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Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi

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Abstract: The dimorphic fungus *Candida albicans* secretes farnesol, which acts as a quorum-sensing molecule and prevents the yeast to mycelium conversion. In this study we examined the effect of farnesol in the filamentous fungus *Aspergillus nidulans*. We show that externally added farnesol has no effect on hyphal morphogenesis; instead, it triggers morphological features characteristic of apoptosis. Additional experiments suggest that mitochondria and reactive oxygen species (ROS) participate in farnesol-induced apoptosis. Moreover, the effects of farnesol appear to be mediated by the FadA heterotrimeric G protein complex. Because *A. nidulans* does not secrete detectable amounts of farnesol, we propose that it responds to farnesol produced by other fungi. In agreement with this notion, growth and development were impaired in a farnesol-dependent manner when *A. nidulans* was co-cultivated with *C. albicans*. Taken together, our data suggest that farnesol, in addition to its quorum-sensing function that regulates morphogenesis, is also employed by *C. albicans* to reduce competition from other microbes.

Introduction

Candida albicans is one of the most frequently isolated fungal pathogens of humans. It is a common cause of nosocomial infections, especially in immunocompromised individuals (Kullberg and Filler, 2002). An important feature of *C. albicans* is its ability to grow as hyphae, pseudohyphae or budding yeasts. Because monomorphic mutants are typically avirulent (Lo *et al*., 1997; Braun *et al*., 2000), it is believed that the yeast to mycelium dimorphism is important for pathogenicity (Saville *et al*., 2003). In this context, *C. albicans* produces farnesol as an extracellular quorum-sensing molecule (Hornby *et al*., 2001). Upon accumulation above a threshold level, farnesol prevents the yeast to mycelium transition and causes the culture to grow as actively budding yeasts without influencing cellular growth rates (Hornby *et al*., 2001). Farnesol has also been found to block biofilm formation by *C. albicans* (Ramage *et al*., 2002). By contrast, in the related yeast *Saccharomyces cerevisiae*, farnesol exhibits a growth inhibitory effect by promoting mitochondrial generation of reactive oxygen species (ROS) (Machida *et al*., 1998; 1999). Farnesol triggers hyperpolarization of the mitochondrial transmembrane potential in yeast through regulation of F_0F_1-ATPase and a corresponding increase of its ATP-hydrolysing activity (Machida and Tanaka, 1999).

Farnesol is a 15-carbon isoprenoid, a class of compounds naturally produced from mevalonate, which is part of the highly conserved sterol biosynthetic pathway. Components derived from this pathway often act as signalling molecules that affect lipid synthesis, protein synthesis and degradation, developmental patterning, meiosis and apoptosis (reviewed by Edwards and Ericsson, 1999). Farnesol is generated within cells by enzymatic dephosphorylation of farnesyl pyrophosphate (FPP). FPP plays an important role as a precursor of protein prenylation, a post-translation modification of proteins (Edwards and Ericsson, 1999). Known farnesylated proteins include Ras and Ras-related GTP-binding proteins (G proteins), which control cell growth, differentiation, proliferation and survival.

The ability to grow as hyphae is a defining feature of the filamentous fungi (Harris *et al*., 2005). Typically, hyphae originate from a swollen spore in a process that superficially resembles the yeast-to-mycelium transition of *C. albicans*. Whether farnesol has a general role in this pivotal stage of hyphal morphogenesis has not yet been determined. Accordingly, to obtain a broader perspective on the role of farnesol in fungal biology, we examined its

effects on the filamentous fungus *Aspergillus nidulans*. We report that exposure to farnesol does not block germ tube formation in this fungus. However, farnesol does cause apoptosis in a manner that depends on mitochondrial function, the production of ROS and heterotrimeric G protein signals. Moreover, our observations suggest that farnesol mediates an unexpected interaction between *C. albicans* and *A. nidulans*. These results reveal a mechanism that may be employed by fungi to antagonize the growth of competitors.

Results

Farnesol induces nuclear condensation independent of mitosis

Germinating *A. nidulans* spores and growing hyphae were exposed to a range of farnesol concentrations (10–250 μM) to determine its effects on hyphal morphogenesis. No obvious effect on the establishment of hyphal polarity was observed. However, at the lowest concentration tested (10 μM), farnesol caused a complete inhibition of hyphal growth (Figure 1). Furthermore, to our surprise, we found that extracellular farnesol triggered rapid DNA condensation. Mitosis in *A. nidulans* is usually completed within 5 min (Bergen and Morris, 1983), such that in an asynchronous population only about 5% of hyphae possess condensed nuclei (Morris, 1975). However, after treatment with farnesol, a dramatic increase in the number of condensed nuclei was observed (Figure 2A). Notably, the percentage of condensed nuclei increased in a dose-dependent manner, such that > 90% of nuclei were condensed following treatment with 250 μM farnesol for 2 h. These effects are likely the result of farnesol treatment because they were suppressed by the presence of 1% BSA, which binds lipophilic molecules such as farnesol (Mosel *et al*., 2005). Note that the growth of the small percentage of hyphae without condensed nuclei was still inhibited, thereby suggesting that they did not represent a farnesol-resistant sub-population.

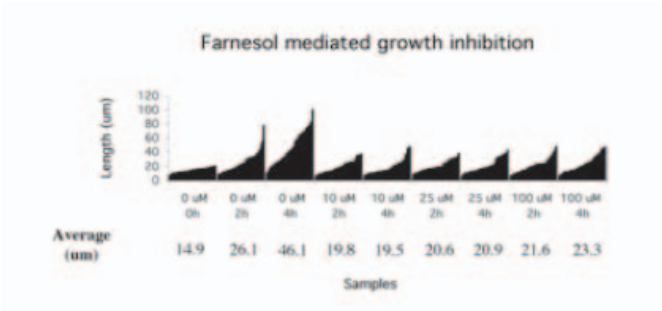

Figure 1. Farnesol inhibits the growth of *A. nidulans* hyphae. Spores of wild-type strain A28 were germinated on coverslips in YGV for 12 h at 30°C, followed by treatment for 0, 2 or 4 h with 0, 10, 25 and 100 μM farnesol. Nuclei were visualized using Hoechst 33258 and individual images from hyphae containing eight or more nuclei were captured using a Photometrics CoolSnap HQ CCD camera. IP Laboratory software was used to obtain lengths of 40 hyphae, from each sample, which were then plotted using Microsoft Excel. The figure presents the length of all 40 hyphae for each condition (obtained from two independent experiments). The average hyphal length for each sample is indicated at the bottom of the figure. Note that the length distributions for hyphae treated with 1 or 10 μM farnesol are identical.

The observed increase in nuclear condensation could reflect the ability of farnesol to trigger mitotic arrest. If so, nuclear condensation should not be observed if the cell cycle is blocked in interphase by a temperature-sensitive *nim* mutation ('never in mitosis'; Morris, 1975). To test this hypothesis, a *nimT23*^{cdc25} mutant was used to arrest the cell cycle in G2 before treatment with farnesol. *nimT23*^{cdc25} encodes the *A. nidulans* homologue of the fission yeast Cdc25 tyrosine phosphatase that triggers mitotic entry by activating the cyclin-dependent kinase Cdc2 (O'Connell *et al*., 1992). Both wild-type and *nimT23*^{cdc25} conidiospores were germinated at 30°C for 12 h and then shifted to restrictive temperature (i.e. 45°C) for 2 h prior to addition of 100 μM farnesol. During continued incubation at 45°C (Figs 2B and 1C), farnesol induced nuclear condensation in both wild-type hyphae (Figure 2B, right panel) and *nimT23*^{cdc25} hyphae arrested in G2 (Figure 2C, right panel). These results show that nuclear condensation induced by farnesol is independent of mitosis.

Farnesol induces phenotypic changes characteristic of apoptosis

We tested the hypothesis that farnesol-induced nuclear condensation is a reflection of apoptotic-like programmed cell death in *A. nidulans*. Consistent with this view, hyphae treated with farnesol exhibited increased uptake of the stain Evan's Blue, which is a characteristic feature of dead fungal cells (Wu and Glass, 2001; Figure S1). Apoptosis is distinguished by a number of morphological changes that occur during cell death. Features conserved from yeast to mammalian cells include DNA condensation and fragmentation, exposure of phosphatidylserine (PS) on the cell surface and production of ROS (Madeo *et al*., 1997; 1999; 2004). We initially tested for DNA fragmentation and PS externalization.

The TUNEL assay was used to determine if farnesol induces DNA fragmentation. This assay uses terminal deoxynucleotidyltransferase to label 3'-OH DNA termini with FITC-conjugated dUTP, which can be directly visualized by fluorescence microscopy. After 2 h treatment with 100 μM farnesol, the majority of hyphae (56%) showed TUNEL positive staining (Figure 3A and B), whereas untreated control hyphae showed no staining.

Mitochondria play a crucial role in apoptosis (Ludovico *et al*., 2002) by releasing pro-apoptotic factors that contribute to both the caspase-dependent and caspase-independent pathways of apoptosis (reviewed by Kroemer and Martin, 2005 and by Bras *et al*., 2005).

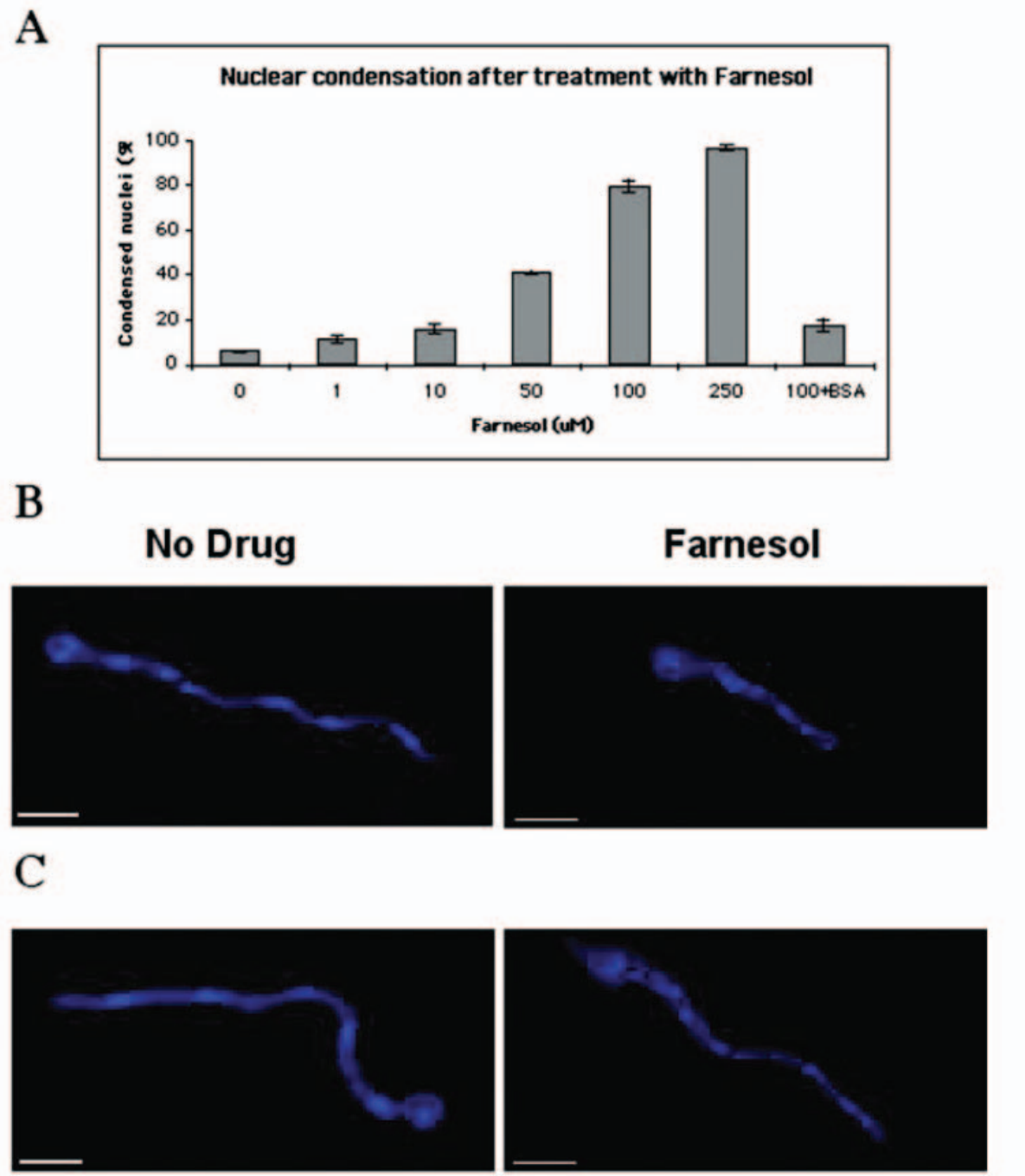

Figure 2. Mitosis-independent nuclear condensation induced by farnesol. (**A**) Percentage of nuclei showing DNA condensation induced by farnesol. Spores of wild-type strain A28 were germinated on coverslips in YGV for 12 h at 30°C, followed by treatment for 2 h with 0, 10, 50, 100 or 250 μM farnesol plus a control using 1% BSA and 100 μM farnesol. Nuclei were visualized using Hoechst 33258 and the proportion containing condensed DNA determined. For each sample, 200 nuclei were examined in two independent experiments, with at least two replicates per experiment. Spores of strains A28 (**B**) and MO73 (**C**) were germinated on coverslips in YGV medium for 12 h at 30°C and then shifted to 45°C for 2 h. One hundred microgram farnesol was added and the strains were incubated at 45°C for an additional 2 h. The germinated spores were stained with Hoechst 33258 and analyzed by laser scanning confocal microscopy. The micrographs show a z series stack of 1 μm sections. Bars, 10 μm.

To confirm the role of mitochondria in farnesol-induced apoptosis, we blocked normal mitochondrial function in wild-type hyphae with oligomycin, a specific inhibitor of mitochondrial F_0F_1-ATPase. Pretreatment with oligomycin markedly inhibited nuclear condensation caused by subsequent treatment with farnesol (Figure 3C–E), and hyphae treated with both oligomycin and farnesol were TUNEL negative (Figure 3C and D). Together, these results demonstrate that normal mitochondrial function is essential for farnesol-induced apoptosis.

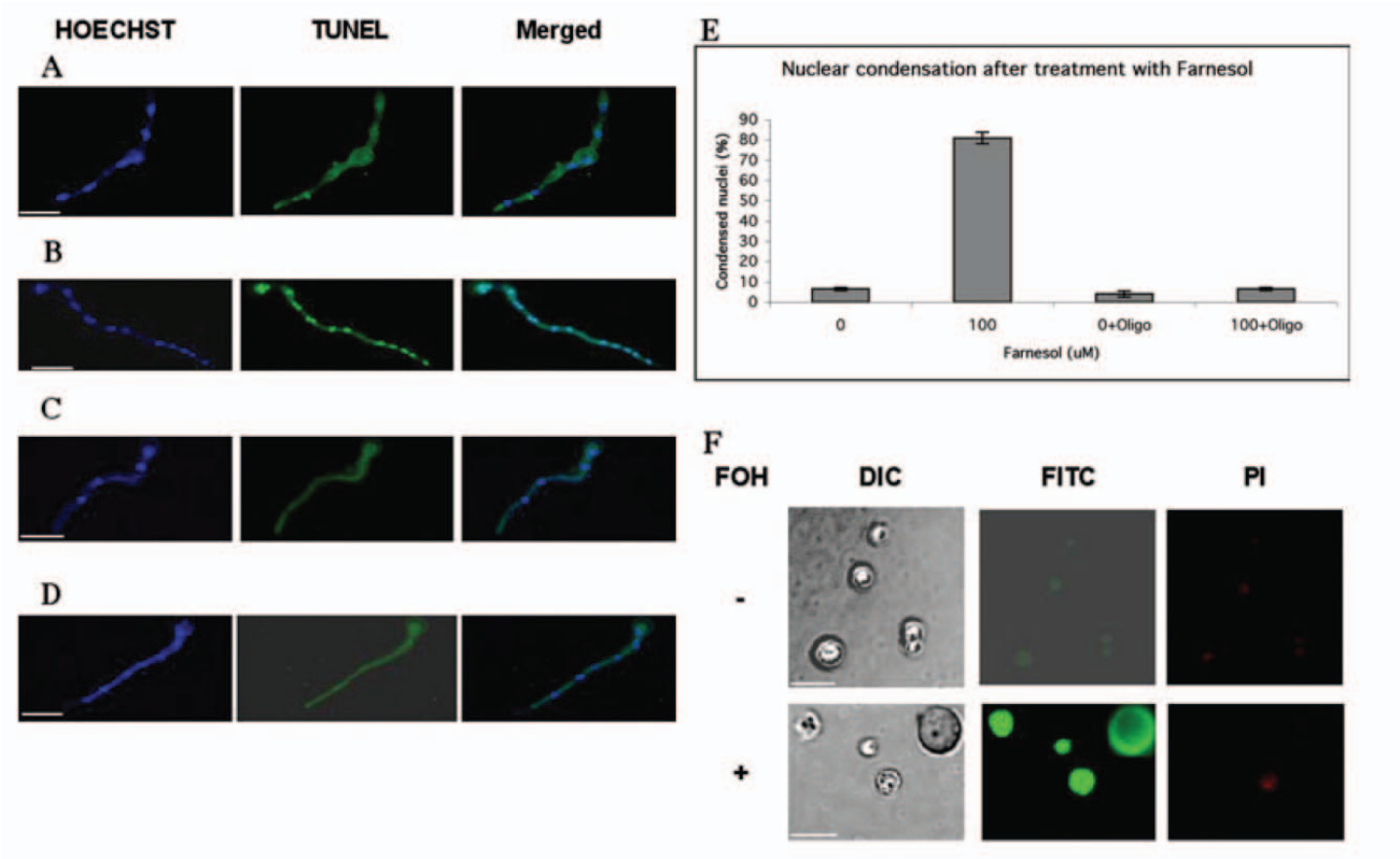

Figure 3. Farnesol-treated hyphae exhibit morphological changes characteristic of apoptosis. **A–D.** TUNEL assay. Spores of strain A28 were germinated on coverslips in YGV medium for 12 h at 30°C. The germinated spores were transferred to YGV containing 0 (A) or 100 μM (**B**) farnesol and incubated at 30°C for an additional 2 h. The germinated spores were then fixed and double stained with Hoechst 33258 and TUNEL. The micrographs represent a z series stack of 1 μm sections obtained with a laser scanning confocal microscope. Bars, 10 μm. The same experiment was repeated with hyphae that were pre-grown for 12 h and then treated with 10 μM oligomycin (Sigma, St Louis) for 4 h. Farnesol was added at 0 (**C**) and 100 μM (**D**), followed by incubation at 30°C for an additional 2 h. Micrographs were obtained as described above. **E.** Percentage of nuclei showing DNA condensation in hyphae treated as described in (**A–D**). For each sample, 200 nuclei were examined in two independent experiments, with at least two replicates per experiment. **F.** Annexin V assay. A28 spores were germinated in YGV medium for 12 h at 30 °C, then treated with 100 μM farnesol and incubated at 30°C for an additional 2 h. Protoplasts were generated from each condition and double stained with FITC-Annexin V and PI. One protoplast showing Annexin V positive and PI negative staining was enlarged for observation (upper right corner). The micrographs represent a z series stack of 1 μm sections obtained with a laser scanning confocal microscope. Bars, 10 μm.

We next used the Annexin V assay to determine if externalization of PS occurs after treatment with farnesol. Normally, PS is only on the inner surface of the cytoplasmic membrane. During apoptosis, plasma membrane asymmetry is lost and PS becomes exposed on the outer leaflet. Annexin V specifically binds to this lipid with high affinity in the presence of 1.8 mM Ca^{2+}. Protoplasts derived from hyphae treated with 100 μM farnesol for 2 h exhibited strong labelling with FITC-conjugated Annexin V (72%; Figure 3F, lower panels), whereas those derived from untreated control cells did not (0%; Figure 3F, upper panels). Propidium iodide (PI) staining showed that the plasma membranes of protoplasts from both treated and control hyphae remained largely intact (Figure 3F, right panels).

Farnesol-induced ROS production is required for apoptosis

A consequence of mitochondrial alterations during apoptosis is disruption of the electron transport chain and subsequent production of ROS (Bras *et al*., 2005). For example, hydrogen peroxide (H_2O_2), which is capable of inducing oxidative stress, triggers apoptosis in *S. cerevisiae* (Madeo *et al*., 1999). To test whether farnesol treatment induces oxidative stress in *A. nidulans*, we monitored intracellular ROS levels after treatment with farnesol by using 2',7'-dichlorodihydrofluorescein diacetate (DCF). DCF is a cell-permeable ROS indicator that penetrates live cells but does not fluoresce unless oxidized by ROS (LeBel *et al*., 1992). Protoplasts produced from cells treated with farnesol as well as from untreated controls were assayed with DCF (Chen and Dickman, 2005). As shown in Figure 4A, farnesol-treated cells displayed intense fluorescence (83%), indicating that ROS is being generated, whereas the untreated controls did not fluoresce (0%).

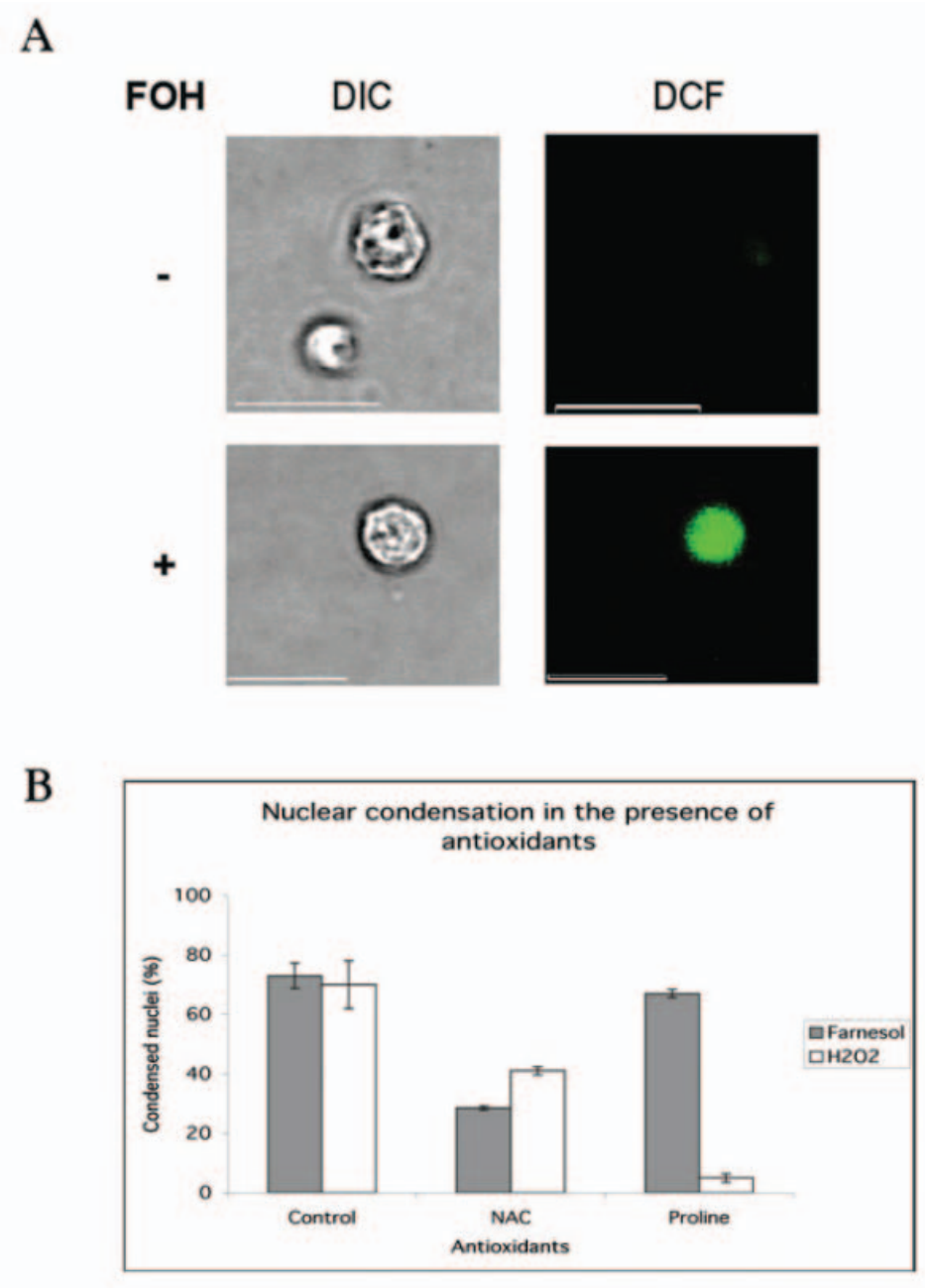

Figure 4. Farnesol induces ROS production and its effects can be reversed by antioxidant. **A.** A28 spores were germinated in YGV medium for 12 h at 30°C and then treated with 100 μM farnesol and incubated at 30°C for an additional 2 h. Protoplasts were generated and incubated with 50 μM DCF. The micrographs represent a z series stack of 1 μm sections obtained with a laser scanning confocal microscope. Bars, 10 μm. **B.** Spores of wild-type strain A28 were germinated in MN for 12 h at 30°C in the absence of antioxidant (control), or in the presence of either 5 mM L-proline or 5 mM NAC. Germlings were then treated with 1 mM H2O2 or 100 μM farnesol and incubated at 30°C for an additional 2 h. The germinated spores were fixed and stained with Hoechst 33258 and the percentage of nuclei showing DNA condensation was determined by fluorescence microscopy. For each sample, 200 nuclei were examined in two independent experiments, with at least two replicates per experiment.

To determine if ROS production participates in farnesol-induced apoptosis, we tested the possibility that scavenging of ROS may suppress the effects of farnesol. As ROS scavengers we used the antioxidants N-acetyl cysteine (NAC) and l-proline. l-proline is known to suppress H_2O_2-induced apoptosis in the fungus *Colletotricum trifolli* (Chen and Dickman, 2005). Because it was not clear that l-proline would behave the same way in *A. nidulans*, we also used H_2O_2 as a control to induce ROS formation in *A. nidulans*. Wild-type hyphae were germinated on coverslips for 12 h in standard minimum media (MN) in the presence of either 5 mM NAC or 5 mM l-proline and then treated with farnesol or H_2O_2 for 2 h. As shown in Figure 4B, NAC partially rescued chromosome condensation in both treatments, whereas l-proline prevented condensation only in cells treated with H_2O_2.

Heterotrimeric G protein signals are required for farnesol-induced apoptosis

The antifungal protein PAF triggers apoptosis in *A. nidulans* via a mechanism that requires the Gβ protein SfaD (Leiter *et al*., 2005). To determine if PAF and farnesol could act by the same mechanism, we examined the effects of Δ*sfaD* and Δ*flbA* mutations on farnesol-induced nuclear condensation. The Δ*sfaD* mutation, which blocks G protein signalling (Rosen *et al*., 1999), conferred complete resistance to farnesol (Table 1). By contrast, the Δ*flbA* mutation, which leads to hyperactivation of the

Table 1. Farnesol-induced nuclear condensation depends on heterotrimeric G protein signals.

Strain/farnesol dose	Nuclei condensation (%)	SD
A28 (wild-type)/0 μM	7.5	2.12132034
A28/10 μM	20.5	3.53553391
A28/100 μM	70.5	6.36396103
tSRB 1.21 (Δ*sfaD*)/0 μM	6.5	2.12132034
tSRB 1.21/10 μM	7.0	1.41421356
tSRB 1.21/100 μM	8.0	1.41421356
rJA5.9 (Δ*flbA*)/0 μM	15.5	0.70710678
rJA5.9/10 μM	78.0	2.82842712
rJA5.9/100 μM	95.5	0.70710678

Spores from strains A28, tSRB 1.21 and rJA5.9 were germinated on coverslips in YGV for 12 h at 30°C, followed by treatment for 2 h with 0, 10 and 100 μM farnesol. Nuclei were visualized using Hoechst 33258 and the proportion containing condensed DNA determined. For each sample, 200 nuclei were examined, and the results represent the average of two independent experiments, with two replicates per experiment.

Gα protein FadA (Yu *et al*., 1996), caused a dramatic increase in sensitivity to farnesol (Table 1). Notably, the level of nuclear condensation observed in Δ*flbA* mutants treated with 10 μM farnesol is equivalent to that seen in wild-type hyphae at a concentration of 100 μM. These observations suggest that the FadA G protein complex is part of the signal transduction pathway that promotes apoptosis in hyphae exposed to farnesol.

Candida albicans and farnesol inhibit growth and development of A. nidulans

To address the possibility that *A. nidulans* secretes farnesol, wild-type hyphae were grown in liquid media, the culture was pelleted, and the cell free supernatant was analysed for farnesol by gas chromatography/mass spectroscopy (GC/MS). Commercial mixed isomer farnesol was used as a control. With a detection limit of 0.005 mg g^{-1}, farnesol was not found in the spent media (Figure S2). Because farnesol is apparently not secreted in detectable quantities by *A. nidulans* under the conditions tested, this fungus is presumably responding to farnesol produced by other fungal species.

The possibility that farnesol from *C. albicans* might affect *A. nidulans* was evaluated in co-culture. Wild-type strains of both fungi were grown in defined glucose, proline and phosphate media (GPP) with added nitrate salts at 30°C for 24 h. As controls, *C. albicans* and *A. nidulans* were grown by themselves, and they were also co-cultured in the presence of 1% BSA, which displays non-specific binding to lipids such as farnesol (Mosel *et al*., 2005). Following growth, the cultures were washed, plated onto YPD media, and incubated at 30°C for 2 days. Pure cultures were recovered when *C. albicans* and *A. nidulans* were grown separately (Figure 5A and B) but only *C. albicans* was recovered when both fungi had been grown together (Figure 5C). Significantly, this effect was reversed by the addition of BSA (Figure 5D). Macroscopic characteristics of the colonies were used to distinguish the two fungi: *C. albicans* colonies were mucoid whereas *A. nidulans* colonies were hyaline and downy in texture (note that *A. nidulans* sporulates poorly in YPD media). Less than 10^3 *A. nidulans* cfu were recovered when co-cultured with *C. albicans*, an inhibition of ≥ 180×. Moreover, in separate experiments, spent media from C. albicans A72 cultures blocked *A. nidulans* colony formation in a dose-dependent manner (Figure S3). These observations demonstrate that *C. albicans* produces a lipophilic compound capable of inhibiting the growth of *A. nidulans*. To confirm that this compound was farnesol, we analysed the supernatants of all four cultures by GC/MS. As expected, the *C. albicans* culture supernatant presented a peak at 11.25 min whose MS peak analysis gave a perfect match (95 degree of similarity) whereas the *A. nidulans* supernatant presented no peak with any similarity to farnesol (data not shown). Peak area for the *C. albicans* supernatant indicated a farnesol concentration of 0.66 μM. Farnesol was also detected in the *C. albicans* and *A. nidulans* co-culture supernatant (0.58 μM) and in a lower concentration in the co-culture with BSA (0.017 μM) (data not shown). These concentrations of farnesol (0.58–0.66 μM) are just below that normally found when *C. albicans* is grown in GPP media (2–4 μM), and are within range of concentrations of commercial farnesol that completely inhibit hyphal growth (1–10 μM; Figure 1and data not shown). Collectively, these results suggest that co-cultivation with *C. albicans* can inhibit the growth of *A. nidulans* in a farnesol-dependent manner.

We also tested *A. nidulans* hyphae for the appearance of nuclear condensation when grown in the presence of *C. albicans*. The fungi were grown separately for 12 h and then added together such that *A. nidulans* was exposed to increasing amounts of the *C. albicans* culture. The co-culture was incubated at 30°C for an additional 2 h and nuclear condensation was measured by microscopy. As expected, the percentage of condensed nuclei increased as a function of *C. albicans* cell density (Figure 5E).

We then inoculated *A. nidulans* on solid media with or without *C. albicans*. As shown in Figure 5F, the presence of *C. albicans* limited the growth of *A. nidulans* and prevented the development of conidiophores. Note that *A. nidulans* did not grow over the *C. albicans* streak line, but could expand in the opposite direction. By contrast, when paired with *S. cerevisiae* (strain BY4741), *A. nidulans* could grow over the streak line (data not shown), thereby suggesting that the inability to grow over *C. albicans* reflects an antagonistic interaction between these fungi. To determine how farnesol travelled from *C. albicans*To *A. nidulans*, a series

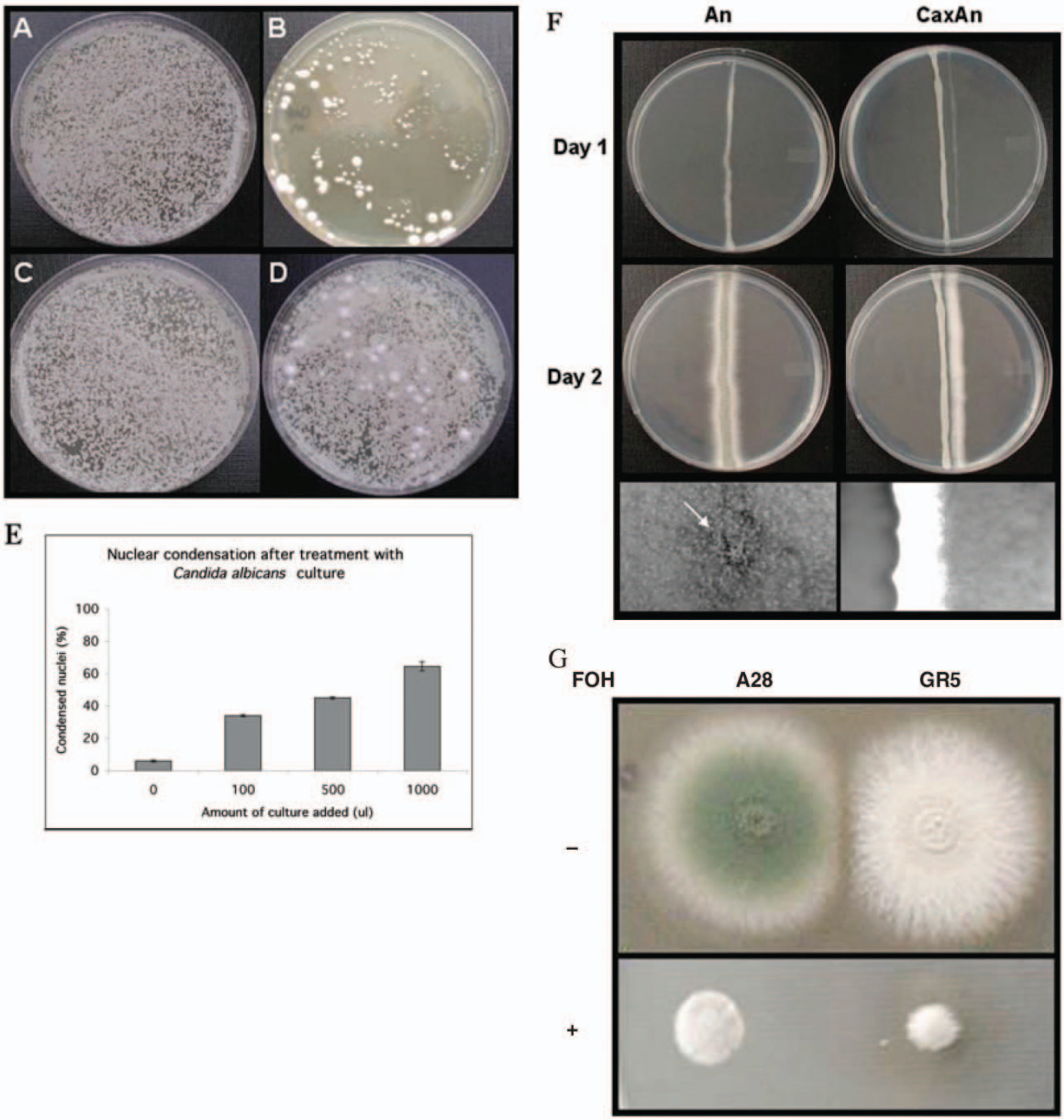

Figure 5. *Candida albicans* and farnesol inhibit growth and development of *A. nidulans*. **A–D.** Strains of (**A**) *C. albicans* (A72) (**B**) *A. nidulans* (A28) (**C**) *C. albicans* and *A. nidulans* and (**D**) *C. albicans* and *A. nidulans* plus 1% BSA were grown in GPP at 30°C, 200 rpm for 24 h. The cultures were washed, diluted and plated onto YPD and incubated at 30°C for 2 days.

E. *A. nidulans* strain A28 was grown in YGV media for 12 h at 30°C, then treated with the indicated amounts of *C. albicans* (strain A72) culture grown separately in GPP for 12 h. The co-culture (final volume 10 ml) was incubated at 30°C for an additional 2 h and treated as described above.

F. *A. nidulans* strain A28 was inoculated in solid YGV media by itself (left panels) or in the presence of *C. albicans* strain A72 (right panels), and incubated at 30°C for 1 (upper panels) or 2 days (middle panels). Note that *A. nidulans* only forms conidia when grown alone. It displays an aconidial phenotype in the presence of *C. albicans*. To better illustrate this phenotype, higher magnification (10x) images obtained with a dissecting microscope are displayed in the lower panels. Arrow indicates conidiophores.

G. *A. nidulans* strains A28 (left) and GR5 (right) were inoculated in CMUU that was left untreated or also contained 500 μM farnesol. Plates were sealed with parafilm to limit farnesol evaporation, and incubated at 30°C for 3 days.

of experiments was done in sealed centre well flasks. Whenever *C. albicans* or farnesol was put in the centre well, *A. nidulans* in the separated outer portion of the flask showed the same developmental phenotype, i.e. no conidiophores (data not shown). This result indicates that farnesol not only diffuses through the media but is also volatile.

Finally, we tested the effect of farnesol on *A. nidulans* growth in solid media. Two *A. nidulans* strains, A28 (green-spored) and GR5 (white-spored), were inoculated onto standard complete media agar plates containing 100–500 μM of farnesol. The plates were sealed to limit farnesol evaporation and incubated at 30°C for 2 days. To our surprise, only the higher farnesol concentration (i.e. 500 μM) was capable of inhibiting *A. nidulans* growth and conidiation (Figure 5G), while lower concentrations of farnesol simply inhibited conidiophore formation (data not shown). The fact that a higher concentration of farnesol was necessary to inhibit *A. nidulans* growth in solid media can be explained by the difference in incubation time. Because the plates were incubated for an extended period (3 days, compared with 2 h of incubation in liquid media), it is possible that prolonged exposure of farnesol to oxygen may lead to loss of its activity.

Discussion

We have demonstrated that the isoprenoid farnesol induces apoptosis in the filamentous fungus *A. nidulans* via a mechanism that requires functional mitochondria, the production of ROS and a heterotrimeric G protein complex. In addition, we have also shown that *C. albicans* inhibits the growth and development of *A. nidulans* in a manner that is likely farnesol-dependent. Our results reveal a possible mechanism that may underlie antagonistic interactions between fungi within microbial communities.

Induction of apoptosis by farnesol

Apoptotic-like programmed cell death has been observed in a number of filamentous fungi, and farnesol joins a growing list of compounds (H_2O_2, amphotericin B, the antifungal protein PAF, sphingoid bases) that trigger this response (Cheng *et al*., 2003; Mousavi and Robson, 2004; Chen and Dickman, 2005; Leiter *et al*., 2005). Characteristic features of apoptosis in filamentous fungi include a requirement for functional mitochondria and, in most cases, ROS production, as well as the absence of dependency on caspase activity. Although the nature of the signalling and effector pathways that regulate fungal apoptosis remain under investigation (Madeo *et al*., 2004), preliminary insights have been obtained. For example, dominant active Ras promotes apoptosis in *C. trifolii* (Chen and Dickman, 2005). Our observations, and those of Leiter *et al*. (2005), also implicate the heterotrimeric Gα protein FadA in promoting apoptosis in *A. nidulans*. Whether Ras and Gα act together in a linear pathway, or via parallel mechanisms, to cause apoptosis is an intriguing issue that merits further investigation. Finally, our recent results also suggest that poly (ADP-ribose) polymerase (PARP) function is required for farnesol-induced apoptosis (C.P. Semighini *et al*., unpubl. obs.).

The ability of farnesol to cause apoptosis is not limited to fungal cells. Farnesol-induced apoptosis has been reported in tobacco cells (Hemmerlin and Bach, 2000) and several tumour-derived cell lines (Adany *et al*., 1994; Haug *et al*., 1994; Voziyan *et al*., 1995; Rioja *et al*., 2000). Farnesol is also capable of arresting the growth of animal cells and *S. cerevisiae* (Chakrabarti and Engleman, 1991; Burke *et al*., 1997; Machida *et al*., 1998; 1999). In both yeast and *A. nidulans*, farnesol treatment induces intracellular ROS generation (Machida *et al*., 1998; 1999). Furthermore, in both cases, the antioxidant NAC can ameliorate the effects induced by farnesol. These results indicate that ROS production is an important factor underlying farnesol-induced apoptosis. However, unlike NAC, l-proline could not reverse the effects of farnesol, though as expected (Chen and Dickman, 2005), it did prevent nuclear condensation in hyphae exposed to H_2O_2. One explanation for this difference is that farnesol may generate a specific intracellular ROS that can only be neutralized by NAC. In this context, it should also be noted that strain GR5 (white-spored) is significantly more sensitive than A28 (green-spored) to farnesol in solid media. The difference between A28 and GR5 may reflect the fact that pigmented strains are more resistant to ROS stress (Langfelder *et al*., 2003). For example, in *Aspergillus fumigatus*, melanin was implicated in both cAMP signalling and ROS detoxification (Liebmann *et al*., 2003).

Antagonistic interactions between fungi and the possible roles of farnesol

Our results show that an antagonistic relationship exists between *C. albicans* and *A. nidulans*. This effect is not unique to *A. nidulans*, as we have recently observed that *C. albicans* A72 also blocks the growth and development of other euascomycete fungi, including *A. fumigatus* and *Fusarium graminearum*. Moreover, as with *A. nidulans*, the response of these two fungi to the presence of *C. albicans* can be mimicked by extracellular farnesol (C.P. Semighini *et al.*, unpubl. obs.). Finally, extracellular farnesol also triggers apoptosis in *A. fumigatus* and *F. graminearum* (C.P. Semighini *et al.*, unpubl. obs.). Taken together, our observations suggest that the response to farnesol may account in part for the antagonistic interactions between *C. albicans* and euascomycetes. Moreover, this response appears to be graded. In particular, lower concentrations of farne-

sol, whether produced by *C. albicans* (0.5–4 μM; Hornby *et al.*, 2001; 2003) or applied externally (1–10 μM), inhibit hyphal growth and colony development. At higher concentrations (50–100 μM), which do not affect the viability or growth of *C. albicans* (Hornby *et al*., 2001), farnesol causes nuclear condensation and cell death. However, it should be emphasized that low but reproducible levels of nuclear condensation could be triggered by farnesol even at a concentration as low as 10 μM (Figure 2A).

Antagonistic interactions between fungi have been well documented in the past. For instance, Waksman (1941) cited at least 13 examples of antagonistic effects of one fungus on another. Notably, the dermatophyte *Trichophyton rubrum* failed to grow when co-cultivated with either *C. albicans* or spent culture media that had been filtered, indicating that the latter secretes a metabolic product with fungistatic action (Jillson and Nickerson, 1948). Our observations suggest that the metabolic product could be farnesol. Whether farnesol-based antagonisms occur in a natural setting remains uncertain. One possibility is that *C. albicans* may use farnesol to eliminate other fungal competitors within the mammalian host environment. This notion is supported by the observation that pure cultures of *C. albicans* are generally isolated from lesions, implying that *C. albicans* exerts an inhibitory effect on the growth of other microbes (Lewis and Hooper, 1943). Alternatively, isolates of *C. albicans* have been recovered from soil samples from New Zealand (di Menna, 1955) and Tennessee (Ajello, 1956), though it is not known if they exist passively following introduction by animal carriers or if they are normal components of soil communities. If *C. albicans* in fact inhabits the soil, it is possible that this fungus secretes farnesol to kill other fungi sharing the same habitat, such as euascomycetes, in order to reduce competition for nutrient sources. A final consideration is that farnesol may be expressed within the host environment to kill immune cells such as macrophages. Many microbes produce virulence factors that induce apoptosis in host cells (reviewed by Mendes-Giannini *et al*., 2000; Moss *et al*., 2005). For example, a 3-oxo-C12 homoserine lactone quorum-sensing molecule from *Pseudomonas aeruginosa* was reported to trigger apoptosis in bone marrow-derived macrophages, as well as in neutrophils and monocytic cell lines (Tateda *et al*., 2003). The significance of this report is that 3-oxo-C12 homoserine lactone has recently been shown (Hogan *et al*., 2004) to have enough structural similarity to farnesol that at high concentrations (200 μM) it can mimic the action of farnesol in *C. albicans*.

We conclude that farnesol, in addition to its quorum-sensing function that regulates morphogenesis, is also employed by *C. albicans*To reduce competition from other microbes. On a more speculative note, farnesol may be used as a virulence factor to induce apoptosis in host cells. In either case, we propose that farnesol significantly enhances the ability of *C. albicans*To colonize new environments. Future experiments will determine how *C. albicans* and filamentous fungi sense farnesol and respond to its presence.

Experimental procedures

Strains, media and reagents

Aspergillus nidulans strains used were A28 (*pabaA6 biA1*; Fungal Genetics Stock Center, University of Kansas Medical Center, Lawrence, KS), MO73 (*nimT23 pabaA6*; Steve Osmani, Ohio State University, Columbus, OH), GR5 (*pyrG89; pyroA4; wA3*, FGSC), tSRB1.21 (Δ*sfaD*::*argB*; *argB2*; Jaeyuk Hu, University of Wisconsin-Madison) and rJA5.9 (Δf*lbA*::*argB*; *argB2*; *pyrG89*; *pyroA*; Jaeyuk Hu, University of Wisconsin-Madison). The *C. albicans* strain used was A72 (Patrick Sullivan, University of Otago, Dunedin, New Zealand; also ATCC MYA-2430). The following media were used: YGV (2% glucose, 0.5% yeast extract, 0.01% vitamins, trace elements), MN (1% glucose, nitrate salts, trace elements, pH 6.5), GPP (2% glucose, 52 mM phosphate and 10 mM proline), YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) and CMUU (1% glucose, 0.1% yeast extract, 0.2% peptone, 0.1% casamino acids, nitrate salts, trace elements, 0.01% vitamins, 5 mM uridine and 10 mM uracil, pH 6.5). Trace elements, vitamins and nitrate salts were used as described by Kafer (1977). Trans-trans farnesol, BSA, oligomycin, l-proline, NAC or H_2O_2 were added to media at the indicated concentration. All chemicals were purchased from Sigma (St. Louis). A 1 M stock solution of farnesol was prepared fresh in DMSO prior to each experiment. The appropriate amount of farnesol was transferred with a pipette from the stock solution to the growth medium. The original flask containing farnesol was de-gassed with nitrogen gas every time after being opened in order to avoid degradation by oxygen.

Measurement of hyphal growth

A28 spores were germinated on coverslips in YGV medium at 30°C for 12 h. The coverslips were treated with 10, 25 and 100 μM farnesol, and incubated at 30°C for 0, 2 or 4 h as indicated. The coverslips were fixed and stained with Hoechst 33258. Slides were viewed using an Olympus BX51 fluorescent microscope and individual images from hyphae containing eight or more nuclei were captured with a Photometrics CoolSnap HQ CCD camera. The length of 40 hyphae for each sample was measured using IPLab software (Scanalytics, Rockville) and plotted with Microsoft Excel.

Chromatin staining

After 12 h of germination on coverslips in YGV medium at 30°C, strains were treated for 2 h as indicated. The coverslips were fixed and stained with Hoechst 33258 (Molecular Probes, Oregon) as described by Harris *et al*. (1994). Slides were viewed using an Olympus BX51 fluorescent microscope and the proportion of nuclei with condensed DNA determined.

TUNEL assay

Spores of strain A28 were germinated on coverslips in YGV medium for 12 h at 30°C. The germinated spores were treated for 2 h as indicated, fixed and double stained with Hoechst 33258 and TUNEL (*In Situ* Cell Death Detection Kit, Roche Diagnostics, Germany) as described by Madeo *et al*. (1997) and Chen and Dickman (2005). Confocal images were obtained with an Olympus FW500/BX61 confocal laser scanning microscope using the following laserlines: 405 nm for Hoechst 33258 and 488 nm for FITC. Images were captured by direct acquisition with a Z step of 1–2 μm and were subsequently processed using ImageJ and Adobe PhotoShop 6.0.

Annexin V staining

A28 spores were germinated in YGV medium for 12 h at 30°C, then treated with 100 μM farnesol and incubated at 30°C for an additional 2 h. Protoplasts were generated from each condition as described by Oakley and Osmani (1993) and double stained with FITC-Annexin V and propidium iodine (PI) using the Annexin V–FITC Apoptosis Detection kit (Oncogene Research Products, Boston) as described by Madeo *et al*. (1997) and Chen and Dickman (2005). Images were obtained by confocal microscopy as described above.

Reactive oxygen species detection

Intracellular ROS levels in *A. nidulans* were monitored with the oxidant-sensitive probe 2',7'-dichlorofluorescin diacetate DCF (Molecular Probes, Oregon) as described by Madeo *et al*. (1997) and Chen and Dickman (2005).

Farnesol detection

Liquid cultures in GPP media were performed at 30°C and 200 rpm for 24 h. Cells were harvested by centrifugation at 4000 *g* for 10 min and the pellet was discarded. The supernatant was sterilized by filtration through a polyethersulfone 0.45 μm membrane (Nalgene, Rochester). Cell-free supernatants were extracted in 1/5 volume ethyl acetate (Sigma, St. Louis), which was collected and removed by rotary evaporation. The residue was resuspended in 1 ml ethyl acetate/hexane (1:4) (Sigma, St. Louis) and transferred to a vial to remove particulate material. The solvent was removed by rotary evaporation and the residue was resuspended in 25 μl ethyl acetate/hexane (1:4). One microlitre was analysed by GC/MS. A Shimadzu GC-17A/MS QP5050A was used for chemical analysis in electron ionization (EI) mode using associated software program GCMS Real Time Analysis ver 1.2. The GC column used an initial temperature of 100°C, held at temperature for 3 min, and then ramped to 300°C over 13 min, with a 1 min hold at 300°C. Helium, at a flow rate of 1 ml min^{-1}, was used as the carrier gas.

Co-culture assay

Strains A72 of *C. albicans* and A28 of *A. nidulans* were grown separately or in co-culture in GPP media plus 0.01% vitamins and nitrate salts at 30°C, 200 rpm for 24 h (initial inoculum of 10^2 cells). The cultures were washed, diluted and plated onto YPD and incubated at 30°C for 2 days.

Evan's Blue staining

A28 spores were germinated on coverslips in YGV medium at 30°C for 12 h. The coverslips were treated with 100 μM farnesol or left untreated. After 2 h of incubation at 30°C, the coverslips were flooded with 1% Evans blue (Sigma, St. Louis) in PBS for 5 min at room temperature, then washed three times with PBS. Both farnesol-treated and untreated hyphae were examined under bright-field illumination with an Olympus BX51 fluorescent microscope.

Conditioned media assay

Candida albicans A72 was grown in GPP media for 16 h at 30°C and 200 rpm. Cells were harvested by centrifugation at 4000 *g* for 10 min and the pellet was discarded. The supernatant was filter sterilized and an aliquot was autoclaved (121°C for 20 min) to volatilize the farnesol. The two sets of conditioned media were added to fresh GPP media (plus 0.01% vitamins and nitrate salts) to give final concentrations of 0, 1:10 and 1:1 (v/v ratio). *A. nidulans* A28 strain was inoculated into the media and grown at 30°C, 200 rpm for 24 h. The cultures were washed, diluted and plated onto solid YGV and incubated at 30°C for 3 days.

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Supplementary material

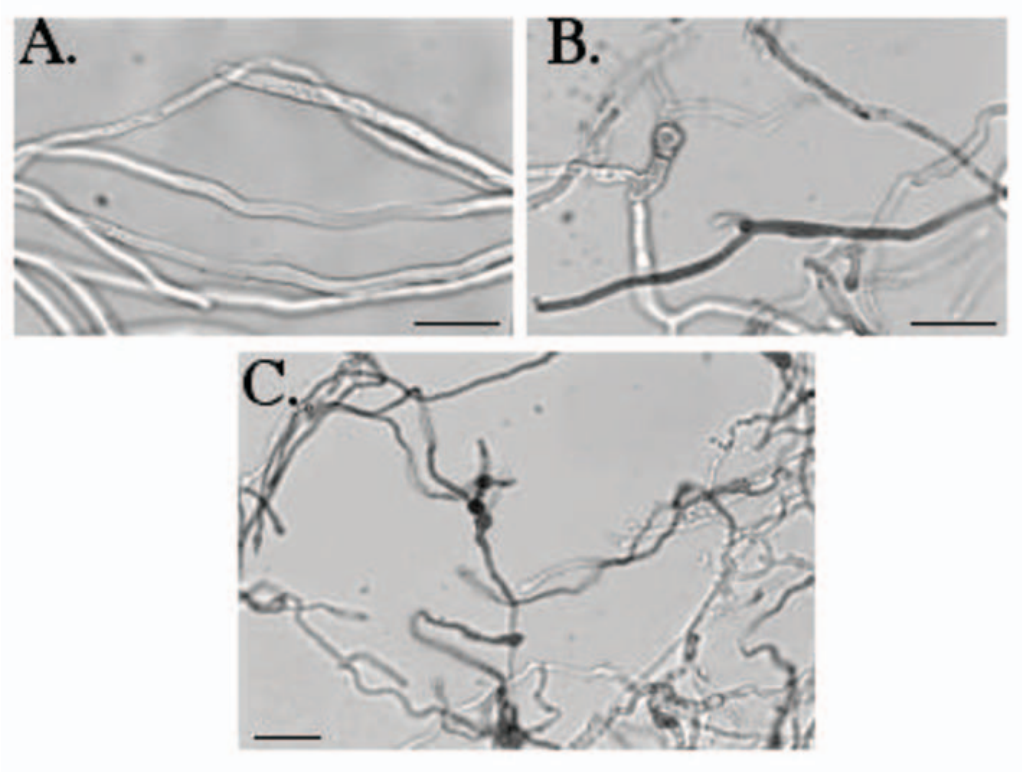

Figure S1. Farnesol treatment causes the death of hyphal cells. Spores of wild-type strain A28 were germinated on coverslips in YGV for 12 h at 30°C, followed by treatment with 0 (A) and 100 uM farnesol (B and C) for 2 h. Hyphae were stained with Evan's Blue (1%) for 5 minutes followed by 3 washes with PBS. Slides were examined under bright-field illumination from an Olympus BX51 fluorescent microscope. Darkened hyphal regions correspond to dead cells stained with Evan's Blue. Bars, 10 um.

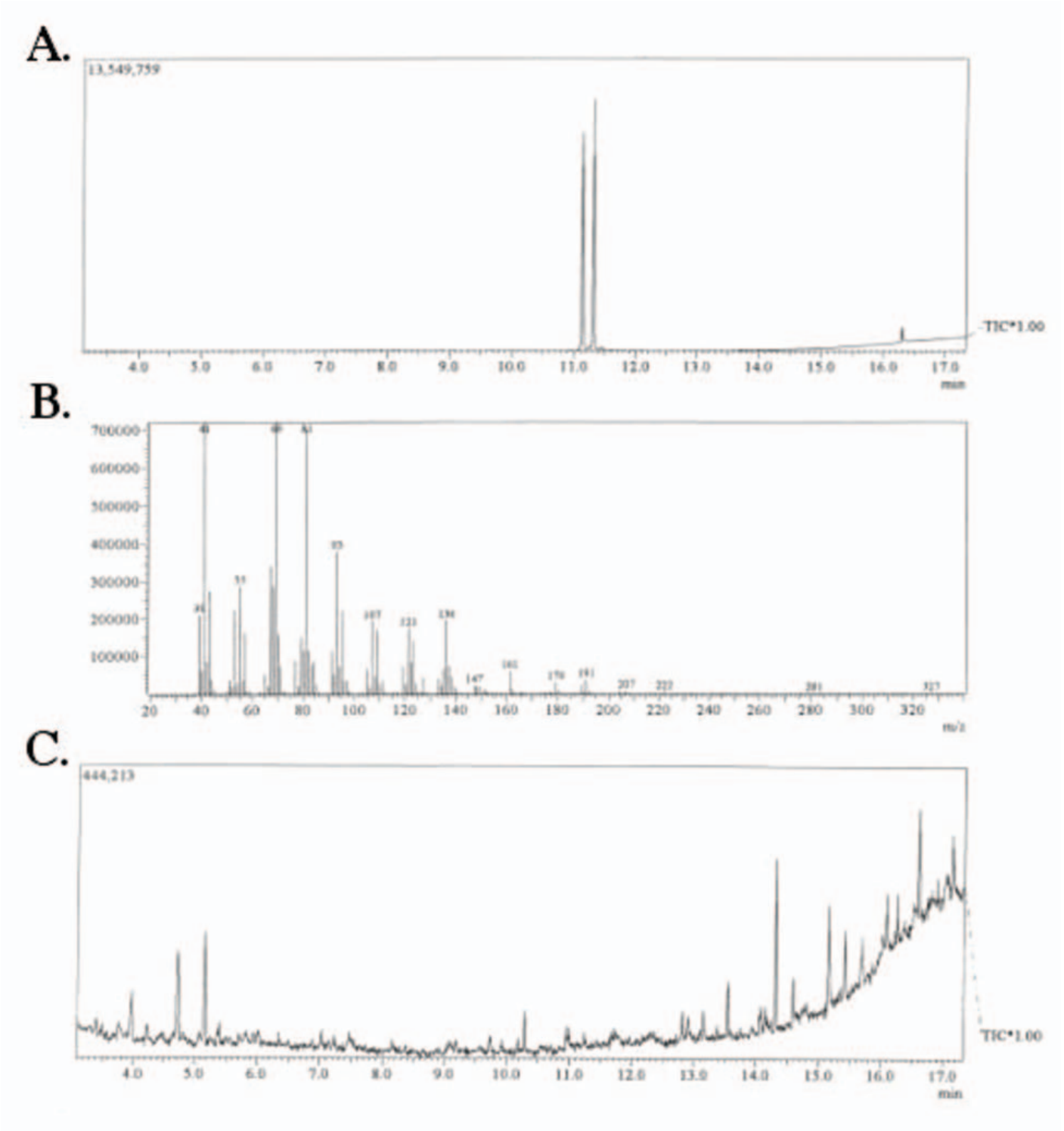

Figure S2. *Aspergillus nidulans.* *A. nidulans* wild-type A28 strain was grown in liquid media at 30°C and 200 rpm for 24 h. Cell-free supernatants were extracted and analyzed by gas chromatography/mass spectroscopy (GC/MS).

(A) GC analysis of a commercial sample of farnesol (mixed isomers, Sigma, St. Louis) showing two peaks A at 11.15 min and B at 11.32 min, which represent two isomers.

(B) MS (EI mode) fragmentation pattern of peak B showing the farnesol characteristic band at m/z 69.

(C) GC of the A28 culture supernatant showing no spectra resembling farnesol or related compounds.

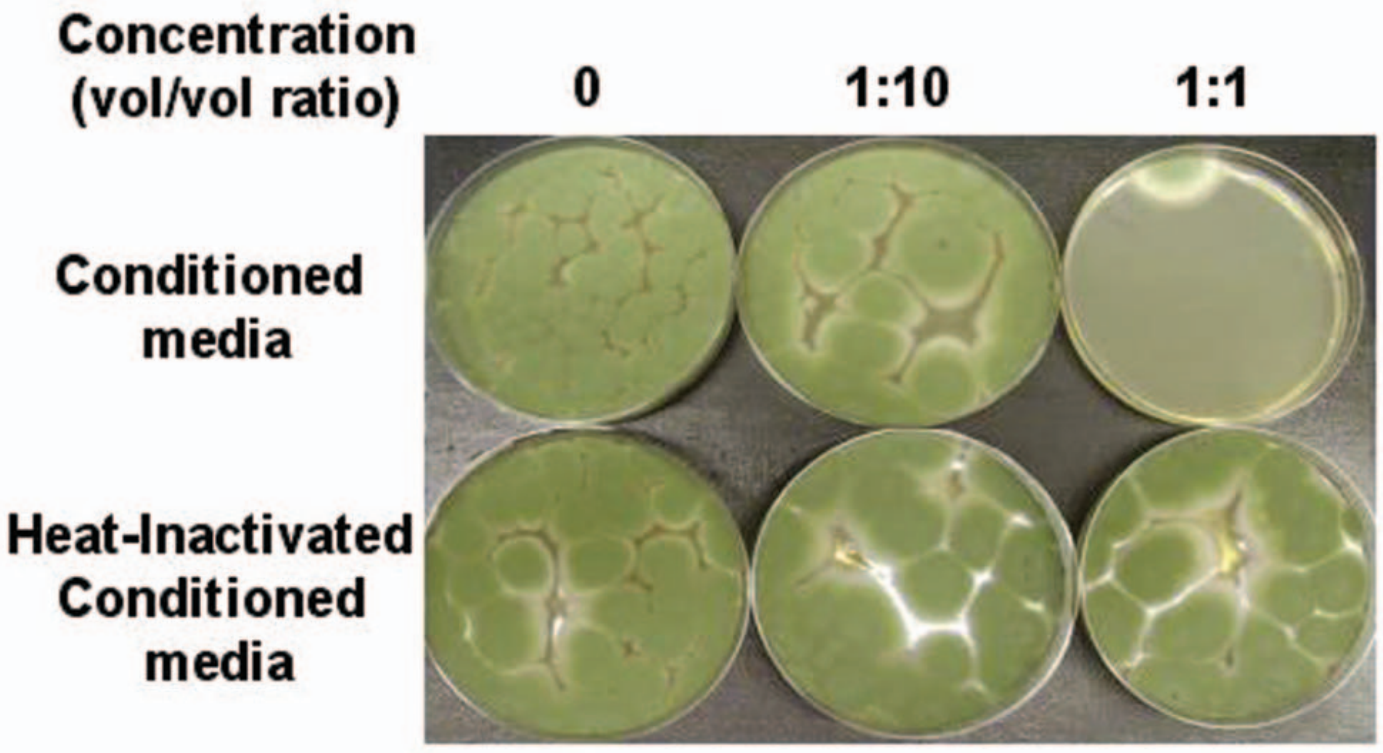

Figure S3. ***Aspergillus nidulans.*** *A. nidulans* A28 strain was inoculated into GPP media containing conditioned media obtained from a C. albicans A72 culture. As a control, part of conditioned media was autoclaved (121°C for 20 minutes) to heat inactivate farnesol. Amounts of conditioned media added were such as to give final concentrations of 0, 1:10 and 1:1 vol/vol ratio. After incubation at 30°C, 200 rpm for 24 h, the cultures were washed, diluted, and plated onto solid YGV and incubated at 30°C for 3 days.