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Use of wild yeasts as a biocontrol agent against toxigenic fungi and OTA production

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ABSTRACT. This study evaluated the antagonistic potential of 32 wild yeast isolates from coffee and cocoa bean fermentation. These yeasts were inoculated in co-cultivation with *Aspergillus carbonarius* (CCDCA 10608 and CCDCA 10408) and *Aspergillus ochraceus* (CCDCA 10612) isolated from grapes and coffee beans. The mycelial growth and ochratoxin A (OTA) production were evaluated, and the spores were counted after cultivation at 28°C for seven days. The yeasts presented higher inhibitory effects (53% in relation to the control) over the mycelial growth of the isolated *A. ochraceus* (CCDCA10612). *Pichia anomala* CCMA0148 and *Saccharomyces cerevisiae* CCMA0159 provided the greatest inhibition of the growth of all fungal strains. All *Pichia* species presented the highest inhibitory effects on the production of spores, and *S. cerevisiae* CCMA 0159 at concentrations of both 10^4 and 10^7 mL^{-1} cells inhibited the production of spores by 100%. *Rhodotorula mucilaginosa* was effective at inhibiting OTA production by the three isolates of *Aspergillus*. *S. cerevisiae* CCMA0159 and *Pichia anomala* CCMA0148 showed high potential as biocontrol agents in the conditions tested.

Keywords: antagonistic activity, ochratoxin A, *Aspergillus*, inhibition, *Pichia* sp., *Saccharomyces* sp.

Uso de leveduras selvagens como agente de biocontrole contra fungos toxigênicos e produção de OTA

RESUMO. Este estudo avaliou o potencial antagônico de 32 isolados de leveduras selvagens de fermentação de café e cacau. Estas leveduras foram inoculadas em co-cultivo com *Aspergillus carbonarius* (CCDCA 10608 e CCDCA 10408) e *Aspergillus ochraceus* (CCDCA 10612) isolado de uvas e grãos de café. O crescimento micelial e a produção de ocratoxina A (OTA) foram avaliados e os esporos foram contados após o cultivo a 28 ° C por sete dias. As leveduras apresentaram maiores efeitos inibitórios (53% em relação ao controle) sobre o crescimento micelial do isolado *A. ochraceus* (CCDCA10612). *Pichia anomala* CCMA0148 e *Saccharomyces cerevisiae* CCMA0159 proporcionaram a maior inibição do crescimento de todas as estirpes fúngicas. Todas as espécies de *Pichia* apresentaram os maiores efeitos inibitórios na produção de esporos e *S. Cerevisiae* CCMA 0159 em concentrações de 10^4 e 10^7 células mL^{-1} inibiram a produção de esporos em 100%. *Rhodotorula mucilaginosa* foi eficaz na inibição da produção de OTA pelos três isolados de *Aspergillus*. *S. cerevisiae* CCMA0159 e *Pichia anomala* CCMA0148 apresentaram alto potencial como agentes de biocontrole nas condições testadas.

Palavras-chave: atividade antagonista, ocratoxina A, *Aspergilllus*, inibição, *Pichia* sp., *Saccharomyces* sp.

Introduction

Fungal producers of mycotoxins can be present in the environment of the preparation and storage stages of coffee bean production. Thus, the relationship of fungi with the quality and security of the final product depends not only on the environmental conditions but also on the management of the culture and the post-harvest processing (Batista, Chalfoun, Prado, Schwan, & Wheals, 2003). Generally, filamentous fungi grow at 25 to 35°C and 0.95 to 0.99 a_w. However, for OTA production, the environmental conditions are 15 to 20°C and 0.95 to 0.98 a_w (Bellí, Marín, Argilés, Ramos, & Sanchis, 2007; Palacios-Cabrera, Taniwaki, Hashimoto, & Menezes, 2005; Esteban, Abarca, Bragulat, & Cabanes, 2006; Leong, Hocking, Varelis, Giannikopoulos, & Scott, 2006; Oueslati et al., 2010; Valero, Oliván, Marín, Sanchis, & Ramos, 2007). Yeasts also grow in these conditions, thus enabling their use in biological control.

Mycotoxins are classified as toxic chemical compounds of low molecular weight resulting from secondary metabolism of certain fungi. Among the mycotoxins found in foods, ochratoxin A (OTA) is

considered a substance with nephrotoxic, hepatotoxic, teratogenic and immunosuppressive effects in several animals, and it can cause tumours in the liver and kidneys. As a result, since 1993, the IARC has classified OTA as a possible carcinogen in humans (IARC, 1993).

Estimates show that 25% of all agricultural products in the world are contaminated by these mycotoxins (Food and Agriculture Organization of the United Nations, 2004), and for special coffee beans, the mycotoxin contamination can reach up to 44% (Batista, Chalfoun, Cirillo, Silva, & Varga, 2009). Many European countries have limits for OTA in food and beverages (Commission Regulation, 2010). In Brazil, some food products have OTA limits; in particular, for toasted and ground coffee, this limit is 10 μg kg^{-1} (ANVISA, 2011). Therefore, preventive strategies are necessary to control the presence of fungi and reduce OTA contamination, such as bio-control agents, fungicides, antioxidants and improvements in agricultural practices (Ponsone, Chiotta, Palazzini, Combina, & Chulze, 2012). The high fungal incidence and mycotoxin production in agricultural products impacts the sanitary quality of the final product, as found in beverages (especially coffee and wine). In coffee, *Aspergillus ochraceus* is an important toxin source dependent on the management of fruits and coffee beans after harvest and storage. The fungal contamination is worst during the coffee harvest period, as the climate is highly humid with increased rainfall (Ahmad & Magan, 2002). In grapes, the more frequent ochratoxigenic fungus is *Aspergillus carbonarius*; wine consumers around the world have highlighted it as an important source of OTA intake by the population (De Curtis, De Felice, Ianiri, De Cicco, & Castoria, 2012).

Several researchers have discussed the control of the growth and degradation of OTA at a laboratory scale with biological resources using different microorganisms or their enzymes, such as bacteria (Shi et al., 2014), yeasts (Gil-Serna, Vázquez, Sardiñas, González-Jaén, & Patiño, 2011; Kapetanakou, Kollias, Drosinos, & Skandamis, 2012; Lutz, Lopes, Rodriguez, Sosa, & Sangorrín, 2013; Medina-Córdova et al., 2016; Núnez et al., 2015; Zhu, Shi, Jiang, & Liu, 2015) and filamentous fungi (Abrunhosa, Paterson, & Venâncio, 2002; Varga, Péteri, Tábori, Téren, & Vágvölgyi, 2005). However, the biocontrol potential of autochthonous agents, such as yeast, which is naturally present in fermented products, is yet unknown.

This study evaluated the antagonistic potential of 32 wild yeasts isolated from coffee and cocoa bean fermentation. *Aspergillus carbonarius* (CCDCA 10608 and CCDCA 10408) and *Aspergillus ochraceus* (CCDCA 10612) isolated from grape and coffee beans were tested against the yeasts. Mycelial growth, spore production and ability to produce ochratoxin A (OTA) were evaluated.

Material and methods

Microorganisms

Thirty-two (32) yeasts isolated from coffee and cocoa beans were tested. These isolates are deposited in the Culture Collection of Agricultural Microbiology (CCMA) at the Department of Biology at Federal University of Lavras (UFLA). The strains of yeast are *Debaryomyces etchellsii* CCMA 0162 (former UFLAYCH 5.56); *Debaryomyces polymorphus* CCMA 0141 (former UFLACF 384), 0142 (former UFLACF 385), 0143 (former UFLACF 650); *Pichia anomala* CCMA 0146 (former UFLACF 507), 0147 (former UFLACF 508), 0148 (former UFLACF 702); *Pichia burtonii* CCMA 0149 (former UFLACF 553), 0150 (former UFLACF 605); *Pichia fermentans* CCMA 0163 (former UFLAYCH 16.2); *Pichia guilliermindii* CCMA 0138 (former UFLACF 381), 0139 (former UFLACF 397), 0140 (former UFLACF 493), 0164 (former UFLAYCH 155); *Pichia holstii* CCMA 0144 (former UFLACF 441.b), 0145 (former UFLACF 957.b); *Pichia jardinii* CCMA 0160 (former UFLACF 933); *Pichia kluyveri* CCMA 0165 (former UFLAYCH 2.2), 0166 (former UFLAYCH 192), 0167 (former UFLAYCH 194), 0168 (former UFLAYCH 1204), 0169 (former UFLAYCH 1207); *Pichia sydowiorum* CCMA 0157 (former UFLACF 732), 0158 (former UFLACF 759); *Rhodotorula mucilaginosa* CCMA 0153 (former UFLACF 640), 0154 (former UFLACF 641), 0155 (former UFLACF 642), 0156 (former UFLACF 693); *Saccharomyces cerevisiae* CCMA 0159 (former UFLACF 856), 0161 (former UFLAYCH 5.5); and *S. kluyveri* CCMA 0151 (former UFLACF 567), 0152 (former UFLACF 768). The fungi *Aspergillus carbonarius* CCDCA 10408 and CCDCA 10608, isolated from grapes, and *Aspergillus ochraceus* CCDCA 10612, isolated from coffee beans, are also deposited.

The yeasts, preserved at -80°C, were reactivated in YEPG medium containing (g L^{-1}) 10.0 yeast extract, 20.0 peptone, 10.0 glucose and 15.0 agar. The cultures were incubated at 28°C for 24h. After this period, the cells were suspended in different concentrations for use in *in vitro* tests.

The isolates of filamentous fungi, preserved in paper filter discs at -15°C, were reactivated by inoculating filter paper with each stock culture in Petri plates containing 20 mL of Malt Extract Agar

(MEA) (g L^{-1}), 20.0 malt extract, 1.0 bacteriological peptone, 20.0 glucose and 20.0 agar. The medium was adjusted to pH 5.6, and the plates were incubated at 28°C for seven days.

In vitro assays

Each preserved yeast isolate was re-suspended in 2 mL of YEPG for 24h, and a serial decimal dilution was performed until 10^4 through 10^7 cells mL^{-1} was obtained. For the fungal isolates, a suspension of spores was obtained from growth in MEA (2%), adding over the colonies 40 mL of sterile distilled water with 0.5% of Tween 80 and filtering through sterile lint. The determination of the final concentration of spores was performed in a Neubauer chamber. The concentration of 10^5 spores mL^{-1} was standardised for all treatments. Each concentration of yeast cells was tested for each fungal isolate to evaluate growth, spore production and mycotoxin production. In total, 192 experimental combinations were carried out to evaluate growth and spore production. Aliquots of 100 μL of the yeast suspension were spread using a Drigalsky handle on Petri dishes containing MEA medium. Aliquots of 10 μL of fungal spore suspension were tested in the centre of the dish. Each assay was performed in triplicate, and the dishes were incubated at 28°C for seven days.

A positive control for the growth of each fungal isolate was conducted by inoculating the suspension of spores at a unique point in the MEA medium under the same conditions as for the assays, although without inoculating the yeast isolates.

Evaluation of vegetative growth

The diameter of each fungal colony was measured to evaluate the mycelial vegetative growth. These measurements were made at two, five and seven days after the inoculation. The mycelial growth was assessed using a calliper rule and considered to be the ray from the centre of the colony multiplied by two. The positive control for growth was evaluated during the same sampling periods. The percentage of growth inhibition was obtained considering that the positive control was 100% of the diameter.

Evaluation of *Aspergillus* spore production

The same treatments for growth inhibition were conducted to analyse spore production. After seven days of cultivation, the spores were counted as described in the section on *in vitro* assays. The percentage of inhibition of spore production was obtained considering the spore concentration in the positive control (i.e., no yeast found in the dishes of the fungus) as 100% for each fungal isolate.

Antagonistic activity of different water activity over OTA production

The tests presenting inhibition in relation to the growth and spore production were selected for evaluation of OTA production in CYA medium (g L^{-1}: 1.0 K_2HPO_4, 1 mL of metallic solution, 5.0 yeast extract, 30.0 saccharose, 15.0 agar), adding 10 mL of concentrated Czapek (30.0 g $NaNO_3$; 5.0 g KCl; 5.0 g $MgSO_4.7H_2O$; 0.1 g $FeSO_4.7.H_2O$; 0.1 g $ZnSO_4.7H_2O$; 0.05 g $CuSO_4.5H_2O$ and 100 mL of sterile distilled water) and YES media (g L^{-1}: 20.0 yeast extract; 150.0 saccharose; 20.0 agar and 0.5 $MgSO_4.7H_2O$), adding 1 mL of a metallic solution (0.10 g $FeSO_4 \cdot 7H_2O$; 1.0 g $ZnSO_4 \cdot 7H_2O$; 0.50 g $CuSO_4 \cdot 5H_2O$) with varying water activity (a_w). The determination of water activity was obtained by adding glycerol and from the reading in Aqualab, model-2 T (Decagon devices Inc., Pullman, WA, EUA). The presence of OTA production was examined using the plug agar method described below.

Determination of the presence of OTA

The agar plug thin layer chromatography (TLC) method was used to determine the presence of OTA. Filtenborg and Frisvad's (1980) experiment showed the inhibition of the development (growth and spore production) of fungi resulting from 81 experimental combinations. The positive control considered only the cultivated fungi.

Tests repeated in the same condition showed high inhibition modifying only the culture medium used. An *A. ochraceus* strain was inoculated into YES medium with water activity of 0.97, 0.96 or 0.94 and an isolate of *A. carbonarius* into CYA medium with water activity of 0.99, 0.96, and 0.94. These media are considered the best substrates for OTA production. All tests were incubated for seven days at 28°C. After this period, a circular cut of approximately 25 mm was made around a fungal colony with agar and put on a previously activated TLC plate (Merck – Silica Gel 60, 20 x 20) containing 20 μL of the OTA standard. The mycelium was removed and elution was performed after 15 min in a glass vat containing TEF – toluene, ethyl acetate and formic acid – 90% (60:30:10) as mobile phase. After elution, the plates were dried in a flow chapel and the confirmation was made in ultraviolet light with λ 366 nm in a CAMAG chromate viewer (UV-BETRACHTER). Isolates producing OTA present a retention factor (Rf) and fluorescence spot similar to those of the OTA standard.

Antagonistic activity by potential yeast strain at cellular level

To evaluate the cell interactions between the fungi and the yeast cells, a 3-mL sample of YEPG

containing 10 μL of suspension at a concentration of 10^5 spores mL^{-1} of *A. carbonarius* CCDCA 10408 and 50 μL of *Rhodotorula mucilaginosa* CCMA 0156 suspension cells at 10^7 cells mL^{-1} was prepared to observe their microscopic interaction. This sample was monitored for 16 h using a Bio Station IM-Q (Nikon) compact incubator system. The machine controls the humidity and temperature and takes pictures using three different axes (XYZ) every 1 min.

Experimental design and statistical treatment of the results

The experiment was carried out in a completely randomised block design; the blocks were the days of evaluation in a 3 × 32 × 2 factorial arrangement, using three isolates of filamentous fungi (A. *ochraceus* and two *A. carbonarius* strains), 32 isolates of yeast (as mentioned before) and two concentrations (10^4 and 10^7 cells mL^{-1}) with three repetitions. Spore production was analysed using a completely randomised block design in a 2 × 32 × 2 factorial arrangement, using two fungal strains instead of three as previously described. The treatment means were compared using the Scott-Knott test at 5% probability. All data were analysed using SISVAR® (Lavras, Brazil) software, version 4.0 (Ferreira, 2008).

Results

Thirty-two yeast strains were evaluated for inhibition of growth, spore production and OTA production. These yeast strains were isolated from coffee and cocoa beans. Independent of their origin, the strains showed a strong inhibition effect on ochratoxigenic *Aspergillus*.

Evaluation of vegetative growth

The mycelial growth inhibition in all treatments in relation to the control at different yeast cell concentrations can be seen in Table 1.

The yeast strains showed specificity in relation to the fungal strains (i.e., the same yeast strain was able to inhibit more or less than three fungal strains). *S. kluyveri* CCMA 0151 strongly inhibited *A. ochraceus* CCDCA 10612 (80%), followed by *A. carbonarius* CCDCA 10608 (41%), but had less effect on *A. carbonarius* CCDCA 10408 (31%) (Table 1).

Generally, the inhibition was more relevant when the concentration of 10^7 cells mL^{-1} was used. The highest yeast cell concentration used was more efficient at inhibiting the growth of the three fungal isolates (Table 2).

Table 1. Average colony diameter of *Aspergillus carbonarius* and *Aspergillus ochraceus* after 7 days *in vitro* antagonistic test with 32 yeast strains. In parenthesis the percentage of inhibition.

Yeast	*A.carbonarius*		*A.ochraceus*
	CCDCA 10408	CCDCA 10608	CCDCA 10612
Debaryomyces etchellsii CCMA 0162	2.46 aC (70%)	2.34 aC (73%)	0.97 bA (82%)
D.polymorphus CCMA 0141	3.76 aB (54%)	3.07 aC (65%)	1.00 bA (82%)
D. polymorphus CCMA0142	2.69 aC (67%)	2.76 aC (68%)	1.35 bA (75.5%)
D. polymorphus CCMA 0143	3.00 aC (63%)	2.67 aC (70%)	0.89 bA (84%)
Pichia anomala CCMA 0146	2.40 aC (70%)	1.47 bD (83%)	0.95 bA (83%)
P. anomala CCMA 0147	3.85 aB (53%)	3.52 aB (60%)	1.02 bA (81.5%)
P. anomala CCMA 0148	1.48 aD (82%)	1.31 aD (85%)	0.82 aA (85%)
Pichia burtonii CCMA 0149	2.81 aC (65%)	1.32 bD (85%)	1.00 bA (82%)
P. burtonii CCMA 0150	4.05 aB (50%)	2.93 bC (67%)	1.16 cA (79%)
Pichia fermentans CCMA 0163	2.06 aD (75%)	1.55 aD (82%)	0.80 bA (85%)
Pichia guilliermindii CCMA 0164	2.69 aC (67%)	2.2 aC (75%)	1.15 bA (79%)
P.guilliermondii CCMA 0138	3.91 aB (52%)	3.68 aB (58%)	1.14 bA (79%)
P. guilliermondii CCMA 0139	4.24 aB (48%)	2.93 bC (67%)	1.21 cA (78%)
P. guilliermondii CCMA 0140	2.17 aD (73%)	2.44 aC (72%)	0.75 bA (86%)
Pichia holstii CCMA 0144	3.08 aC (62%)	2.88 aC (67%)	0.97 bA (82%)
P. hostilii CCMA 0145	1.86 aD (77%)	1.49 aD (83%)	0.79 bA (85%)
Pichia jardinii CCMA 0160	1.90 aD (77%)	1.66 aD (81%)	0.88 bA (84%)
Pichia kluyveri CCMA 0168	3.12 aC (62%)	2.86 aC (67%)	0.91 bA (83%)
P. kluyveri CCMA 0169	2.99 aC (63%)	2.32 aC (74%)	1.15 bA (79%)
P. kluyveri CCMA 0166	2.78 aC (66%)	2.37 aC (73%)	0.83 bA (85%)
P. kluyveri CCMA 0167	2.75 aC (66%)	2.43 aC (72%)	1.13 bA (79%)
P. kluyveri CCMA 0165	1.82 aD (78%)	1.39 aD (84%)	0.79 bA (85%)
Pichia sydowiorum CCMA 0157	2.58 aC (68%)	1.58 bD (82%)	1.24 bA (77,5%)
P. sydowiorum CCMA 0158	4.00 aB (51%)	2.93 bC (67%)	1.02 cA (81,5%)
Rhodotorula mucilaginosa CCMA 0153	2.75 aC (66%)	2.25 aC (74%)	0.92 bA (83%)
R.mucilaginosa CCMA0154	2.50 aC (69%)	2.44 aC (72%)	1.00 bA (82%)
R.mucilaginosa CCMA 0155	2.60 aC (68%)	1.92 aD (78%)	1.10 bA (80%)
R.mucilaginosa CCMA 0156	5.73 aA (30%)	5.76 aA (34%)	1.37 bA (75%)
Saccharomyces cerevisiae CCMA 0159	1.58 aD (81%)	1.40 aD (84%)	0.66 bA (88%)
S. cerevisiae CCMA 0161	3.47 aB (57%)	3.18 aC (64%)	1.00 bA (82%)
S.kluyveri CCMA 0152	2.62 aC (68%)	1.38 bD (84%)	0.73 bA (86%)
S. kluyveri CCMA 0151	5.60 aA (31%)	5.15 aA (41%)	1.10 bA (80%)

Means followed by the same capital letter in the columns did not differ according to the Scott-Knott test ($p > 0.05$). Means followed by the same lower case letter in the rows did not differ according to the Scott-Knott test ($p > 0.05$).

The inhibition action for some yeast isolates did not differ in relation to the two cell concentrations

used (e.g., *D. etchellsii* CCMA 0162, *D. polymorphus* CCMA 0143, *P. burtonii* CCMA 0149, *P. fermentans* CCMA 0163, *P. kluyveri* CCMA 0169, *R. mucilaginosa* CCMA 0155, *S. kluyveri* CCMA 0151). Thus, the lower concentration of cells may be used in the future since the same inhibitory effect will be observed on mycelial growth (Table 2).

Table 2. Average colony diameter of *Aspergillus* strains after 7 days in *in vitro* antagonistic test with 32 yeast strains in both concentration cells. In bold, the yeasts strains showed significance statistical.

Yeast	Concentration (10^4 cells mL^{-1})	Concentration (10^7cells mL^{-1})
D. etchellsii CCMA 0162	2.19 aD	1.66 aB
D. polymorphus CCMA 0141	3.45 aB	1.77 bB
D. polymorphus CCMA 0142	2.81 aC	1.73 bB
D. polymorphus CCMA 0143	2.36 aD	2.01aB
Pichia anomala CCMA 0146	1.95 aD	1.26 bC
P. anomala CCMA 0147	3.52 aB	2.07 bB
P. anomala CCMA 0148	1.53 aE	0.88 bC
Pichia burtonii CCMA 0149	1.90 aE	1.52 aC
P. burtonii CCMA 0150	3.22 aB	2.20 bB
Pichia fermentans CCMA 0163	1.44 aE	1.51 aC
Pichia holstii CCMA 0144	2.96 aC	1.66 bB
Pichia guilliermindii CCMA 0164	2.39 aD	1.64 bB
P. guiilliermondii CCMA 0140	2.09 aD	1.48 aC
P.guilliermondii CCMA 0138	3.71 aB	2.11 bB
P.guilliermondii CCMA 0139	3.25 aB	2.34 bB
P. hostilii CCMA 0145	1.58 aE	1.17 aC
Pichia jardinii CCMA 0160	1.73 aE	1.22 aC
Pichia kluyveri CCMA 0166	2.33 aD	1.65 bB
P. kluyveri CCMA 0168	2.89 aC	1.71 bB
P. kluyveri CCMA 0165	1.56 aE	1.11 aC
P. kluyveri CCMA 0169	2.37 aD	1.93 aB
P.kluyveri CCMA 0167	2.40 aD	1.80 aB
Pichia sydowiorum CCMA 0157	2.06 aD	1.53 aC
P. sydowiorum CCMA 0158	3.37 aB	1.93 bB
R. mucilaginosa CCMA 0153	2.44 aD	1.51 bC
R. mucilaginosa CCMA 0154	2.20 aD	1.76 aB
R. mucilaginosa CCMA 0155	2.11 aD	1.65 aB
R. mucilaginosa CCMA 0156	4.77 aA	3.80 bA
Saccharomyces cerevisiae CCMA 0159	1.38 aE	1.05 aC
S. cerevisiae CCMA 0161	2.96 aC	2.13 bB
Saccharomyces kluyveri CCMA 0151	4.21 aA	3.68 aA
S. kluyveri CCMA 0152	1.83 aE	1.32 aC

Means followed by the same capital letter in the columns did not differ according to the Scott-Knott test ($p > 0.05$), analysis of the concentration in relation to the yeast. Means followed by the same lower case letter in the rows did not differ according to the Scott-Knott test ($p > 0.05$), analysis of the yeast in relation to the concentration.

Higher values of inhibition (85%) were recorded for *A. ochraceus* CCDCA 10612. In this context, the mycelial growth of isolate *A. ochraceus* CCDCA 10612 was completely inhibited by the yeast *Pichia holstii* CCMA 0145 (Figure 1).

Considering the different yeasts with the two isolates of *A. carbonarius*, it was observed that the inhibition depends on the isolate used. The yeasts *Debaryomyce spolymorphus* CCMA 0141, *Pichia guilliermondii* CCMA 0140, *R. mucilaginosa* CCMA 0155, *Pichia anomala* CCMA 0148 and *S. kluyveri* CCMA 0152 did not have the same inhibition performance against the two *Aspergillus carbonarius* isolates CCDCA 10408 and CCDCA 10608 (Figure 1). *A. carbonarius* CCDCA 10608 was more sensitive to inhibition than *A. carbonarius* CCDCA 10408, with some yeast strains highlighted. For example, *Pichia burtonii* CCMA 0149 (53%), *Saccharomyces kluyveri* CCMA 0152 (47%) and *Pichia anomala* CCMA 0146 (39%) inhibited more mycelial growth (Figure 1).

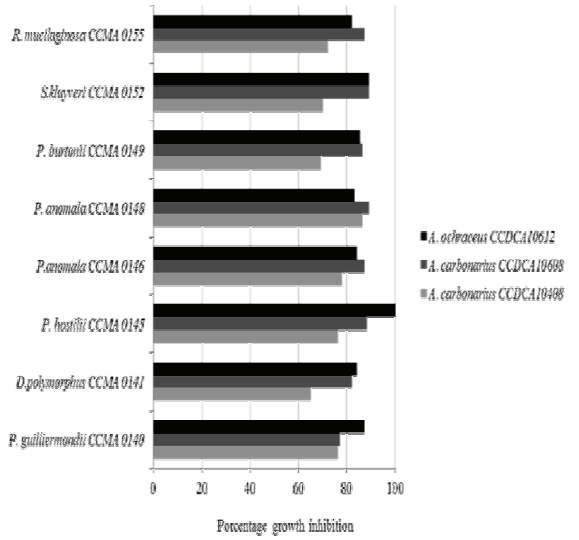

Figure 1. Porcentage growth inhibition from *Aspergillus carbonarius* (CCDCA 10408 and CCDCA 10608) and *A. ochraceus* CCDCA 10612 by co-cultivation with yeasts strains of isolated that showed better inhibition effect at concentration of 10^7 cells mL^{-1} after 7 days.

Evaluation of the spore production

It was not possible to evaluate the quantity of spore production by *A. ochraceus* CCDCA 10612 (in light microscopic) because the spores were too small, smooth and similar in colour to the yeasts and buds. However, the spores of *A. carbonarius* are dark brown and very rough, so it is easy distinguish them from yeast cells.

The yeast strains that inhibited the *A. carbonarius* strains growth did not show the same efficiency against spore production (Table 3). Moreover, the percentage of inhibition by each yeast isolate differed in relation to the fungal strains.

The spore production of *Aspergillus carbonarius* CCDCA 10408 was less inhibited than that of *A. carbonarius* CCDCA 10608 at both yeast cell concentrations. *A. carbonarius* CCDCA 10608 was more sensitive to antagonistic action, showing a similar effect at some yeast cell concentrations, although some yeasts inhibited spore production at higher cell concentrations (e.g., *D. polymorphus* CCMA 0141, *P. sydowiorum* CCMA 0157, *R. mucilaginosa* CCMA 0155, *S. kluyveri* CCMA 0152) (Table 3).

The inhibition action for some yeast isolates did not differ in relation to the two cell concentrations

used (e.g., *P. holstii* CCMA 0145, *S. cerevisiae* CCMA 0159). Thus, the lower concentration of cells may be used in future since the same inhibitory effect will be observed on spore production (Table 3).

Table 3. Spores production (log mL^{-1}) and % of inhibition of the production of spores (in parenthesis) from isolate *A. carbonarius* CCDCA 10608 and CCDCA 10408 by yeasts strains at the concentrations 10^4 and 10^7 cells mL^{-1}. Some isolates were omitted because showed inhibition rate less than 21%.

Yeast	*Aspergillus carbonarius*		
	Cells mL^{-1}	CCDCA 10608	CCDCA 10408
D. etchellsii CCMA 0162	10^4	0.00 (100%)	5.65 (21.53%)
	10^7	0.00 (100%)	6.43 (10.70%)
D. polymorphus CCMA 0141	10^4	6.49 (4.28%)	6.53 (9.21%)
	10^7	0.00 (100%)	6.83 (4.73%)
P. anomala CCMA 0146	10^4	0.00 (100%)	5.92 (17.71%)
	10^7	0.00 (100%)	0.00 (100%)
P. anomala CCMA 0148	10^4	0.00 (100%)	0.00 (100%)
	10^7	0.00 (100%)	5.77 (19.86%)
P. burtonii CCMA 0149	10^4	0.00 (100%)	6.47 (10.14%)
	10^7	0.00 (100%)	6.46 (10.28%)
P. fermentans CCMA 0163	10^4	0.00 (100%)	5.32 (26.11%)
	10^7	6.35 (6.35%)	5.98 (16.95%)
P. holstii CCMA 0145	10^4	0.00 (100%)	0.00 (100%)
	10^7	0.00 (100%)	0.00 (100%)
P. jadinii CCMA 0160	10^4	0.00 (100%)	5.30 (26.39%)
	10^7	0.00 (100%)	0.00 (100%)
P. kluyveri CCMA 0165	10^4	0.00 (100%)	4.69 (34.86%)
	10^7	0.00 (100%)	5.71 (20.70%)
P. kluyveri CCMA 0169	10^4	0.00 (100%)	7.12 (1.12%)
	10^7	6.47 (4.58%)	6.97 (3.20%)
P. sydowiorum CCMA 0157	10^4	6.14 (9.44%)	6.11 (15.14%)
	10^7	0.00 (100%)	6.23 (13.47%)
P. sydowiorum CCMA 0158	10^4	6.53 (3.69%)	6.47 (10.14%)
	10^7	0.00 (100%)	5.97 (17.09%)
R. mucilaginosa CCMA 0155	10^4	6.51 (3.99%)	5.93 (17.64%)
	10^7	0.00 (100%)	5.90 (18.06%)
S. cerevisiae CCMA 0159	10^4	0.00 (100%)	0.00(100%)
	10^7	0.00 (100%)	0.00 (100%)
S. kluyveri CCMA 0151	10^4	0.00 (100%)	6.81 (5.42%)
	10^7	6.56 (3.15%)	6.13 (14.87%)
S. kluyveri CCMA 0152	10^4	6.51 (3.99%)	6.59 (8.47%)
	10^7	0.00 (100%)	0.00 (100%)
Control	10^5	6.78 (0.00%)	7.20 (0.00%)

Determination of the presence of OTA

Different yeast strains were assayed for inhibition of toxin production according to reduction of growth and spore production (Table 2 and 3). The control fungal strains – *A. carbonarius* CCDCA 10608, CCDCA 10408 and *A. ochraceus* CCDCA 10612 – produced OTA only when cultivated in CYA medium at 0.99 a_w and YES at 0.98 a_w (Table 4), respectively. The *A. ochraceus* strain was strongly inhibited by the yeast strains tested; however, at 0.964 a_w, *Saccharomyces kluyveri* CCMA 0151 induced OTA production.

Most yeasts were able to inhibit *A. carbonarius* mycotoxin production, except *D. polymorphus* CCMA 0142, *P. anomala* CCMA 0148, *P. guillermondii* CCMA 0140, *P. kluyveri* CCMA 0165, *P. sydowirum* CCMA 0158, *R. mucilaginosa* CCMA 0153 and CCMA 0156 and *Saccharomyces kluyveri* CCMA 0151, which stimulated mycotoxin production (Table 4). *P. kluyveri* CCMA 0165 showed different inhibition action based on the tested cell concentration, while *R. mucilaginosa* CCMA 0153 (10^7 cells mL^{-1}) inhibited *A. carbonarius* CCDCA 10608 and induced mycotoxin in *A. carbonarius* CCDCA 10408 at 0.960 a_w (Table 4).

Table 4. Evaluation of OTA production by *A. carbonarius* CCDCA 10608 and CCDCA 10408, *A. ochraceus* CCDCA 10612 and in co-cultivation with different yeasts isolates (10^4 and 10^7 cells mL^{-1}) in CYA media with different activity water (a_w).

Aspergillus carbonarius CCDCA10608 CYA media				
Yeast	Cells mL^{-1}	0.984 a_w	0.960 a_w	0.939 a_w
Pichia anomala CCMA 0146	10^4	-	-	-
P. anomala CCMA 0148	10^4	-	-	-
P. burtonii CCMA 0149	10^4	-	-	-
P. guillermondii CCMA 0140	10^7	-	+	-
P. kluyveri CCMA 0165	10^7	-	-	-
Pichia sydowirum CCMA 0158	10^7	++	+	-
R. mucilaginosa CCMA 0153	10^7	-	-	+
R. mucilaginosa CCMA 0156	10^7	-	-	-
S. cerevisiae CCMA 0159	10^4	-	-	-
S. kluyveri CCMA 0152	10^4	-	-	-
S. kluyveri CCMA 0152	10^7	-	-	-
A.carbonarius CCDCA 10608 (Control)	10^5	++	-	-
Aspergillus carbonarius CCDCA10408 CYA media				
Yeast	Cells mL^{-1}	0.984 a_w	0.960 a_w	0.939 a_w
D.polymorphus CCMA 0142	10^7	-	++	-
P.guillermondii CCMA 0140	10^4	-	-	-
P. holstii CCMA 0145	10^4	-	-	-
P. anomala CCMA 0148	10^4	-	-	-
P. anomala CCMA 0148	10^7	++	-	-
P. fermentans CCMA 0163	10^4	-	-	-
P. jardinii CCMA 0160	10^7	-	-	-
P. kluyveri CCMA 0165	10^4	-	-	-
P. kluyveri CCMA 0165	10^7	++	-	-
R.mucilaginosa CCMA 0153	10^7	-	++	-
R. mucilaginosa CCMA 0156	10^4	++	-	-
R. mucilaginosa CCMA 0156	10^7	-	-	-
S. cerevisiae CCMA 0159	10^4	-	-	-
S. cerevisiae CCMA 0159	10^7	-	-	-
A.carbonarius CCDCA 10408 (Control)	10^5	+++	-	-
Aspergillus ochraceus CCDCA 10612 YES media				
Yeast	Cells mL^{-1}	0.976 a_w	0.964 a_w	0.945 a_w
Pichia burtoni CCMA 0149	10^4	-	-	-
P. guilliermondii CCMA 0138	10^4	-	-	-
P. kluyveri CCMA 0164	10^4	-	-	-
P. kluyveri CCMA 0166	10^4	-	-	-
P. kluyveri CCMA 0168	10^4	-	-	-
R.mucilaginosa CCMA 0156	10^4	-	-	-
R. mucilaginosa CCMA 0156	10^7	-	-	+
*S.kluyveri*CCMA 0151	10^4	-	++	-
S. kluyveri CCMA 0151	10^7	-	-	-
A.ochraceus CCDCA 10612 (Control)	10^5	+++	-	-

Signs -: absence of OTA production; +, ++ and +++: OTA production in different intensities when compared to the OTA standard.

Antagonistic activity at the cellular level of *Aspergillus carbonarius* CCDCA 10408 co-cultivated with *Rhodotorula mucilaginosa* CCMA 0156

During the 16h of inoculation, the spore germination of *A. carbonarius* CCDCA 10408 co-cultivated with *R. mucilaginosa* CCMA 0156 was observed through an image capture system coupled with an optical microscope (Figure 2). The temperature presented some variation, from 22.9 to 27.1°C, and the humidity ranged from 36.7 to 37.1°C.

At 20 min. after inoculation, it was observed that some yeast cells were already budding (Figure 2a). After 11 min., spores started germinating (though not simultaneously) (Figure 2b); after approximately 16h of incubation, a high number of yeast cells, spores germinated and spores not yet germinated were observed (Figure 2d).

Discussion

The majority of papers published on pest control are based on yeast or bacteria strains as deterring agents. It is important to focus on microorganisms such as fungi (producers of mycotoxin) because the best way to reduce contamination is by preventing the development of fungi pre- and post-harvest (including the storage period). Many chemical agents are used for this purpose; however, recent research has indicated that biological agents such as yeasts (especially the autochthonous strains) are more effective. This work is innovative because we evaluated growth, spore production and OTA production in a favourable a_w context. Most importantly, the yeast strains tested are considered enhancers of coffee beverage quality; therefore, they could be inoculated and act as mycotoxin fungal biological control and flavour enhancers of the coffee beverage. The growth conditions for yeast and ochratoxigenic fungi are very similar, as are their incidence in coffee, so yeasts are appropriate to use for biocontrol directly in field conditions.

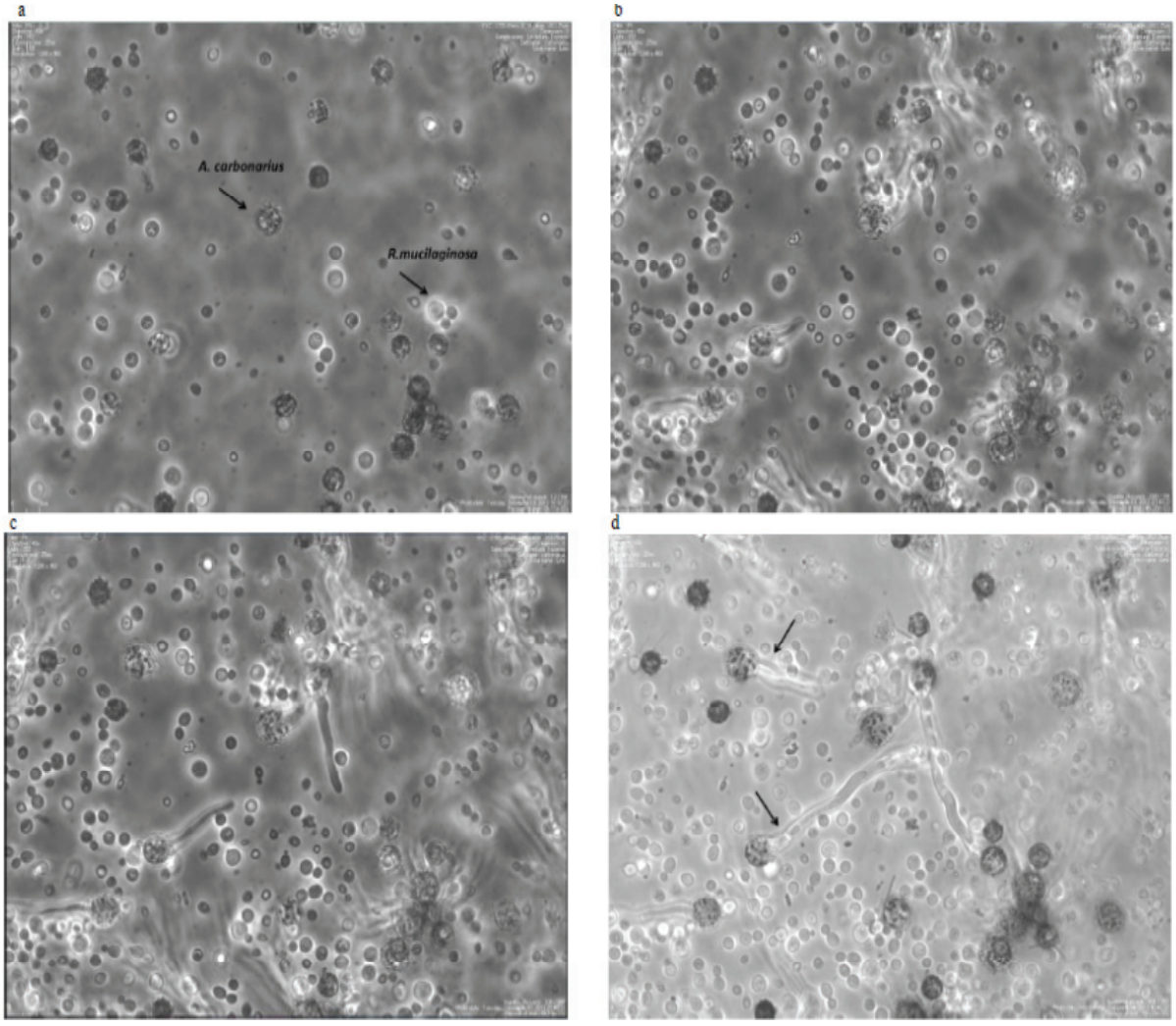

Figure 2. Antagonistic activity at cellular level of *Aspergillus carbonarius* CCDCA 10408 co-cultivated with *Rhodotorula mucilaginosa* CCMA 0156, increased 40x, 10 μm. (a) Yeast cells and spores of the fungi after inoculation. (b) Increase on the number of yeast cells and beginning of germination of *A. carbonarius* spores (4 hours and 39 minutes). (c) Germination of *A. carbonarius* spores (5 hours and 19 minutes). (d) Observation on the increase of hyphae emitted by the spore of *A. carbonarius;* it was possible to observe the formation of nuclei after approximately 16 h of incubation. There is also a spore that did not present a very former pressive growth of the hyphae. Images obtained using BioStation IM-Q (Nikon). The pictures are in an augmentation of 40x.

Two concentrations of yeast cells were tested (10^4 and 10^7 cells mL^{-1}) for growth inhibition. The efficiency of the inhibition effect was dependent on the strain (i.e., both cell concentrations were effective). Thus, for some yeast strains (e.g., *D. polymorphus* CCMA 0143, *Pichia burtonii* CCMA 0149, *P. kluyveri* CCMA 0165, *R. mucilaginosa* CCMA 0154), the action mechanism is not nutrient or space competition, as reported by Bleve, Grieco, Cozzi, Logrieco, and Visconti (2006), Masoud and Kaltoft (2006) and Zhu et al. (2015); other mechanisms could include killer toxin production (Banjara, Nickerson, Suhr, & Hallen-Adams, 2016; Grzegorczyk, Żarowska, Restuccia, & Cirvilleri, 2017), volatile compound production (Núnez et al., 2015) or adsorption to yeast cell walls, as observed in *Rhodotorula* by Var, Erginkaya, and Kabak (2009) and *Debaryomyce shansenii* by Gil-Serna et al. (2011).

Among the fungal strains, *A. ochraceus* was the most inhibited. This phenomenon is correlated with that observed by Ramos, Silva, Batista, and Schwan (2010) and Zhu et al. (2015). Spore production was strongly inhibited by 16 yeast strains, above 21% at both cell concentrations. A decrease in spore production is important because the spores can contain mycotoxin (Guzmán-de-peña & Herrera, 1997). Kapetanakou et al. (2012) observed that the greatest reduction in OTA occurred at low pH. In our work, pH was evaluated (data not shown) during the incubation period of the tests, with almost 16 yeasts exhibiting high expression of inhibition and a final pH of approximately 4. Therefore, we can infer that the decrease in spore production, and consequently in OTA production, was influenced by the pH value. Moreover, the strategy of strain inoculation (yeasts and fungi) differs in the level of its inhibition effect on spore production. Ramos et al. (2010) inoculated yeast isolates concomitantly with the inoculation of filamentous fungi (*A. carbonarius* and *A. ochraceus*) 4 cm apart, allowing both isolates to develop before direct contact of the yeast colony with the filamentous fungus. This may allow a greater inhibitory effect of the yeast over sporulation than over mycelial development. We found that inhibition of both mycelial growth and the production of spores occurred in most of the assays. Controlling the production of spores is a relevant factor because these structures produce and accumulate toxins, and they are agents of the species' dispersion (Guzmán-de-peña & Herrera, 1997). In relation to coffee cultivation, the control of the production of spores is reflected directly in the dissemination of such fungus in the farm produce and harvest of the year, as well as in the post-harvest period, thus decreasing the sanitary quality of the product. A lack of control of sanitary quality reflects directly on the health of the consumer and on the trade of the product (Silva, Batista, & Schwan, 2008; Duarte, Pena, & Lino, 2009).

A. ochraceus was more sensitive than *A. carbonarius* strains in terms of growth and OTA production. This sensitivity might be due to the lower level of OTA production in *A. ochraceus* than in *A. carbonarius* (Zhu et al., 2015). The biocontrol of OTA production by yeasts in *A. westerdijkiae* (former *A. ochraceus*) is at a transcriptional level, as observed by Gil-Serna et al. (2011). In *A. carbonarius* strains, we observed a different level of inhibition in all tested yeasts. Different expression levels of genes for mycotoxin production is a normal characteristic in different strains reflecting a contrasting ability to produce OTA (Botton et al., 2008). It is essential to find potent biocontrol agents for *A. carbonarius* strains because 75% – 100% of the strains are OTA producing (Romero et al., 2005).

OTA production is influenced by environmental factors, such as water activity (a_w), temperature and substrate. a_w may be one of the most important factors influencing the growth, germination and establishment of fungi in substrates rich in nutrients (Bouras, Kim, & Strelkov, 2009; Passamani et al., 2014). The fungal strains used as treatment control for OTA evaluation did not produce OTA in lower a_w, and in a general way, the best environmental condition for OTA production is the same for growth (Bellí, Ramos, Coronas, Sanchis, & Marín, 2005). OTA production was found to depend on the species and was influenced by the different yeasts used in co-cultivation with fungi. *P. kluyveri* CCMA 0165 stimulated OTA in high a_w (0.99) when 10^7 cell mL^{-1} was used but not in 10^4 cell mL^{-1}, suggesting a stress condition in the fungal cells. This phenomenon is uncommon and new experiments should be done to understand it better.

Conclusion

This study opens new possibilities for using yeast strain enhancers of sensorial quality in coffee beverages as biocontrol agents for two different fungal species in coffee beans. Some yeast strains were more effective at inhibiting growth, spore and OTA at low cell concentrations, which is advantageous for biological control. In situ tests should be conducted to establish possible interactions with natural microbiota present in coffee fruits as well as with environmental conditions. Yeasts of genera *Pichia*, *Debaryomyces*, *Saccharomyces* and *Rhodotorula* could be used as biocontrol agents since the tests conducted certify that OTA is not produced at different a_w levels.

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