International Journal of Food Microbiology 284 (2018) 1–10

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

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

Maria Grazia Farbo[a,1], Pietro Paolo Urgeghe[a,1], Stefano Fiori[a,1], Angela Marcello[a], Stefania Oggiano[a], Virgilio Balmas[a], Zahoor Ul Hassan[b], Samir Jaoua[b], Quirico Migheli[a,c,*]

[a] Dipartimento di Agraria, Università degli Studi di Sassari, Viale Italia 39, I-07100 Sassari, Italy
[b] Department of Biological & Environmental Sciences, College of Arts and Sciences, Qatar University, P.O. Box: 2713, Doha, Qatar
[c] Unità di Ricerca Istituto Nazionale di Biostrutture e Biosistemi, Università degli Studi di Sassari, Viale Italia 39, I-07100 Sassari, Italy

ARTICLE INFO

Keywords:
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.

* Corresponding author at: Dipartimento di Agraria, Università degli Studi di Sassari, Viale Italia 39, I - 07100 Sassari, Italy.
E-mail address: qmigheli@uniss.it (Q. Migheli).
[1] The first three authors have equally contributed to the present work.

https://doi.org/10.1016/j.ijfoodmicro.2018.06.023
Received 2 September 2017; Received in revised form 25 June 2018; Accepted 29 June 2018
Available online 30 June 2018
0168-1605/

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 (Morath et al., 2012; Passoth et al., 2006; Romano et al., 2015; Wriessnegger 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 and Geisen, 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 and Geisen, 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 and Keller, 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.

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	

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