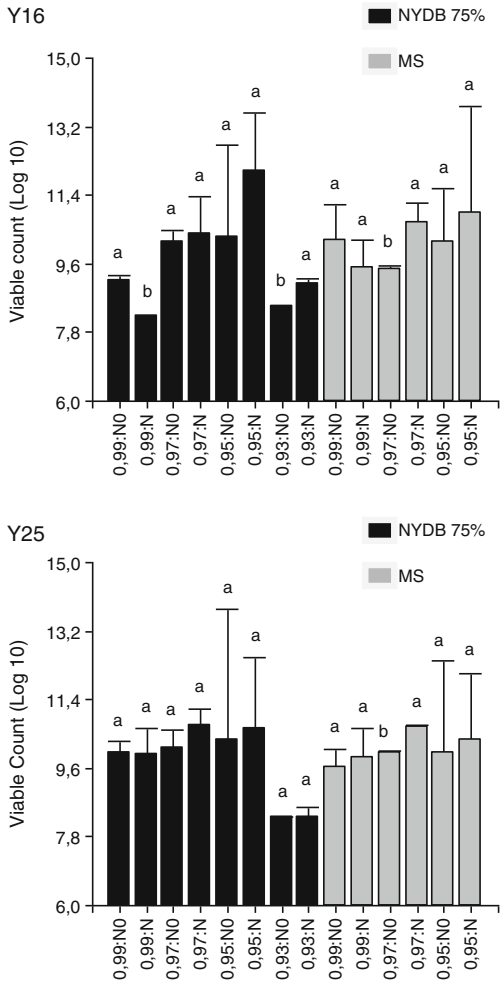

Fig. 1 Survival of yeast cells growth in NYDB 75% and MSB media at different water activities (a_w; 0.99, 0.97, 0.95 and 0.93), after 60 min at 45 °C. N_0initial count prior to heat shock exposure, *N* yeast count following heat shock exposure. Data with the same *lowercase letter* for N_0 and N at the same a_w and in the same medium are not significantly different according to Duncan's multiple range test ($p<0.05$)

Discussion

The search for a culture medium for biomass production of specific yeast that can be used as a biocontrol agent in stored

Table 7 Ecological similarity and co-existence of adapted *Kluyveromyces* spp. isolates paired with *Aspergillus flavus* RCM 89 at different water activities

Water activity	Competence[a]	Co-existence[b]	Competent isolates (%)
0.99	100	0	100
0.97	100	0	100
0.95	100	0	100
0.93	100	0	100

[a] Percentage of paired yeast–-*A. flavus* RCM89 with niche overlap index (NOI) between 0.9 and 1

[b] Percentage of paired yeasts–*A. flavus* RCM89 with NOI of <0.9

Table 8 Effect of adapted yeast–*Aspergillus* interactions on growth rate in different growth media and at different water activities

Interactions	Growth rate means (mm day^{-1}) in MEA			
	Water activity			
	0.99 A	0.97 A	0.95 A	0.93 A
A. flavus RCM89 (control)	5.41 a	5.19 a	4.90 a	4.24 a
A. flavus RCM89–isolate Y16	0.37 b	0.24 b	2.23 b	1.29 b
A. flavus RCM89–isolate Y25	0.42 b	0.55 b	2.26 b	1.02 c
	Growth rate means (mm day^{-1}) in MMEA			
	Water activity			
	0.99 B	0.97 B	0.95 B	0.93 A
A. flavus RCM89 (control)	4.65 a	3.64 a	3.33 a	2.68 a
A. flavus RCM89–isolate Y16	3.45 b	3.08 b	2.38 b	1.97 b
A. flavus RCM89–isolate Y25	3.30 b	3.33 b	1.98 b	1.55 c

MEA Malt extract agar, *MMEA* maize meal extract agar

For control and interactions at the same a_w for the same medium: data followed by the same lowercase letter are not significantly different according to Duncan's multiple range test ($p<0.05$). For different media at the same a_w: data followed by the same capital letter are not significantly different according to Duncan's multiple range test ($p<0.05$)

maize is a preliminary step for the optimization of the formulation process. The yeast cultures assayed in our study all showed good growth in all of the media tested. However, isolates grown on NYDB 75% showed rapid growth rate and high biomass production. All of the media tested, except MSB, contain yeast extract, which supports rapid growth and high cell yield of yeasts (Costa et al. 2001; Wan and Tian 2004). However, yeast extract is considered too expensive for an industrial process (Nohata and Kurane 1997). Peptone has the same problem. Peptone is a constituent of culture media NYDB 75%, NYDB 50%, GYP and YMB. The MSB medium, which has molasses (20 g l^{-1})+soy meal (10 g l^{-1}) as constituents, is a relatively low-cost culture medium because the molasses can be industrial grade. Molasses media showed a good yield efficacy in studies on the biocontrol agent *Pantoea agglomerans* (Costa et al. 2001). This substance has been widely used in the production of different microorganisms (Reed and Nagodawithana 1991; Kinay and Yildiz 2008; Sartori et al. 2011), and its effectiveness is considered to be due to its high sugar content (Costa et al. 2001). The other constituent of the MSB medium is soy meal which contains protein, carbohydrate and fat (Genta and Alvarez 2006). As such, soy meal is a good source of nitrogen and carbon (Navaratnam et al. 1996).

Another aspect examined in our study was the osmoadaptation of the yeast isolates on media of low a_w and thermotolerance. This manipulation could enhance yeast tolerance to water potential stress in the postharvest ecosystem. The application of physiological methods is necessary to improve the response of yeast isolates to environmental stress. These methods have been shown to increase the performance

Table 9 Effect of adapted yeast–*Aspergillus* interactions on aflatoxin B_1 accumulation in different growth media and at different water activities

Interactions	Water activity	AFB_1 in MEA (ng g^{-1} medium)	AFB_1 in MMEA (ng g^{-1} medium)
A. flavus RCM89	0.99	1,642.3	53.23
A. flavus RCM89–isolate Y16	0.99	61.37 (96)	12.61 (76)
A. flavus RCM89–isolate Y25	0.99	1.60 (99)	3.93 (93)
A. flavus RCM89	0.97	232.81	ND
A. flavus RCM89–isolate Y16	0.97	4.76 (98)	ND
A. flavus RCM89–isolate Y25	0.97	ND	ND
A. flavus RCM89	0.95	ND	ND
A. flavus RCM89–isolate Y16	0.95	ND	ND
A. flavus RCM89–isolate Y25	0.95	ND	ND
A. flavus RCM89	0.93	ND	ND
A. flavus RCM89–isolate Y16	0.93	ND	ND
A. flavus RCM89–isolate Y25	0.93	ND	ND

AFB_1, Aflatoxin B1, *ND* not detectable

Values given in parenthesis are the percentage of AFB_1 inhibition

of biological control agents (Teixidó et al. 2005). Some authors have also shown that microbial cells that have induced tolerance to one type of stress may provide cross-protection against other stresses (Teixidó et al. 2005; Mitchell et al. 2009; Liu et al. 2011). Sartori et al. (2010) showed that osmotic tolerance helps to maintain survival after thermal shock in cells of *Bacillus amyloliquefaciens* and *Microbacterium oleovorans*. Therefore, in bacteria such as *Kluyveromyces* spp. Y16 and Y25, osmotic tolerance is linked to thermotolerance. The three isolates of *Kluyveromyces* spp. tested here showed a high viability after heat shock exposure to 45 °C and 0.95 a_w. Cañamás et al. (2008) showed that only a few degrees above the optimal growth temperature (25 °C) for *Candida sake* were enough to induce heat-tolerance responses. The response to heat stress is usually characterized by the translation of general or specific proteins. Moreover, heat stress generates physiological changes that confer an ability for the organism to survive in adverse environmental conditions (Ang et al. 1991). The manipulation of the growth of microorganisms modifying water stress significantly affects the intracellular accumulation of polyols and sugar content. One predominant sugar that has been found to accumulate in yeast when the cells were grown in a synthetic medium with low water activity was trehalose (Teixidó et al. 1998). In a previous study, *Kluyveromyces* spp. Y16 was found to accumulate higher amounts of trehalose than glucose when grown in meal extract broth at 0.95 and 0.93 a_w (Montemarani et al. 2012). Trehalose acts as a metabolite that provides protection against several stress situations (Kwon et al., 2003; Gancedo and Flores, 2004), such as extreme temperature, dehydration and/or osmotic stress (Van Laere 1989; Meikle et al. 1991; Piper 1993). Exposure to high temperatures trigger a rapid molecular response in yeast cells. The highly conserved heat shock proteins (Hsps) are a specific set of proteins whose synthesis is induced when cells are put under a sublethal thermal shock (Lindquist and Craig 1988). Trehalose, together with Hsp104p, acts synergistically to confer thermoprotection (Feldmann 2010).

Ecophysiological manipulation of yeasts and filamentous fungi cells must not affect biocontrol potential. Moreover is expected to improve the ability to control (Magan 2001; Teixidó et al. 2006).

The ecological similarity and coexistence obtained with the NOI between these yeast isolates and *Aspergillus* section *Flavi* have been previously studied (La Penna et al. 2004). In that study, *Kluyveromyces* isolates had similar C-source utilization patterns to *Aspergillus* section *Flavi* and were able to compete for the same maize C-source. However, the impact of ecophysiologically adapted cells on the NOI has not been evaluated for La Penna et al. (2004). In our work, we observed a similar result of competence when physiologically adapted yeasts were paired with the pathogen. Consequently, we can infer that the physiological adaptation did not affect the ecological similarity between biocontrol agents and pathogen.

The adapted Y16 and Y25 isolates had a negative influence on *A. flavus* RCM89 mycelial growth and AFB_1 accumulation levels in vitro. Similar results were observed in a previous study in which non-adapted Y16 and Y25 isolates showed reduced growth and AFB_1 of *Aspergillus* section *Flavi* (La Penna and Etcheverry 2006). The adapted yeasts evaluated in our study did not stimulate AFB_1 accumulation, although this effect of stimulation has been observed in other interacting yeast–filamentous fungi cultures (Wicklow et al. 1980; Cuero et al. 1987).

Our results have important implications in optimizing the formulation process of proven biocontrol agents against *A. flavus*, in addition to enhancing survival under environmental stress conditions. We are currently conducting experiments in microcosms of maize on the biocontrol potential of physiologically modified *Kluyveromyces* spp. Y16 and Y25.

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