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Research Article

Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*

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Abstract

The effects of volatile compounds produced during coffee processing by *Pichia anomala, P. kluyveri* and *Hanseniaspora uvarum* on growth of *Aspergillus ochraceus* and production of ochratoxin A (OTA) were studied. On malt extract agar (MEA) and on coffee agar (CA), exposure of *A. ochraceus* to the gaseous phase of malt yeast glucose peptone (MYGP) plates inoculated with *P. anomala, P. kluyveri* and *H. uvarum* inhibited fungal growth, with the two *Pichia* spp. showing the strongest effect. The main esters and alcohols produced by the three yeasts were ethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate, ethyl propionate and isoamyl alcohol. The individual esters and alcohols were found to affect fungal growth. The most effective compound in inhibiting fungal growth was 2-phenyl ethyl acetate; which at 48 μg/l headspace completely inhibited growth of *A. ochraceus*. Exposure of *A. ochraceus* to the gaseous phase of MYGP plates inoculated with *P. anomala, P. kluyveri* and *H. uvarum* prevented production of OTA. On CA medium, only the headspace of *P. anomala* and *P. kluyveri* prevented OTA production. Furthermore, when *A. ochraceus* was exposed to the headspace of the individual volatile compounds, 2-phenyl ethyl acetate was the most effective in preventing OTA production. Prevention of OTA seems to be due to reduction of fungal biomass. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: coffee; *Pichia anomala*; *Pichia kluyveri*; *Hanseniaspora uvarum*; *Aspergillus ochraceus*; OTA; volatile compounds

Introduction

The coffee plant belongs to the genus *Coffea* of the family Rubiaceae. *Coffea arabica* and *Coffea canephora* var. *robusta* are the two species that have commercial importance. Coffee cherries are processed by dry or wet method to separate the beans from the pulp. The dry method is mainly used for Robusta coffee, which has a thin pulp that allows direct drying (Fowler *et al*., 1998). In wet processing of coffee, which is mainly used for Arabica coffee, the ripe coffee cherries are pulped followed by fermentation and drying (Fowler *et al*., 1998).

The microbiota during coffee processing is composed of Gram-negative and Gram-positive bacteria, yeasts and filamentous fungi (Silva *et al*., 2000). In coffee samples collected at different steps of the wet processing of Arabica coffee in Tanzania three predominant yeast species were isolated in a previous study (Masoud *et al*., 2004). They included *Pichia anomala*, which was found in high numbers at the beginning of fermentation and during the drying process, *P. kluyveri*

which predominated during the whole process and *Hanseniaspora uvarum* which was detected in high numbers during fermentation but decreased markedly in the drying process (Masoud *et al*., 2004).

Ochratoxin A (OTA) is a secondary metabolite of some toxigenic species of *Aspergillus* and *Penicillium*, which has been shown to be genotoxic, nephrotoxic, teratogenic, immunotoxic and possibly carcinogenic (Petzinger and Ziegler, 2000). Levi *et al*. (1974) were the first to report occurrence of OTA in coffee beans. Since then, several studies have detected both OTA-producing fungi and OTA in green coffee beans (Levi, 1980; Mislivec *et al*., 1983; Micco *et al*., 1989; Studer-Rohr *et al*., 1995; Nakajima *et al*., 1997; Romani *et al*., 2000; Ottender and Majerus, 2001; Pittet and Royer, 2002). The main filamentous fungi that have been found in coffee with the potential to produce OTA were *A. ochraceus, A. carbonarius* and *A. niger* (Nakajima *et al*., 1997; Joosten *et al*., 2001; Ngabirano *et al*., 2001; Pitt *et al*., 2001). The origin of OTA in coffee is not known. Bucheli *et al*. (1998) studied the accumulation of OTA during storage of green coffee beans and found no growth of OTA-producing fungi and no consistent production of OTA at different storage conditions. It appeared that accumulation of OTA in coffee beans occurred before storage and it was suggested to be linked mainly to post-harvest conditions of coffee processing (Bucheli *et al*., 1998, 2000). However, for Brazilian coffee, it was found that the highest levels of contamination with OTA and OTA-producing fungi occurred in coffee beans during drying and storage (Taniwaki *et al*., 2003). Recently, Palacios-Cabrera *et al*. (2004) reported that storage of contaminated green coffee beans with *A. ochraceus* at high humidity resulted in high levels of OTA at changing temperatures within the range 14–15 °C compared to a constant temperature of 25 °C.

It has been reported that some yeasts can inhibit growth of filamentous fungi. *Pichia anomala* was shown to be antagonistic to several fungi including *Botrytis cinerea* responsible for grey mould disease in grape-vine (Masih *et al*., 2000), plant pathogenic fungi such as *Rhizoctonia solani, Fusarium equiseti, Botrytis fabae* and *Phytophthora infestans* (Walker *et al*., 1995), *Penicillium roqueforti* and *A. candidus* (Björnberg and Schnürer, 1993) and *Penicillium verrucosum* (Petersson *et al*., 1998). It has been found that *P. anomala* and *P. kluyveri* were able to inhibit growth and OTA production by *A. ochraceus* when grown together on malt extract agar (MEA) or on coffee agar (CA) (unpublished results).

For biological control purposes in solid state fermentations, the antimicrobial effects of volatiles produced by some microorganisms against other microorganisms could be of a particular interest. It has been reported that growth of a number of plant pathogenic fungi can be inhibited by volatile compounds produced by *Muscodor albus* (Strobel *et al*., 2001; Mercier and Jiménez, 2004) and by *Gliocladium* spp. (Stinson *et al*., 2003). *Trichoderma* spp. were also found to produce volatile compounds, which were effective against wood decay fungi (Wheatley *et al*., 1997). The effect of ethyl acetate, which is a major volatile compound produced by *P. anomala*, on growth of *Penicillium roqueforti* was investigated by Fredlund *et al*. (2004), who found that fungal growth was inhibited when plates inoculated with *Penicillium roqueforti* were exposed to gaseous phase containing ethyl acetate.

The aim of the present study was to determine the spectrum of volatiles produced by *P. anomala, P. kluyveri* and *H. uvarum* during coffee processing and the effects of the individual compounds on growth and OTA production by *A. ochraceus*.

Materials and methods

Cultures

Yeasts used in this study were obtained from coffee samples collected from the Arusha region, Tanzania (Masoud *et al*., 2004). They included six strains of *P. anomala* (S12, S13, S14, S15, S16 and S17), four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4 and S13Y4) and two strains of *H. uvarum* (S3Y8 and S15Y2). A non-fermentative laboratory strain of *Debaryomyces hansenii* CBS798 (CBS, Baarn and Delft, The Netherlands) was included as a control. Two strains of *A. ochraceus* (B677, B722) with the potential to produce OTA were studied. They were also isolated from coffee samples collected from Arusha region, Tanzania (Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition and Food, Karlsruhe, Germany).

Culture media

Malt yeast glucose peptone agar (MYGP) was prepared by dissolving 3 g malt extract (Difco, Detroit, MI, USA), 3 g yeast extract (Difco), 5 g bactopeptone (Difco), 10 g D(+)-glucose monohydrate (Merck, Darmstadt, Germany) and 20 g agar (Difco) in 1 l distilled water and the medium was adjusted to pH 5.6 by 1 M NaOH (Merck). For malt extract agar (MEA), 20 g malt extract (Difco), 10 g D(+)-glucose monohydrate (Merck), 5 g bactopeptone (Difco) and 20 g agar (Difco) were dissolved in 1 l distilled water and the medium was adjusted to pH 5.6 by 1 M NaOH (Merck). Diluent saline peptone (SPO) was prepared by dissolving 8.5 g NaCl (Merck), 0.3 g disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) (Merck) and 1 g bactopeptone (Difco) in 1 l distilled water. SPO was adjusted to pH 5.6 by the addition of 1 M HCl and 1 M NaOH. Coffee agar (CA) was prepared by adding 20 g grounded green coffee beans (Levi Farm, Arusha, Tanzania) and 20 g agar (Difco) to 1 l distilled water. The diluent and all substrates were autoclaved at 121 °C for 15 min.

Effect of exposure of *A. ochraceus* to volatiles produced by *P. anomala, P. kluyveri* and *H. uvarum* on fungal growth and OTA production

The yeasts were propagated in 25 ml MYGP broth at 25 °C for 48 h. After propagation, the cells were harvested by centrifugation at 3000 × *g* for 10 min, and resuspended in SPO. Cell concentrations were estimated by microscopy using a counting chamber (Neubauer) and the suspensions were diluted to final concentrations of 10^6 cells/ml; after which 100 µl each yeast suspension were spread on the MYGP plates.

Malt extract agar plates inoculated with *A. ochraceus* after an incubation period of 7 days at 30 °C were used to harvest fungal spores. Spores were suspended in SPO and the concentration was estimated by microscopy as just described and the suspensions were diluted in SPO to 10^6 spores/ml; 100 µl spore suspensions were spread onto MEA plates. The plates inoculated with yeasts and fungal spores were left to dry for 4 h at 25 °C. The lids were removed and the MYGP plate inoculated with yeast was inverted facing down the MEA plate inoculated with *A. ochraceus* and the two plates were sealed with tape and three layers of parafilm and incubated at 30 °C for 7 days. A plate inoculated with *A. ochraceus* sealed with yeast free agar plates was included as well. Six trials were done for each yeast; three plates to estimate fungal growth and three plates to detect OTA as described below. The same experiment was carried out on CA medium in which both of the yeasts and fungi were grown on CA.

Growth of *A. ochraceus* was estimated by measuring the dry weight of the fungal biomass. The agar with fungal growth was cut and placed in 200 ml distilled water, which was then heated in a microwave oven until the residual agar was melted. The fungal biomass was recovered on a pre-weight filter paper washed with distilled water and dried at 80 °C for 24 h, whereafter, the dry weight of the fungal biomass was determined (Fredlund *et al.*, 2004).

Identification and quantification of volatile compounds produced by yeasts

The volatiles produced by four strains of *P. anomala* (S12, S13, S16 and S17), two strains of *P. kluyveri* (S7Y1 and S8Y4), one strain of *H. uvarum* (S15Y2) and a laboratory strain of *D. hansenii* (CBS 798) were identified. MYGP plates were inoculated with 100 µl of 10^6 cells/ml of each yeast, respectively, and left to dry for 4 h at 25 °C. Then the plates were sealed with tape and three layers of parafilm and incubated at 25 °C for 48 h. A yeast-free MYGP plate was used as a control. To analyse volatiles in the headspace above the plates inoculated with the yeasts, a hole was made in the top plate and capped with a rubber septum to allow sampling from the headspace with a syringe. The volatiles were collected in a trap of 250 mg Tenax GR (mesh size = 60/80, Buchem bv, Apeldoorn, The Netherlands) using a gas pump (Handy check-8000, PBI Dansensor, Denmark) at a flow rate of 100 ml/min for 3 min. Desorption of volatiles was done thermally by an ATD 400 automatic thermal desorption system (Perkin-Elmer, Buckinghamshire, UK). Desorption temperature of the first trap to the second cold trap (30 mg Tenax GR, 5 °C) was 250 °C for 15 min with a helium flow of 60 ml/min. Desorption temperature of the cold trap was 300 °C for 4 min with a helium flow of 31 ml/min and an outlet split ratio of 1 : 30. Separation was performed by a gas chromatography–mass

spectrometry (HP G1800 A GCD system, Palo Alto, CA) under the following conditions: column, DB Wax form, J&W Scientific, CA (30 m × 0.25 μm film thickness); carrier gas, helium; flow rate, 1 ml/min (constant); column pressure (constant), 48 kPa; oven programme, 45 °C for 10 min, then rising at 6 °C/min to 240 °C, constant at 240 °C for 30 min. The mass selective detector was operated in the electron ionization mode (ionization energy, 70 eV) and the *m/z* (mass/charge) ratio range was 10–425. Identification was done by probability-based matching with mass spectra in the G1035A Wiley library (Hewlett-Packard, Palo Alto, CA).

Quantification of volatiles was based on comparisons of retention times and integrated peak areas of 14 reference compounds (see Table 1). A 10 μl aliquot of each reference compound was dissolved in 10 ml heptane. A quantity of 2 μl of the reference compound in heptane solution was injected into the GC–MS with the same flow, time and temperature conditions as just described. The peak area obtained for each reference compound was used to calculate the concentration of the compound in the headspace. The experiment was done in triplicate.

Effect of individual volatile compounds on growth and OTA production by *A. ochraceus*

The effect of the 14 individual volatile compounds identified in the headspace of yeasts cultures (Table 1) on growth of *A. ochraceus* and OTA production were investigated at concentrations of 4, 8 12, 24, 48 and 96 μg/l headspace. To control the concentration of volatiles in the headspace, 2 μl each volatile compound purchased as a pure chemical, were added to 20 ml distilled water and placed in a Petri dish, which was sealed with tape and three layers of parafilm and left for 2 h at 25 °C, whereafter the concentrations of volatiles were determined by headspace analysis as described above. To obtain 4, 8, 12, 24, 48 and 96 μg/l headspace over plates, the amounts of each volatile compound in distilled water that corresponded to the above concentrations in the headspace were calculated from the peak area obtained for each reference compound, as described above, assuming a linear relation between the concentrations in distilled water and in the headspace.

Aspergillus ochraceus was inoculated at a rate of 100 μl of 10^6 spores/ml on MEA plates, which were left to dry. Then each plate was placed facing down another plate containing distilled

Table 1. The profiles of volatile compounds identified for four strains of *P. anomala* (P.a), two strains of *P. kluyveri* (P.k), one strain of *H. uvarum* (H.u) and one strain of *D. hansenii* (D.h) in headspace above malt yeast glucose peptone (MYGP) plates after 48 h of incubation at 25 °C

	Concentrations of volatiles in the headspace above yeast cultures (μg/l)[1]							
Compound	**P.a S12**	**P.a S13**	**P.a S16**	**P.a S17**	**P.k S8Y4**	**P.k S7Y1**	**H.u S15Y2**	**D.h CBS 798**
Ethyl acetate	65.0	42.0	31.0	74.0	69.0	54.0	49.0	—[2]
Ethyl propionate	10.0	14.0	12.0	6.0	2.0	5.0	3.0	—
Propyl acetate	8.0	10.0	3.0	14.0	—	—	2.0	—
Isobutyl acetate	50.0	37.0	23.0	62.0	34.0	53.0	21.0	—
Propyl propionate	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	—	—
Butyl acetate	2.0	≤1.0	≤1.0	5.0	≤1.0	≤1.0	≤1.0	—
Isobutyl alcohol	3.0	4.0	≤1.0	5.0	≤1.0	≤1.0	≤1.0	6.0
Isopentyl acetate	3.0	4.0	≤1.0	5.0	4.0	3.0	—	—
Butanoic acid octyl ester	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	—
Isoamyl alcohol	7.0	4.0	6.0	10.0	7.0	5.0	≤1.0	3.0
Acetoin	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	—
2–phenyl ethyl acetate	23.0	19.0	17.0	27.0	16.0	17.0	5.0	—
Phenyl ethyl alcohol	3.0	≤1.0	≤1.0	4.0	≤1.0	≤1.0	≤1.0	—
Isobutyl propanoate	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	—

[1] Average values for three trials for each volatile compound determined for each yeast. Standard deviations for all trials were in the range 0.5–2.0.
[2] Not detected.

water with the concentration estimated for each volatile compound, respectively. The two plates were sealed as just described and incubated at 30 °C for 7 days. Six trials were done for each volatile to estimate fungal growth and OTA formation in triplicates. The dry weight of the fungal biomass was determined as described above and OTA was detected as described below.

Determination of OTA in MEA and CA by thin layer chromatography (TLC)

The ability of *A. ochraceus* to produce OTA on MEA and CA plates when exposed to yeasts or volatiles was investigated by thin layer chromatography (TLC) as described by Samson *et al.* (2002). Agar plugs were aseptically removed from mould colonies on MEA and CA plates inoculated with *A. ochraceus* and one drop of chloroform : methanol mixture (1 : 2) was added to each plug. The plug was placed onto a TLC plate silica gel 60 (Merck Art 5721) with mycelium side towards the gel. 10 μg/l OTA in toluene : acetic acid (99 : 1) was added to TLC plates as a standard. Then the TLC plates were developed in toluene : acetone : methanol (5 : 3 : 2) and left to dry in a fume hood for 10 min. The TLC plates were examined visually under UV light at 366 nm wave length. The OTA detection limit of this analysis was determined to be 6 μg/l.

Results

Effect of exposure of *A. ochraceus* to volatiles produced by *P. anomala, P. kluyveri* and *H. uvarum* on fungal growth

As seen from Figure 1, exposure of *A. ochraceus* B722 grown on MEA to the headspace of MYGP plates inoculated with yeasts inhibited fungal growth. Growth of *A. ochraceus* B722 on CA was also inhibited when exposed to CA plates inoculated with strains of the three yeast species; however, the inhibition was less pronounced. On both MYGP and CA media, strains of *P. anomala* and *P. kluyveri* showed stronger inhibition than *H. uvarum*. When yeasts were grown on MYGP, the strongest inhibition of fungal growth was observed for *P. anomala* S12 and S17. The inhibition amounted to 70% compared to the control. On CA, *P. anomala* S17 was observed to inhibit

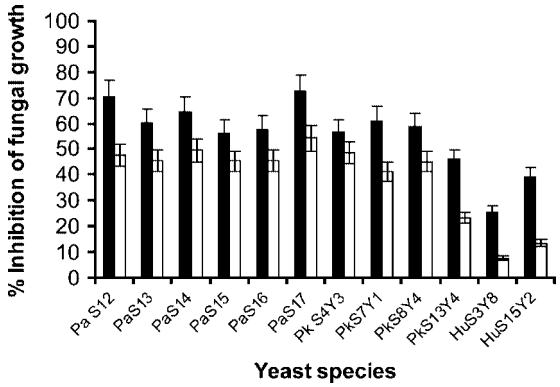

Figure 1. Growth inhibition of *A. ochraceus* B722 exposed to volatiles produced by six strains of *P. anomala* (Pa S12, Pa S13, Pa S14, Pa S15, Pa S16 and Pa S17), four strains of *P. kluyveri* (Pk S4Y3, Pk S7Y1, Pk S8Y4 and Pk S13Y4) and two strains of *H. uvarum* (Hu S3Y8 and Hu S15Y2). *A. ochraceus* grown on MEA was exposed to yeasts grown on MYGP (black bars). *A. ochraceus* grown on CA was exposed to yeasts grown on CA (white bars). Inhibition is expressed as the percentage reduction of the fungal biomass compared to the control (mould grown on plates exposed to yeast-free medium). Indicators above bars represent standard deviations

growth of *A. ochraceus* B722 by about 55%. The remaining strains of *P. anomala* and three strains of *P. kluyveri*, S4Y3, S7Y1 and S8Y4, grown on CA, inhibited fungal growth by 40–50%. For the two strains of *H. uvarum* grown on MYGP, the inhibition of fungal growth was about 25% and 40%. When grown on CA medium, the two strains of *H. uvarum* S3Y8 and S15Y2 showed weak inhibition, with percentages of growth inhibition of ca. 10%. For all trials, similar observations were made for *A. ochraceus* B677 (results not shown).

Identification and quantification of volatile compounds produced by yeasts

The major volatile compounds produced by the yeasts on MYGP agar are shown in Table 1. The profiles of volatile compounds produced by *P. anomala, P. kluyveri* and *H. uvarum* were similar. However, the concentrations of volatiles such as isoamyl alcohol, ethyl propionate, isobutyl acetate, isopentyl acetate and 2-phenyl ethyl acetate in the headspace above cultures of *H. uvarum* on MYGP plates were lower than those above cultures of *P. anomala* and *P. kluyveri*. The major volatile produced by the three yeasts was ethyl acetate, followed by isobutyl acetate, 2-phenylethyl acetate,

ethyl propionate and isoamyl alcohol. No ester was found in the headspace above MYGP agar inoculated with *D. hansenii*. For this yeast, only isobutyl alcohol and isoamyl alcohol were detected in the headspace.

Effect of the individual volatile compounds on growth of *A. ochraceus*

The effect of exposure of *A. ochraceus* B722 inoculated on MEA plates to the headspace of different concentrations of esters and alcohols produced by *P. anomala, P. kluyveri* and *H. uvarum* are shown in Table 2. Differences between volatiles in their ability to affect fungal growth were evident. Ethyl acetate, the main ester produced by the three yeasts (Table 1), started to inhibit growth of *A. ochraceus* at a concentration of 48 μg/l headspace with a 15% reduction of the dry weight of fungal mycelia compared with the control. However, 2-phenyl ethyl acetate started to inhibit fungal growth at 4 μg/l headspace and the percentage of inhibition increased strongly with increasing headspace concentration. At 48 μg/l headspace, 2-phenyl ethyl acetate was able to completely inhibit fungal growth under the conditions of the trial. Other volatiles, such as ethyl propionate, isobutyl acetate, isoamyl alcohol, isobutyl alcohol and phenyl ethyl alcohol, were found to reduce growth of *A. ochraceus* at initial concentrations of 12 and 24 μg/l headspace and the reduction increased with increasing volatile concentrations (Table 2).

Effect of exposure of *A. ochraceus* to the headspace of agar plates inoculated with yeasts on OTA formation

The effect of exposure of MEA plates inoculated with *A. ochraceus* B722 to the headspace of MYGP plates inoculated with strains of *P. anomala, P. kluyveri, H. uvarum* and *D. hansenii* on the production of OTA is shown in Figure 2. All strains of *P. anomala, P. kluyveri* and *H. uvarum* were found to prevent production of OTA by *A. ochraceus* at a detection limit of 6 μg/l. Exposure of *A. ochraceus* to the headspace of plates inoculated with *D. hansenii* did not prevent production of OTA. When both yeasts and *A. ochraceus* B722 were grown on CA, the headspace of *P. anomala* and *P. kluyveri* prevented OTA production; while the headspace of *H. uvarum* and *D. hansenii* did not prevent OTA production (Table 3). Although exposure of *A. ochraceus* B722 to the headspace of MYGP and CA agar inoculated with the strains

Table 2. The effect of individual volatile compounds on growth of *A. ochraceus* B722 at the headspace concentrations stated

	Dry weight of fungal biomass at different concentrations of volatiles in the headspace above plates[1]						
Compound	**0**	**4 μg/l**	**8 μ g/l**	**12 μ g/l**	**24 μ g/l**	**48 μ g/l**	**96 μ g/l**
Ethyl acetate	61[2]	62 (–)[3]	61 (–)	62 (–)	61(–)	52 (15%)	33 (46%)
Ethyl propionate	59	61 (–)	60 (–)	58 (–)	52 (11%)	50 (15%)	38(36%)
Isobutyl acetate	62	61 (2%)	62 (–)	57 (8%)	54 (13%)	30 (52%)	17 (73%)
Butyl acetate	61	62 (–)	60 (2%)	60 (2%)	59 (3%)	54 (11%)	35 (43%)
Propyl acetate	58	60 (–)	59 (–)	58 (–)	58 (–)	60 (–)	32 (45%)
Propyl propionate	62	60 (3%)	59 (5%)	62 (–)	61 (2%)	55 (11%)	39 (37%)
Isopentyl acetate	59	59 (–)	58 (–)	60 (–)	60 (–)	51(14%)	34 (42%)
Isobutyl propionate	60	58 (3%)	60 (–)	59 (2%)	58 (3%)	49 (18%)	29 (52%)
Isoamyl alcohol	62	60 (3%)	58 (6%)	56 (10%)	52 (16%)	41 (34%)	17 (73%)
2-Phenyl ethyl acetate	61	54 (12%)	39 (36%)	32 (48%)	16 (74%)	0 (100%)	0 (100%)
Isobutyl alcohol	59	61 (–)	60 (–)	53 (10%)	48 (19%)	25 (58%)	16 (73%)
Phenyl ethyl alcohol	61	59 (3%)	61(–)	54 (12%)	39 (36%)	17 (72%)	0 (100%)
Butanoic acid octyl ester	61	63 (–)	59 (3%)	58 (5%)	60 (2%)	54 (11%)	43 (30%)

[1] Average values of dry weight for three trials for each concentration of each volatile tested. Standard deviations for all trials were in the range 1–2.5.

[2] Dry weight of fungal biomass not exposed to volatiles.

[3] The values between brackets are the percentages of reduction of the dry weight of the fungal biomass compared to the dry weight of fungal biomass not exposed to volatiles. (–), no reduction in the dry weight of fungal biomass.

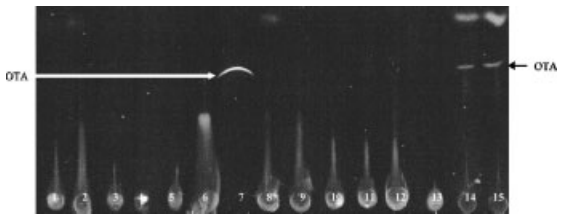

Figure 2. Production of OTA by *A. ochraceus* B722 grown on MEA plates exposed to the headspace of yeasts grown on MYGP plates. The lane numbers refer to *A. ochraceus* exposed to plates inoculated with: lane 1, *P. anomala* S12; lane 2, *P. anomala* S13; lane 3, *P. anomala* S14; lane 4, *P. anomala* S15; lane 5, *P. anomala* S16; lane 6, *P. anomala* S17; lane 7, OTA standard (10 μg/l); lane 8, *P. kluyveri* S4Y3 *P*; lane 9, *P. kluyveri* S7Y1; lane 10, *P. kluyveri* S8Y4; lane 11, *P. kluyveri* S13Y4; lane 12, *H. uvarum* S3Y8; lane 13, *H. uvarum* S15Y2; lane 14, *D. hansenii*; lane 15, MYGP without yeast (control)

Table 3. Ochratoxin A production (≥6 μg/l) by *A. ochraceus* B722 and B677 on malt extract agar (MEA) and coffee agar (CA) when exposed to agar plates inoculated with *P. anomala, P. kluyveri, H. uvarum* and *D. hansenii* grown on malt yeast glucose peptone (MYGP) and CA media

	***A. ochraceus* B722**		***A. ochraceus* B677**	
Yeast	**MYGP[1]**	**CA[2]**	**MYGP[1]**	**CA[2]**
Control[3]	+[4]	+	+	+
P. anomala S12	−[5]	−	−	−
P. anomala S13	−	−	−	−
P. anomala S14	−	−	−	−
P. anomala S15	−	−	−	−
P. anomala S16	−	−	−	−
P. anomala S17	−	−	−	−
P. kluyveri S4 Y8	−	−	−	−
P. kluyveri S7 Y1	−	−	−	−
P. kluyveri S8 Y4	−	−	−	−
P. kluyveri S13 Y4	−	−	−	−
H. uvarum S3Y8	−	+	−	+
H. uvarum S15Y2	−	+	−	+
D. hansenii CBS 798	+	+	+	+

[1] *A. ochraceus* grown on MEA exposed to MYGP plate inoculated with yeasts.
[2] *A. ochraceus* grown on CA exposed to CA plates inoculated with yeasts.
[3] *A. ochraceus* exposed to MYGP and CA plates without yeasts.
[4] OTA ≥6 μg/l.
[5] OTA <6 μg/l.

of *P. anomala* and *P. kluyveri* did not inhibit fungal growth completely (Figure 1), production of OTA by *A. ochraceus* B722 under the same experimental conditions was under the detection limit of 6 μg/l. Similar observations were made for *A. ochraceus* strain B677, as shown in Table 3.

Effect of exposure of *A. ochraceus* to individual volatile compounds on OTA formation

The exposure of *A. ochraceus* B722 to different concentrations of the individual volatile compounds similar to those produced by the three yeasts on OTA formation showed that the most effective compound which affected OTA formation was 2-phenyl ethyl acetate; OTA was not detected at a concentration of 24 μg/l headspace or higher (results not shown). Phenyl ethyl alcohol and isobutyl alcohol affected OTA production at 48 and 96 μg/l headspace concentrations (results not shown). Only at 96 μg/l headspace of isobutyl acetate and isoamyl alcohol, OTA was not detected. The remaining volatiles, ethyl acetate, ethyl propionate, butyl acetate, propyl acetate, propyl propionate, isopentyl acetate, isobutyl propionate and butanoic acid octyl ester were found to have no effect on OTA formation by *A. ochraceus* in the range of concentrations investigated (results not shown).

Discussion

In the present work, exposure of *A. ochraceus* to the headspace of MYGP or CA plates inoculated with strains of *P. anomala, P. kluyveri* and *H. uvarum*, the predominant yeasts involved in coffee processing, was found to inhibit mould growth. Differences were observed in the degrees of growth inhibition among yeast species as well as between strains of the same species. Two strains of *P. anomala* S12 and S17 showed the strongest effect on growth of *A. ochraceus*, followed by the remaining strains of *P. anomala* and strains of *P. kluyveri*. Growth inhibition of *A. ochraceus* caused by the headspace of strains of *H. uvarum* was comparatively weak, especially on CA medium. Furthermore, exposure of *A. ochraceus* to the headspace of *P. anomala, P. kluyveri* and *H. uvarum* grown on MYGP plates prevented production of OTA. On the other hand, exposure of *A. ochraceus* to MYGP plates inoculated with *D. hansenii* did not affect OTA production. When yeasts were grown on CA medium, only exposure of *A. ochraceus* to the headspace of *P. anomala* and *P. kluyveri* prevented OTA production. Similar findings were obtained on the effects of co-culturing of *P. anomala, P. kluyveri* and *H.*

uvarum with *A. ochraceus* on MEA and CA substrates, as mentioned above. Although exposure of *A. ochraceus* to the headspace of CA plates inoculated with *P. anomala* and *P. kluyveri* showed less inhibition of mould growth than when the two yeasts were grown on MYGP plates, exposure of the fungus to the headspace of CA plates with the two yeasts prevented OTA formation. This could be due to reduction of fungal biomass where OTA was under the detection limit, i.e. <6 μg/l. It might also be because volatiles produced by the investigated yeasts can inhibit biosynthesis of OTA by *A. ochraceus* under conditions where fungal growth takes place.

The effect of exposure of *A. ochraceus* grown on MEA to the headspace of MYGP plates inoculated with *P. anomala, P. kluyveri* and *H. uvarum* was found to be stronger in reduction of fungal biomass compared to exposure of *A. ochraceus* grown on CA to yeasts grown on CA medium. It was also found that *P. anomala, P. kluyveri* and *H. uvarum* have a stronger inhibitory effect on growth and OTA production by *A. ochraceus* on MEA than on CA (unpublished results). The reason could be that the three yeasts were found to show weaker growth on CA than on MYGP. De Maria *et al*. (1994) found that in Arabica fermented green coffee beans the main carbohydrates were polysaccharides, sucrose and trace amounts of galactose, arabinose, mannose, glucose and xylose. It was also reported that in Arabica coffee beans the concentrations of glucose and fructose by the end of maturation and at the time of picking of the cherries, i.e. before fermentation, were about 0.03% and 0.04% dry weight, respectively (Rogers *et al*., 1999). The three yeasts were found to produce acetate esters, which are produced from alcohols and acetyl co-enzyme A by the alcohol acetyltransferase (Yoshioka and Hashimoto, 1981). It has been reported that production of esters and alcohols by *Saccharomyces cerevisiae* increases when glucose or fructose are used as a sole carbon source compared to maltose, and increasing the concentrations of glucose increased the amounts of esters and alcohols produced (Younis and Stewart, 1998). This might explain the lower antagonist activity of the yeasts when grown on CA, with its low content of carbohydrates. Furthermore, it has been reported that the number and quantities of volatiles, which included alcohols, esters and ketones, produced by the antagonist fungus *Muscodor albus* grown on low nutrient medium, were also found to be less than those produced on a more enriched medium with a high level of carbon source (Ezra and Strobel, 2003). It has also been found that exposure of plant pathogenic fungi to the antagonist fungi *M. albus* (Ezra and Strobel, 2003) and *Trichoderma* spp. (Wheatley *et al*., 1997) grown on a medium with a low sugar content was less effective in inhibiting fungal growth than when the two antagonists were grown on a more sugar-enriched medium.

The major esters produced by *P. anomala, P. kluyveri* and *H. uvarum* were ethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate and ethyl propionate. On the other hand, only isobutyl alcohol and isoamyl alcohol at 3 and 6 μg/l were detected in the headspace of plates inoculated with a laboratory strain of *D. hansenii*. Exposure of *A. ochraceus* to this yeast species did not affect fungal growth (unpublished results) or OTA production. With the exception of ethyl acetate, the estimated quantities of esters and alcohols in the headspace above yeast cultures showed that *H. uvarum* produced the lowest amounts compared to *P. anomala* and *P. kluyveri*, which might explain the weak antagonist activity of this yeast species.

The main esters and alcohols produced by the three yeasts were individually tested against growth of *A. ochraceus* and OTA formation. Various inhibition levels were observed among the tested compounds. When *A. ochraceus* was exposed to 48 μg/l headspace of ethyl acetate, the fungal biomass reduction was only 15%. At 96 μg/l headspace, which is higher than that estimated in the headspace above yeast cultures, the biomass reduction increased to 46%. Furthermore, OTA formation was not affected when the fungus was exposed to the various headspace concentrations of ethyl acetate. This means that ethyl acetate is not the major ester responsible for inhibition of *A. ochraceus*. On the contrary, 2-phenyl ethyl acetate reduced the biomass of *A. ochraceus* at an initial headspace concentration of 4 μg/l and the percentage of biomass reduction increased with increasing headspace concentration. The growth of *A. ochraceus* was inhibited completely at 48 μg/l headspace of 2-phenyl ethyl acetate. Furthermore, OTA was not detected when *A. ochraceus* was exposed to a concentration of 24 μg/l headspace of 2-phenyl ethyl acetate. It seems that 2-phenyl ethyl acetate plays a major role in the antagonist

activity of the tested yeasts against *A. ochraceus*. It has been reported that 2-phenyl ethyl acetate and phenyl ethyl alcohol were among the bioactive compounds produced by the antagonist fungi *M. albus* (Strobel *et al*., 2001) and *Gliocladium* spp. (*Stinson et al*., 2003).

It was observed that when the percentage of reduction of the fungal biomass caused by the individual volatiles was more than 50% (Table 3), OTA was not detected on TLC plates (results not shown). It seems that reduction of the fungal biomass is the main reason for the absence of OTA.

For the purpose of preventing production of OTA in coffee, the present work demonstrated the possibility of using *P. anomala* and *P. kluyveri* in biological control of OTA-producing fungi during coffee fermentation. From this work and a recent study (unpublished results), it appears that two mechanisms are involved, i.e. an effect of volatiles and a competition for nutrients. Further studies are needed on the effects of *P. anomala* and *P. kluyveri* on other OTA-producing fungi present in coffee. In addition, studies should be conducted of interactions between the two yeasts and OTA-producing fungi *in vivo*, i.e. during coffee processing.

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