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8-Carbon oxylipins inhibit germination and growth, and stimulate aerial conidiation in *Aspergillus nidulans*☆

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ABSTRACT

Germination of *Aspergillus nidulans* conidia in liquid cultures was progressively inhibited at inoculum loads above 1×10^5 conidia mL^{-1}. High conidial densities also inhibited growth of neighbouring mycelia. The eight-carbon oxylipin 1-octen-3-ol was identified as the main inhibitor in a fraction also containing 3-octanone and 3-octanol.

These three oxylipins also increased the conidiation rate of dark-grown surface cultures, but had no effect on liquid cultures. 3-octanone was the most conidiogenic compound. The action of 3-octanone required functional forms of developmental activators *fluG*, *flbB-D* and *brlA*, and was not additive to the conidiogenic effect of stress stimuli such as osmotic stress or carbon starvation.

Oxylipins were produced shortly after hyphae made contact with the atmosphere and were most effective on aerial mycelia, indicating that they perform their signalling function in the gas phase.

Introduction

The fungal colony (mycelium) is an intricate network of cells that combines highly polarised growing cells (hyphae) with asexual and sexual reproductive structures. The processes that govern the stages of mycelium development respond to environmental cues, as well as endogenous autoregulatory signals, of which only a few examples have been documented (Ugalde 2006). Among the former, the emergence to the atmosphere has been reported as a powerful natural stimulus of asexual spore production (Morton 1961), although the precise mechanism by which it is sensed has not been clearly established. Aerial emergence is often accompanied by exposure to light, and this further stimulates conidiation in the case of *Aspergillus nidulans*, where the VeA (velvet) protein plays a major regulatory role (revised in (Calvo 2008)). Blue and red light-sensing systems interact with VeA in the nucleus, where it accumulates in the dark (Stinnett *et al.* 2007; Purschwitz *et al.* 2008). VeA also integrates signalling of secondary metabolite and mycotoxin biosynthesis (Bok & Keller 2004; Bayram *et al.* 2008; Calvo 2008).

☆ Dedicated to Prof. A.P.J. Trinci.

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Abbreviations: 8CO, Eight-carbon oxylipin; flb, Fluffy low bristle expression mutant; SDO, Saturating dissolved oxygen; VOC, Volatile organic compound

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Conidia normally germinate under favourable growth conditions, but also produce signals that inhibit conidial germination in overcrowded conditions, termed auto-inhibitors (Allen 1957), which ensure efficient substrate colonisation. These compounds include Volatile Organic Compounds (VOCs) that may be species-specific, as in many obligate biotrophs, or interspecific, as in saprotrophs ((Ugalde 2006) and references therein). The eight-carbon oxylipin (8CO) 1-octen-3-ol was reported to act as a specific germination auto-inhibitor in the food spoiling fungus *Penicillium paneum*, and was also attributed the role of inducing micro cycle conidiation in spores which had undergone germination under crowding conditions (Chitarra *et al.* 2004). Recent studies in *Trichoderma* spp. have also shown that 1-octen-3-ol, and other 8COs such as 3-octanone and 3-octanol were produced in conidiating cultures and interestingly, induced conidiation in non-conidiating neighbouring cultures (Nemcovic *et al.* 2008).

Auto-inhibition of germination resulting from high conidial densities was reported in the model organism *A. nidulans* over four decades ago (Trinci & Whittaker 1968), although the chemical signal involved in the process was not specified. In this investigation, we report that 1-octen-3-ol is the principal germination auto-inhibitor in overcrowding conditions. In addition, each 8CO inhibited vegetative growth and promoted conidiogenesis, with 3-octanone as the most active agent in this capacity. We provide further evidence that 8COs are produced in the mycelium shortly after contact with the atmosphere and that VeA-complex is involved in its biosynthesis.

Materials and methods

Strains and culture conditions

The *Aspergillus nidulans* strains used in this study (Table 1) were cultivated on minimal medium (MMA) as described by (Pontecorvo *et al.* 1953) and (Cove 1966). Solid cultures were prepared with 1.5 % (w/v) agar. Liquid cultures consisted of 100 ml MMA (without agar) in 500 mL conical flasks shaken at 150 rpm in an orbital incubator. All cultures were incubated at 37 °C in the dark.

Hyperaeration experiments were carried out in cultures that had previously been cultivated for 18 h as above by pumping air through a 0.45 μm pore size filter (Millex-GR, Millipore, Bedford MA) into the medium at a rate of 200 mL min^{-1} for 1 h.

Table 1 – *Aspergillus nidulans* strains used in this study.

Strain	Genotype	Source
WIM126	*pabaA1*; *yA2*; *veA+*	Butnick *et al.* 1984
FGSC26	*biA1*; *veA1*	Fungal Genetics Stock Center
TTA127.4	*ΔfluG::trpC*; *biA1*; *veA1*	Lee & Adams 1994
RRMD3.4	*pyrG89*; *pyroA*; *veA+*	Stinnett *et al.* 2007
MAD2446	*pyrG89*; *wA4*; *inoB2*, *pyroA4*; *hhoA::mCh::pyroA*; *(Myc)3-PacC(PacC900)*; *veA1*	Etxebeste *et al.* 2009
BD234	*ΔfluG::trpC*; *pyroA4*; *veA+*	This study
BD239	*ΔnkuA::argB*, *pyroA4*; *ΔflbC::pyrG*; *veA+*	
BD215	*pyrG89*; *ΔflbB::pyrG*, *pyroA4*; *veA+*	
BD276	*pyrG89*; *pyroA4*; *ΔflbD::pyrG*, *veA+*	
BD400	*pyrG89*; *pyroA4*; *ΔbrlA::pyrG*, *veA+*	

Monitoring of germination, growth and conidiation levels

Spore suspensions were obtained from surface cultures incubated for 7 d by adding 10 mL of distilled water with 0.02 % (v/v) Tween solution and gentle mechanical removal with a sterile glass rod. The suspension was then filtered through Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 4000*g* for 5 min in order to concentrate the spores. When the action of 8COs was monitored, the spore suspension was first washed free of auto-inhibitors by centrifugation and resuspension in 0.02 % Tween solution as above, for three successive cycles, before addition of the compound or extract. Germination of conidia was monitored in liquid medium at different spore or auto-inhibitor concentrations by microscopic observation of at least 500 sporelings in each of three measurements.

Growth inhibition by spore suspensions or after addition of 8COs was monitored as described (Chitarra *et al.* 2004). Briefly, one Petri dish was prepared containing a top and a bottom layer. The topside contained the spore suspension or filter paper soaked with the tested compound. On the bottom side of the plate, a mycelial plug was placed that had been obtained from the periphery of a 48 h old culture. The plate was incubated for 40 h. The diameters of colonies on the bottom layer (excluding the central plug) were measured.

Inhibition of germination and growth caused by 1-octen-3-ol was analysed in liquid MMA. To avoid the auto-inhibitory effect of conidia crowding, 10^5 conidia ml^{-1} of RRMD3.4 or MAD2446 strains were inoculated in 20 ml of MMA in the presence or absence of 4 mM 1-octen-3-ol and incubated in an orbital shaker at 37 °C. Whole cultures were filtered through 0.45 μm pore size filter and resuspended in 100 μl of MMA for microscopic observation. Images of conidia, using Nomarski optics, were taken at $t = 0$, $t = 4$, $t = 6$ and $t = 16$ h. Conidial swelling was determined by measuring the cell diameter using ImageJ package software. Cell volume was estimated considering conidia as spheres. To determine the lifting of conidial germination arrest after removal of 1-octen-3-ol, cultures that had been exposed to 1-octen-3-ol for 4 h were filtered and transferred to fresh medium and further incubated for 2, 4 and 6 h before conidial volumes were measured. MAD2446 was used to observe nuclear morphology and distribution by visualisation of a cherry-mRFP tagged version of Histone 1 protein, HhoA. Strain and protocols for HhoA-mCh observation are described in Etxebeste *et al.* (2009), (see Table 1 and below).

The level of conidiation of surface colonies was determined, using the methodology described by (Calvo *et al.* 1999). Briefly, 8CO pure compounds were loaded onto 12.5 mm diameter sterile filter paper discs and placed onto 55 mm diameter Petri dishes containing 15 mL of MMA with a lawn of 10^5 conidia. These conidia had previously been washed free of auto-inhibitor. The level of conidiation was

Table 2 – Effect of conidia concentration on germination and growth.

Conidial concentration (conidia mL^{-1})	1×10^3	1×10^4	1×10^5	1×10^6	1×10^7	1×10^8
% Germination[a]	91.3 ± 7.1	92.5 ± 7.4	83 ± 7.0	72 ± 6.1	17 ± 0.3	8 ± 0.3
Kr ($\mu m\ h^{-1}$)[b]	280 ± 31	283 ± 33	260 ± 21	204 ± 14	195 ± 16	189 ± 13

a The germination rate of conidia of strain RRMD3.4 at different concentrations in liquid medium, after 11 h of incubation.
b Radial extension rate (Kr) of colonies from the same strain cultivated for 48 h in the presence of different concentrations of conidia as described in Materials and methods.

determined by collecting conidia as above and performing a total count by microscopic observation in a Thoma cell.

Extraction and analysis of 8COs

Extraction of 8COs from conidia was conducted from a suspension of 10^{11} conidia from 7-d old MMA surface cultures of strain FGSC26 (Table 1) in 10 mL of H_2O:CH_3CN (95:5) and further shaking at 250 rpm for 10 min at 25 °C. The suspension was then centrifuged at 2500*g* for 5 min. The supernatant was separated and the precipitated spores were washed again as above twice. Washing solutions were mixed resulting in a total of 30 mL of 8COs-extract, which was filtered through a nylon filter (0.45 μm pore size) to eliminate residual spores.

Extraction of 8COs from mycelium biomass was carried by preparing liquid cultures (3×100 mL) inoculated with 10^6 conidia mL^{-1} from strains RRMD3.4 (*veA*+) and FGSC26 (*veA*1) (Table 1), and incubated for 18 h. The biomass was filtered through 47 mm diameter, 0.45 μm pore size nitrocellulose membrane filters. The filters now overlaid by a thin layer of biomass were placed on fitting MMA plates and incubated for 1 h under white light followed by increasing periods of incubation in the dark. Parallel controls were incubated in the dark following (Mooney & Yager 1990). Biomass from each filter incubated at different regimes of light and dark were removed and added to 30 mL of ethyl-acetate, incubated at 250 rpm for 10 min at room temperature and filtered through a 0.45 μm pore size nitrocellulose membrane as above. The resulting solution was extracted with 15 mL (3 extractions × 5 mL) of diethyl ether. The ether was allowed to evaporate leaving an orange oily residue which was resuspended with acetonitrile to a volume of 120 μl. Extracts were analysed by HPLC injecting 10 μl in a 10×250 mm Hypersil-ODS (SUPELCO) column using 100 % acetonitrile as the mobile phase at a flow rate of 2.2 mL/min. Quantitative determinations were conducted using external standard curves obtained with purified standards (Sigma–Aldrich).

Qualitative identification of VOCs other than 8COs was carried out from extracts obtained as above, diluted in acetonitrile and analysed in an Agilent GC/MS instrument fitted with a 300×0.25 mm HP-5MS capillary column and a Triple Axis Detector. Compound identifications were performed with Wiley 8 and NIST05 mass spectrum libraries.

Light microscopy

Liquid cultures were examined with a Nikon Optiphot microscope in bright-field or fluorescence mode. Images were recorded with a Nikon FX-35DX camera attached to the microscope. Epifluorescence and Nomarski optics observations were done using a Nikon 80i microscope equipped with specific filters for mRFP visualisation and images were captured with an ORCA-ER digital camera (Hamamatsu Photonics) and processed using Metamorph software (Universal Image).

Results

1-octen-3-ol is the main auto-inhibitor of germination and growth

The germination response of *Aspergillus nidulans* conidia under crowding conditions initially described by Trinci & Whittaker (1968) corresponded to a currently unavailable strain. Hence, the process was fully reassessed with alternative strains and methods. Table 2 shows the percentage of germinating conidia of the *veA* wild-type strain RRMD3.4 at increasing concentrations in liquid MMA. These results indicate that conidial densities above 1×10^5 conidia mL^{-1} resulted in increasing inhibitory levels, reaching a maximum at conidial concentrations exceeding 1×10^8 conidia mL^{-1}. These results are comparable to those described in the first study (Trinci & Whittaker 1968). In addition to these effects on germination, high conidial densities on the upper surface of MMA plates also inhibited the radial growth of mycelial plugs inoculated centrally on MMA plates (Table 2).

In order to identify the active agent responsible for the described auto-inhibitory effects, spore suspensions were subjected to solvent extraction as described in Materials and methods and an HPLC analysis protocol. Fig 1 shows a profile of the extracted chemicals with a predominant peak with a retention time (t_R) of 7.6 min. This t_R coincided with that of a 1-octen-3-ol standard. This compound had been reported as the germination and growth auto-inhibitor contained in *Penicillium paneum* conidia (Chitarra *et al.* 2004), and as an endogenous volatile produced in *A. nidulans* (Fischer *et al.* 1999) as well as other related fungi (Fischer *et al.* 2000; Jelen 2003; Karlshøj *et al.* 2007). Fractions corresponding to the two other major peaks ($t_R = 6.2$ and 8.5 min) were separated and identified as 3-octanol and 3-octanone, respectively, using standards.

All three 8COs were assayed for their capacity to inhibit germination in liquid medium at a concentration previously reported to be effective (4 mM as in (Chitarra *et al.* 2004)). After 10 h of incubation, the germination levels in the presence of 1-octen-3-ol were of 12 ± 0.8 % in contrast to the control, which germinated to 98 ± 7 %. Addition of 3-octanone and 3-octanol to the medium were much less effective, reaching germination levels of 67 ± 7 % and 73 ± 6 % respectively. Hence, it was

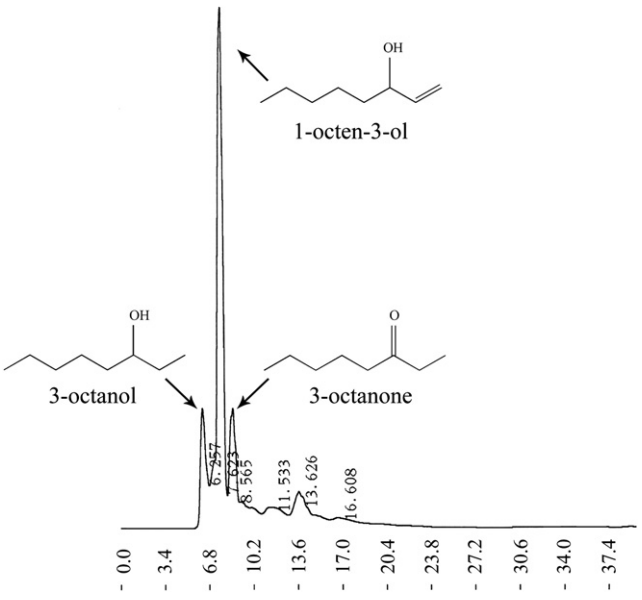

Fig 1 – Reverse phase HPLC separation of the ether extract from a conidial suspension of strain FGSC26, as described in Materials and methods. The major peak, with a t_R of 7.6 min corresponded to 1-octen-3-ol. Peaks with a t_R of 6.2 and 8.5 min corresponded to 3-octanol and 3-octanone respectively, as determined by the t_R values of the corresponding standards.

considered that 1-octen-3-ol was the principal inhibitory component in the fraction. A quantitative assessment of activity using increasing doses of 1-octen-3-ol (1, 2.5, 4.0, and 5.0 mM) yielded germination rates after 10 h of incubation of 87 ± 6.1 %, 36 ± 4.3 %, 14 ± 2.1 %, and 1 ± 0.3 %, respectively. The germination level of controls was of 95 ± 5.3 %.

The inhibitory effect of 1-octen-3-ol on colony growth was assessed using centrally inoculated agar plugs in a Petri dish containing different concentrations of commercial 8COs and equivalent amounts of 8CO-extracts from mycelial cultures. In these experiments the light-dependent, *veA*$^+$, and light-independent, *veA1*, conidiating strains WIM126 and TTA127.4, were respectively used. For both strains, inhibition was observed at a range between 0.06 mM and 11.70 mM in surface cultures (Fig 2A). The response pattern of the 8CO-extract and 1-octen-3-ol were comparable with responses being observed at a minimum concentration of 0.065 mM, and the maximum inhibition observed at 11.65 mM.

Microscopic examination of both conidia and hyphae exposed to 1-octen-3-ol at a concentration of 4 mM in liquid medium, revealed reversible effects which could not be associated to cell damage or stress. Normally germinating conidia displayed a three-fold increase in volume after 4 h of incubation in MMA. Those conidia exposed to 4 mM 1-octen-3-ol did not undergo conidial swelling after 4 h, and remained quiescent throughout the experimental period (up to 16 h, Fig 2B). This inhibitory effect of 1-octen-3-ol was shown to be reversible, as conidia treated as above that were subsequently washed of the inhibitor underwent swelling after 4 h of incubation, and after 6 h, the swelling was comparable to that observed in untreated controls (Fig 2B), with no observable changes in spore viability (not shown). Examination of vegetative hyphae in liquid MMA supplemented with 4 mM 1-octen-3-ol revealed no visible signs of stress such as vacuolisation, cell or tip swelling, or nuclear degradation after 4 h of incubation.

The combined results indicated that 1-octen-3-ol was the principal agent responsible for inhibition of germination and growth in the 8CO fraction, following a pattern which resembled the one previously described for *P. paneum* (Chitarra *et al.* 2004).

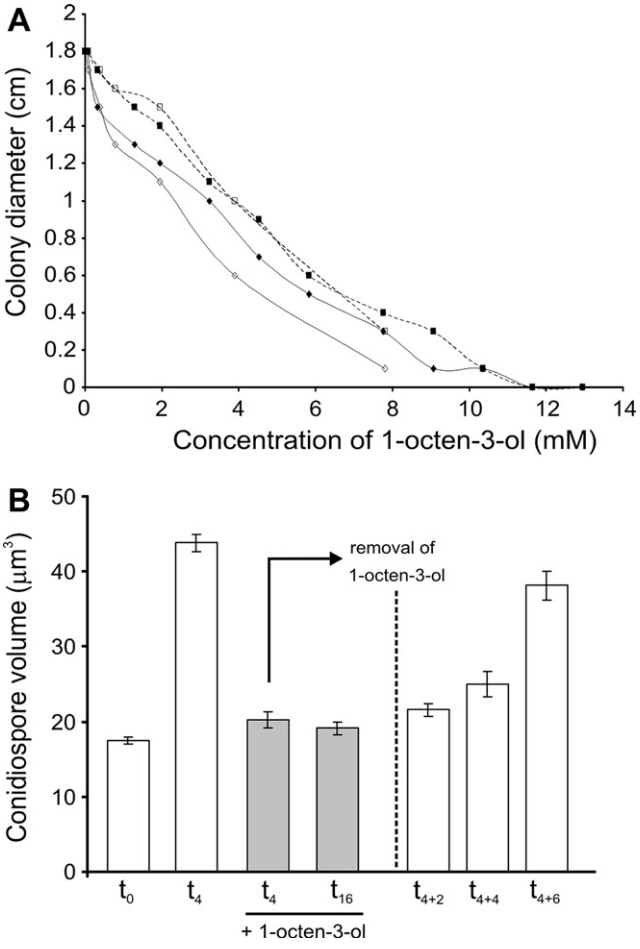

Fig 2 – The inhibitory effect of 1-octen-3-ol. (A). The pattern of growth inhibition recorded in TTA127.4 (◇, ◆) and WIM126 (□, ■) colonies exposed to increasing concentrations of the obtained extract (white) and pure 1-octen-3-ol (black) for 40 h of incubation. (B). The inhibitory effect in conidium isotropic growth. In liquid medium strain RRMD3.4 (*veA*$^+$) increases conidia volumes after 4 h (t_4) incubation at 37 °C, being this the isotropic growth phase of conidium germination. (t_0) indicates conidia volumes prior to inoculation. Addition of 4 mM 1-octen-3-ol (grey bar) prevents conidia swelling. Isotropic growth of conidia following removal of the inhibitor after 4 h incubation was measured at 2 h (t_{4+2}), 4 h (t_{4+4}) and 6 h (t_{4+6}). Standard errors are shown; number of conidia measured ($n = 50$) for each observation.

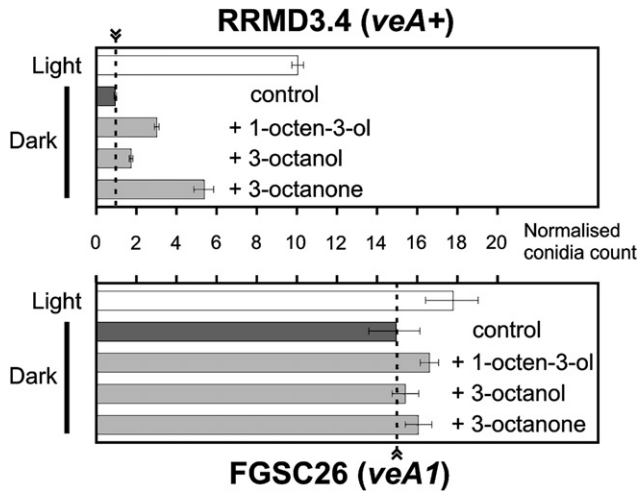

Fig 3 – Conidial production of wild-type and mutant *veA* strains and the effects of 8COs. The *veA*+ strain RRMD3.4 and the *veA*1 strain FGSC26 were cultured under light and dark conditions (bars in white and black, respectively), as well as under dark conditions in the presence of 1 mg of 1-octen-3-ol, 3-octanol and 3-octanone (bars in grey). Conidia counts, quantifying conidia mm^{-2} and normalising the resulting values were carried out according to Calvo *et al.* (1999).

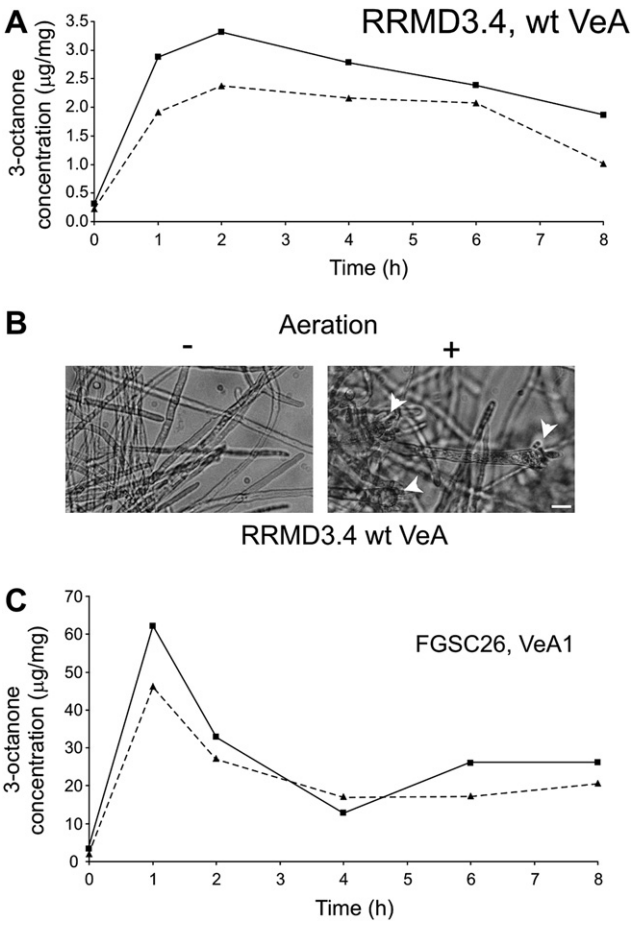

Fig 4 – Effect of aeration on cell morphology and effect of *veA*1 loss of function mutation on 3-octanone production. (A). The amount of 3-octanone, expressed as μg of 3-octanone per mg of mycelium, produced by mycelium grown in liquid medium, filtered and exposed to the air at time intervals with (■) and without (▲) light exposure in *veA*$^{+}$ strain RRMD3.4. (B). The morphology of hyphae from strain RRMD3.4 after 18 h of pre-cultivation in liquid medium followed by forced aeration (2.0 vvm) for 24 h (+) or continued culture conditions as described in Materials and methods (−). Arrowheads indicate emerging conidiophore structures. Scale bar = 5 μm. (C). The amount of 3-octanone, expressed as μg of 3-octanone per mg of mycelium, produced by mycelium grown in liquid medium, filtered and exposed to the air at time intervals with (■) and without (▲) light exposure in *veA*1 strain FGSC26.

8COs promote aerial conidiation

Given that 8COs had been reported to exert a positive effect on conidiation in other filamentous fungi, we examined the effects of the 8CO fraction on surface culture conidiation in an *Aspergillus nidulans* strain showing a light-dependent conidiation response, RRMD3.4, and a strain that conidiates independently of illumination, FGSC26. Conidial production of the *veA*$^{+}$ strain RRMD3.4 was profuse on exposure to white light, with respect to dark-grown cultures (Fig 3). When 1-octen-3-ol (1 mg) or 3-octanol (1 mg) was added to the plates, the rate of conidial production in the absence of light was increased to a low extent. However, an increase of five fold was observed with 3-octanone (1 mg; Fig 3). Higher doses did not increase the conidiation response. In addition, the combination of 3-octanone treatment with exposure to light did not result in conidiation levels surpassing those obtained under light incubation alone. Addition of each of the oxylipins to cultures of the constitutively conidiating *veA*1 mutant FGSC26 did not alter the conidial output of the strain significantly over the levels registered in the absence of the oxylipin (Fig 3).

Since 3-octanone was the most active conidiation inducer, all subsequent assays were conducted with this compound. In order to elucidate the stage at which 3-octanone affected conidiation, we administered 1 mg to a series of null mutants (Table 1) of Upstream Developmental Activators (*UDA*s), reported to regulate conidiation at early stages (Yu *et al.* 2006; Etxebeste *et al.* 2010). The results indicated that 3-octanone was unable to induce the conidiation response in wild-type *veA* strains bearing null mutations in any of the *UDA* genes: *fluG*, or *flbB, flbC* or *flbD* (not shown, strains BD234, BD215, BD239 and BD276, respectively; Table 1). Similar experiments using a null mutant of the central regulatory pathway regulatory gene *brlA* (strain BD400; Table 1) yielded a similar result (not shown). In addition, surface cultures subjected to stress conditions which are known to act as independent conidiation stimuli such as culture with 0.6 mM KCl or carbon and nitrogen starvation (Etxebeste *et al.* 2008) presented no significant increases in conidiation when added 3-octanone (not shown). Finally, the addition of 3-octanone at 0.5 and 1 mg mL^{-1} to a 24 h mycelium grown in liquid media in a shaken flask did not result in induction of conidiation in the subsequent 48 h of incubation (not shown).

The combined results indicated that 3-octanone enhanced the conidiation response in aerial cultures only, partially bypassing or inactivating the blocking effect exerted under

darkness by the nuclear Velvet (VeA)-complex (Stinnett et al. 2007; Purschwitz et al. 2008). Conversely, 3-octanone appeared not to act as a separate inducer of conidiation, since it was not effective under submerged conditions and did not exacerbate the conidiation response to stress stimuli.

The production of 8COs is associated with the emergence to the atmosphere

In order to determine the growth conditions in which 8COs were produced, and more specifically 3-octanone, mycelium from liquid cultures of strain RRMD3.4 was collected by filtration, placed onto MMA-containing Petri dishes and then incubated at increasing time intervals followed by the extraction of 8COs to determine the levels of 3-octanone produced. To verify whether light irradiation modified 3-octanone production, a set of plates were irradiated at various inductive time intervals with light according to a classic protocol (Mooney & Yager 1990). Initial tests had shown that 8COs were not efficiently extracted as in conidial suspensions, with a water: acetonitrile mixture. In turn, mycelial extractions required ethyl-acetate, suggesting that the 8CO fraction was intracellular, rather than superficial. The results in Fig 4A reveal that 3-octanone was produced within the first 2 h of exposure of the mycelium to the atmosphere, and an increase of approximately 25 % was registered in illuminated plates irrespective of the duration of the period of light exposure (thus only the results corresponding to continuous exposure are presented). These results indicated that the major stimulus for 3-octanone synthesis was the contact with the atmosphere. Further observations demonstrated that the greater production levels detected under illumination were likely due to comparatively higher mycelium drying rates rather than to photoinduction phenomena, as illuminated mycelia presented consistently lower wet weights with respect to mycelia incubated in the dark (not shown).

In order to ascertain whether 8CO emissions were solely dependent on oxygen availability, strain RRMD3.4 was cultured in liquid medium and placed under saturating dissolved oxygen (SDO) conditions for 1 h. After this treatment, the mycelium was filtered and the 8CO fraction was extracted and analysed by HPLC, with no significant differences in the 8CO profiles (not shown). A more detailed GC-MS analysis of the fractions (Appendix) revealed increases in the content of various VOCs, but no significant production of 8COs. Thus, it was concluded that saturating oxygen concentrations alone were not sufficient for 8CO production.

In the process of these experiments, we observed that extended culturing under SDO conditions for 24 h resulted in a submerged conidiation response (Fig 4B). This latter observation led us to consider that SDO conditions may affect the functionality of VeA-complex. To determine a role of VeA in octanone production, we conducted an 8CO production assay under identical conditions to those shown in Fig 4A, with *veA1* strain FGSC26. Levels of 3-octanone produced exceeded by over 20 fold those recorded for the wild-type strain (Fig 4C), and as observed before, the results did not support the existence of photoinduction.

Overall, the results support the view that oxylipin production is a process that requires the emergence of hyphae to the atmosphere and is negatively regulated by VeA. Oxygen availability alone is not sufficient to stimulate 3-octanone synthesis but promotes conidiation in liquid medium. Integration of SDO and VeA signalling therefore remains a regulatory process as yet not clarified.

Discussion

1-octen-3-ol, 3-octanone and 3-octanol are eight-carbon volatiles resulting from the oxidation and cleavage of linoleic acid (Combet et al. 2009). They are classified as oxylipins, a group of molecules that also includes 18C derivatives of linoleic acid oxidation (collectively called *psi*-factor), which regulate the balance between asexual and sexual development in *Aspergillus nidulans* (Tsitsigiannis & Keller 2007). 1-octen-3-ol was described as the molecule responsible for the density-dependent auto-inhibition of spore germination in *Penicillium paneum* (Chitarra et al. 2004). This phenomenon, also known as the "crowding effect", has been described in many fungi, including *A. nidulans* (Trinci & Whittaker 1968), although the chemical nature of the responsible agent had not been identified to date.

This study has shown that 1-octen-3-ol is the principal cell density-dependent auto-inhibitor signal in *A. nidulans*, effective at a range of concentrations comparable to that observed in the food spoiling fungus *P. paneum* (Chitarra et al. 2004). This feature, as well as the widespread occurrence of 8COs as fungal VOCs (Fischer et al. 1999), would suggest that the mode of action of 8COs might be common among a range of saprophytic filamentous fungi, with subsequent important ecological implications. Indeed, these compounds could play a fungistatic role not only between colonies of related species, but also more interestingly, among different fungal species in enclosed terrestrial microenvironments, in which communication through volatile signals is, most probably, more effective than by diffusion through liquids, biofilms or semisolid substrates. Although this study was limited to their effects on conidial germination, hyphal extension and conidial production, the possible extension of the signalling role of 8COs to meiospore (ascosoore) germination is an interesting and important aspect which remains to be explored.

The mechanism by which 8CO compounds inhibit both germination and growth has been proposed to involve reversible physical changes in plasma membrane function (Chitarra et al. 2005). However, a more specific signalling mode of action cannot be ruled out, especially in view of the evidence presented in this and former studies (Chitarra et al. 2004), whereby growth inhibition is accompanied by an increase in conidiation of aerial cultures, and the effects observed are reversible. Indeed, the less volatile but structurally related 18C oxylipins, are also involved in developmental regulation within the colony (Calvo et al. 1999; Calvo et al. 2001; Tsitsigiannis & Keller 2007). We postulate that 8COs could function in overcrowding signalling by partly sharing mechanistic features with their intracolonial *psi*-factor counterparts. The morphogenetic response to 8COs of signified mutants in the balance of *psi* factor synthesis (PpoA and PpoC; (Tsitsigiannis et al. 2004a, 2004b)), and hence the mitospore/meiospore production balance, would probably help verify the plausibility of this speculation.

Regarding the production of 8COs, this study has ascertained that large amounts of 8COs are present in spores and are extractable through mild methods using aqueous acetonitrile mixtures. This would suggest that 8COs are already present in mature spores, and lightly bound to the spore surface, in a similar way to that described for the volatile auto-inhibitors of rust urediospores (Allen 1972; Macko & Staples 1973). In contrast, mycelial 8COs could not be efficiently extracted with these methods, and required ethyl-acetate extraction, suggesting an intracellular location.

This opening study could not unveil the details of 8CO synthesis, but our observations suggest that the process is finely regulated and closely related to the formation of aerial hyphae and conidiation. The reactions that yield 8COs through the oxidative cleavage of linoleic acid are catalysed by lipoxygenases, for a full description consult (Combet *et al.* 2009). A gene encoding such an enzyme (*Aflox*) has been described in *Aspergillus flavus*, and the deletion of this gene abolished the inhibition of germination associated to the "crowding effect" (Horowitz-Brown *et al.* 2008). Thus, AFlox activity most likely is involved in the oxidative cleavage of linoleic acid to form 8COs in *A. flavus*. However, our searches have yielded no homologues of this gene in the *A. nidulans* genome, and we postulate that the reaction may be performed in this species by another mechanism, possibly involving PpoC or another related desaturase enzyme. The VOC profiles of the abovementioned Δ*ppoA* and Δ*ppoC* mutants, and their corresponding crowding effect phenotypes would provide valuable evidence to evaluate this hypothesis.

The few references cited in the field of fungal 8CO biology throughout this study illustrate the need for further investigations to clarify important aspects, which were encountered here. Our observations show that high availability of oxygen alone is not sufficient to generate 8COs. Other regulatory controls, likely related to aerial emergence therefore influence the process. Moreover, the high content of 8COs detected in conidia would indicate that 8CO production, or the combination of all the elements for their immediate production on hydration of conidia, is a major feature of the later stages of conidium maturation.

Finally, our preliminary results involving 8COs and VeA cross-regulation suggest the absence of a direct effect of 8COs in VeA function, but on the contrary, 8CO production may well be under the control of VeA, possibly *via* repression. Thus, the regulatory role of VeA and its consequences in conidiation induction may show to be even more complex than presently envisaged (Calvo 2008).

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Appendix

List of the VOCs extracted from the mycelium of *Aspergillus nidulans* grown in shaken liquid cultures that experiment most significant changes after 1 h under SDO conditions (aeration at 2 vvm). The Confidence column represents the degree of confidence in the identification by comparison of obtained mass spectra with those of the reference libraries used.

Compound	Molecular formula	Confidence (%)	Presence
2,6-di-tert-butyl-4-methylene-2,5-cyclohexadien-1-one	$C_{15}H_{22}O$	91	In both samples, largely increased in SOD sample
1-hydroxy-4-methyl-2,6-di-tert- butylbenzene	$C_{15}H_{24}O$	98	
2,6-di-tert-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	$C_{15}H_{24}O_2$	97	
Glycerol	$C_3H_8O_3$	83	In control only

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