Effect of yeast volatile organic compounds on ochratoxin A-producing Aspergillus carbonarius and A. ochraceus

Elsevier Editorial System(tm) for
International Journal of Food Microbiology
Manuscript Draft

Manuscript Number: FOOD-D-17-00886R1

Title: Effect of yeast volatile organic compounds on ochratoxin A-producing Aspergillus carbonarius and A. ochraceus

Article Type: Full Length Article

Keywords: mycotoxin; food safety; gene expression; 2-phenylethanol; post-harvest; biological control; antifungal compounds.

Corresponding Author: Professor Quirico Migheli,

Corresponding Author's Institution: Università degli Studi di Sassari

First Author: Maria Grazia Farbo

Order of Authors: Maria Grazia Farbo; Pietro Paolo Urgeghe; Stefano Fiori; Angela Marcello; Stefania Oggiano; Virgilio Balmas; Zahoor Ul Hassan; Samir Jaoua; Quirico Migheli

Abstract: Many foods and beverages in temperate and tropical regions are prone to contamination by ochratoxin A (OTA), one of the most harmful mycotoxins for human and animal health. Aspergillus ochraceus and Aspergillus carbonarius are considered among the main responsible for OTA contamination. We have previously demonstrated that four low or non-fermenting yeasts are able to control the growth and sporulation of OTA-producing Aspergilli both in vitro and on detached grape berries: the biocontrol effect was partly due to the release of volatile organic compounds (VOCs). Aiming to further characterise the effect of VOCs produced by biocontrol yeast strains, we observed that, beside vegetative growth and sporulation, the volatile compounds significantly reduced the production of OTA by two A. carbonarius and A. ochraceus isolates. Exposure to yeast VOCs also affected gene expression in both species, as confirmed by downregulation of polyketide synthase, non-ribosomal peptide synthase, monooxygenase, and the regulatory genes laeA and veA. The main compound of yeast VOCs was 2-phenylethanol, as detected by Headspace-Solid Phase Microextraction-Gas Chromatography-Tandem Mass Spectrometry (HS-SPME-GC-MS) analysis. Yeast VOCs represent a promising tool for the containment of growth and development of mycotoxigenic fungi, and a valuable aid to guarantee food safety and quality.

Cover Letter

UNIVERSITÀ DEGLI STUDI DI SASSARI
Dipartimento di Agraria – Sezione di *Patologia vegetale ed Entomologia*
Via Enrico De Nicola, 9 - 07100 Sassari
ITALIA

Quirico Migheli - Tel: ++39 079 229295; Fax: ++39 079 229316; e-mail: qmigheli@uniss.it

---

Professor Luca Cocolin

Editor-in-Chief, International Journal of Food Microbiology

Dear Professor Cocolin,

we are pleased to submit a revised version of manuscript entitled: "Effect of yeast volatile organic compounds on ochratoxin A-producing *Aspergillus carbonarius* and *A. ochraceus*", by Maria Grazia Farbo, Pietro Paolo Urgeghe, Stefano Fiori, Angela Marcello, Stefania Oggiano, Virgilio Balmas, Zahoor Ul Hassan, Samir Jaoua, and myself.
In this revised version, we have met all the reviewers' requirements, as indicated in the detailed response to reviewers. Changes in text are highlighted in red.

We hope that this new version shall be suitable for publication in *IJFM*.

On behalf of the authors,

Yours faithfully,

Professor Quirico Migheli

Author's response to Reviewers' comments:

Reviewer #1: The authors analyzed volatile organic compounds (VOCs) produced by four yeast species that they previously isolated. The major VOC was identified to be 2-phenylethanol, a compound known to inhibit fungal growth and development, and production of secondary metabolites. The VOCs of the yeasts reduced both vegetative growth and conidiation as well as ochratoxin A (OTA)-production of Aspergillus carbonarius and Aspergillus ochraceus. The significant reduction in OTA production was correlated with decreased expression of OTA biosynthesis genes and other related regulatory genes. The work is straightforward. Results may aid current biocontrol of toxigenic fungi in food and feed.

The following are some suggestions for authors' consideration in revising the manuscript-

1. Results presented in Table 2 are somewhat redundant since Figure 1 shows time-course changes of radial growth of colonies. Moreover, Table 4 also shows colony diameters after seven days at 25 °C on PDA. If necessary, briefly state the "reduction" in the text.

R: Table 2 was deleted and the results summarised in the text. Figure 1 was retained as it shows the growth inhibition over 10 days of incubation. Table 4 (now renamed as Table 3) was retained since it allows comparing growth inhibition and OTA inhibition within the same experiments.

2. Lines 281-286 can be presented in the introduction section. No need to describe the general information about VOCs in the discussion section.

R: Done.

3. Lines 316-319 basically are results and can be merged into section 3.3. Give it a strong start by directly discussing the major compound, 2-phenylethanol.

R: Done.

4. OTA production is a major focus of this study. Consider move the part describing OTA pathway and gene structure/organization (lines 333-346) to the introduction section. Discuss only how LaeA and VeA may regulate OTA production.

R: The part related to the description of OTA pathway has been moved to the Introduction section. In the Discussion section, the main differences between A. carbonarius and the less studied A. ochraceus are highlighted, with focus on the genes investigated in each species.

Reviewer #2: The manuscript Number FOOD-D-17-00886

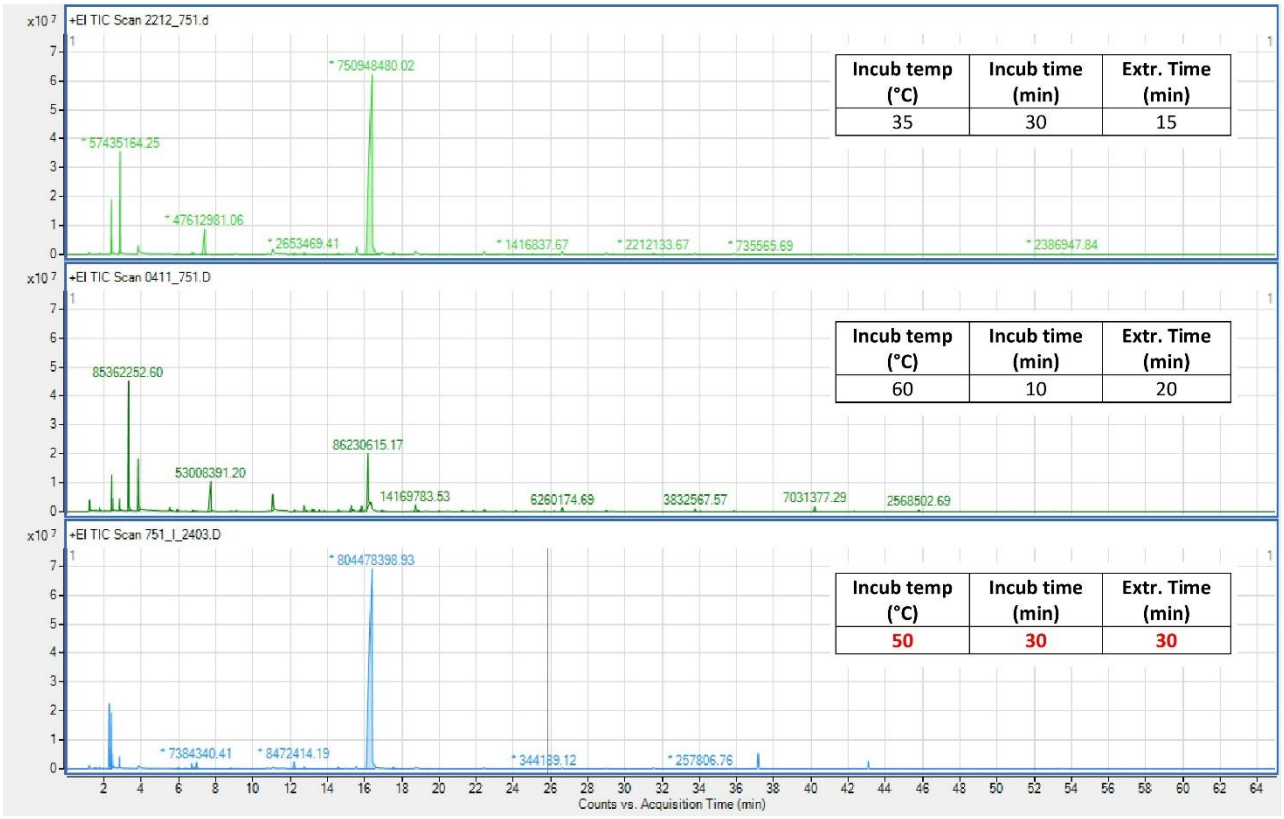
Title: Effect of yeast volatile organic compounds on ochratoxin A producing Aspergilli

General comments:
The manuscript FOOD-D-17-00886 describes the control effect that VOCs produced by yeasts have on vegetative growth and sporulation by both A. carbonarius and A. ochraceus. The volatile compounds significantly reduced the production of OTA by both species and affected gene expression in A. carbonarius. The manuscript is well presented and the results well presented. There are though some questions and comments that would need to be addressed by the authors before publication is granted.

Specific comments:

With regard to the analysis of the VOCs: The extraction and trapping of the VOCs has been done at a much higher temperature than the experiments were run, 50oC. In this case, could it be possible that we are overestimating the importance of 2-phenylethanol? Would the proportion of volatiles be the same at lower temperatures as the ones used to grow the fungi?

R: We agree that the temperature of extraction and the time of extraction can influence the volatile pattern one can obtain during the subsequent GC-MS analysis, therefore an optimization of these parameters is advisable. To this aim, during the optimization step we tested some different time-temperature combinations, as reported in this Figure:



As it can be seen, performing the incubation either at 35°C or 50°C did not result in any significant difference, being the 2-phenylethanol the main peak of the chromatogram, while increasing the incubation temperature to 60 °C caused a depletion of its peak area (please note that some peaks in the region of 2 minutes of retention time are referable to stationary phase bleeding). Our objective was to obtain a fairly balanced profile, trying to enhance the response of main peaks, and taking also into account that this is a semi-quantitative analysis, being the instrumental response in GC-MS strongly dependent on the structure of single analytes.

The volatile analysis has been performed at a specific time. Does the pattern of VOC from the yeasts change during the time the experiment was running? Have the authors been able to check this?
R: We have not checked whether the VOCs pattern may change over time. Based on the biocontrol data, we decided to measure VOCs after 7 days of yeast growth.

Some of the volatiles the authors have mentioned are not related at all with living organisms. For example, styrene is normally found in laboratory samples since most plastics will contain styrene. Thus, when you heat at 50oC it will be quite common to find styrene in the samples.

R: We thank the reviewer for this suggestion. We have carefully revised Table 5 by removing the analytes that are unlikely to be related to yeast metabolism or YPD. We have also corrected some mistakes in the mean calculations.

The paper looks unbalanced from the point of view that only the gene expression from A. carbonarius has been considered. Are VOC modifying the expression of the OTA synthetic pathway in general or is just an effect on this species. This is a very important lack of content in the paper. Nothing has been mentioned in the discussion about why this has happened. This data will need to be included in order to balance the content.

R: The manuscript has been integrated with new expression studies on *A. ochraceus*. Based on available literature, the expression of different genes involved in OTA biosynthesis was evaluated in *A. carbonarius* MPVA566 (*acOTApks* and *acOTAnrps*) and in *A. ochraceus* MPVA703 (*ao-pks* and *nrps-west*) by qRT-PCR. An *acpks* gene as well as two regulatory genes (*laeA* and *veA*) were considered in *A. carbonarius*, whereas two monooxygenase genes (*p450-H11* and *p450-B03*) and a halogenase gene (*hal-west*) were evaluated in *A. ochraceus*. The expression levels of the target genes were normalised by the expression of the reference genes *18S* and *g3pdh* for *A. carbonarius* and *A. ochraceus,* respectively, these being the most stable among four potential reference genes (namely, β- tubulin, actin, *18S* and *g3pdh*) used in preliminary studies.

The explanation of the statistical methods used looks very short and succinct. Have the authors checked the normality and homoscedasticity of the datasets? Where all datasets normally distributed allowing for the use of ANOVA?

R: Normality of residuals and homoscedasticity of variances were indeed verified prior to performing ANOVA. The section has been completed and rephrased as follows: “Data from each experiment were subject to one-way analysis of variance (ANOVA) followed by multiple comparisons by Tukey’s test, using the Statgraphics Centurion XVI software. Prior to performing ANOVA, normality of residuals was verified by the Shapiro-Wilk test, whereas assumption of homoscedasticity was checked by the Levene test.”

The title is quite broad taking into account that only 2 species of Aspergillus has been used and only one has been fully studied.

R: The title has been changed and now refers solely to the two species of *Aspergillus* considered in this study.

***Highlights (for review)**

- Food and feed can be contaminated by ochratoxin A (OTA) produced by *Aspergillus* spp.
- Non- or low-fermenting yeast selected strains are able to control OTA-producing *Aspergillus ochraceus* and *A. carbonarius*
- Yeast volatile organic compounds (VOCs) are responsible for reduction of vegetative growth and sporulation
- Yeast VOCs also reduce OTA production and OTA-biosynthetic gene expression
- The main component of yeast VOCs is 2-phenylethanol

Effect of yeast volatile organic compounds on ochratoxin A-producing *Aspergillus carbonarius* and *A. ochraceus*

Maria Grazia Farbo[1§], Pietro Paolo Urgeghe[1§], Stefano Fiori[1§], Angela Marcello[1], Stefania Oggiano[1], Virgilio Balmas[1], Zahoor Ul Hassan[2], Samir Jaoua[2], Quirico Migheli[1,3]

[1] *Dipartimento di Agraria, Università degli Studi di Sassari, Viale Italia 39, I-07100 Sassari, Italy.*

[2] *Department of Biological & Environmental Sciences, College of Arts and Sciences, Qatar University, P.O. Box: 2713, Doha, Qatar.*

[3] *Unità di Ricerca Istituto Nazionale di Biostrutture e Biosistemi, Università degli Studi di Sassari, Viale Italia 39, I-07100 Sassari, Italy.*

[§] The first three authors have equally contributed to the present work.

Corresponding author:

Quirico Migheli, Dipartimento di Agraria, Università degli Studi di Sassari

Viale Italia 39, I - 07100 Sassari, Italy

Phone: +39 079 229295; Fax: +39 079 229316; E-mail: qmigheli@uniss.it

Key words: mycotoxin, food safety, gene expression, 2-phenylethanol, post-harvest, biological control, antifungal compounds

Abstract

Many foods and beverages in temperate and tropical regions are prone to contamination by ochratoxin A (OTA), one of the most harmful mycotoxins for human and animal health.

Aspergillus ochraceus and *Aspergillus carbonarius* are considered among the main responsible for OTA contamination. We have previously demonstrated that four low or non- fermenting yeasts are able to control the growth and sporulation of OTA-producing *Aspergilli* both *in vitro* and on detached grape berries: the biocontrol effect was partly due to the release of volatile organic compounds (VOCs). Aiming to further characterise the effect of VOCs produced by biocontrol yeast strains, we observed that, beside vegetative growth and sporulation, the volatile compounds significantly reduced the production of OTA by two *A. carbonarius* and *A. ochraceus* isolates. Exposure to yeast VOCs also affected gene expression in both species, as confirmed by downregulation of polyketide synthase, non-ribosomal peptide synthase, monooxygenase, and the regulatory genes *laeA* and *veA*. The main compound of yeast VOCs was 2-phenylethanol, as detected by Headspace-Solid Phase Microextraction-Gas Chromatography-Tandem Mass Spectrometry (HS-SPME-GC-MS) analysis. Yeast VOCs represent a promising tool for the containment of growth and development of mycotoxigenic fungi, and a valuable aid to guarantee food safety and quality.

1. Introduction

After aflatoxins, ochratoxin A (OTA) is the second most frequent mycotoxin found in food and feed products (European Commission, 2012). The OTA structure consists in the amino acid phenylalanine linked by an amide bond to a pentaketide dihydroisocoumarin (Huffman et al., 2010). Some species of *Aspergillus* and *Penicillium* are the main source of OTA in warm and tropical regions, and in particular *Aspergillus carbonarius* (Bainier) Thom. is considered one of the most relevant OTA producers in food and feed (Abarca et al., 2003; Duarte et al., 2010; Kogkaki et al., 2015). OTA is classified as a group 2B carcinogen by the World Health Organization (Cabañes et al., 2013; IARC, 1993; JECFA,

2008; Van der Merwe et al., 1965). Studies are still under way to confirm whether OTA is responsible for the so-called Balkan Endemic Nephropathy (Castegnaro et al., 1998; Krogh, 1978). In most countries, strict regulatory limits are set for the presence of OTA in food commodities. The European Union has set the maximum OTA level at 2 mg/kg in wine, grape juice, and other grape products, and at 3 mg/kg for all products derived from cereal, including processed cereal products and cereal grains for human consumption (Duarte et al., 2010; European Commission, 2012).

Inhibiting the growth of OTA-producing fungi on sensitive commodities is by far the most reliable method to prevent OTA contamination of food and feed. Fungicides can control the growth of OTA-producing fungi and OTA contamination, but the European Union has established a strict legislation concerning the maximum residue levels of pesticides in agricultural commodities. Moreover, continuous application of specific active substances favours the selection of resistant OTA-producing *Aspergillus* spp. (Malandrakis et al. 2013; Zhang et al., 2016), and often growth inhibition achieved by improper use of fungicides can be accompanied by an unwanted induction of toxin biosynthesis (Schmidt-Heydt et al. 2013).

Many studies were focused on alternative biological control methods, which may be most appropriate to reduce infection and mycotoxin production by different fungal pathogens both in the field and during the postharvest phases.

Among the biological antagonists, yeasts are particularly promising in different commodities, as they have several properties that can be manipulated to improve their use and efficiency. Many yeast species have simple nutritional requirements, they are adapted to colonise wounds as well as dry surfaces and can grow quickly on a broad range of substrates in bioreactors. Furthermore, yeasts do not produce allergenic compounds or secondary metabolites like many filamentous fungi or bacterial antagonists do (Droby et al., 2009; Janisiewicz et al., 2010; Liu et al., 2013).

A number of yeast strains were selected and evaluated for use as a pre- or postharvest biological treatment of grape against OTA-producing *Aspergilli* (Bleve et al., 2006; Cubaiu et al., 2012; De Curtis et al., 2012; Ponsone et al., 2011; Zhu et al., 2015).

Besides other mechanisms of action, the biocontrol ability of some antagonistic yeast strains has been at least partly attributed to the production of volatile organic compounds (VOCs). VOCs are typically lipophilic substances with low molecular weight (<300 Da), high vapour pressure and low polarity (Werner et al., 2016), and they are able to inhibit mycelial growth and sporulation in many fungi (Buzzini et al., 2005; Chang et al., 2015; Di Francesco et al., 2015; Fiori et al., 2014; Huang et al., 2012; Parafati et al., 2017). VOCs may derive from different biosynthetic pathways, hence the term "volatilome" has been proposed to describe their broad chemical complexity (Maffei et al., 2011). So far, the production of volatile compounds by industrially relevant yeasts has been mainly explored for technological purposes (Passoth et al., 2006; Romano et al., 2015; Wriessenegger and Pichler 2013).

Yeast VOCs may also modulate the expression of genes involved in the OTA biosynthesis (Chang et al., 2015). Hence, VOCs released by selected yeasts deserve attention for their ability to reduce spore germination, mycelial growth, and mycotoxin production in preventive food safety strategies.

The biosynthetic pathway of OTA, as described by Huff and Hamilton (1979) has not yet been fully explained and only few genes were discovered so far (Abbas et al., 2009; Bacha et al., 2009; Gallo et al., 2014; Geisen et al., 2006; Karolewiez et al., 2005; Niessen et al., 2005; O' Callaghan et al., 2003; O' Callaghan et al., 2013; Wang et al., 2015). According to the OTA structure, the biosynthesis pathway includes a polyketide synthase (*pks*) and a non-ribosomal peptide synthase family (*nrps*), but also other genes such as regulators (Bayram et al., 2008) and monooxygenases (O' Callaghan et al., 2006) are likely to play a key role in the mycotoxin production.

In *A. carbonarius*, the *acpks* gene encodes a conserved kethosyntase and acyl transferase domains (Gallo et al., 2009). The *ac*OTA*pks* gene encodes a component of the PKS family, and contains a methyltransferase domain responsible for the addition of a methyl group to the OTA polyketide structure (Gallo et al., 2014). Another gene implicated in OTA biosynthesis in *A. carbonarius*, *AcOTAnrps*, is located about 900 nt upstream of *pks* and is transcribed in the same direction, differently from *Penicillium nordicum*, where OTA *pks* and *nrps* genes are transcribed in the opposite direction (Gallo et al., 2009, 2012; Karolewiez et al., 2005). Furthermore, in *A. carbonarius* two other genes are implicated in the regulation of OTA biosynthesis, *lae*A and *ve*A. *LaeA* encodes a methyltransferase, and was described for the first time in *Aspergillus nidulans*, in *Aspergillus terreus* and in *Aspergillus fumigatus* (Bok et al., 2004; Linde et al., 2016). *VeA* codes for a regulatory protein, which is transported from the cytoplasm to the nucleus in response to illumination. These two highly conserved proteins are considered as global regulators in fungi, modulating the sporulation capacity and mycotoxin production in *Aspergillus* spp. (Bayram et al., 2008). Deletion of these genes induces a drastic decrease of OTA production and a downregulation in the *nrps* gene expression (Crespo-Sempere et al., 2013).

We have previously demonstrated that four low or non- fermenting yeasts are able to control the growth and sporulation of OTA-producing *A. carbonarius* both *in vitro* and on detached grape berries (Fiori et al., 2014). This biological effect was at least partly due to the release of VOCs. The objectives of the present study were: 1) to further determine the effect of VOCs produced by selected yeast strains on vegetative growth and sporulation of OTA-producing *A. carbonarius*, and *A. ochraceus*; 2) to identify the main component(s) of VOCs released by yeasts; 3) to evaluate the capability of yeast VOCs to inhibit OTA production; 4) to evaluate the effect of yeast VOCs on the expression level of key genes in the OTA biosynthetic pathway.

2. Materials and Methods

2.1. Fungal and yeast strains and culture conditions

A. carbonarius Bainier Thom. MPVA566 and *A. ochraceus* G. Wilh. MPVA703 (courtesy of Professor Paola Battilani, Università Cattolica del Sacro Cuore, Piacenza, Italy) strains are maintained in the mycological collection of the Dipartimento di Agraria, Università di Sassari (Italy). The two strains were tested for their potential to produce OTA on PDA (potato dextrose agar; Sigma-Aldrich, St. Louis, MO, USA), after incubation at 25 °C for 7 days, as described by Bragulat et al. (2001).

Four yeast strains, namely two non-fermenting (*Cyberlindnera jadinii* 273 and *Candida friedrichii* 778) and two low-fermenting (*Candida intermedia* 235 and *Lachancea thermotolerans* 751) were selected based on their ability to control the growth and sporulation of *A. carbonarius* (Fiori et al., 2014) and for their OTA-adsorption properties (Farbo et al., 2016).

2.2. Inhibition of fungal growth and OTA production by yeast strains

A spore suspension (10^5 spores/mL) of each strain of *A. carbonarius* MPVA566 and *A. ochraceus* MPVA703, grown on PDA for seven days at 25 °C, was prepared in Ringer's solution containing 0.1% Tween 20 (Sigma) to prevent spore clumping.

The four yeasts were routinely grown on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar; Sigma-Aldrich, St. Louis, MO, USA) and stored at 4 °C until use. Two days before each trial, yeast were grown on PDA agar at 25 °C and a loopful of fresh cells was further grown overnight at 25°C in 100 mL YPD broth (1% yeast extract, 2% bacteriological peptone, 2% dextrose; Sigma-Aldrich, St. Louis, MO, USA). Cells were recovered by centrifugation, washed, resuspended in Ringer's solution (0.9% NaCl) and counted in a Thoma haemocytometer to obtain a final concentration of $1x10^8$ CFU/mL.

To evaluate the efficacy of yeast VOCs against *Aspergillus* spp., 100 µL of yeast cell suspension (10^8 CFU/mL) was evenly spread on YPD agar plates by using a disposable sterile inoculation loop and incubated at 25 °C for 24 h. After 24 h, the lid of the plate was replaced by a base plate containing PDA. Twenty µL of a spore suspension (10^7 CFU/mL) of each pathogen were spotted onto the centre of the PDA plate. The two base plates were sealed immediately with a double layer of Parafilm® and one layer of Scotch® tape and incubated at 25 °C for 7-10 days. The plates were examined daily to measure radial growth and any change in morphology of *Aspergillus* colonies was recorded. A control treatment was represented by plates inoculated only with *Aspergillus*. There were three replicates for each treatment and the experiment was repeated three times.

2.3. Analysis of yeast VOCs

A loopful of freshly grown yeast cells was transferred into a 20 mL amber headspace vial containing 5 mL of YPD agar slant and incubated at 26 °C for seven days. Yeast VOCs composition was qualitatively evaluated by head-space solid phase microextraction (HS-SPME) followed by gas chromatography coupled with mass spectrometry (GC-MS) analysis. Prior to extraction the vials were equilibrated under agitation for 30 min at 50 °C. The extraction of VOCs was performed on a 1-cm PDMS 50/30 Stableflex SPME fiber (Supelco, Milano, Italy). The extraction time was fixed at 30 min, after which it was desorbed for 2 min into a Gerstel CIS6 PTV injector operating at 250 °C in a splitless mode. Before and after each extraction, the fiber underwent a bake-out step for 5 min at 250 °C on a Gerstel bake-out station.

The GC-MS analysis was carried out using an Agilent 7890 GC equipped with a Gerstel MPS autosampler, coupled with an Agilent 7000C MSD detector. The chromatographic separation was performed on a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.17 µm) with the following temperature program: 60 ºC hold for 3 min, then increased to

210 ºC at a rate of 4 ºC/min, then held at 210 ºC for 15 min, then increased to 280 ºC at a rate of 10 ºC/min. Helium was used as carrier gas, at a constant flow of 1 mL/min. The data were analysed using a MassHunter Workstation B.07.00 SP1. The identification of the components was performed by comparing their retention indices with the built-in libraries or literature data and by matching their spectra on mass spectral libraries (NIST/EPA/NIH 2008).

2.4. OTA extraction and HPLC analysis

OTA production was assessed after seven days of incubation according to the method described by Bragulat et al. (2001). Briefly, three PDA agar plugs (6 mm diameter each) were removed with a sterile cork borer, across the diameter of the mycelium. Samples were weighed, placed into a 2.0 mL Eppendorf tube, 1 mL methanol was added and the tubes were briefly hand-shaken. Then, they were left still for one h, extracts were filtered through a 0.2 µm filter (Millex, Millipore Co., Bedford, Mass.) and stored at 4°C until HPLC analysis. OTA was analysed using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump with integrated vacuum degasser (G1311C), autosampler (G1329B), column oven (G1316A) and fluorescence detector (G1321A). Separation of OTA was performed on a Zorbax column SB-C18, 4.6 x 150 mm, 5-Micron (Agilent, Santa Clara, CA, USA). The isocratic mobile phase composition was a mixture of acetonitrile, water and acetic acid (49.5:49.5:1 v/v/v, respectively). The constant flow and column temperature were set to 1 mL/min and 25°C, respectively. The excitation and the emission wavelengths were set to 333 and 460 nm, respectively. OTA identification was achieved by comparing the retention time of the pure standard (5.9 min) with that of the target peak on samples. The injection volume was 100 µL. A linear external calibration over a range from 0.6 to 60 µg/L, with a mean correlation coefficient of 0.99995, was used for the quantification step. Results were then expressed as ng per g of sample.

In a preliminary experiment, a time course of mycelium growth and OTA production by *Aspergilli* cultured on PDA at 25°C, with no contact with yeast cells, was assessed by evaluating the radial growth and by analysing 3 plugs cut at 1, 2, 3 and 7 days from duplicate colonies as described previously.

2.5. Gene expression analysis in A. carbonarius MPVA566 and A. ochraceus MPVA703

The expression of different genes involved in OTA biosynthesis was evaluated in *A. carbonarius* MPVA566 (*acOTApks* and *acOTAnrps*) and in *A. ochraceus* MPVA703 (*ao-pks* and *nrps-west*) by qRT-PCR. An *acpks* gene as well as two regulatory genes (*laeA* and *veA*) were considered in *A. carbonarius*, whereas two monooxygenase genes (*p450-H11* and *p450-B03*) and a halogenase gene (*hal-west*) were evaluated in *A. ochraceus*.

The expression levels of the target genes were normalised by the expression of the reference genes *18S* and *g3pdh* for *A. carbonarius* and *A. ochraceus*, respectively, and hence expressed relatively to the control. The two genes *18S* and *g3pdh* were chosen as reference being the most stable among four potential reference genes (namely, β- tubulin, actin, *18S* and *g3pdh*) used in preliminary studies (not shown). For gene expression analysis, 20 µL of spore suspension (10^7 CFU/mL) of *A. carbonarius* MPVA566 or *A. ochraceus* MPVA703 were inoculated in PDB and incubated at 25 °C for five days as previously described. After 120 h, the mycelium was carefully harvested, ground in liquid nitrogen and stored at - 80 °C. Total RNA was extracted from frozen mycelium using the GeneMATRIX® Universal RNA Purification Kit (URx Ltd, Gdansk, Poland) following the manufacturer's protocol and then reverse-transcribed into cDNA using the iScript® cDNA Sythesis Kit (Bio-Rad, Hercules, CA, USA).

Gene-specific primers are listed in Table 1. The PCR efficiencies for each primer pair were determined by a 5-fold serial cDNA dilution of cDNA, and calculated from the slopes of the curve realised by the CFX96 Touch Real time PCR detection system software (version

3.1, 2010; Bio-Rad). The reactions were held using CFX96 Touch Real Time PCR detection system Bio-Rad (version 3.1, 2010) using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), by starting with 10 ng/µL of synthesised cDNA. The PCR programme was as follows: 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 58 °C for 1 min. The melting curve was defined by 0.5°C increments of the slow ramp rate between 55 °C and 95 °C after the real-time PCR cycles. Specificity of the PCR amplification was confirmed by dissociation curve analysis. Real time PCR derived data as normalised expression ($\Delta\Delta C_q$) were quantified by using the measured expression level of one reference gene (target) as a normalisation factor. Reaction were prepared in triplicate 96-well reaction plates, and three replicates of control sample without cDNA were also included in the runs.

2.6. Statistical analysis

Data from each experiment were subject to one-way analysis of variance (ANOVA) followed by multiple comparisons by Tukey's test, using the Statgraphics Centurion XVI software. Prior to performing ANOVA, normality of residuals was verified by the Shapiro-Wilk test, whereas assumption of homoscedasticity was checked by the Levene test.

3. Results

3.1. Yeast VOCs reduce vegetative growth and sporulation of A. carbonarius *and* A. ochraceus

All the four yeast strains were able to inhibit mycelium development of both *A. carbonarius* MPVA566 and *A. ochraceus* MPVA703. Colony diameter was reduced to 30-50% compared to the untreated control after 10 days of incubation with no contact between bionts (Figure 1).

Aspergillus spp. colonies exposed to yeast VOCs did not sporulate, and were characterised by a white mycelium. The colony border was undefined, with elongated

hyphae irregularly branched compared to unexposed control (Figure 2). Both *A. carbonarius* MPVA566 and *A. ochraceus* MPVA703, upon exposure to VOCs emitted by *L. thermotolerans* 751, produced hyphae containing coagulated cytoplasm, with swollen hyphal tips exhibiting frequent lysis (Figures 2-3).

3.2. Yeast VOCs inhibit OTA production in vitro

A preliminary experiment was set to monitor the time course of OTA production by the two *Aspergilli*. After seven days of growth on PDA medium at 25 °C, *A. carbonarius* MPVA566 produced 8,316.6 ng/g of OTA, whereas *A. ochraceus* MPVA703 produced 29,679.9 ng/g (Table 2).

Three separate experiments were set up to evaluate the ability of yeast strains to inhibit both growth and OTA production when co-cultured with *A. carbonarius* MPVA566 or *A. ochraceus* MPVA703. The reduction of radial growth after 7 days incubation on PDA at 25 °C ranged from 56 to 74%, with *C. intermedia* 253 and *L. thermotolerans* 751 being the most effective inhibitors of colony growth (Table 3).

The vegetative growth inhibition was accompanied by a striking reduction of OTA production as an effect of VOCs release by the yeast strains: in *A. carbonarius* MPVA566 the OTA content released in the medium dropped from 7,613-13,883 ng/g in the unexposed control to 0.1-135 ng/g upon exposure to yeast volatiles, whereas in *A. ochraceus* MPVA703 OTA release was reduced from 19,609-42,960 to 2.7-940 ng/g. VOCs emitted by *L. thermotolerans* 751 provided the most consistent inhibition of OTA production in both *Aspergillus* species (Table 3).

3.3. 2-phenylethanol is the main component in yeast VOCs

The HS-SPME/GC-MS analysis allowed to identify about 20 compounds, belonging to different chemical classes, such as alcohols, aldehydes, hydrocarbons and terpenes.

Table 4 summarizes, for each of the four tested yeast strains, the absolute area (x 10^6) of each analyte. Most of these volatiles showed a negligible area, and were also detected in the control headspace, possibly due to the presence of yeast extract in the YPD medium. The main component of all the yeast volatile fraction was 2-phenylethanol, which was not detectable in the YPD medium. Other volatile compounds showed a significantly lower or negligible area, compared with 2-phenylethanol; most of these compounds were also found in the control YPD agar medium headspace (Figure 4).

3.4. Aspergillus carbonarius *and* Aspergillus ochraceus *genes involved in OTA biosynthesis are downregulated by yeast VOCs*

In *A. carbonarius* MPVA566, yeast VOCs dramatically affected the expression level of all the tested OTA biosynthetic genes *Ac*OTA*pks*, *AcOTAnrps*, *acpks*, as well as the two regulatory genes *veA* and *laeA* (Figure 5). The expression of *acpks* was almost totally abolished (99%) when the fungus was co-cultured with *C. intermedia* 253, *C. jadinii* 273, *L. thermotolerans* 751 and *C. friedrichii* 778. Similarly, *acOTApks* and *AcOTAnrps* expression levels were both equally reduced by 99.9% by the four yeasts. Expression of the regulatory genes *veA* and *laeA* was affected by 97% by *C. intermedia* 253 and by 99.9% by the other three yeast strains (Figure 5).

In *A. ochraceus* MPVA703 the expression level of tested OTA biosynthetic genes was far less influenced by yeast VOCs, with the strongest reduction of *pks* expression observed when the fungus was co-cultured with *C. intermedia* 253 (37%) and *L. thermotolerans* 751 (25%). *L. thermotolerans* 751 was also the major inhibitor of *nrps* (35%) and one of the strongest repressors of H11 and B03 (both 30%) while the *hal* gene resulted the most affected by all four yeasts, with a reduction of its expression level ranging from 27% (by *L. thermotolerans* 751) to 38% (by *C. intermedia* 253; Figure 6).

4. Discussion

The presence of OTA in food and feed chain is a major concern for food safety and health authorities (Ponsone et al., 2011). In a previous investigation (Fiori et al., 2014), we proved the high antagonistic efficacy of two non-fermenting (*Cyberlindnera jadinii* 273 and *Candida friedrichii* 778) and two low-fermenting yeasts (*Candida intermedia* 235 and *Lachancea thermotolerans* 751) in controlling *A. carbonarius in vitro* and on grape berries. The biological effect was at least partly ascribed to the release of VOCs. The present work focuses on the chemical composition and role of VOCs produced by these four yeast strains and on their inhibitory effect on *A. carbonarius* and *A. ochraceus* growth and sporulation, OTA production, and OTA biosynthetic gene expression.

Since the first exploitation in biocontrol of the VOCs producer *Muscodor albus* (Worapong et al., 2001; Strobel et al., 2001), a panoply of VOCs produced by isolated soil microbes showed antifungal activity against plant pathogens (Kanchiswamy et al., 2015; Werner et al., 2016; Zeilinger et al., 2016). Many biocontrol yeast strains have the ability to produce VOCs and in some instances the volatiles were shown to be implicated as main drivers of the antagonistic capacity. For instance, VOCs emitted by *Candida intermedia*, *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima* inhibited *Botrytis cinerea* growth *in vitro* and reduced disease on strawberries inoculated with this pathogen (Suwannarach et al., 2010; Huang et al., 2011; Parafati et al., 2015). A mixture of VOCs produced by *Candida sake* was able to reduce the incidence of apple rot caused by *Penicillium expansum* and *B. cinerea* (Arrarte et al., 2017). VOCs released by different strains of *Pichia anomala, Pichia kluyveri*, and *Hanseniaspora uvarum* inhibited *A. ochraceus* growth and OTA production during processing of *Coffea arabica* (Masoud et al., 2005; Masoud & Kaltoft, 2006). On the contrary, the role of VOCs released by biocontrol strains of *Candida*

zemplinina, *Saccharomyces cerevisiae*, *P. kluyveri*, and *Metschnikowia aff. fructicola* in the *in vitro* and *in vivo* inhibition of *A. carbonarius* was not proven (Zhu et al., 2015).

The present study demonstrates that the production of VOCs, and particularly 2-phenylethanol, plays an essential role in the antagonistic activity of four yeasts strains against *A. carbonarius* MPVA566 and *A. ochraceus* MPVA703. All the experiments were performed under airtight conditions, and any contact between *Aspergilli* and yeast strains was prevented. Therefore, the antifungal activity of yeasts can be definitely attributed to their VOCs release. While all the four yeast tested were biologically active, two strains (*C. intermedia* 253 and *L. thermotolerans* 751), were most effective in reducing mycelial growth, sporulation and *in vitro* OTA production by *A. carbonarius* and *A. ochraceus*. Remarkably, in both *Aspergilli*, hyphae containing coagulated cytoplasm, with swollen hyphal tips and frequent lysis were observed upon exposure to *L. thermotolerans* 751 volatilome. To the best of our knowledge, this study reveals for the first time the ability of the yeast species *C. jadinii*, *C. friedrichii*, *C. intermedia* and *L. thermotolerans* to reduce the production of OTA by two *Aspergilli* through the release of VOCs.

The GC-MS analysis allowed us to identify several compounds, including alcohols, aldehydes, hydrocarbons and terpenes, with a marked variability on their absolute area. The foremost compound detected in our work, 2-phenylethanol, has been previously observed as the main volatile produced by other yeasts, such as *S. cerevisiae* with a potential to control the pathogen *Sclerotinia sclerotiorum in vitro* and in bean seeds (Fialho et al., 2010), *Kloeckera apiculata* controlling *P. italicum* mold in citrus fruit (Liu et al., 2014), and *P. anomala* able to inhibit spore germination and aflatoxin production by *Aspergillus flavus* (Chang et al. 2015; Hua et al., 2014). This compound is present in nature, particularly in extracts of perfumed flowers and essential oils, and represents one of the nine most dominant volatile compounds of the rose scent (Yan et al. 2011). Chang et al. (2015) reported that 2-phenylethanol has a lethal effect against *A. flavus* and inhibits

365 the production of aflatoxin at sublethal dosage. High concentrations of 2-phenylethanol
366 may cause alterations in amino acid and protein biosynthesis, in mitochondria and the
367 nuclei of both fungi and bacteria (Rosenkranz, et al., 1965; Liu et al., 2014).
368 Our results indicate that yeast volatiles are able to downregulate key genes implicated in
369 OTA biosynthesis. In *A. carbonarius*, so far the best described OTA-producing *Aspergillus*
370 species, expression of all the tested OTA biosynthetic (*Ac*OTA*pks*, *AcOTAnrps*, *acpks*) as
371 well as the two regulatory genes *veA* and *laeA* was almost completely suppressed by
372 yeast VOCs. In *A. ochraceus*, instead, knowledge of the OTA biosynthetic gene
373 organisation is less understood. Therefore, alongside the polyketide synthase gene *pks*, a
374 non-ribosomal peptide synthase *nrps* and a halogenase protein that could be responsible
375 for chlorination in OTA biosynthesis (Gil-Serna et al., 2018) were evaluated in the present
376 study using primers previously tested on the phylogenetically similar species *A.*
377 *westerdijkiae*. In addition, we tested the expression of two putative p450 type
378 monooxygenase genes, namely *p450-H11* and *p450-B03*, known for having a role in other
379 mycotoxin biosynthetic pathways (O'Callaghan et al., 2006). Compared to *A. carbonarius*,
380 the expression level of tested OTA biosynthetic genes in *A. ochraceus* was less affected,
381 with inhibition levels comprised between 25 and 38%. Whether this discrepancy indicates
382 a differential sensitivity to VOCs by the two species/tested isolates or is dependent on the
383 primers used is still questionable.
384 In a previous report (Chang et al., 2015) based on *A. flavus* exposed to low concentrations
385 of 2-phenylethanol, this compound decreased the aflatoxin production by reducing the
386 expression levels of all aflatoxin gene pathway. This specific effect on secondary
387 metabolism may explain the fact that while vegetative growth was reduced by 50-70%,
388 OTA release in the medium was almost completely repressed upon exposure to yeast
389 VOCs.

Overall, the present report provides additional evidence to the suitability of selected yeast strains able to produce VOCs as important tools for postharvest management of mycotoxin-producing fungi. Acting as biodegradable fumigants, VOCs leave no residues and do not need any direct contact between yeast cells and the surface of the target commodity (Parafati et al. 2017). To further develop yeast VOCs application in postharvest disease control, it will be crucial to increase their stable production over time by identifying optimal growing and storage conditions. Moreover, there is a need to develop new carriers for efficient and inexpensive application and compatibility with standard postharvest handling pipeline (Parafati et al. 2017). Finally, those compounds that are exclusively responsible for fungal inhibition should be further characterised to allow the design of improved artificial VOCs mixtures with high efficiency and low toxicity towards non-target organisms.

Acknowledgments

This publication was made possible by NPRP grant # 8-392-4-003 from the Qatar National Research Fund (a member of Qatar Foundation). The findings achieved herein are solely the responsibility of the authors. We acknowledge constructive comments provided by anonymous reviewers.

References

Abarca, M.L., Accensi, F., Bragulat, M.R., Castella, G., Cabañes, F.J., 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. J. Food Prot. 66, 504-506.

414 Abbas, A., Valez, H., Dobson, A.D.W., 2009. Analysis of the effect of nutritional factors on
415 OTA and OTB biosynthesis and polyketide synthase gene expression in *Aspergillus*
416 *ochraceus*. Int. J. Food Microbiol. 135, 22-27.
417 Arrarte, E., Garmendia, G., Rossini, C., Wisniewski, M., Vero, S., 2017. Volatile organic
418 compounds produced by Antarctic strains of *Candida sake* play a role in the control of
419 postharvest pathogens of apples. Biol. Control 109, 14-20.
420 Bacha, N., Atoui, A., Mathieu, F., Liboz, T., Lebrihi, A., 2009. *Aspergillus westerdijkiae*
421 polyketide synthase gene “aoks1” is involved in the biosynthesis of ochratoxin A. Fungal
422 Genet. Biol. 46, 77-84.
423 Bayram, Ö., Krappmann, S., Ni, M., Bok, J.W., Helmstaedt, K., Valerius, O., Braus-
424 Stromeyer, S., Kwon, N.J., Keller, N.P., Yu, J.H., Braus, G.H., 2008. VelB/VeA/LaeA
425 complex coordinates light signal with fungal development and secondary metabolism.
426 Science 320, 1504-1506.
427 Bleve, G., Grieco, F., Cozzi, G., Logrieco, A., Visconti, A., 2006. Isolation of epiphytic
428 yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. Int. J.
429 Food Microbiol. 108, 204–209.
430 Bok, J.W., Keller, N.P., 2004*. LaeA*, a regulator of secondary metabolism in *Aspergillus*
431 *spp*. Eukaryot. Cell 3, 527-535.
432 Bragulat, M.R., Abarca, M.L., Cabañes, F.J., 2001. An easy screening method for fungi
433 producing ochratoxin A in pure culture. Int. J. Food Microbiol. 71, 139-144.
434 Buzzini, P., Romani, S., Turchetti, B., Vaughan, A., Pagnoni, U.M., Davoli, P. 2005.
435 Production of volatile organic sulfur compounds (VOCs) by basidiomycetous yeasts.
436 FEMS Yeast Res. 5, 379-385.
437 Cabañes, F.J., Bragulat, M.R., Castellá, G., 2013. Characterization of nonochratoxigenic
438 strains of *Aspergillus carbonarius* from grapes. Food Microbiol. 36, 135-141.

439 Castegnaro, M., Mohr, U., Pfohl-Leszkowicz, A., Estève, J., Steinmann, J., Tillmann, T.,
440 Michelon, J., Bartsch, H., 1998. Sex- and strain-specific induction of renal tumors by
441 ochratoxin A in rats correlated with DNA adduction. Int. J. Cancer 77, 70-75.
442 Chang, P.K., Hua, S.S.T., Sarreal, S.B.L., Li, R.W., 2015. Suppression of aflatoxin
443 biosynthesis in *Aspergillus flavus* by 2-phenylethanol is associated with stimulated growth
444 and decreased degradation of branched-chain amino acids. Toxins 7, 3887-3902.
445 Crespo-Sempere, A., Marin, S., Sanchis, V., Ramos, A.J., 2013. Vea and LaeA
446 transcriptional factors regulate ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J.
447 Food Microbiol. 166, 479-486.
448 Cubaiu, L., Abbas, H., Dobson, A.D.W., Budroni, M., Migheli, Q., 2012. A *Saccharomyces*
449 *cerevisiae* wine strain inhibits growth and decreases ochratoxin A biosynthesis by
450 *Aspergillus carbonarius* and *Aspergillus ochraceus*. Toxins 4, 1468-1481.
451 De Curtis, F., de Felice, D.V., Ianiri, G., De Cicco, V., Castoria, R., 2012. Environmental
452 factors affect the activity of biocontrol agents against ochratoxigenic *Aspergillus*
453 *carbonarius* on wine grape. Int. J. Food Microbiol. 159, 17-24.
454 Di Francesco, A., Ugolini, L., Lazzeri, L., Mari, M., 2015. Production of volatile organic
455 compounds by *Aureobasidium pullulans* as a potential mechanism of action against
456 postharvest fruit pathogens. Biol. Control 81, 8-14.
457 Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest
458 biocontrol research: Is it time for a new paradigm? Postharvest Biol. Technol. 52, 137-145.
459 Duarte, S.C., Pena, A., Lino, C.M., 2010. A review on ochratoxin A occurrence and effects
460 of processing of cereal and cereal derived food products. Food Microbiol. 27, 187-198.
461 European Commission (EC), 2012. Rapid Alert System for Food and Feed (RASFF).
462 Annual Report. Directorate-General for Health and Consumers. Available:
463 http://ec.europa.eu/food/food/rapidalert/docs/rasff_annual_report_2012_en.pdf.

Farbo M.G., Urgeghe P.P., Fiori S., Marceddu S., Jaoua S., Migheli Q., 2016. Adsorption of ochratoxin A from grape juice by yeast cells immobilised in calcium alginate beads. Int. J. Food Microbiol. 217, 29-34.

Fialho, M.B., Toffano, L., Pedroso, M.P., Augusto, F., Pascholati, S.F., 2010. Volatile organic compounds produced by *Saccharomyces cerevisiae* inhibit the *in vitro* development of *Guignardia citricarpa*, the causal agent of citrus black spot. World J. Microbiol. Biotechnol. 26, 925-932.

Fiori, S., Urgeghe, P.P., Hammami, W., Razzu, S., Jaoua, S., Migheli, Q., 2014. Biocontrol activity of four non- and low-fermenting yeast strains against *Aspergillus carbonarius* and their ability to remove ochratoxin A from grape juice. Int. J. Food Microbiol. 189, 45-50.

Gallo, A., Perrone, G., Solfrizzo, M., Epifani, F., Abbas, A., Dobson, A.D., Mulè, G., 2009. Characterisation of a *pks* gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. Int. J. Food Microbiol. 129, 8-15.

Gallo, A., Bruno, K.S., Solfrizzo, M., Perrone, G., Mulè, G., Visconti, A., Baker, S.E., 2012. New insight into the ochratoxin A biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. Appl. Environ. Microbiol. 78, 8208-8218.

Gallo, A., Knox, B.P., Bruno, K.S., Solfrizzo, M., 2014. Identification and characterization of the polyketide synthase involved in ochratoxin A biosynthesis in *Aspergillus carbonarius*. Int. J. Food Microbiol. 179, 10-17.

Geisen, R., Schmidt-Heydt, M., Karolewiez, A., 2006. A gene cluster of the ochratoxin A biosynthetic genes in *Penicillium*. Mycotoxin Res. 22, 134-141.

Gil-Serna, J., García-Díaz, M., González-Jaén, M.T., Vázquez, C., Patiño, B., 2018. Description of an orthologous cluster of ochratoxin A biosynthetic genes in *Aspergillus* and *Penicillium* species. A comparative analysis. Int. J. Food Microbiol. 268, 35-43.

Hua, S.S.T., Beck, J.J., Sarreal, S.B. L., Gee, W., 2014. The major volatile compound 2-phenylethanol from the biocontrol yeast, *Pichia anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*. Mycotoxin Res., 30, 71-78.

Huang, R., Li, G.Q., Zhang, J., Yang, L., Che, H.J., Jiang, D.H., Huang, H.C., 2011. Control of postharvest *Botrytis* fruit rot of strawberry by volatile organic compounds of *Candida intermedia*. Phytopathology 101, 859-869.

Huang, R., Che, H.J., Zhang, J., Yang, L., Jiang, D.H., Li, G.Q., 2012. Evaluation of *Sporidiobolus pararoseus* strain YCXT3 as biocontrol agent of *Botrytis cinerea* on post-harvest strawberry fruits. Biol. Control 62, 53-63.

Huff, W.E., Hamilton, P.B., 1979. Mycotoxins - Their biosynthesis in fungi: ochratoxins-metabolites of combined pathways. J. Food Prot. 42, 815-820.

Huffman, J., Gerber, R., Du, L., 2010. Recent advancements in the biosynthetic mechanisms for polyketide-derived mycotoxins. Biopolymers 93, 764-776.

IARC, 1993. Ochratoxin A. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. 56. IARC, Lyon, France.

Janisiewicz, W.J., Kurtzman, C.P., Buyer, J.S., 2010. Yeasts associated with nectarines and their potential for biological control of brown rot. Yeast 27, 389-398.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2008. Safety Evaluation of Certain Mycotoxins in Food. WHO Food Additives Series 59. Available http://whqlibdoc.who.int/publications/2008/9789241660594_eng.pdf.

Kanchiswamy, C.N., Malnoy, M., Maffei, M.E., 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Front. Plant. Sci. 6, 151.

Karolewiez, A., Geisen, R., 2005. Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and characterization of the ochratoxin polyketide synthase gene. Syst. Appl. Microbiol. 28, 588-595.

515 Kogkaki, E.A., Natskoulis, P.I., Magan, N., Panagou, E.Z., 2015. Effect of interaction
516 between *Aspergillus carbonarius* and non-ochratoxigenic grape-associated fungal isolates
517 on growth and ochratoxin A production at different water activities and temperatures. Food
518 Microbiol. 46, 521-527.
519 Krogh, P., 1978. Casual associations of mycotoxic nephropathy. Acta Path. Micro. Im. A
520 269, 1-28.
521 Linde, T., Zoglowek, M., Lübeck, M., Frisvad, J.C., Lübeck, P.S., 2016. The global
522 regulator LaeA controls production of citric acid and endoglucanases in *Aspergillus*
523 *carbonarius*. J. Ind. Microbiol. Biotechnol. 43, 1139-1147.
524 Liu, J., Sui, Y., Wisniewski, M., Droby, S., Liu, Y., 2013. Review: Utilization of antagonistic
525 yeasts to manage postharvest fungal diseases of fruit. Int. J. Food Microbiol. 167, 153-
526 160.
527 Liu, P., Cheng, Y., Yang, M., Liu, Y., Chen, K., Long, C.A., Deng, X., 2014. Mechanisms of
528 action for 2-phenylethanol isolated from *Kloeckera apiculata* in control of *Penicillium* molds
529 of citrus fruits. BMC Microbiol. 14, 242.
530 Maffei, M.E., Gertsch, J., Appendino, G., 2011. Plant volatiles: production, function and
531 pharmacology. Nat. Prod. Rep. 28, 1359-1380.
532 Malandrakis, A.A., Vattis, K.N., Doukas, E.G., Markoglou, A.N., 2013. Effect of
533 phenylpyrrole-resistance on fitness parameters and ochratoxin production in *Aspergillus*
534 *carbonarius*. Int. J. Food Microbiol. 165, 287-294.
535 Masoud, W., Poll, L., Jakobsen, M., 2005. Influence of volatile compounds produced by
536 yeasts predominant during processing of *Coffea arabica* in East Africa on growth and
537 ochratoxin A (OTA) production by *Aspergillus ochraceus*. Yeast 22, 1133-1142.
538 Masoud, W., Kaltoft, C.H., 2006. The effects of yeasts involved in the fermentation of
539 *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus*
540 *ochraceus*. Int. J. Food Microbiol. 106, 229-234.

Morath, S.U., Hung, R., Bennett, J.W., 2012. Fungal volatile organic compounds: a review with emphasis on their biotechnological potential. Fungal Biol. Rev. 26, 73-83.

Niessen, L., Schmidt, H., Mühlencoert, E., Färber, P., Karolewiez, A., Geisen, R., 2005. Advances in the molecular diagnosis of ochratoxin A–producing fungi. Food Addit. Contam. 22, 324-334.

O'Callaghan, J., Caddick, M.X., Dobson, A.D.W., 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. Microbiology 149, 3485-3491.

O'Callaghan, J., Stapleton, P.C., Dobson, A.D.W., 2006. Ochratoxin A biosynthetic genes in *Aspergillus ochraceus* are dfferentially regulated by pH and nutritional stimuli. Fungal Genet. Biol. 43, 213-221.

O'Callaghan, J., Coghlan, A., Abbas, A., García-Estrada, C., Martín, J.F., Dobson, A.D.W., 2013. Functional characterization of the polyketide synthase gene required for ochratoxin A biosynthesis in *Penicillium verrucosum*. Int. J. Food Microbiol. 161, 172-181.

Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G., 2015. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. Food Microbiol. 47, 85-92.

Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G., 2017. Performance evaluation of volatile organic compounds by antagonistic yeasts immobilized oh hydrogel spheres against gray, green and blue postharvest decays. Food Microbiol. 63, 191-198.

Passoth, V., Fredlund, E., Druvefors, U.Ä., Schnürer, J., 2006. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. 6, 3-13.

Ponsone, M.L., Chiotta, M.L., Combina, M., Dalcero, A., Chulze, S., 2011. Biocontrol as a strategy to reduce the impact of ochratoxin A and *Aspergillus* section *Nigri* in grapes. Int. J. Food Microbiol. 151, 70-77.

566 Romano, A., Capozzi, V., Spano, G., Biasioli, F., 2015. Proton transfer reaction-mass
567 spectrometry: online and rapid determination of volatile organic compounds of microbial
568 origin. Appl. Microbiol. Biotechnol. 99, 3787-3795.
569 Rosenkranz, H.S., Carr, H.S., Rose, H.M., 1965. Phenethyl alcohol. I. Effect on
570 macromolecular synthesis of *Escherichia coli*. J. Bacteriol. 89, 1354-1369.
571 Schmidt-Heydt, M., Stoll, D., Geisen, R., 2013. Fungicides effectively used for growth
572 inhibition of several fungi could induce mycotoxin biosynthesis in toxigenic species. Int. J.
573 Food Microbiol. 166, 407-412.
574 Strobel, G.A., Dirkse, E., Sears, J., Markworth, C., 2001. Volatile antimicrobials from
575 *Muscodor albus*, a novel endophytic fungus. Microbiology 147, 2943-2950.
576 Suwannarach, N., Bussaban, B., Hyde, K.D., Lumyong, S., 2010. *Muscodor cinnamon*, a
577 new endophytic species from *Cinnamomum bejolghota*. Mycotaxon 114, 15-23.
578 The National Center for Biotecnology Information advances science and health by
579 providing access to biomedical and genomic information. Available online: http://
580 www.ncbi.nlm.nih.gov/
581 Van den Doo, H., Kratz, P.D., 1963. A generalization of the retention index system
582 including linear temperature programmed gas-liquid partition chromatography. J.
583 Chromatography A 11, 463-471.
584 Van der Merwe, K.J., Steyn, P.S., Fourie, L., Scott, D.B., Theron, J.J., 1965. Ochratoxin A,
585 a toxic metabolite produced by *Aspergillus ochraceus* Wilh. Nature 205, 1112-1113.
586 Wang, L., Wang, Y., Wang, Q., Liu, F., Selvaraj, J.N., Liu, L., Xing, F., Zhao, Y., Zhou, L.,
587 Liu, Y., 2015. Functional characterization of new polyketide synthase genes involved in
588 ochratoxin A biosynthesis in *Aspergillus ochraceus* fc-1. Toxins 7, 2723-2738.
589 Werner, S., Polle, A., Brinkmann, N., 2016. Belowground communication: impacts of
590 volatile organic compounds (VOCs) from soil fungi on other soil-inhabiting organisms.
591 Appl. Microbiol. Biotechnol. 100, 8651-8665.

592 Worapong, J., Strobel, G.A., Ford, E.J., Li, J.Y., Baird, G., Hess, W.M., 2001. *Muscodor*
593 *albus* anam. gen. et sp. nov., an endophyte from *Cinnamomum zeylanicum*. Mycotaxon
594 79, 67-79.
595 Wriessnegger, T., Pichler, H., 2013. Yeast metabolic engineering-targeting sterol
596 metabolism and terpenoid formation. Prog. Lipid Res. 52, 277-293.
597 Yan, H., Zhang, H., Wang, Q., Jian, H., Qiu, X., Wang, J., Tang, K., 2011. Isolation and
598 identification of a putative scent-related gene RhMYB1 from rose. Mol. Biol. Rep. 38,
599 4475-4482.
600 Zeilinger, S., Gruber, S., Bansal, R., Mukherjee, P.K., 2016. Secondary metabolism in
601 *Trichoderma* - Chemistry meets genomics. Fungal Biol. Rev. 30, 74-90.
602 Zhang, H., Apaliya, M.T., Mahunu, G.K., Chen, L., Li, W., 2016. Control of ochratoxin A-
603 producing fungi in grape berry by microbial antagonists: a review. Trends Food Sci. Tech.
604 51, 88-97.
605 Zhu, C., Shi, J., Jiang, C., Liu, Y., 2015. Inhibition of the growth and ochratoxin A
606 production by *Aspergillus carbonarius* and *Aspergillus ochraceus in vitro* and *in vivo*
607 through antagonistic yeasts. Food Control 50, 125-132.
608

609 **Figure captions**

610 Figure 1. Biocontrol activity of four yeast strains (253, *Candida intermedia*; 273,
611 *Cyberlindnera jadinii*; 751, *Lachancea thermotolerans*; 778, *Candida friedrichii*) against
612 *Aspergillus ochraeus* (A) or *Aspergillus carbonarius* (B). Results are expressed as colony
613 diameter (cm) over 10 days of incubation at 25 °C. Vertical bars indicate standard error (n
614 = 5).

615

616 Figure 2. Colony growth and morphology of *Aspergillus carbonarius* MPVA566 (A) and
617 *Aspergillus ochraceus* MPVA703 (F) after seven days of incubation on PDA alone, or in
618 the presence of VOCs produced by yeast strains *Candida intermedia* 253 (B, G),
619 *Cyberlindnera jadinii* 273 (C, H), *Lachancea thermotolerans* 751 (D, I), or *Candida*
620 *friedrichii 778* (E, J). Microscopic detail (10X) of the colony margin is displayed below each
621 *Aspergillus*-yeast combination.

622 Figure 3. Microphotography (40X) of *A. ochraceus* MPVA703 marginal hyphae exposed to
623 VOCs emitted by *Lachancea thermotolerans* 751: the presence of coagulated cytoplasm
624 (white arrows) and swollen hyphal tips exhibiting lysis (black arrows) is shown.

625 Figure 4. HS-SPME/GC-MS chromatograms of yeast strains, highlighting the peak of 2-
626 phenylethanol, the main compound released by the *Candida friedrichii* 778 (A), *Lachancea*
627 *thermotolerans* 751 (B), *Candida intermedia* 235 (C), *Cyberlindnera jadinii* 273 (D) and not
628 detectable in YPD medium (E).

629

630 Figure 5. Relative expression analysis by qRT-PCR of *Ac*OTA*nrps*, *Ac*OTA*pks*, *acpks*,
631 *laeA* and *veA* genes in *A. carbonarius* MPVA566 after 5 days growth on PDB at 25 °C.

Figure 6. Relative expression analysis by qRT-PCR of *pks*, *nrps*, *p450-H11*, *p450-B03* and *hal* genes in *A. ochraceus* MPVA703 after 5 days growth on PDB at 25 °C.

658 Table 1. List of the primers used in this study.

Primer name	Sequence (5'→3')	References
acpks-F	GAGTCTGACCATCGACACGG	Gallo *et al*., 2009
acpks-R	GGCGACTGTGACACATCCAT	
acOTApks-F	CGTGTCCGATACTGTCTGTGA	Gallo *et al*., 2014
acOTApks-R	GCATGGAGTCCTCAAGAACC	
acOTAnrps-F	ATCCCCGGAATATTGGCACC	Gallo *et al*., 2012
acOTAnrps-R	CCTTCGATCAAGAGCTCCCC	
laeA-F	CACCTATACAACCTCCGAACC	Crespo-Sempere *et al*., 2013
laeA-R	GGTTCGGCCAACCGACGACGC	
veA-F	TCCCGGTTCTCACAGGCGTA	Crespo-Sempere *et al*., 2013
veA-R	GCTGTCCTTGGTCTCCTCGTA	
18S-F	GCAAATTACCCAATCCCGAC	NCBI
18S-R	GAATTGCCGCGGCTGCTG	
ao-pks2-F	TTCTCTACTGCGCTTCTCACATC	O'Callaghan *et al.,* 2006
ao-pks2-R	AACATCATAGCCATAAGAGGTCAACA	
nrps-west-F	GCTTGCTGACAAGCCGATGAC	Gil-Serna *et al.,* 2018
nrps-west-R	GGTCGTCAGTCGCTCATCCA	
ao-p450-B03-F	CTCGGTGACATCAGGGGTATC	O'Callaghan *et al.,* 2006
ao-p450-B03-R	AGCGTATTCAGTCACTCATTCAGA	
ao-p450-H11-F	AGAACGGGATGCCAAAACAGTGAG	O'Callaghan *et al.,* 2006
ao-p450-H11-R	AAGAATGCGAGGGATGGGATAACC	
hal-west-F	AGGAGGGAGAGGATGGGTTC	Gil-Serna *et al.,* 2018
hal-west-R	GCTCTTGCTGAAGGCGACAG	
g3pdh-F	TCGTCAACGGCAAGAAGATT	O'Callaghan *et al.,* 2006
g3pdh-R	TAGCAAGGGGAGCAAGGCAGT	

659

660

661

Table 2. Time course of OTA production by *Aspergillus carbonarius* MPVA566 and *Aspergillus ochraceus* MPVA703 incubated on PDA medium for 1-7 days. Results are expressed as colony diameter (cm) and OTA concentration (ng/g) ± standard error. Values in the same column followed by different letters indicate significant differences as per Tukey's test ($P < 0.05$).

Day	Colony diameter (cm)		OTA (ng/g)	
	A.carbonarius	*A.ochraceus*	*A.carbonarius*	*A.ochraceus*
1	1.2 ± 0	0.9 ± 0	10.2 ± 3.0^{c}	13.7 ± 3.1^{b}
2	1.8 ± 0	1.4 ± 0	111.7 ± 36.1^{c}	29.6 ± 1.0^{b}
3	2.3 ± 0	2.0 ± 0	3,059.9 ± 25.5^{b}	4,624.8 ± 226.6^{b}
7	4.6 ± 0	5.0 ± 0	8,316.6 ± 90.4^{a}	29,679.9 ± 2,421.8^{a}

Table 3. Growth of *Aspergillus carbonarius* MPVA566 and *Aspergillus ochraceus* MPVA703 and their ochratoxin A (OTA) production in PDA medium when incubated alone or in co-culture with antagonistic yeast strains after seven days at 25 °C. Data from three independent experiments are expressed as the colony diameter (cm), whereas OTA production is expressed in ng/g ± standard error. Values in the same column followed by different letters indicate significant differences as per Tukey's test ($P < 0.05$).

Tested combinations	Experiment 1		Experiment 2		Experiment 3	
	Colony diameter (cm)	OTA (ng/g)	Colony diameter (cm)	OTA (ng/g)	Colony diameter (cm)	OTA (ng/g)
A. carbonarius	5 ± 0^{a}	13883 ± 2197	5 ± 0^{a}	7866 ± 1214^{a}	5 ± 0^{a}	7613.9 ± 643^{a}
C. intermedia 253	1.3 ± 0.2^{c}	< 0.9	2.8 ± 0.2^{bc}	135 ± 16.3^{b}	2.5 ± 0.2^{b}	0.1 ± 0.1^{b}
C. jadinii 273	2.2 ± 0.3^{b}	8.9 ± 1.5	3.2 ± 0.2^{bc}	93.4 ± 28.1^{b}	2.3 ± 0.2^{b}	13.7 ± 6.0^{b}
L. thermotolerans 751	1.9 ± 0.1^{bc}	< 0.4	3.4 ± 0.1^{b}	13.6 ± 15.5^{b}	2.2 ± 0.2^{b}	< 0.8
C. friedrichii 778	2.1 ± 0.3^{b}	< 0.2	2.6 ± 0.1c	51 ± 14.3^{b}	2.0 ± 0.3^{b}	2.4 ± 1.4^{b}
A. ochraceus	4.5 ± 0^{a}	42960 ± 4112^{a}	4.5 ± 0^{a}	28269 ± 3811^{a}	4.5 ± 0^{a}	19609 ± 10492^{a}
C. intermedia 253	1.3 ± 0.1^{c}	3.2 ± 0.1^{b}	1.7 ± 0.1^{b}	14.1 ± 6.8^{b}	1.7 ± 0.2^{b}	4.5 ± 0.8^{a}
C. jadinii 273	1.6 ± 0.1^{b}	2.7 ± 0.1^{b}	2.2 ± 0.2^{b}	939.7 ± 145.9^{b}	1.8 ± 0.2^{b}	75.2 ± 31.7^{a}
L. thermotolerans 751	1.4 ± 0.1^{bc}	3.0 ± 0.3^{b}	1.9 ± 0.1^{b}	7.5 ± 2.3^{b}	1.6 ± 0.3^{b}	5.0 ± 0.5^{a}
C. friedrichii 778	1.5 ± 0^{bc}	3.4 ± 0.8^{b}	2.1 ± 0.1^{b}	50.3 ± 16.0^{b}	1.3 ± 0.3^{b}	5.3 ± 0.8^{a}

675 Table 4. VOCs produced by yeast strains.

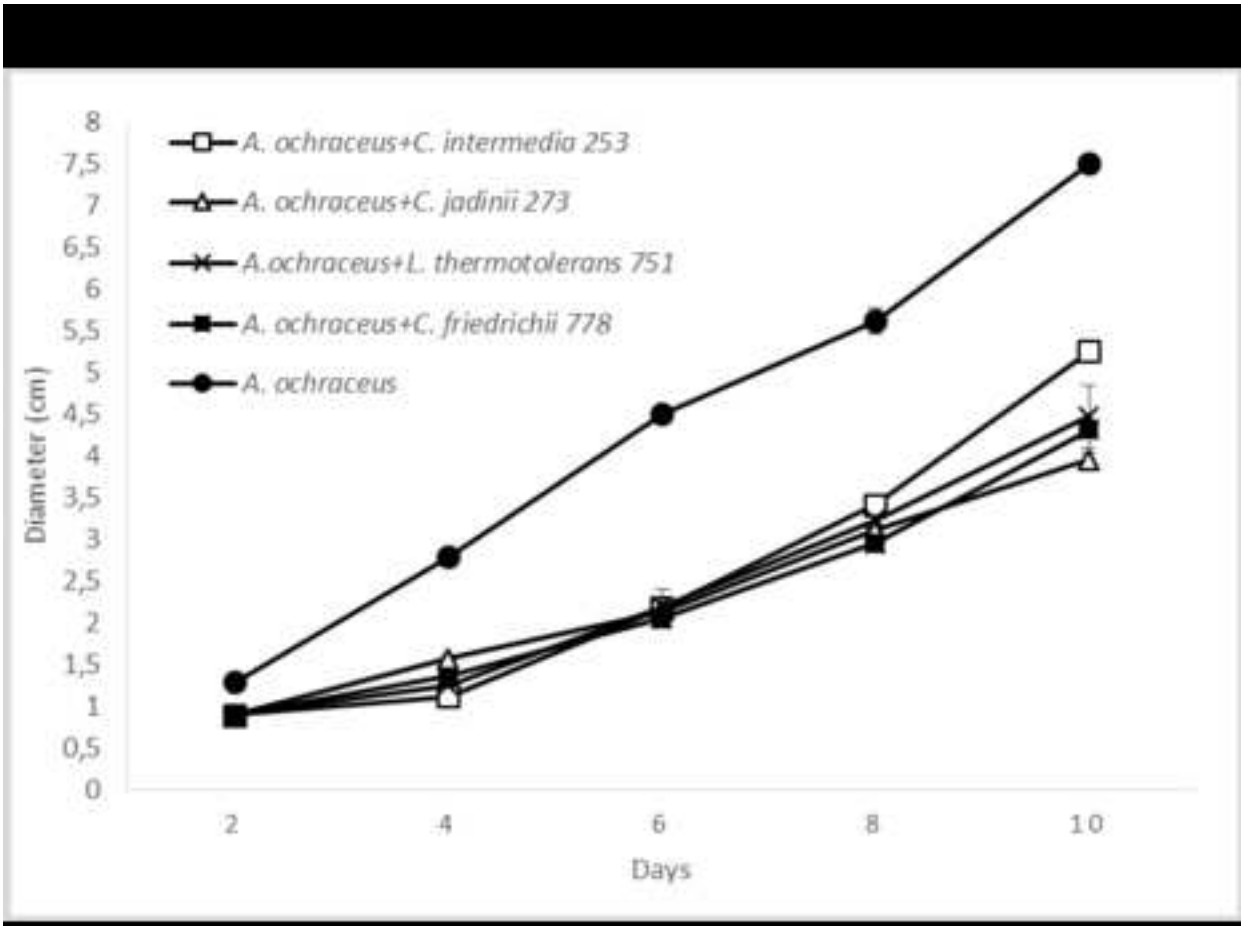
Cpd ID	Name	LRI	Area (x10^6, n=3)											
			273			253			751			778			YPD		
1	Methanethiol	641	0.44	±	0.09	1.50	±	1.08	0.87	±	0.46	0.72	±	0.59	0.13	±	0.11
2	Ethanol	643	0.32	±	0.13^{ab}	0.26	±	0.05^{b}	ND			0.21	±	0.02^{b}	0.61	±	0.17^{a}
3	2-Butanone	663	ND			0.23	±	0.07	0.17	±	0.10	0.21	±	0.06	0.27	±	0.10
4	3-Methylbuthanal	681	ND			0.13	±	0.03	0.38	±	0.21	0.22	±	0.05	0.51	±	0.26
5	3-Methyl-butanol	726	11.80	±	4.30	10.30	±	11.19	20.06	±	10.75	9.99	±	3.41	7.57	±	0.54
6	2-Methylbutanol	729	3.50	±	2.21	Tr			4.93	±	3.79	3.09	±	0.93	4.16	±	0.48
7	Dimethyl disulfide	737	0.50	±	0.22^{b}	3.00	±	1.37^{a}	tr			0.76	±	0.13^{b}	0.93	±	0.97^{ab}
8	2-Heptanone	883	ND			1.67	±	0.95	0.71	±	0.47	0.71	±	0.13	0.90	±	0.65
9	2-Heptanol	895	0.92	±	0.73	Tr			0.46	±	0.34	0.44	±	0.03	ND		
10	2,5-Dimethylpyrazine	905	8.27	±	2.80^{ab}	7.20	±	1.07^{b}	7.36	±	1.09^{ab}	8.05	±	0.75^{ab}	11.52	±	1.23^{a}
11	Benzaldehyde	950	0.52	±	0.14^{b}	0.49	±	0.06^{b}	1.07	±	0.63^{ab}	1.83	±	0.51^{a}	1.23	±	0.54^{ab}
12	D-Limonene	1019	0.45	±	0.10	0.49	±	0.13	0.41	±	0.02	0.52	±	0.05	1.02	±	1.23
13	2-Ethylhexanol	1024	7.76	±	2.38^{ab}	4.17	±	0.74^{b}	6.49	±	2.24^{ab}	9.95	±	1.37^{a}	10.82	±	0.11^{a}
14	Benzeneacetaldehyde	1035	0.39	±	0.02^{b}	7.09	±	5.37^{a}	2.28	±	0.98^{ab}	1.66	±	0.57^{ab}	0.68	±	0.39^{b}
15	Acetophenone	1057	0.25	±	0.13^{c}	1.08	±	0.43^{ab}	0.30	±	0.11^{c}	0.50	±	0.09^{bc}	1.42	±	0.28^{a}
16	3-ethyl-2,5-dimethylpyrazine	1072	3.02	±	0.14	3.31	±	0.32	2.66	±	1.23	3.48	±	0.22	3.35	±	0.16
17	2-Phenylethanol	1110	508.67	±	57.46	499.46	±	136.90	581.97	±	283.88	306.55	±	32.89	ND		
18	Camphor	1134	2.52	±	0.05	2.16	±	0.16	1.56	±	1.07	2.46	±	0.22	2.94	±	0.71
19	2,5-dimethyl-3-(3-methylbutyl)pyrazine	1310	0.31	±	0.06	0.39	±	0.07	0.26	±	0.08	0.33	±	0.03	0.26	±	0.02
20	Nerylacetone	1439	ND			0.94	±	0.08	1.13	±	0.93	1.29	±	0.31	ND		
21	Stilbene	1495	0.43	±	0.12^{a}	0.38	±	0.03^{a}	0.21	±	0.14^{b}	0.30	±	0.01^{ab}	0.31	±	0.06^{ab}

676

Presented data show mean ± standard deviation of the mean for 3 replicate experiments, for each yeast. Values within a row not sharing a common superscript differ significantly ($P < 0.05$).

Figure 1

Click here to download high resolution image



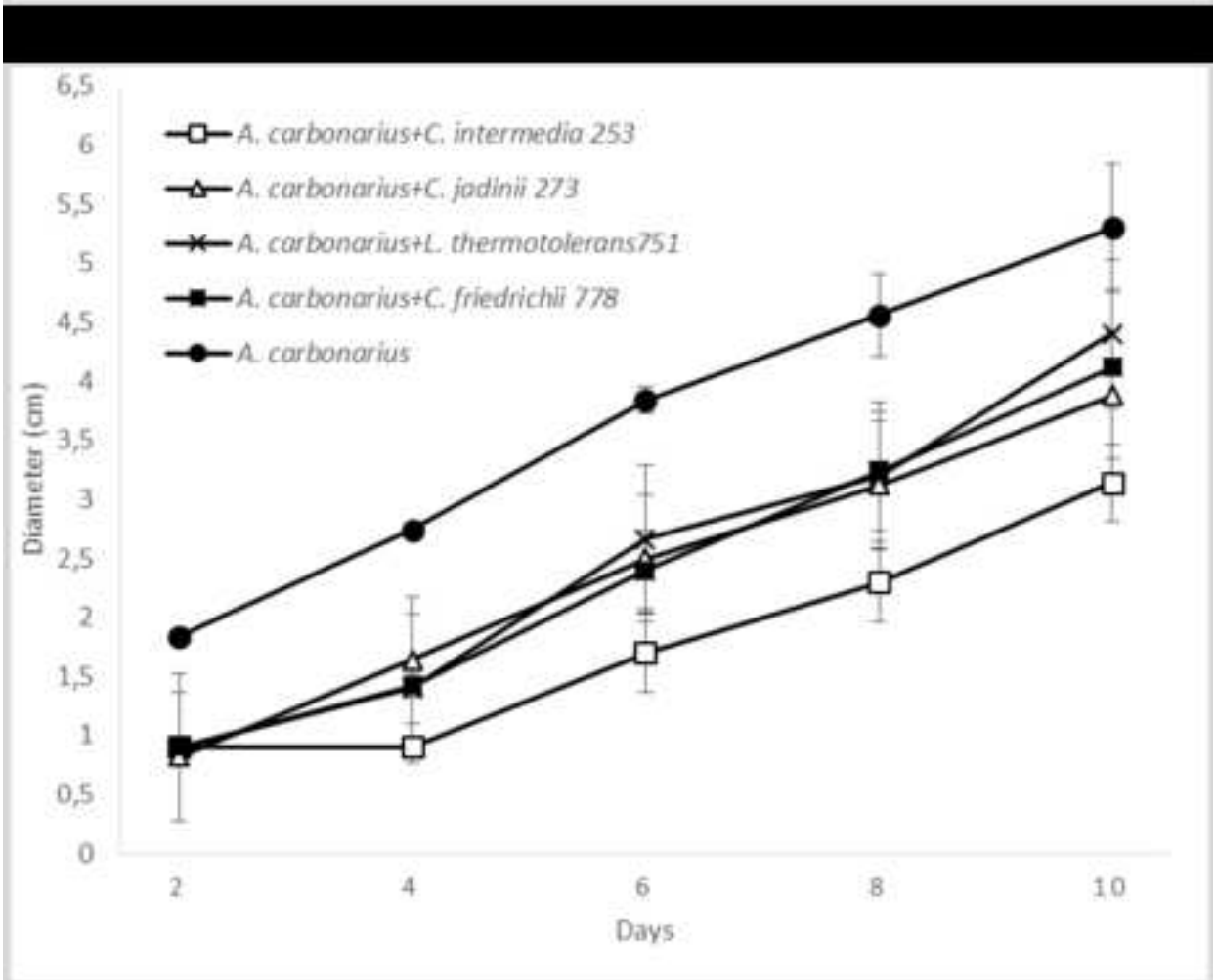


Figure 2

Click here to download high resolution image

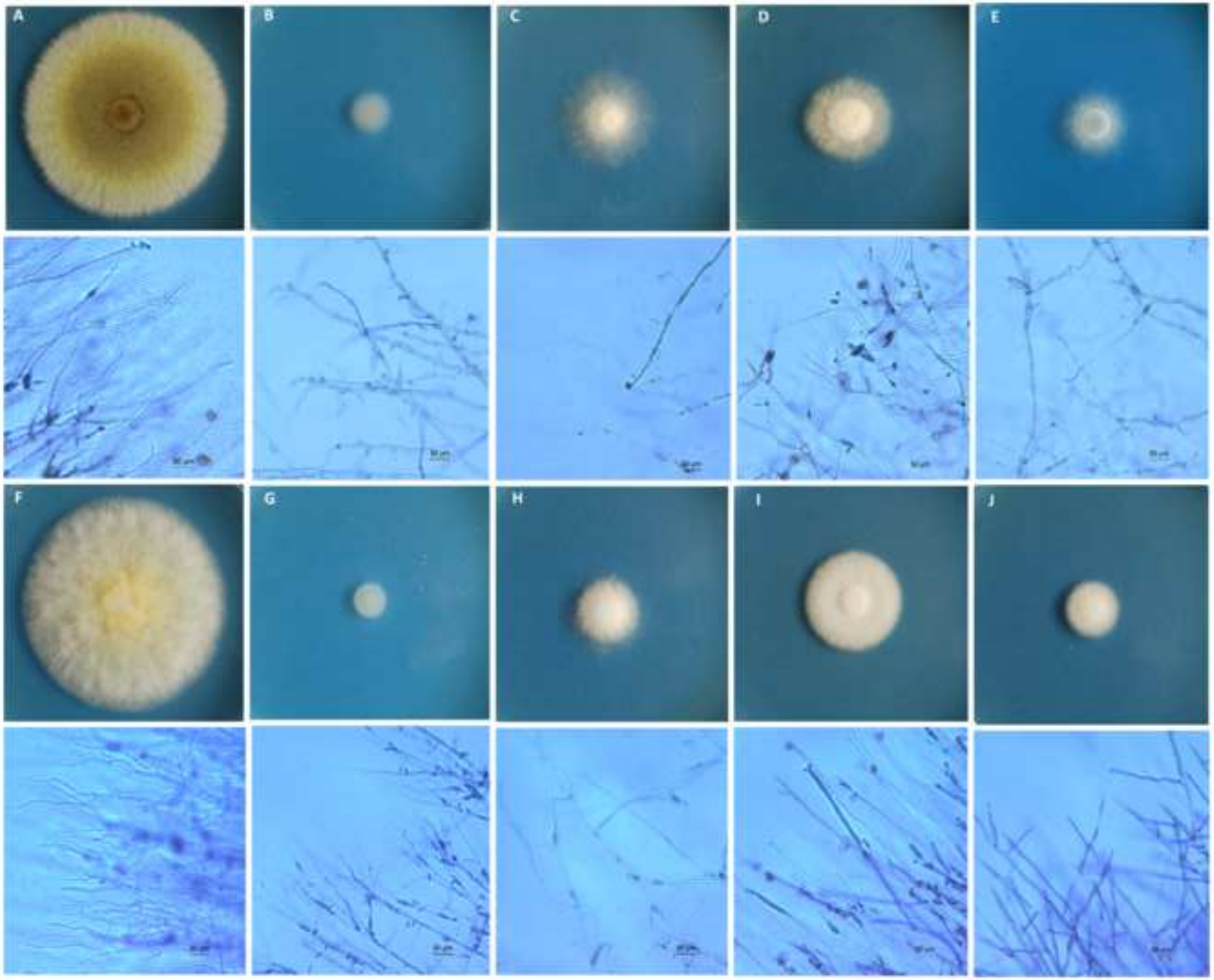


Figure 3

Click here to download high resolution image

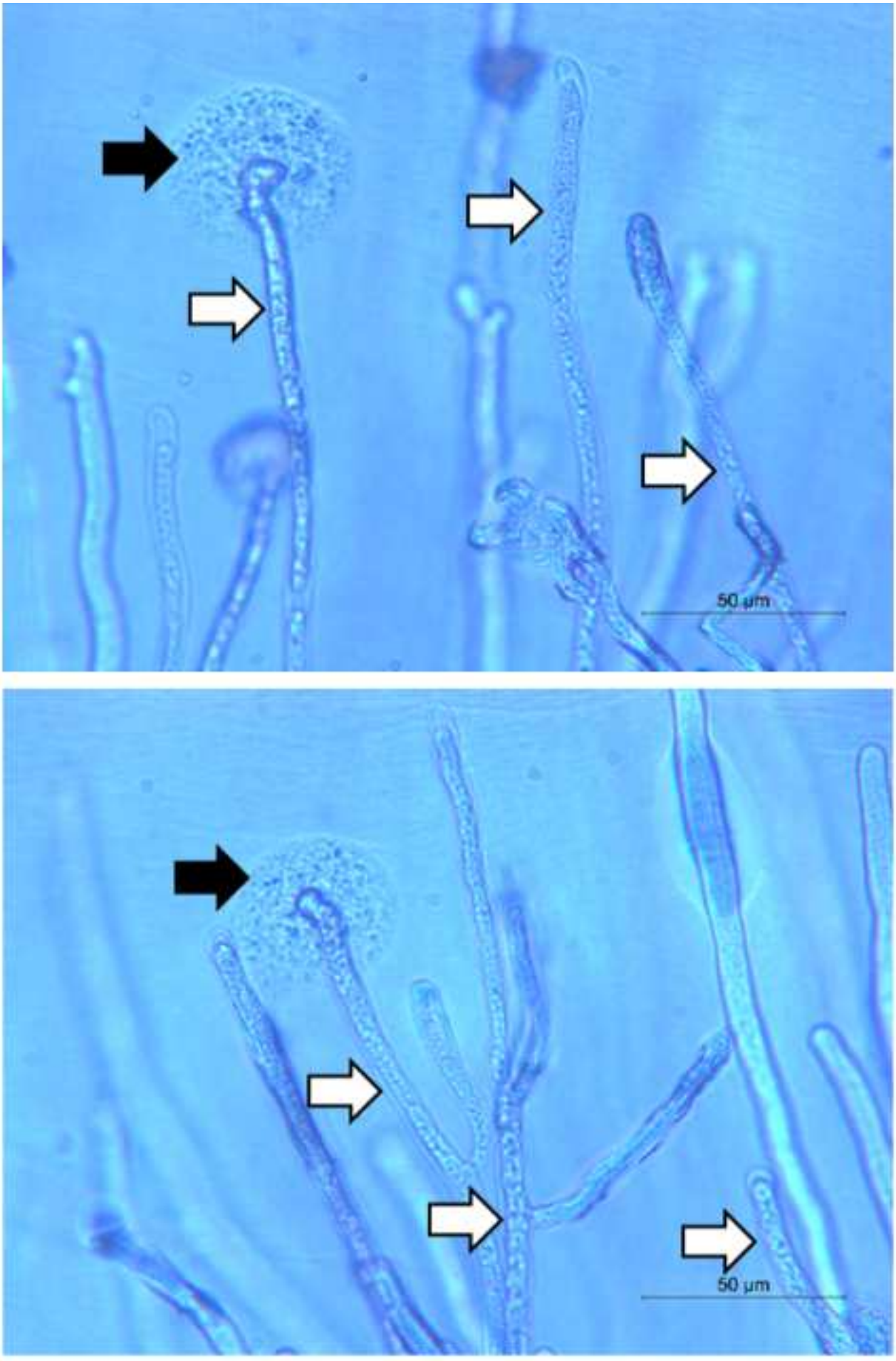


Figure 4

Click here to download high resolution image

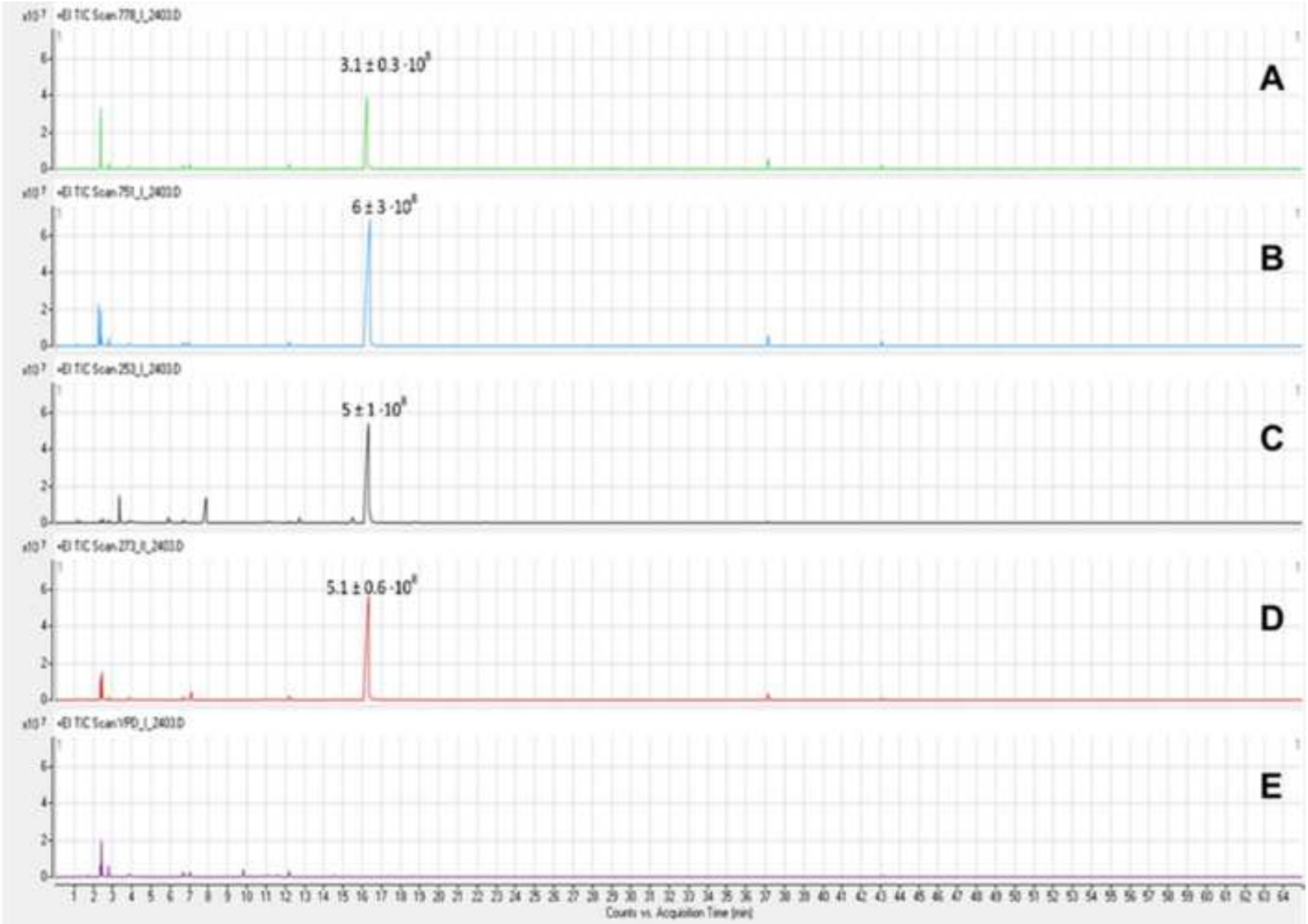


Figure 5

Click here to download high resolution image

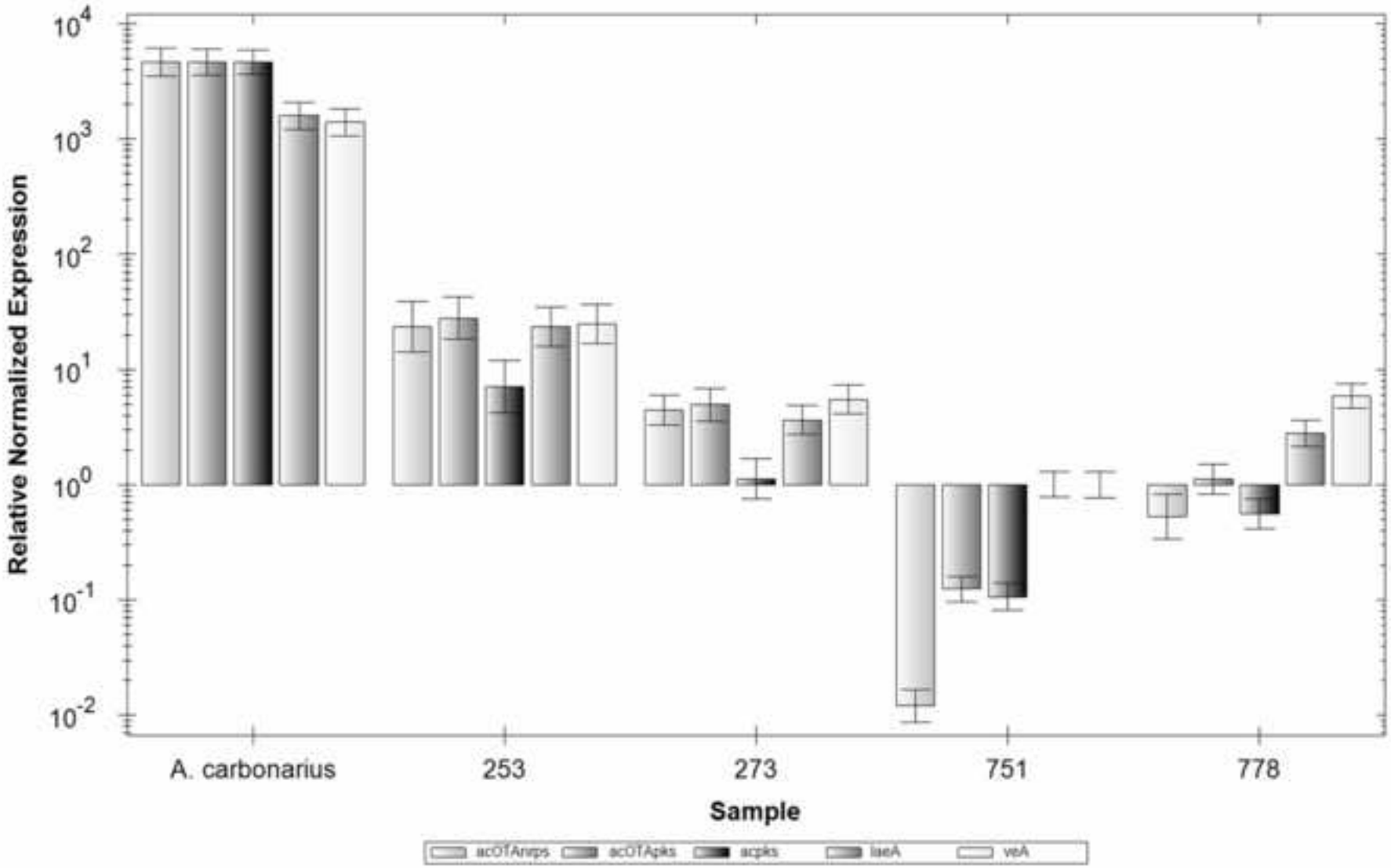


Figure 6

Click here to download high resolution image