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Control of toxigenic *Aspergillus* spp. in dried figs by volatile organic compounds (VOCs) from antagonistic yeasts

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ABSTRACT

Aspergillus flavus and *Aspergillus niger* are fungi which can contaminate dried figs before and after harvest and consequently produce aflatoxins (AFs) and ochratoxin A (OTA). Many approaches have been applied to minimise the growth of these filamentous fungi, mainly involving the use of synthetic fungicides which are limited due to their negative impact on human health and the environment. In this context, biocontrol is a recent approach that needs to be explored. This study evaluated the potential of three volatile organic compounds (VOCs), octanoic acid (OA), 2-phenylethyl acetate (2PEA) and furfuryl acetate (FA), produced by *Hanseniaspora uvarum* and *Hanseniaspora opuntiae* yeasts on the growth, germination, gene expression and production of AFs and OTA by *A. flavus* M144 and *A. niger* M185 on dried fig-based agar and the incidence rates in dried figs. Two of the three VOCs evaluated (2PEA and FA) effectively controlled *A. flavus* M144 and *A. niger* M185 by using at least amounts of 50 μL (715 μL/L in the headspace) for FA and 100 μL (1430 μL/L in the headspace) for 2PEA in dried figs. One of the mode of actions of both compounds consists in early repressing the expression of genes involved in the biosynthesis of AFs (*aflR*) and OTA (*pks*) of *A. flavus* and *A. niger*, respectively. The results of this study support the application of 2PEA and FA at the early post-harvest stages of dried figs to control mycotoxin accumulation.

1. Introduction

Dried fig is the product obtained by dehydrating the fruits of the fig tree (*Ficus carica* L.). According to FAOStat (2020) (http://www.fao.org/faostat/en/#home), the main exporter countries in 2020 were Turkey, with more than 85.000 t, followed by Afghanistan and Germany. The production of dried figs is carried out in a traditional way; handling and harvesting of the crop are prone to fungal contamination as dehydration is a lengthy process and starts with fruit ripening and senescence in the tree and continues on the ground. Once the figs are on the ground, they are manually harvested and placed on wooden trays or cement floors. The dehydration process of figs ends with their drying under the sun for 3–5 days (Javanmard, 2010; Villalobos et al., 2019) until they reach a moisture content ≤26%, according to Standard DDP-14 (UNECE, 2016). After drying, the figs can be stored for several months until commercialisation.

The production process, the long periods of drying and storing, as well as the physicochemical characteristics of dried figs favour filamentous fungi contamination and mycelia development (Flaishman et al., 2008). The mould population on dried figs varies, with counts ranging from 1.46 to 6.74 $\log_{10}$ CFU/g (Javanmard, 2010; Öztekin et al., 2006; Villalobos et al., 2016, 2019). This mould population is mainly composed of different species of the genera *Cladosporium*, *Acremonium*, *Mucor*, *Fusarium*, *Penicillium* and *Aspergillus* (Galván et al., 2021; Heperkan et al., 2012a; Javanmard, 2010; Villalobos et al., 2016). The presence of mycotoxigenic species in dried figs is common, resulting in contamination with different mycotoxins. The most important and frequent mycotoxins encountered in dried figs are aflatoxins (AFs) and ochratoxin A (OTA) (Bakirci, 2020; Bircan, 2009; Senyuva et al., 2005), although other important mycotoxins have also been reported. For example, Wang et al. (2018) detected toxins of *Alternaria* (alternariol and alternariol monomethyl ether), ochratoxin B, HT-2 toxin and

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beauvericin (BEA), besides B and G-group AFs (AFB_1, AFB_2, AFG_1 and AFG_2) in dried figs from different areas of China. In samples from Tunisia and Spain (most of them imported from Turkey), the most prevalent mycotoxins were enniatins and BEA, belonging to the *Fusarium* toxin group (Azaiez et al., 2015). Besides, OTA, AFG_1, HT-2 toxin and diacetoxyscirprenol were identified in such samples (Azaiez et al., 2015). Karbancıoglu-Güler and Heperkan (2009) determined that more than 71% of dried figs samples from the Aegean region (Turkey) presented detectable amounts of fumonisin B_1. The contamination of dried figs with fumonisins, OTA and cyclopiazonic acid, in co-occurrence with AFs, has also been reported from this region (Heperkan et al., 2012a; Heperkan et al., 2012b).

From the point of view of toxicology and toxin regulation, the most concerning mycotoxins in dried figs are AFs and OTA. The European Commission (EC) has set a maximum tolerable limit of 6 μg/kg of AFB_1 and 10 μg/kg for the sum of AFB_1, AFB_2, AFG_1 and AFG_2 in dried fruits (EC, 2012). Although to date, the EC has not established a maximum limit of OTA for marketed dried figs, the European Food Safety Authority indicates that figs are important contributors to OTA dietary exposure (EFSA, 2020). The great public health impact of AFs and OTA contamination in dried figs has been supported by 138 notifications in the Rapid Alert System for Food and Feed (RASFF) portal (https://webgate.ec.europa.eu/rasff-window/screen/list) since January 2020 to January 2022; most of the figs were from Turkey. For this reason, it is important to control and minimise the presence of AF- and OTA- producing species in figs.

So far, there are numerous methods to control pathogenic mould growth and mycotoxin contamination in dried figs and other dried products, with biocontrol and biological detoxification being an emerging and prominent strategies. Efficient biodegradation of AFB_1 has been reported in non-toxigenic strains of *Aspergillus niger* (Qiu et al., 2021). Also, lactic-acid bacteria, specifically *Lactobacillus kefiri*, reduced *A. flavus* and *A. carbonarius* growth and AFB_1, AFB_2 and OTA contamination in inoculated almonds and peanuts (Taheur et al., 2019). Among the microorganisms that can act as biopreservatives, yeasts are the most suitable ones to counteract risks provoked by the growth of filamentous fungi (Sharma et al., 2009). Ruiz-Moyano et al. (2016) reported the antagonistic capacity of two yeast strains, *H. opuntiae* L479 and *M. pulcherrima* L672, isolated from fig and breva crops to control the development of common postharvest pathogens.

Among the mechanism of action of antagonistic microbes (Spadaro and Droby, 2016), the application of antagonistic microbial producers of antifungal volatile organic compounds (VOCs) or the compounds themselves is one of the less explored ways to mitigate toxigenic moulds, although the results, so far, are promising. For instance, *Candida nivariensis* reduced *A. flavus* growth (64.9%) and AF production (78.4%) in corn grains associated with the production of VOCs, with the main one being 1-pentanol (Jaibangyang et al., 2020). In another study, non-toxigenic *A. flavus* strains produced antifungal VOCs such as 3-octanone and trans-2-methyl-2-butenal, which resulted in a significant reduction in AF production (Moore et al., 2021). Tejero et al. (2021) identified several VOCs produced by *Hanseniaspora uvarum* and *Hanseniaspora opuntiae* which, *in vitro*, diminished *A. flavus* growth and AF production.

In this context, the aim of this work was to evaluate the effects of three antifungal VOCs produced by the antagonistic yeasts *H. uvarum* and *H. opuntiae* in controlling the development of *Aspergillus* spp. in dried figs to prevent AF and OTA accumulation in dried figs.

2. Materials and methods

2.1. Moulds strains and inoculum

Two common fungal pathogens of dried figs, belonging to the genus *Aspergillus*, were selected for this study: *A. flavus* M144 (previously characterised by Galván et al. (2021) and *A. niger* M185. These moulds were isolated from dried figs (*Ficus carica* L.) harvested from fields in Extremadura (Spain), following to protocol described by Ruiz-Moyano et al. (2009). Mould identification was carried out as described by Galván et al. (2021).

The fungal inocula were prepared by growing both mould strains on potato dextrose agar (PDA, Scharlab, Barcelona Spain) plates at pH 3.5 with a sterilised solution of tartaric acid at 10% (w/v) and incubated for 7 days at 25 °C. Subsequently, 10 mL of sterile deionised water was added to each plate, and the conidia were freed by rubbing the surface with a glass rod. The conidia concentration was adjusted to 10^5 conidia/mL using a microscope (Olympus CX 400, Tokyo, Japan) and a Neubauer chamber.

2.2. Dried fig-based medium preparation

The dried fig samples used in this study are of the Calabacita variety and were obtained from the Scientific and Technological Research Centre of Extremadura, Finca La Orden-Valdesequera (CICYTEX), Guadajira, Spain. A dried fig-base (DFB) medium was essentially prepared following the protocol described by Galván et al. (2021), but the percentage of lyophilised dried figs in the media was 10%.

2.3. Volatile organic compounds

Three antifungal VOCs were used in this work, namely octanoic acid (OA), 2-phenethyl acetate (2PEA) and furfuryl acetate (FA). All compounds were purchased from Sigma-Aldrich (San Luis, EEUU) and were previously identified as produced from antagonistic yeast isolates and potentially associated with antimould properties (Ruiz-Moyano et al., 2020). To evaluate the antagonistic activities of the three VOCs against two moulds, *A. flavus* M144 and *A. niger* M185, *in-vitro* assays with DFB plates and *in-vivo* assays with dried figs were carried out.

2.4. In-vitro antifungal activity of volatile organic compounds

2.4.1. "Conidial" and conidial suspensions

To determine the involvement of the VOCs in the inhibition of conidium germination, 100 μL of conidium suspensions were seeded on a PDA plate. After that, VOCs were applied as described in Section 2.4, and the plates were incubated at 25 °C for 12 h. Subsequently, a glass slide was placed on top of the agar and observed under 40× magnification with sub-stage illumination (DMLS, Leica, Buccinasco, MI, Italy), using a Leica DM 2000 LED microscope. For each plate, the percentage of spore germination was estimated from the observation of 100 spores. When the germ tube was at least as long as the width of the spore, it was considered germinated (Gougouli and Koutsoumanis, 2013). The lengths of 50 germinative tubes per plate were measured and compared with those of the control plates. Three plates were performed for treatment, and the assay was repeated twice.

2.4.2. Effects of VOCs on lag phase, growth rate, relative gene expression and aflatoxin production

2.4.2.1. Effects of VOCs on lag phase and growth rate. The *in-vitro* evaluation of VOCs effects on lag phase and growth rate was carried out by using DFB plates. To perform the assay, a 1 × 1-cm square of sterilised filter paper was placed on one side of the plate, on which different quantities of 10, 25, 50 and 100 μL (which are 143, 357, 715 and 1430 μL/L of headspace) of the different three VOCs were added. In the centre of the plate, 5 μL of the spore solution of each mould was inoculated. Three control samples were made with each mould without adding VOCs. Subsequently, the lid of the petri dish was discarded, and the bottom of another empty petri dish was placed on the top of the previous one, and both parts were fixed with parafilm. To avoid an increase in the CO_2 concentration to detrimental levels (Contarino et al., 2019), four holes were drilled in the sides just between the two plates with an

incandescent seeding loop. The plates were then incubated at room temperature (≈ 25 °C) for 8 days. The doses of each compound in each of the moulds were studied in triplicate, and the assay was repeated twice.

Radial growth was daily recorded by measuring two right-angled diameters with a scaled rule. Growth curves were obtained by plotting the mycelial diameter (mm) against the incubation period (days). The lag phase (λ; days) was determined from linear regression by equating the formula of the regression line with the original inoculum size (diameter, mm), and the growth rate (μ; mm/day) was determined from the slope of the growth curve (Rodríguez et al., 2014).

2.4.2.2. Quantification of the relative gene expression. The two moulds *A. flavus* M144 and *A. niger* M185 were sampled on days 5, 7 and 8 of the incubation periods for gene expression analysis. After each incubation time, mycelium was scraped from the surface, collected under sterile conditions, flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

- RNA extraction

The RNA was extracted from frozen mycelium using the Spectrum™ Plant Total RNA kit (SigmaAldrich), following the manufacturer's instructions. The RNA concentration (ng/μL) and purity (A260/A280 ratio) were determined spectrophotometrically in a NanoDrop (Thermo Scientific™ NanoDrop 2000). Samples were diluted to a concentration of 0.1 μg/μL and treated with DNAse I (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove genomic DNA traces. The obtained RNA was kept at −80 °C until the reverse transcription (RT) reaction.

- Two-step reverse-transcription real-time PCR

First, the RT reaction was conducted using 5 μL of total RNA (≅ 100 ng) with the PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan), as described by the manufacturer. Complementary DNA (cDNA) samples were stored at −20 °C until analysis. The real-time PCR (qPCR) reactions were performed in the 7300 Real-Time PCR System (Applied Biosystems, Foster City, California, USA), using the SYBR® Green technology. Amplification of the *aflR*, *pks* and *β-tubulin* genes was carried out following the methodology described by Rodríguez et al. (2012) and the methodology described in this study. Briefly, the reaction mixture for the amplification of each gene consisted of 6.25 μL of NZY qPCR Green Master Mix 2x (NZYTech, Lisbon, Portugal), 2.5 μL of cDNA template (2 ng/μL) and different concentrations of each primer. For the *aflR* gene, the final concentration of the primer pair AflRTaq1 and AflRTaq2 was 300 nM each, for the primers pks-F1/pks-R1, which amplify the *pks* gene, the concentration as 300 nM each, whereas for the primers F-TUBjd/R-TUBjd, which amplify the *β-tubulin* gene, the concentration was 400 nM each. The thermal cycling conditions for amplification of the three genes included one initial denaturation step at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After the final PCR cycle, melting curve analyses of the PCR products were conducted, and the values of the melting temperatures were checked to ensure the reliability of the results and the specificity of the primers. The quantification cycle (Cq), the cycle in which the fluorescence reaches a defined threshold, was automatically obtained by the instrument, using the default parameters of the 7300 Fast System Software (Applied Biosystems).

- Calculation of the relative gene expression

The relative quantification of the expression of the *aflR* and *pks* genes was calculated using the $2^{-\Delta\Delta CT}$ method described elsewhere (Livak and Schmittgen, 2001). The *β-tubulin* gene was used as an endogenous control to normalise the amount of target cDNA added to each reaction. The calibrator used for the calculations was the non-treated control sample (*A. flavus* or *A. niger*) at each sampling time.

2.4.2.3. Aflatoxin and ochratoxin A analysis. The AF content produced by *A. flavus* M144 and the OTA content produced by *A. niger* M185 were determined on days 5, 7 and 8 of incubation. Agar plates were immediately stored at −20 °C until extraction.

- Standards and solvents

All solvents used were of HPLC grade and purchased from Thermo Fisher Scientific. The AF calibrations were carried out with a standard mixture of AFB_1, AFB_2, AFG_1 and AFG_2, purchased from Sigma-Aldrich, and OTA calibrations were performed with a standard from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

- Mycotoxin extraction

Both AFs and OTA were extracted according to the method described by Ruiz-Moyano et al. (2009) with modifications. The contents of an agar plate with the whole colonies were transferred to a plastic filter bag and homogenised with 80 mL of chloroform. The protocol was followed stepwise, except that filtering was performed at the end, once the residue was resuspended in 1 mL methanol and 1 mL water, through a 0.22-μM pore size filter into HPLC vials for quantification.

- Mycotoxin quantification

The quantification of AFs and OTA was performed using an Angilet 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with an Agilent 1200 FLD detector (G1321A); excitation and emission wavelengths were 330 and 460 nm, respectively, using a C-18 column (15 cm × 4.6 mm, 5 μm particle size; Supelco, Bellefonte, PA). The injection volume was 100 μL. To quantify AFs, a post-column derivatisation with pyridinium bromide at 0.05% (*w/v*; Sigma), pumped at 0.3 mL/min, was performed. Separation of AFs was achieved with a mobile phase containing a mixture of MeOH: ACN: water (20:20:60 *v/v/v*), which was delivered at an isocratic flow rate of 1 mL/min. The OTA quantification was performed with the mobile phase composed of water: ACN: acetic acid (41:57:2) at an isocratic flow rate of 1 mL/min.

2.5. Antifungal activity of volatile organic compounds on dried figs

Based on the results of the *in-vitro* assay, a further experiment was carried out on dried figs with the three VOCs. The dried figs were immersed in a spore suspension of 10^5 spores/mL for 2 min, and each compound was studied in triplicate with each of the moulds. Each replicate consisted of 20 dried figs, which were placed in 3-L plastic boxes with the respective VOC in glass culture dishes, with 4.3 mL of each VOC to reproduce the same concentrations as used in Section 2.4. Three control samples were run with each mould without adding VOCs. Plastic boxes were incubated at 25 °C for 30 days.

A daily visual assessment was carried out, where the percentage of dried figs with the presence or absence of mould contamination in each plastic box was quantified.

2.6. Statistical analysis

The growth parameters, spore germination, AF and OTA production and relative gene expression in the *in-vitro* assay, as well as the percentage damage in the *in-vivo* assay, were analysed using one-way ANOVA. Differences between mean values were separated by Tukey's honestly significant difference test ($p \leq 0.05$). All analyses were performed in SPSS for Windows version 21.0.

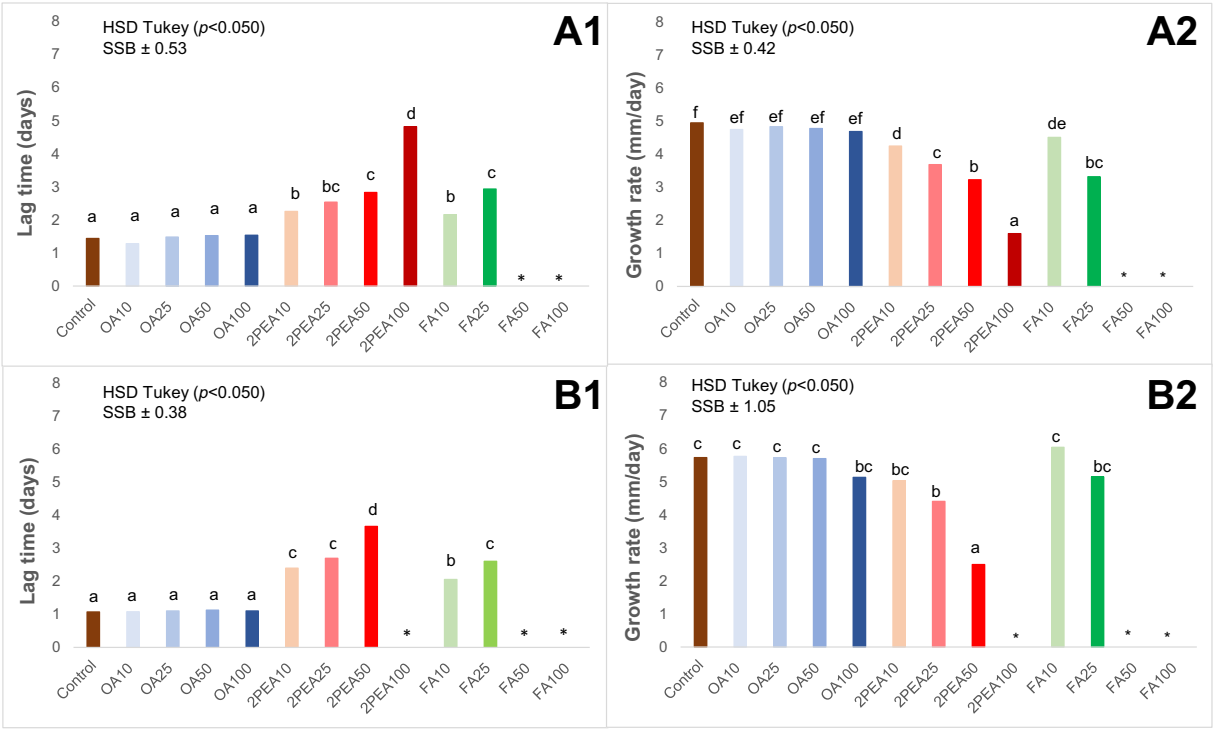

Fig. 1. Growth parameters in dried fig-based agar at 25 °C of (A) *A. flavus* M144 and (B) *A. niger* M185, represented by values of (1) lag phase (days) and (2) growth rate (mm/day). OA: octanoico acid; 2PEA: 2-phenylethyl acetate; FA: furfuryl acetate. The number (10, 25, 50, 100) indicates the amount (μL) of compounds applied in the plates. "*" indicates complete inhibition of mycelial growth. SSB: Statistical Significance Bar.

3. Results and discussion

3.1. Effects of VOCs on lag phase and growth rate

In recent years, several studies have reported the production of VOCs by antagonistic microorganisms which control the development of *Aspergillus sp.* Bacterial species such as *Streptomyces alboflavus*, *Streptomyces yanglinensis* and *Bacillus licheniformis* presented high efficacy for the growth inhibition of *Aspergillus ochraceus*, *A. westerdijkiae*, *A. carbonarius*, *A. niger* and *A. flavus*. The antagonistic activities have been related to compounds of different chemical families, such as dimethyl trisulfide, benzenamine, methyl 2-methylbutyrate, 2-phenylethanol, β-caryophyllene and 3-methyl-1-butanol (Lyu et al., 2020; Ul Hassan et al., 2019; Yang et al., 2018). Some species of yeasts produce VOCs with similar properties, such as *Candida nivariensis*, which produces 1-pentanol (Jaibangyang et al., 2020), or *Candida intermedia* and *Lachancea thermotolerans*, which produce 2-phenylethanol (Farbo et al., 2018). In recent works, *H. uvarum* and *H. opuntiae*, which produce 2PEA, FA and OA, were effective in the control of *Botrytis cinerea* and *A. flavus* (Ruiz-Moyano et al., 2020; Tejero et al., 2021). The effects of these three VOCs on the *in-vitro* growth of *A. flavus* and *A. niger* on DFB agar are shown in Figs. 1 and 2. The lag phase prior to the growth of *A. flavus* M144 in the control plates was 1.44 ± 0.07 days. The application of OA, in any of the different amounts, did not modify this parameter ($p > 0.050$). The application on 2PEA and FA extended the lag phase in a dose-dependent manner ($p < 0.001$). The lowest amounts of VOCs presented values of 2.26 ± 0.24 and 2.16 ± 0.08 days for 2PEA and FA, respectively, whereas 100 μL of 2PEA resulted in 4.82 ± 0.26 days of lag phase, and 25 μL of FA delayed the start of the mycelium growth to 2.93 ± 0.14 days (Fig. 1.A1). Higher amounts of FA (50 and 100 μL) did not allow mycelial growth (Fig. 2). Similar results were obtained in the case of *A. niger* M185. The lag phase in control plates was 1.08 ± 0.06 days, achieving values of 3.66 ± 0.22 days after treatment with 50 μL of 2PEA and 2.61 ± 0.15 days with 25 μL of FA (Fig. 1.B1).

The graphs showed, after a lag phase, a linear trend with time. The growth rate of *A. flavus* M144 in the control plates was 4.95 ± 0.10 mm/day and was not affected by treatment with OA ($p > 0.050$). However, treatment with amounts ≥25 μL of 2PEA and FA reduced or even impeded mycelial development ($p \leq 0.001$). In the case of *A. niger*, its growth rate was 5.73 ± 0.03 mm/day in control plates. The growth rate was reduced to 5.13 ± 0.08 mm/day with 100 μL of OA and was completely inhibited with 100 μL of 2PEA and 50 μL of FA. The overall lack of antifungal activity of OA was unexpected because of the previous reports about its control capacity for hyphal growth and cell aggregation in *Candida albicans* (Lee et al., 2021) and the disorder in cell membranes of several filamentous fungi (Pohl et al., 2011). The compound 2PEA is a highly valued flavouring compound used in the fragrance, cosmetic and food industries. However, its antifungal properties have been less exploited than those of its precursor phenylethyl alcohol, which is used as a disinfectant, pest control agent, cleaning agent and in personal care products (Martínez-Avila et al., 2018). Finally, some furan derivates with microbial origin have been associated with antifungal activities, such as 2,3-dihydrofuran produced by atoxigenic strains of *A. flavus* (Moore et al., 2021) and furan-tetrahydro-2,5-dimethyl produced by *Bacillus subtillis* and effective against *Botrytis cinerea* (Mu et al., 2017).

Treatment with 2PEA and FA produced changes at the macroscopic level (Fig. 2) on *A. flavus*. The mycelia presented a white colour, and sporulation was reduced or inhibited, mainly in FA applications. Similar effects have been observed when *A. carbonarius* and *A. ochaceus* were exposed to VOCs produced by *Lachancea thermotolerans*, *Candida intermedia*, *Candida friedrichii* and *Cyberlindnera jadinii* (Farbo et al., 2018).

3.2. Effects of VOCs on conidium germination

The influences of the three compounds on spore germination are shown in Table 1. Control treatments of *A. flavus* M144 presented 74.08 ± 8.11% of germinated spores after 12 h of incubation, with a medium germ tube size of 22.45 ± 2.74 μm. A non-significant ($p > 0.050$)

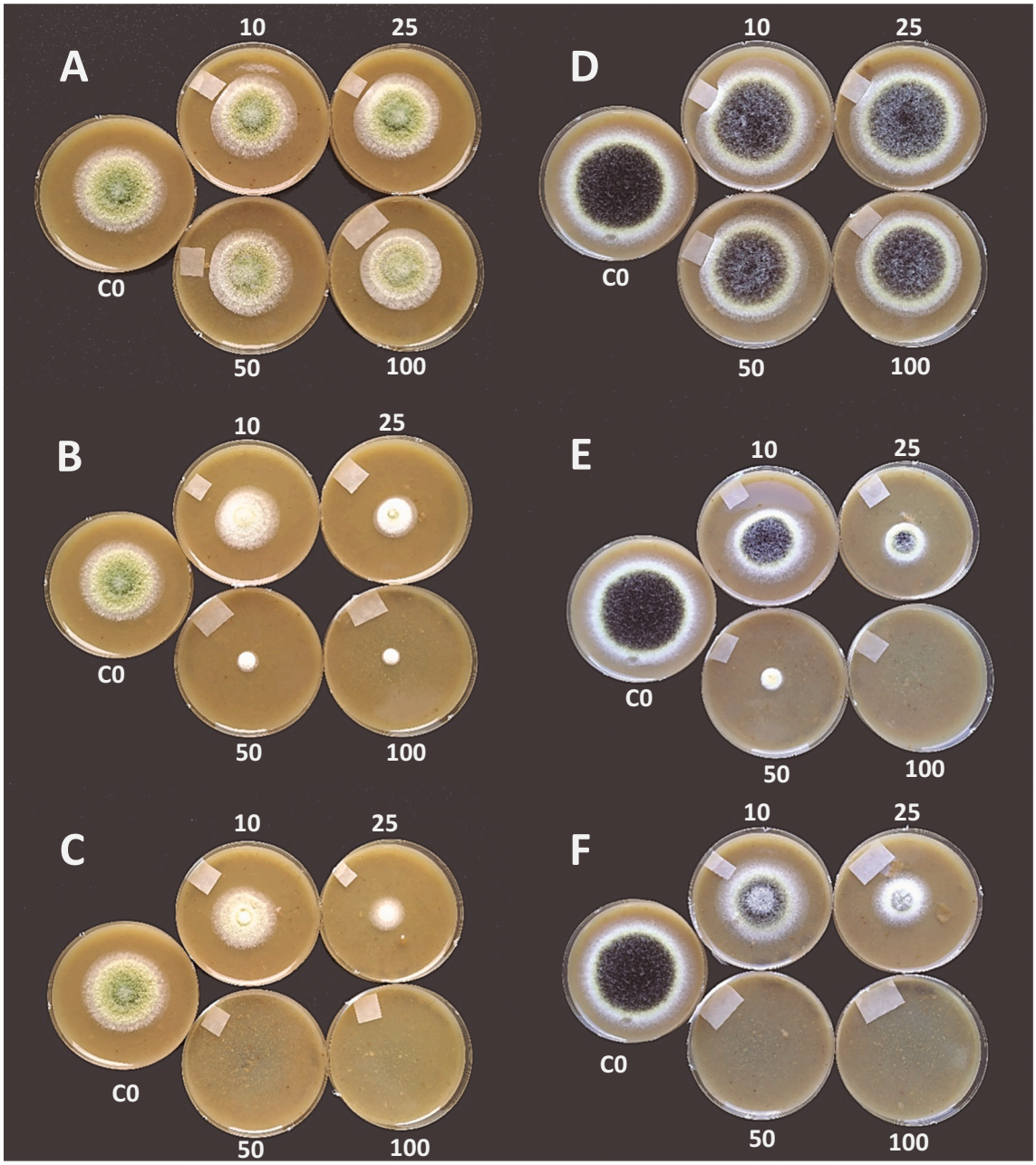

Fig. 2. Growth on dried fig-based agar at 25 °C *of A. flavus* M144 (A, B, C) and *A. niger* M185 (D, E, F) against the three volatile organic compounds, octanoic acid (A and D), 2-phenylethyl acetate (B and E) and furfuryl acetate (C and F) on day 5 of the study at the four treatments studied (10, 25, 50 and 100) and the control (C0).

decrease in the percentage of germination and the size of the germ tube was observed when OA was applied. Germination diminished ($p \leq 0.050$) to values of 49.10 ± 3.41% and 25.63 ± 3.26% with 10 and 100 μL of 2PEA, respectively. However, the size of the germ tube was not significantly modified when 2PEA was added, with mean values ranging from 20.23 to 18.36 μM. The FA inhibited the germination from 33.46 ± 7.30% at 10 μL to complete the inhibition at 100 μL. The size of the germ tube was approximately half (11.34 ± 0.38 μM) of that of the control at 10 μL of FA.

Regarding *A. niger* M185, it presented 85.23 ± 4.30% of germination, with a size of germ tubes of 17.41 ± 1.11 μm. The addition of OA reduced mean values of % germination (71.15 ± 2.57% at 100 μL) and size of the germ tube (13.86 ± 2.57 μM at 100 μL), but no statistical differences were found ($p > 0.050$). The addition of 2PEA allowed a significant reduction of the percentage of germination with treatments ≥50 μL, but germ tube size was not impacted. Finally, FA diminished the germination at all treatments and completely inhibited the germination of *A. niger* M185 at ≥50 μL.

The inhibition of conidium germination is an important mechanism of controlling toxigenic fungi because it impedes colonisation and toxin production in the agri-food products. In this sense, VOCs produced by *Streptomyces alboflavus* TD-1, mainly dimethyl trisulfide and benzenamine, strongly inhibited the spore germination of *A. ochraceus* (Yang et al., 2018). In the abovementioned study, dimethyl trisulfide and benzanamide completely inhibited mycelial growth at 10 and 1000 μL/L, respectively. In our case, the concentrations for the complete inhibition of spore germination with FA were 715 μL/L for *A. niger* and 1430 μL/L for *A. flavus*. In a previous study, phenylethyl alcohol and 1-pentanol produced by *Enterobacter asburiae* Vt-7 presented the minimal inhibitory concentrations of spore germination for *A. flavus* of 100 and

Table 1
Effects of treatment with octanoic acid (OA), 2-phenethyl acetate (2PEA) and furfuryl acetate (FA) at different doses of application (μL) and control (C0) on spore germination of *A. flavus* M144 and *A. niger* M185.

Strain	Compound	Treatment (μL)	Size of germ tube (μm)	% Germination
A. flavus M144	C0	0	22.45[a]* ± 2.73	74.08[a] ± 8.11
	OA	10	19.11[a] ± 1.34	74.62[a] ± 2.81
	OA	25	19.22[a] ± 0.63	69.86[a] ± 2.94
	OA	50	17.90[a] ± 2.03	68.12[a] ± 2.35
	OA	100	18.53[a] ± 1.34	61.61[a] ± 4.85
	2PEA	10	20.23[a] ± 1.32	49.10[b] ± 3.41
	2PEA	25	19.51[a] ± 0.47	34.65[b] ± 0.77
	2PEA	50	18.83[a] ± 1.95	33.43[b] ± 7.45
	2PEA	100	18.36[a] ± 3.23	25.63[c] ± 3.25
	FA	10	11.34[b] ± 0.36	33.46[b] ± 7.30
	FA	25	11.49[b] ± 0.18	18.81[c] ± 1.91
	FA	50	7.02[b] ± 1.04	9.19[c] ± 1.06
	FA	100	0.00[c] ± 0.00	0.00[d] ± 0.00
A. niger M185	C0	0	17.41[a] ± 1.11	85.23[a] ± 4.30
	OA	10	17.11[a] ± 0.51	85.63[a] ± 5.72
	OA	25	16.91[a] ± 1.29	78.46[a] ± 2.14
	OA	50	14.12[a] ± 0.84	74.92[a] ± 0.53
	OA	100	13.86[a] ± 2.57	71.15[a] ± 5.53
	2PEA	10	14.25[a] ± 2.12	84.39[a] ± 1.16
	2PEA	25	13.31[a] ± 1.91	77.63[a] ± 3.45
	2PEA	50	12.11[ab] ± 2.06	55.12[b] ± 10.11
	2PEA	100	15.19[a] ± 2.03	27.94[c] ± 4.39
	FA	10	10.61[b] ± 1.37	66.20[b] ± 7.40
	FA	25	8.64[b] ± 0.3	57.29[b] ± 9.06
	FA	50	0.00[c] ± 0.00	0.00[d] ± 0.00
	FA	100	0.00[c] ± 0.00	0.00[d] ± 0.00

* In a column, mean values with different letters indicates statistical differences ($p \leq 0.050$) for each mould specie.

200 μL/L, respectively (Gong et al., 2019).

3.3. Effects of VOCs on gene expression

The effects of the three VOCs, at different amounts, on the relative expression of the *aflR* gene by *A. flavus* at 5, 7 and 8 days of incubation are shown in Fig. 3A. The expression of the regulatory *aflR* gene is one of the main factors in the AF pathway (Georgianna and Payne, 2009), and its expression has been associated with AF production under different environmental and nutritional conditions (Peromingo et al., 2017; Tejero et al., 2021). The relative expression of the target gene was evaluated and compared with that of the control batch (*A. flavus* inoculated on DFB agar plates in the absence of compounds) at each incubation time. The impact of the FA at 50 and 100 μL on the AF-related gene expression could not be evaluated since no *A. flavus* growth was observed. First, changes in the relative *aflR* gene expression could be observed during the incubation time; these results coincided with previous studies that demonstrated that the expression of this regulatory gene may fluctuate over time, depending on experimental conditions (Lozano-Ojalvo et al., 2013; Peromingo et al., 2017; Tejero et al., 2021). On day 5, overexpression of the AF regulator gene was provoked by the three VOCs at all the amounts tested, except in the case of OA at 10 μL. The impact on gene expression was more pronounced in FA and 2PEA than in OA. Regarding OA, amounts ranging from 25 to 100 μL had a similar influence on the expression of the *aflR* gene (≈1 $\log_2$). With respect to 2PEA, the lowest and the highest amounts of this compound increased the expression of the target gene more significantly (≈ 4 $\log_2$) than the intermediate quantities (≈ 1–2 $\log_2$). Finally, in relation to FA, the two quantities evaluated, 10 and 25 μL, stimulated the expression of the *aflR* gene at levels of 3 and 5 $\log_2$, respectively. After 7 days of incubation, an inhibition of the AF regulator gene expression was only observed when OA was applied at 25 μL, and there was no effect either at 50 or 100 μL. The remaining compounds and concentrations stimulated the expression of the mycotoxin-biosynthetic gene at levels of about 1

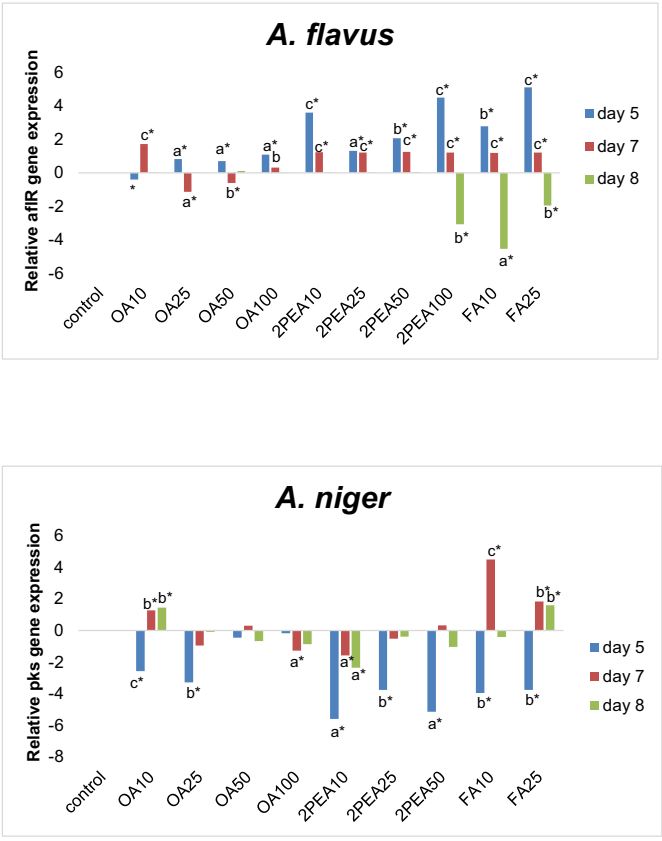

Fig. 3. Effects of volatile organic compounds on the relative expression of the *aflR* gene in *A. flavus* (A) and the *pks* gene in *A. niger* (B) on days 5, 7 and 8. Calibrators (non-treated control samples at each incubation time) always take the value of 0. OA: octanoic acid; 2PEA: 2-phenylethyl acetate; FA: furfuryl acetate. The number next to the compounds (10, 25, 50, 100) indicates the amount (μL) of compounds applied on the plates. [a,b,c] Different letters above the bars indicate significant differences ($p \leq 0.050$) within-day among the different compounds and concentrations. (*) Asterisk means significant difference ($p \leq 0.050$) regarding the calibrator.

$\log_2$. On the last day of incubation (day 8), no influence of most combinations of VOCs × amounts on gene expression was found, only at the highest 2PEA amount tested (100 μL) and the two of FA quantities (10 and 25 μL), which noticeably decreased the expression of the target gene at levels of ≈ 2–4 $\log_2$. Overall, it is important to point out that in general, the expression of this mycotoxin biosynthetic gene in the presence of VOCs was more repressed at longer incubation periods. Tejero et al. (2021) also found that two yeasts (*H. opuntiae* L479 and *H. uvarum* L793), producers of VOCs, were able to inhibit the expression of *aflR* gene by *A. flavus* at day 9 of incubation.

The influence of the combinations of the three VOCs × four amounts on the relative expression of the *pks* gene by *A. niger* at 5, 7 and 8 days of incubation is displayed in Fig. 3B. The *pks* gene is an important gene intimately linked to OTA synthesis (Gil-Serna et al., 2018; O'Callaghan et al., 2003). The relative expression of the target gene was evaluated and compared with that of the control batch (*A. niger* inoculated on DFB agar plates in the absence of compounds) at each incubation time. The impacts of 2PEA at 100 μL and FA at 50 and 100 μL on the OTA-related gene expression could not be evaluated since no *A. niger* growth was observed. At the shortest incubation time (day 5), the expression of the *pks* gene dropped when the three VOCs at the different amounts were applied, with the exception of OA at 50 and 100 μL, which had no effect on gene expression. The highest effect on *pks* gene expression was found when either 2PEA or FA was used, which provoked a considerable decline in the expression (≈4–6 $\log_2$). On day 7 of incubation, repression

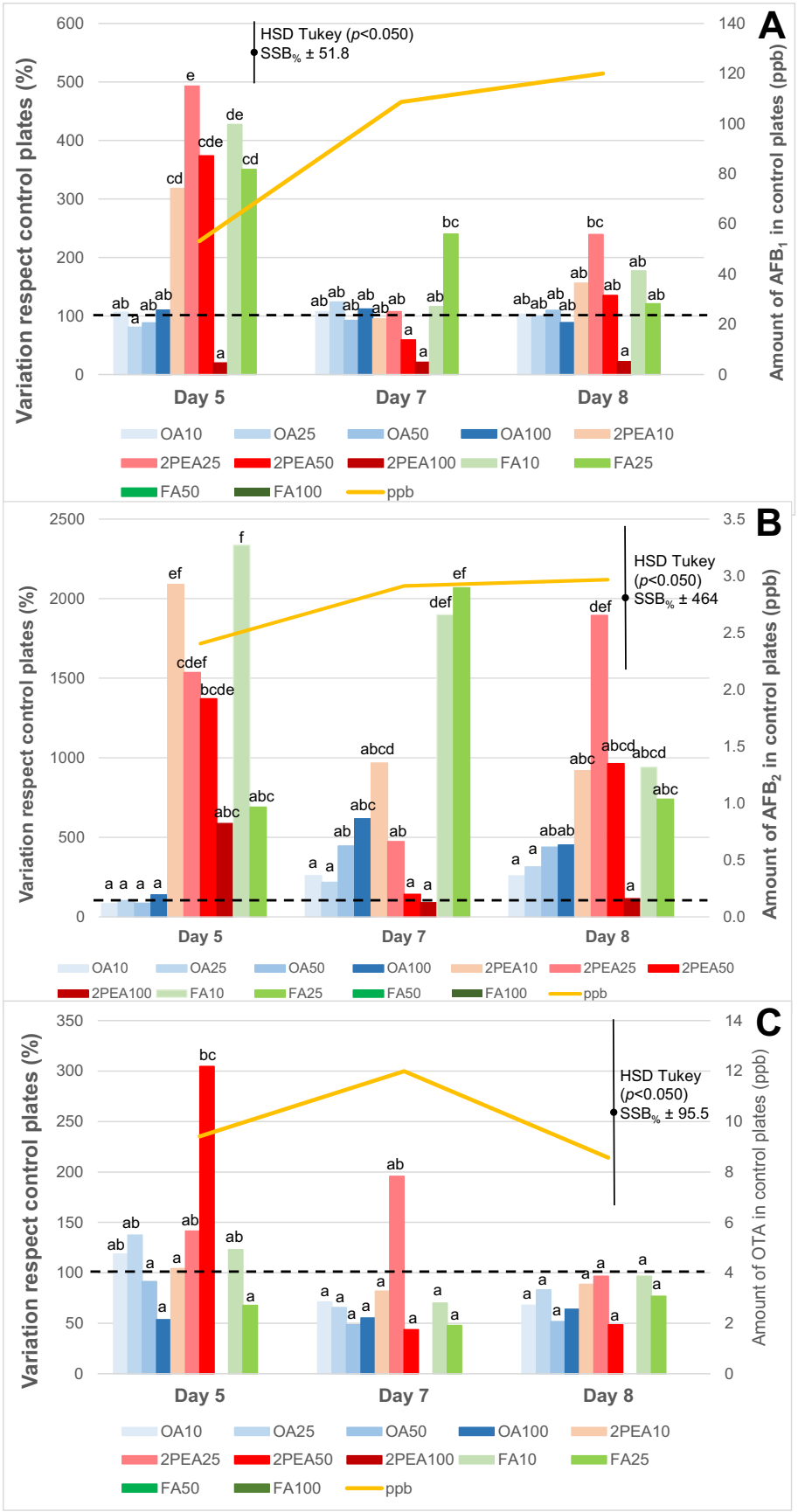

Fig. 4. Variation (%) with respect to control plates of aflatoxin B_1 (A) and aflatoxin B_2 (B) by *A. flavus* M144 and ochratoxin A (C) by *A. niger* M185 on days 5, 7 and 8 of the study of the three volatile organic compounds (OA: octanoic acid; 2PEA: 2-phenylethyl acetate; FA: furfuryl acetate). The number next to the compounds (10, 25, 50, 100) indicates the amount (μL) of compounds applied on the plates. The amounts of aflatoxin B_1, B_2 and ochratoxin A of the control plates (ppb) are shown on the yellow line (A, B, C). ^{a,b,c,d,e,f} Different letters above the bars indicate significant differences ($p < 0.050$) within-day among the different compounds and concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of gene expression was encountered when OA and 2PEA were applied at the highest (100 μL) and lowest (10 μL) amounts, respectively, at levels of about 1 log_2. Activation of the expression of the *pks* gene was observed when spreading out FA at 10 and 25 μL and OA at 10 μL. At the last sampling day, only three combinations VOCs × amount exerted a significant impact on gene expression, with two of them (OA at 10 μL and FA at 25 μL) increasing it and the other one (2PEA at 10 μL) reducing it. Taking into account the results for the ochratoxigenic species in relation to gene expression studies, a contrasting effect regarding aflatoxigenic species behaviour was found. The most pronounced inhibition of the *pks* gene expression was observed at a shorter incubation period (day 5). No previous studies regarding the influences of antifungal compounds on this OTA-biosynthetic gene expression have been conducted so far, rendering any comparisons impossible.

3.4. Effects of VOCs on aflatoxin and ochratoxin A production

The presence of AFs and OTAs during the production and storage of dried figs is a major concern for health and food safety authorities. Some yeast strains can produce VOCs, and in several cases, volatile compounds have been reported as the main factors responsible for the antagonistic capacity (Farbo et al., 2018). During the *in-vitro* assays using DFB agar plates, AFB_1 and AFB_2 produced by *A. flavus* M144 and OTA synthesised by *A. niger* M185 were significantly influenced by the three VOCs, 2PEA, FA and OA, produced by *H. uvarum* and *H. opuntiae* (Fig. 4). The percentages of mycotoxin amount variation with respect to the control DFB agar plates for AFB_1 ranged from 19.74 to 493% (Fig. 4A). The mean AFB_1 concentrations for *A. flavus* M144 (without VOCs exposure) were 53.16 ± 22.69 ppb on day 5, 108.68 ± 14.74 ppb on day 7 and 120.03 ± 30.73 ppb on day 8 (Fig. 4A). The effects of the VOCs varied at different application concentrations. Generally, the OA had no effect on AFB_1 produced by *A. flavus* compared to the control. The highest variation, with respect to the control plates, of AFB_1 was observed with 2PEA at 25 μL on days 5 and 8, with 492.97 and 239.77%, respectively, and with FA at 25 μL on day 7 (239.89%). However, the AFB_1 content was significantly diminished on days 5, 7 and 8 with 2PEA at 100 μL ($p \leq 0.050$); the percentages of variation, with respect to the control, were 19.74, 20.93 and 22.10%, respectively. In the case of AFB_2 production (Fig. 4B), the percentages of variation regarding the DFB control agar ranged from 83.56 to 2336.48%. The mean AFB_2 concentrations for the *A. flavus* M144 control agar plates on days 5, 7 and 8 ranged between 2.40 ± 1.24 and 2.87 ± 2.14 ppb. The highest percentages of variation, with respect to the control, were found on day 5 with FA at 10 μL, with 2335.48%, on day 7 with FA at 25 μL, with 2067.52%, and on day 8 with 2PEA at 25 μL, with 1793.59% ($p \leq 0.050$).The addition of FA at 50 and 100 μL effectively inhibited the production of AFB_1 and AFB_2 by *A. flavus* M144. Chang et al. (2015) demonstrated that the volatile 2-phenylethanol, produced by *Pichia anomala*, can reduce AF production when applied to pistachios. In a similar study of Wang et al. (2019), cinnamaldehyde inhibited aflatoxin production in *A. flavus*. Moore et al. (2021) reported that the volatile compounds decane and 2,3-dihydrofuran reduced the AF production by three aflatoxigenic strains and completely inhibited the production of cyclopiazonic acid by one strain.

Regarding OTA, in terms of variation with respect to the DFB control plates (Fig. 4C), the percentages varied from 43.72% in 2PEA at 50 μL on day 7 to 304.69% in 2PEA at 50 μL on day 5. The average OTA concentrations for *A. niger* M185 control plates were 9.41 ± 2.72 ppb on day 5, 12.00 ± 1.12 ppb on day 7 and 8.57 ± 0.52 ppb on day 8 (Fig. 4C). The greatest variation with respect to the OTA control plates was observed with 2PEA at 50 μL on day 5 and at 25 μL on day 7 ($p \leq 0.050$). The OTA production was effectively inhibited with FA at 50 and 100 μL and 2PEA at 100 μL on all days of the study. In other studies, VOCs produced by different strains of *Pichia anomala*, *Pichia kluyveri* and *Hanseniaspora uvarum* inhibited the growth of *A. ochraceus* and OTA production during the production of *Coffea arabica* (Masoud and Kaltoft, 2006; Masoud et al., 2005). Farbo et al. (2018) demonstrated that 2-phenylethanol, produced for four yeasts, plays an important role in the antagonistic activity against *A. carbonarius* MPVA566 and *A. ochraceus* MPVA703.

Table 2
Effects of treatment with octanoic acid (OA), 2-phenethyl acetate (2PEA) and furfuryl acetate (FA) and control (C0) on the incidence rates of *A. flavus* M144 and *A. niger* M185 on days 5, 6, 7, 8, 9 and 30.

Day	Compound	Incidence rate of *A. flavus* M144	Incidence rate of *A. niger* M185
5	C0	$96.25^{a} \pm 4.14$*	$52.50^{a} \pm 16.77$
5	OA	$70.00^{a} \pm 10.00$	$72.50^{a} \pm 12.50$
5	2PEA	$0.00^{b} \pm 0.00$	$1.67^{b} \pm 2.36$
5	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
6	C0	$94.86^{a} \pm 6.12$	$65.42^{a} \pm 26.90$
6	OA	$97.22^{a} \pm 2.78$	$91.67^{a} \pm 8.33$
6	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
6	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
7	C0	$96.25^{a} \pm 6.50$	$70.83^{a} \pm 26.46$
7	OA	$100^{a} \pm 0.00$	$94.44^{a} \pm 5.56$
7	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
7	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
8	C0	$100^{a} \pm 0.00$	$86.63^{a} \pm 10.52$
8	OA	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$
8	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
8	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
9	C0	$100^{a} \pm 0.00$	$94.44^{a} \pm 5.56$
9	OA	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$
9	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
9	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
30	C0	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$
30	OA	$100^{a} \pm 0.00$	$100^{a} \pm 0.00$
30	2PEA	$0.00^{b} \pm 0.00$	$2.08^{b} \pm 2.95$
30	FA	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$

* In a column, mean values with different letters indicates statistical differences ($p \leq 0.050$) for each mould specie.

3.5. Antifungal activities of volatile organic compounds on dried figs

Based on the *in-vitro* results, the highest amount of VOCs was selected for the *in-vivo* test (100 μL; 1430 μL/L of headspace). Since lower concentrations of VOCs could stimulate mycotoxin production, according to the results displayed in Fig. 4, the sporestatic treatment is the best choice in the use of VOCs as biofumigants. Table 2 shows the evolution of the percentages of incidence of *A. flavus* M144 and *A. niger* M185 on inoculated dried figs exposed to OA, 2PEA and FA. After 5 days of storage, control treatments achieved 96.25 ± 4.14% of infection for *A. flavus* M144, whereas a complete incidence was observed at day 8. The application of 1430 μL/L of OA produced a non-significant delay of infection at 5 days of storage ($p > 0.050$), with an incidence of 70.00 ± 10.00%. However, differences disappeared on the next day of exposure (day 6). This result could be associated with the effects of OA on spore germination and the size of the germ tube observed in *in-vitro* analyses. In the case of *A. niger* M184, a certain non-significant stimulus ($p > 0.050$) of mould development was observed at the first days of storage. Control treatments produced 52.50 ± 16.77% of infection in 5 days, whereas 72.50 ± 12.50% of infected figs were observed in OA treatments. These differences were observed until day 9, when both treatments achieved 100% incidence. Although the antifungal properties of OA on *A. flavus* and *A. niger* have previously been established (Huang et al., 2010), presenting minimal inhibitory concentrations similar to those of conventional antifungals such as sorbic acid, in our case, the application of OA did not effectively control the development of *Aspergillus sp.* in figs (Fig. 5).

The application of 2PEA and FA could effectively control both strains of *Aspergillus*. The 2PEA completely inhibited infections by *A. flavus* M144 (Table 2) during the 30 days of storage, and no symptoms of mycelium development were observed (Fig. 5). In contrast, 2.08 ±

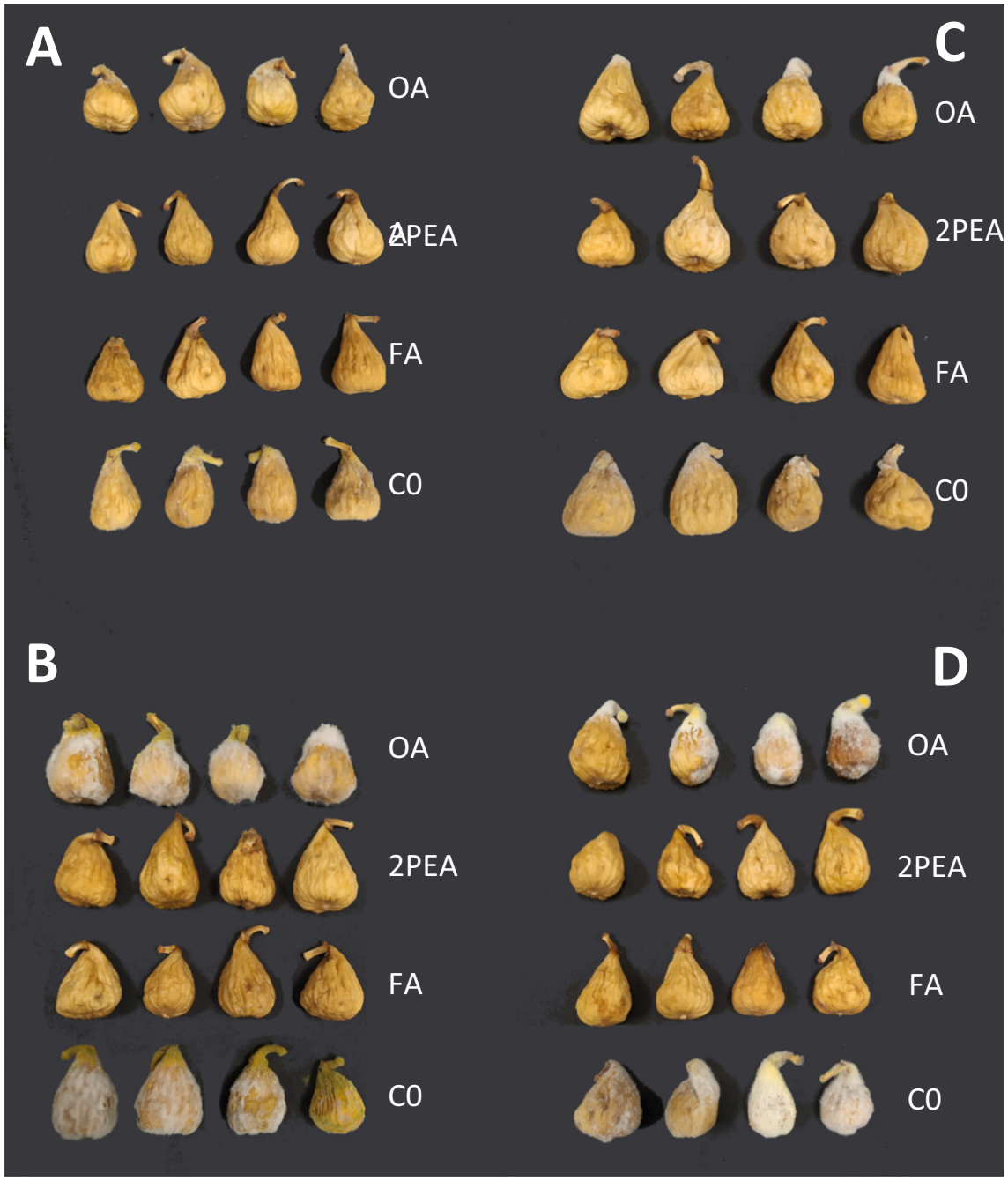

Fig. 5. Growth at 25 °C of *A. flavus* M144 (A and B) and *A. niger* M185 (C and D) on dried figs against the three volatile organic compounds, octanoic acid (OA), 2-phenylethyl acetate (2PEA) and furfuryl acetate (FA), on days 6 (A and C) and 9 (B and D) of the study at the concentration of 1413 μL/L of headspace and the control (C0).

2.95% of the figs presented a visible mycelium when *A. niger* M184 was inoculated. The application of FA avoided mycelium appearance during the 30 days of storage for both *Aspergillus* species (Table 2; Fig. 5).

4. Discussion

The findings of the *in-vitro* (growth, germination, mycotoxin biosynthetic-gene expression and toxin production) and *in-vivo* analyses (incidence rate) carried out in this study proved that two of the three VOCs tested (2PEA and FA) were effective in the control of *A. flavus* and *A. niger* either in a dried fig model system or in dried figs. In addition, the highest effect of VOCs on AFs and OTA reduction was observed on day 8, when the synthesis of VOCs by two yeasts (*H. opuntiae* L479 and *H. uvarum* L793) reaches its maximum (Tejero et al., 2021). In the case of aflatoxigenic species, this coincides with the fact that both compounds repress the expression of the regulator gene at this time, in contrast to the ochratoxigenic species. Probably, the early inhibition of the *pks* gene expression of *A. niger* by the action of VOCs is sufficient to keep the OTA levels low. The results of this study support the application of 2PEA and FA at the early post-harvest stages of dried figs to control mycotoxin accumulation.

Previous studies have pointed out the convenience of using VOCs as biofumigants because of their rapid conversion to gaseous state (Passone and Etcheverry, 2014) and the absence of organoleptic modifications on products (Schotsmans et al., 2008). In this sense, this work has proved the high effectivity of FA and/or 2PEA to control *A. flavus* and *A. niger*

infections in dried figs. Moreover, these compounds presented pleasant fruity and floral odours, respectively; and are considered as safe (GRAS) flavouring agents by the Flavor and Extract Manufacturers' Association (FEMA) and the Food and Drug Administration (FDA). Thus, there are no safety concerns when using biofumigants to control toxigenic moulds during dried fig production and commercialisation.

The selection of the concentrations of these compounds used in biofumigation treatments is key in order to ensure mycotoxin concentrations are kept below legal limits. It has been found that at low concentrations of 2-PEA and FA there is an increase in aflatoxins and OTA synthesis. In this regard, Klich (2007) reported that certain biological treatments using yeasts, and chemicals such as pesticides and volatile compounds have similar effects on mycotoxin production in *Aspergillus* spp. Specifically, among the volatile compounds tested, C6 to C9 alkenals stimulate aflatoxins synthesis at low concentrations (Zeringue, 1991), and similar effect was found when applying 3-methyl-1-butanol (Greene-McDowelle et al., 1999). Therefore, the most relevant findings of the current study related to the use of VOCs, should be focused on their ability to inhibit spore germination, as shown in *in vitro* and *in vivo* tests at the highest concentrations tested. Additionally, these findings also question the suitability of using the VOC-producing yeasts of the *Hanseniaspora* genus in products susceptible to contamination by toxigenic fungal species. De Paiva et al. (2017) and Ruiz-Moyano et al. (2016, 2020) showed the feasibility of using antifungal VOC-producing yeasts on several fruit species. By contrast, the concentrations of VOCs produced by these antagonists may cause the opposite effect after application on dried figs, which means increasing mycotoxin synthesis. Therefore, 2-PEA and FA can be considered as potential treatments for the control of *Aspergillus* spp. growth in dried figs; however, the concentrations applied must ensure spore inhibition to avoid potential risks of mycotoxin formation.

CRediT authorship contribution statement

Ana Isabel Galván: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Alejandro Hernández:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition. **María de Guía Córdoba:** Writing – review & editing, Funding acquisition. **Alberto Martín:** Visualization, Writing – review & editing. **Manuel Joaquín Serradilla:** Writing – review & editing. **Margarita López-Corrales:** Project administration, Funding acquisition. **Alicia Rodríguez:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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