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In vitro evaluation of essential oils against *Aspergillus carbonarius* isolates and their effects on Ochratoxin A related gene expression in synthetic grape medium

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ABSTRACT

Clove, mandarin, cinnamon and lemongrass essential oils (EOs) were tested for their efficacy against 2 wild isolates (*Ac29*, *Ac28*) and a reference strain (5010) of *Aspergillus carbonarius*. Two different assays were performed on Malt Extract Agar (MEA) and Czapek Yeast Extract Agar (CYA) solid cultures, as well as on synthetic grape medium (SGM) liquid cultures. The impact of EOs on fungal growth and toxin production was measured after 7 days of incubation at 25 °C. Furthermore, the effect of clove, lemongrass and mandarin EOs on *A. carbonarius* OTA related genes *Ac*OTA*nrps*, *Ac*OTA*pks* and *laeA* was investigated by relative expression using Real Time PCR on SGM cultures. Results showed that in solid cultures, complete fungal inhibition was obtained at 100–300 μl/l of EOs revealing a dose dependant effect of clove, cinnamon and lemongrass, whereas mandarin was proved to be less effective. EOs could not inhibit OTA production but only reduce it by 15–98% depending on the type and concentration of EO, even though in certain cases of low concentrations, EOs showed to enhance toxin production. In liquid cultures, a major effect on growth inhibition and OTA reduction was observed for all EOs. Regarding gene expression, down regulation of *Ac*OTA*nrps* was observed in the majority of treatments that could be associated with toxin reduction. Moreover, *Ac*OTA*pks* exhibited some differential expression but *laeA* did not show any differences in the transcriptional profile. The above gene expression results may indicate a possible mode of action of EOs in OTA biosynthetic pathway.

1. Introduction

The ability of different filamentous fungal species to excrete mycotoxins has drawn the attention of the scientific community as a serious potential health issue. Their presence as natural contaminants of agricultural products is still a major food safety problem. Research on mycotoxin contamination is a major priority for scientists worldwide, since the specific toxins are stable and resistant to heat or acidic environment and could remain in the food chain during distribution and storage for a long period causing serious safety issues. *Aspergillus* genera belong to the most important fungal group, as they can biosynthesize toxic secondary metabolites. *Aspergillus carbonarius* has been defined as one of the most important opportunistic pathogen in grapes, since it has the highest incidence of ochratoxin-producing isolates within the *Aspergillus* group (Stefanaki, Foufa, Tsatsou-Dritsa, & Dais, 2003) and high potential of toxin production. Ochratoxin A (OTA) is considered to be a prevalent and most toxic mycotoxin of the ochratoxins group, since it is rated as potential carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1993), nephrotoxic (JECFA, 2008) with immunotoxic and hepatotoxic impact to humans and animals. Mycotoxins may reach consumers by direct contamination of plant material or carry over their metabolites to food products. The European Union has established strict legislation for many products, including grapes, concerning the use of chemicals and maximum residue levels of pesticides (MRLs). Therefore, over the last few decades the interest of scientists has been focused on alternative applications of natural antimicrobials to control fungal growth on grapes and grape products.

It has been demonstrated that essential oils (EOs) contain diverse bioactive components that prevent moulds and their toxic metabolites, having at the same time the advantage of non-

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phytotoxicity and biodegradation. The Federal Regulation Code has recognized, EOs of clove, cinnamon, lemongrass, etc., as safe (GRAS) in the United States. Several experiments with EOs have reported that clove, cinnamon and lemongrass are promising candidates for the inhibition of foodborn pathogens and spoilage microorganisms. (Soliman & Badeaa, 2002; Tian et al., 2012). There are also reports on the inhibition of spore formation in *Aspergillus* species by lemongrass, cinnamon and clove (Paranagama, Abeysekera, Abeywickrama, & Nugaliyadde, 2003; Pawar & Thaker, 2006), spore germination (Tzortzakis & Economakis, 2007), or interaction with the cells of hyphae as reported for *Citrus sinensis* on *A. niger* (Sharma & Tripathi, 2006). Low molecular weight and highly lipophilic compounds of EOs pass easily through cell membranes and disrupt the fungal cell. On the other hand, regarding the effect of EOs on mycotoxin biosynthesis there are studies indicating actions like inhibition of lipid peroxidation during aflatoxin B1 formation (Bluma, Amaiden, Daghero, & Etcheverry, 2008). Moreover, a gene expression study has reported that a-terpinen affected genes involved in ergosterol biosynthesis in *Saccharomyces cerevisiae* (Parveen et al., 2004).

OTA is a polyketide mycotoxin linked to the amino acid phenylalanine. This molecular structure indicates that enzymatic reactions are needed for metabolite biosynthesis and even today this pathway has not been completely elucidated. However, a number of putative pathways have been proposed and recently the role of two genes has been revealed, namely NRPSs and PKSs that have been confirmed to be key enzymes of OTA biosynthesis gene cluster (Gallo et al., 2009; Gallo et al., 2012, 2014). Moreover, LaeA protein has been reported to govern secondary metabolites production on *Aspergillus* genome (Bok & Keller, 2004) and recently *laeA* gene has been correlated with the regulation of OTA biosynthesis in *A. carbonarius* (Crespo-Sempere, Marin, Sanchis, & Ramos, 2013).

During the last decay many studies have examined the ecophysiology of *A. carbonarius*, however very few deal with agents for growth inhibition and toxin control such as gallic acid, *Matricaria chamomilla* essential oil, natamycin and pine resin (Romero, Alberto, & Vaamonde, 2010; Tolouee et al., 2010; Kogkaki, Natskoulis, & Panagou, 2016). Moreover, there are few publications on the use of EOs as anti-ochratoxigenic agents (Hua et al., 2014; Santos et al., 2010) and even less on their impact on the growth and toxin production of *A. carbonarius* (Garcia, Garcia-Cela, Ramos, Sanchis, & Marin, 2011; Passone, Giardi, & Etcheverry, 2012).

Thus, the objective of this work was to determine the efficacy of the EO of clove, mandarin, cinnamon and lemongrass in the control of *A. carbonarius* growth in different media and investigate any potential influence on the expression of OTA related genes. To our knowledge this is the first report on EOs which along with intraspecific OTA production investigates also their effect on *A. carbonarius* gene expression.

2. Materials and methods

2.1. Essential oils

Four EOs were evaluated in the present study, namely *Eygenia cariophyllus* (clove oil, 87% eugenol), *Citrus reticulata* (mandarin, 89% limonene), *Cinnanomum cassia* (cinnamon, 78% e-cinnamaldehyde) and *Cymbopogon citratus* (lemongrass, 45% geranial, 25% neral) purchased from Pranarôm Intenational S.A. (Ghislenghien, Belgium). The concentration of the main active compounds for each EO was provided by the manufacturer.

2.2. Fungal isolates and sample preparation

Two different wild isolates of *A. carbonarius* (*Ac*28 and *Ac*29) from the fungal collection of the Laboratory of Microbiology and Biotechnology of Foods (LMBF) of the Agricultural University of Athens were used in this work. The fungi have been previously isolated from grapes, characterized by molecular methods (Kizis, Natskoulis, Nychas, & Panagou, 2014) and their OTA potential has been reported elsewhere (Lappa, Kizis, Natskoulis, & Panagou, 2015a). In addition, a strain of *A. carbonarius* ITEM 5010 kindly provided by Prof. Tsitsigiannis from the Laboratory of Phytopathology of the same University was used as reference. The study was performed on 3 different growth media: (a) Malt extract agar (MEA, malt extract, 20 g; peptone, 1 g; glucose, 20 g; bacteriological agar, 20 g; distilled water, *c.* 1000 ml) which favors fungal sporulation, (b) Czapek Yeast extract Agar (CYA, SD, Oxoid) which is recommended for the determination of OTA in *A. carbonarius* (Bragulat, Abarca, Cabanes, 2001), and (c) Synthetic Grape Juice Medium (SGM) that simulates grape composition between véraison and ripeness (Delfini, 1982). The pH of the SGM was adjusted to 3.8 with 2M KOH. MEA and CYA were used for the preparation of solid media whereas SGM for liquid media. Spore suspensions of each *A. carbonarius* isolate were prepared by collecting spores from 7-day old fungal colonies grown on MEA at 25 °C. Conidia were harvested from sub-cultures in an aqueous solution of 0.01% Tween 80 (Merck, Schuchardt, Germany) by scraping the surface of the mycelium with a sterile glass rod. The final spore suspensions were assessed using a haemocytometer (Brand, Wertheim, Germany) and adjusted by appropriate dilutions to approximately 10^6 spores ml^{-1}. EOs were initially dissolved in sterile Tween 80 (0.01% aqueous solution) that was used as a nonionic emulsifier to facilitate dispersion into the media (Fu et al., 2007). Further on, EOs were individually diluted in autoclaved media under constant agitation, dispersed into petri dishes, and allowed to solidify within 5 min. Plates containing 50–1000 μl/l of EOs (Table 1) were prepared by adding the respective amount of EO directly into the autoclaved medium that was further centrally single spotted with 10^3 spores on the surface. All plates were sealed using Parafilm to minimize desiccation of the medium and loss of the EO. For gene expression studies, SGM was used in shaking flask cultures. Specifically, conidia were inoculated into 100 ml Erlenmeyer flasks containing 50 ml of the medium supplemented with 50–100 μl/l of EOs and sealed with Parafilm (Table 1). Incubation was carried out for 7 days at 25 °C in a rotating shaker at 100 rpm. Control samples (i.e., cultures without EOs) were also used. Cultures showing no growth were incubated for 7 more days and then transferred to plates without EO to determine fungicidal or fungistatic effects.

For kinetic studies in solid media cultures, colony radius was observed on a daily basis and recorded at right angles with the aid of a ruler, while for liquid cultures mycelium was harvested from broth and fungal biomass weighed. The growth of fungal cultures containing different concentrations of EOs was compared with the control culture. All the assays for fungal growth and OTA

Table 1
Concentrations of essential oils employed in the experimental work.

Essential oils	Concentrations (μl/l)	
	Solid media	Liquid media
Lemongrass	50, 100, 200, 300	50, 75, 100
Clove	50, 100, 200, 300	50, 75, 100
Cinnamon	50, 75, 100, 150	50, 75, 100
Mandarin	400, 700, 1000	400, 700, 1000