Annals of Microbiology, **59 (1)** 33-38 (2009)

Effect of farnesol on morphogenesis in the fungal pathogen *Penicillium expansum*

Pu LIU, Boxun DENG*, Chao-An LONG, Xiaofang MIN

National Centre of Citrus Breeding, Key laboratory of Horticultural Plant Biology of Ministry of Education, Huazhong Agricultural University, Wuhan, Hubei 430070, P. R. China

Received 3 October 2008 / Accepted 5 February 2009

Abstract - Farnesol as a quorum-sensing molecule secreted by *Candida albicans* prevents the yeast to mycelium conversion and causes the cultures to grow as activity budding yeasts, but has no effect on cellular growth rates. A growth inhibitory effect on *Penicillium expansum* was found in our study by addition of exogenous farnesol to cultures, just like which was reported on *Aspergillus nidulans*. The result implied that farnesol triggered apoptosis-like phenotype in *P. expansum*, including nuclear condensation, DNA fragmentation and the integrity of plasma membranes. Besides, farnesol induced the morphology of hypha changes when it was exposed to *P. expansum* spores suspension.

Key words: apoptosis; citrus isolated; spores; quorum-sensing (QS).

INTRODUCTION

Microbes through cell-cell communication are referred to as quorum sensing (QS) (Bassler, 2002). In natural habitats, microbes can exist in highly ordered communities composed of multiple species. These communities are constructed such that each species carries out a specific subset of functions that, collectively, allow the conglomerate to thrive. Successful associations of this type require effective intra- and inter-species cell-cell communication (Bassler, 2002). In bacteria, the auto-stimulatory compounds are usually small molecules, and the population density is monitored by the concentration of QS molecules which are released by bacteria. In Gram-negative bacteria, these compounds are often acylhomoserine lactones, while in Gram-positive bacteria, modified peptides are usually as QS molecules for it (Chen *et al*., 2004).

In fungi, *Candida albicans* was the first investigated microbe for QS. Some compounds were already described as quorum sensing molecules (QSM) in *C. albicans*, namely, tyrosol (Chen *et al*., 2004) and farnesol. Specifically, farnesol was first identified as extracellular QSM in eukaryotic microbes (Hornby *et al*., 2001).

Farnesol is generated by dephosphorylation of farnesyl pyrophosphate (FPP), a key metabolic intermediate in the highly conserved sterol biosynthesis pathway in eukaryotes. In dimorphic fungus *C. albicans*, farnesol is secreted as a QSM that prevents the yeast to mycelium conversion (Semighini *et al.*, 2006). Recently it has been reported that external farnesol induced apoptosis-like phenotype in a number of filamentous fungus, e.g., *Aspergillus nidulans*, *Fusarium graminearum* and *Candida dubliniensis* (Henriques *et al*., 2007; Lorek *et al*., 2008; Semighini *et al*., 2006, 2008).

The fungal pathogen *P. expansum* is the causative agent of blue mold rot in fruits (e.g., apple, citrus and pear), which causes substantial economic losses during a long-term storage of harvested fruit (Conway *et al.,* 2005; Droby, 2006).

In *A. nidulans*, farnesol have no effect on hyphal morphogenesis, instead, it triggers morphological features characteristic of apoptosis, and exhibits promoting the generation of reactive oxygen species (ROS) (Semighini *et al*., 2006, 2008). Our observation suggests that external farnesol induces the morphology of hypha changes and hyphal apoptosis-like phenotype in *P. expansum*.

MATERIALS AND METHODS

Isolate acquisition, medium and reagents. The strain PE-L was obtained from citrus *unshiu Marcov* (2002; Wuhan, China) with evident development of green and blue rot. With the aid of a loop, spores were collected and inoculated in a Petri dish with Potato Dextrose agar (PDA) and incubated at 28 °C until mould development. The isolate was re-inoculated as many times as necessary, in order to obtain a pure culture. Then, a monosporic culture of the isolate was obtained. Initially, the strain PE-L was identified on the basis of morphological features in PDA and Czapack medium, according to manual of fungi's identification (Wei, 1979).

* Corresponding Author. Phone: +86-27-87286926;
Fax: +86-27-87282010; E-mail: lljdbx@mail.hzau.edu.cn

Spore suspensions were obtained by washing the spores from plates of a 7 d old culture with sterile bi-distilled water plus 0.05% (w/v) Triton X-100. Spore concentrations were determined by a hemocytometer, and adjusted to required (1.0 x 10^6 conidia/ml) with sterile bi-distilled water and stored at 4 °C. The PDA and PD liquid medium (PDB) were used in all experiments. The PDB was treated by centrifugation at 10,000 *x g* for 10 min to wipe off impurity.

Trans-trans farnesol (Sigma, St Louis, USA) stock solution was prepared fresh in methanol. The original flask containing farnesol was de-gassed with nitrogen gas each time after being opened in order to avoid degradation by oxygen.

Molecular identification of strain PE-L. In order to confirm the accuracy of morphological identification, the isolated strain was subjected to molecular analysis with fungus-specific universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990). Genomic DNA of the strain was obtained following the methodology previously described by Yang *et al*. (2006). Genomic DNA was used for PCR amplification. All amplification reactions were carried out in volumes of 50 µl containing 2.0 µl of template DNA, 1 µl of each primer (20 µM), 5.0 µl of 10X PCR buffer, 5.0 µl of $MgCl_2$ (25 mM), 2.0 µl of dNTPs (10 mM), 2.0 µl of BSA and 0.4 µl of Taq DNA polymerase (5 U/µl). PCR was carried out using the following condition: initial denaturation at 94 °C for 4 min; 37 cycles of denaturation (94 °C for 1min), annealing (54 °C for 1 min), and extension (72 °C for 1 min); and a final extension step at 72 °C for 10 min. A negative control was performed with each run by replacing the template DNA with sterile water in the PCR mixture.

PCR products were detected in 2% agarose ethidium bromide gels in 1×TAE buffer (Tris–acetate 40 mM and EDTA 1.0 mM). The ITS sequence was identified by searching databases using the BLAST sequence analysis tool (http://www.ncbi.nlm.nib.gov/BLAST). The ITS sequences was compared using nucleotide-nucleotide BLAST (blastn).

Antagonistic assays. The PDA (containing 25-400 µM farnesol) was poured into Petri plates (20 ml/plate). Spore suspension (10 µl, approximately 1.0 x 10^4 spores) was added into the beforehand prepared hole (5 mm diameter) and incubated at 28 °C after sealing the plates with Parafilm™ (Pechiney Plastic Packaging Chicago, IL). The area of lawn was examined 5 d later.

PE-L spores were germinated in PDB at 28 °C for 0, 2, 6 and 12 h, and followed treatment of 100 and 200 µM farnesol for 2 h. The treated suspension was spread on PDA Petri dishes. The activity of spores was assayed by status of colony forming units (CFU) per plates after being incubated at 28 °C for 72 h. The inhibitory effect was acquired by compared the number of CFU/plate with control.

Sporulation assays. The 100 µl of spore suspension (1.0 x 10^4 spores/ml) was spread on PDA (containing farnesol or not) Petri dishes, and incubated at 28 °C after sealing the plates with ParafilmTM. Colony morphology and the formation of spores were examined 5 d later.

Spores germination assays. Spores were germinated in PDB (containing 25-400 µM farnesol or not) and then incubated at 28 °C on a rotary shaker. The morphological features characteristic of germinated spores were examined by microscope every 12 h.

Apoptosis-like phenotype assays. PE-L spores were germinated in PDB at 28 °C for 12 h. The suspensions were treated with 50-400 µM farnesol, and incubated on a rotary shaker with 177 rpm at 28 °C for 2 h. The mycelium was fixed in 3.7% formaldehyde at least for 30 min, and then stained with Hoechst 33258 and PI as described by Harris *et al*. (1994). Slides were viewed by Nikon 80i fluorescent microscope, and proportion of nuclei with condensed DNA was counted.

After homogenization of the hyphae in liquid N_2, the genome DNA was extracted using the method described by Wilson *et al*. (2004) and Yang *et al*. (2006). The extraction was dissolved in 35 µl TE buffer (containing 40 µg/ml RNase A) and incubated at 37 °C for 2 h. Finally, the DNA extraction (each 10 µl) was monitored by electrophoresis on 2% agarose gel in 1×TAE buffer for 2 h at 120 V cm^{-1}, and the gel was stained by EB and visualized through UV light.

Germinated spores were treated with different concentrations of farnesol for 2 h at 28 °C, and then fixed and stained with TUNEL (Promega, USA) as described by Madeo *et al*. (1997) and Semighini *et al*. (2006). Slides were viewed by fluorescent microscope, and proportion of positive cells was counted.

RESULTS

Identification by ITS sequence analysis

According to manual of fungi's identification (Wei, 1979), on the basis of morphological features the isolated strain belonged to *P. expansum*, then, the result was confirmed by the rDNA-ITS molecular analysis by the fungus-specific universal primers pairs ITS1-ITS4. The 528 bp ITS region was successfully amplified from genomic DNA. The output of the BLAST search of the ITS sequence of PE-L showed 99.62% sequence identity with the type strain of *P. expansum* (GenBank accession no. AY373912, AJ60895 and AF218786), 99.61% with *P. expansum* (No. AJ 005676), 99.25% with *P. expansum*.wb342 (No. AF455466) and 99.25% with *P. italicum* ATCC 48114 (No. AY373920). In conclusion, the results indicate that the isolated strain PE-L belong to *Penicillium expansum.*

Farnesol inhibits germ tube growth

Previous reports showed that external farnesol inhibited the germination of *F. graminearum* (Semighini *et al*., 2008). In order to monitor the effect of farnesol in *P. expansum*, PE-L in different phases were exposed to farnesol for 2 h. The result showed that farnesol effectively inhibited spores growth after spores had been incubated in PDB for 2, 6 and 12 h (Fig. 1). However, no obvious inhibition was found if farnesol was added at the same time as the spores were incubated. In other words, spores are transitioning from being uncommitted to being committed if farnesol inhibits *P. expansu*m growth. Spores in different germination phases have different sensitivities to farnesol, and the dormancy spores were more resistant to farnesol than the active.

Oxygen depresses the effect of farnesol

To determine the stabilization of farnesol exposed to oxygen, spore suspension (mixing farnesol) was spread on Petri dishes, and the plates were not sealing with Parafilm™ to ensure air free exchange. No obvious effect of farnesol on spore germination was observed. Meanwhile, the external farnesol mixed with PDA medium, and spore suspension was spread on farnesol mixed PDA plates. The result showed that farnesol was obviously induced the morphology of colonies change. Farnesol degraded by oxygen was the main reason for the disparity between farnesol mixed with and spread on PDA medium.

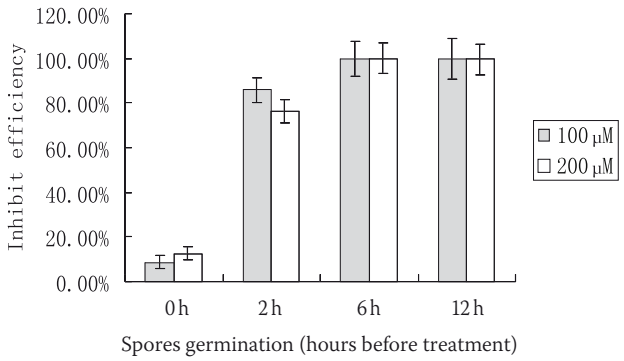

FIG. 1 - Inhibition efficiency of farnesol (100 and 200 μM) to hyphae. Spores of *Penicillium expansum* strain PE-L were germinated in PDB for 0, 2, 6 and 12 h at 28 °C, followed by treatment with farnesol or not for 2 h. CFU per plate and the inhibition incidence were determined after treated hyphae were germinated in PDA medium for 3 days at 28 °C. Each experiment at least two replicates.

Farnesol impairs development of *Penicillium expansum*

To determine whether farnesol inhibited colonies sporulation or not, different concentrations of farnesol were used in this experiment. At the presence of lower concentrations of farnesol (< 50 μM), the colonies exhibited some residual sporulation, while in higher concentrations (≥ 50 μM) of farnesol, the sporulation was almost completely inhibited and the results (100 and 200 μM) were shown as Fig. 2C and 2F.

In Fig. 2, the size of colonies was much smaller than control, meanwhile, the colonies of farnesol treated showed yeast character (distinct circular colony appear unity white patches, with a glossy surface) instead of mold (often appear whitish grey, with fuzzy edges, and they usually turn into a different color, from the center outwards) (Fig. 2A, 2D and 2G). The microscope observation suggested that the colonies exhibited an enhanced formation of aerial hyphae, and exhibited the "fluffy" phenotype resembling cotton appearance (Fig. 2C and 2F). We observed that, naturally, *P. expansum* colonies substrate hyphae showed yellow in medium (Fig. 2H), but there was no obviously color change in treated hyphae (Fig. 2B and 2E). The area of lawn of treated was obviously smaller than control indicating that the growth of hyphae was effectively arrested by farnesol (Fig. 3).

Spores were exposed to farnesol to monitor its effects on hyphal morphogenesis. We found that extra-cellular farnesol did not trigger the stagnation of spore germination. Instead, the change of hyphal polarity (growth direction) was observed under microscope at the lowest concentration (50 μM). After spores germinating in 400 μM farnesol for 12 and 24 h (Fig. 4A), the shape of primary hyphae suggested that the conversion of hyphal growth direction took place and the branch were formed earlier than the control (Fig. 4A1 and 4A2). In Fig. 4A1, arrow showed

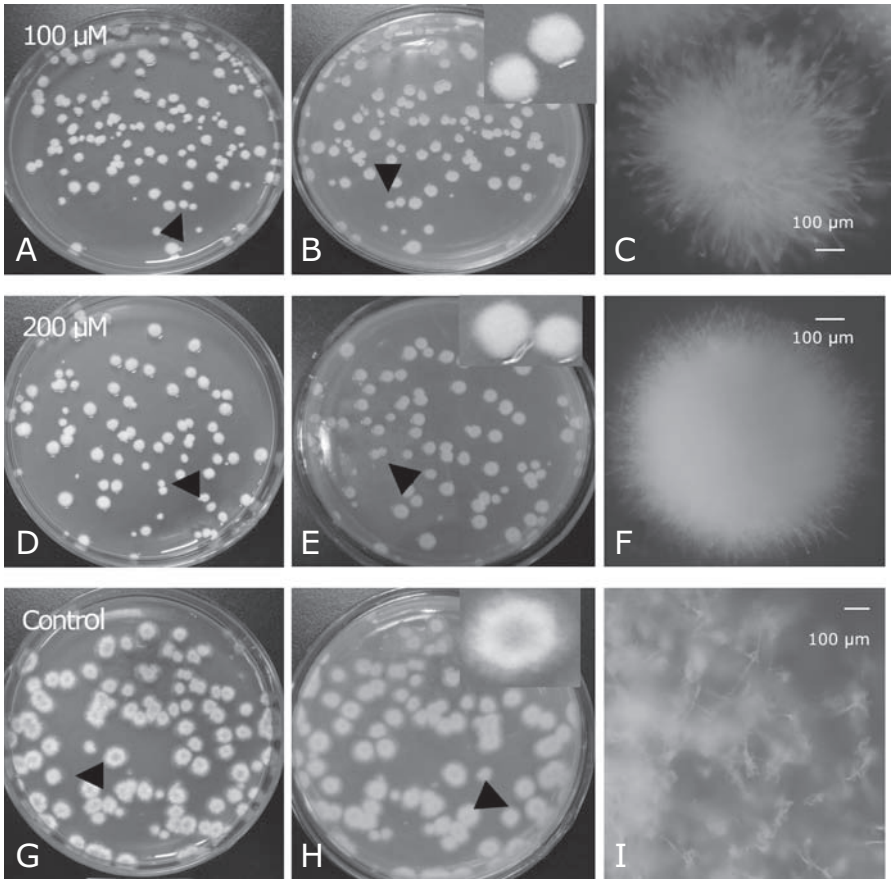

FIG. 2 - Effect of farnesol (100 and 200 μM) on the colonies morphogenesis of *Penicillium expansum*. Spores of strain PE-L were germinated in PDA medium (containing farnesol or not). Plates were sealed with parafilm to limited farnesol evaporation. Each experiment at least two replicates. The colonies were showed after 5 days incubation at 28 °C. A, D and G: face; B, E and H: inverse, the middle panels' colonies were magnified from arrow's colonies. C, F and I: higher magnification (100x) images obtained with a dissecting microscope to better visualize the colonies morphogenesis.

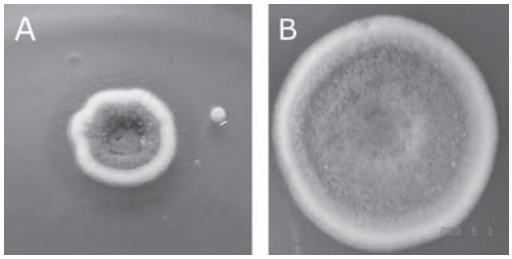

FIG. 3 - Colonies' outspread inhibition by farnesol (400 μM) in solid medium. Spores (10^4 spores/hole) of *Penicillium expansum* were germinated in PDA containing farnesol (A) or not (B, Control). Plates were sealed with parafilm to limited farnesol evaporation. The plates were incubated for 5 d at 28 °C. Each experiment at least three replicates.

that branches were induced at 12 h. In Fig. 4A2, 4A3, 4B2 and 4B3, white panels showed that the distance between hypha's node-node was distinctly shorter than control. The hyphae appeared as spotted deer's buckhorn at 36 h. The growth of hyphae formed branches resembling yeast's budding, but it had no obvious effect on cellular growth rates. The result was similar to the result of exterior farnesol to *C. albicans*, but not to that of *A. nidulans*. In Fig. 4A5, the increased aberrance hyphae (arrow) reflected that farnesol triggered the stagnation of the hyphae growth and induced the appearance of enlarged cell vacuole lead to hyphal swell, which implied that the hyphae are carrying through programmed cell death.

Farnesol induces apoptosis in *Penicillium expansum*

The Hoechst 33258 was used to test nuclear condensation, and PI was also used to determine the integrity of plasma membranes. The percentage of condensed nuclei increased in a dose-dependent manner whereas the control showed just 5% nuclei condensation (Table 1). Meanwhile, PI stain showed that the plasma membranes remained integrity regardless of the treatment (Fig. 5). Agarose electrophoresis testified that genome DNA was slightly degraded (Fig. 6) after treated with 50-400 μM farnesol. Besides, a corresponding fraction of hyphae displayed TUNEL positive (This assay used terminal deoxynucleotidyltransferase to label 3'-OH DNA termini with FITC-conjugated dUTP, which can be directly visualized by fluorescence). In Table 2, obviously enhanced percentage of positive of cells suggested that farnesol induced programmed cell death via apoptosis in *P. expansum*.

Cell death of fungi can be classified into three main categories: autophagy, apoptosis and necrosis. The first two are programmed and genetically regulated, and the third is environmentally induced by physical or chemical injuries (Lu, 2006). DNA fragmentation, nuclear condensation and the integrated plasma membranes suggested apoptosis-like phenotype may appeared when hyphae treatment with farnesol.

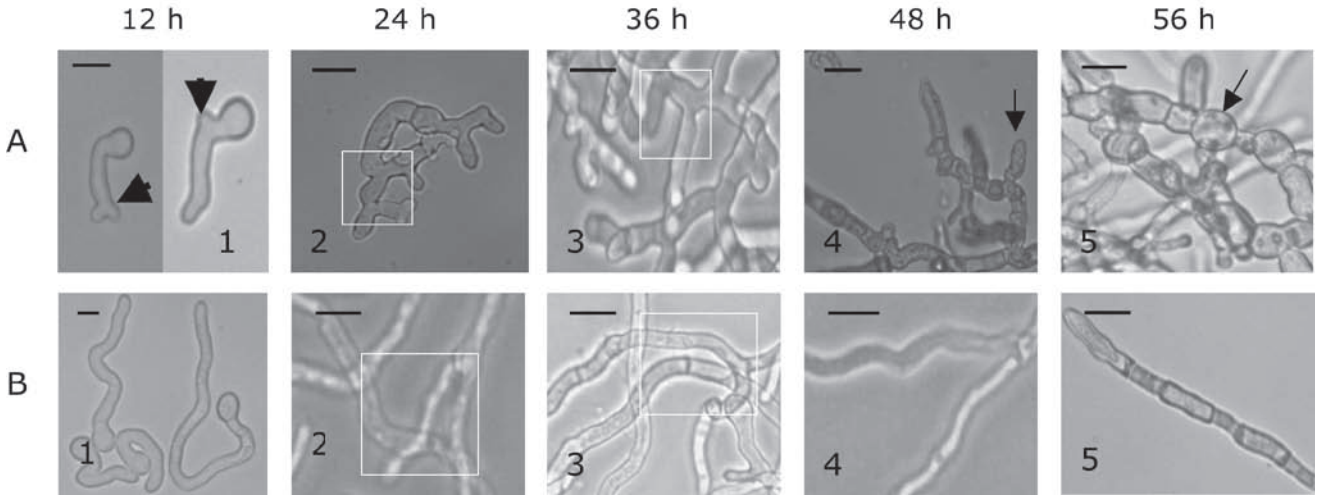

FIG. 4 - Effect of farnesol on hyphal morphogenesis in liquid medium. Spores of *Penicillium expansum* strain PE-L were germinated in PDB containing farnesol (A) or not (B, Control) and incubated at 28 °C for 12, 24, 36, 48 and 56 h. The hyphae were visualized by microscope. Two independent experiments for each sample. A1: arrows indicate the change of hyphal polarity; A4 and A5: arrow indicates the change of hyphal morphogenesis. A2, A3, B2 and B3: white panels indicate the branches of hyphae and its distances. Bars: 10 μm.

TABLE 1 - Nuclear condensation of hyphae of *Penicillium expansum* induced by farnesol

Farnesol (μM)	Control	25	50	100	200	400
Condensed nuclei (%)	5	48.5	93	97	98	98

Germinated hyphae were treated with farnesol for 2 h, then fixed and stained with Hoechst 33258. Slides viewed by fluorescent microscope and the proportion containing condensed DNA determined; 200 nuclei per sample were examined and the results represent the average of two independent experiments, with three replicates per experiment.

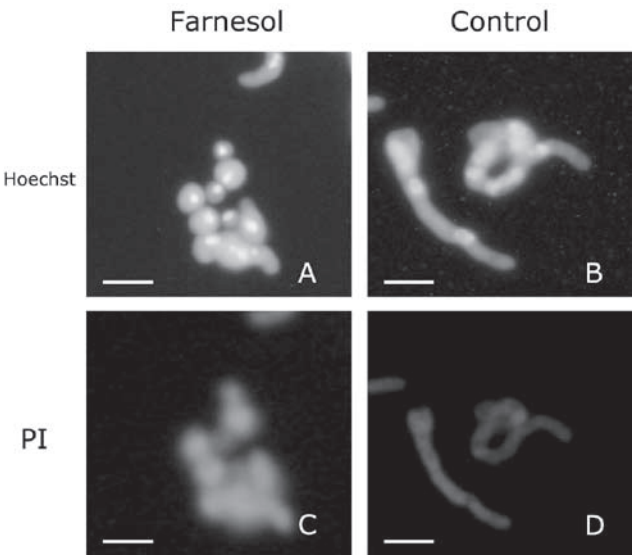

FIG. 5 - Nuclear condensation induced by farnesol. Spores were germinated in PDB at 28 °C for 12 h, followed by treatment for 2 h with 200 μM farnesol or not. Nuclei were visualized using Hoechst 33258 and PI. Two replicates for per experiment. Bars: 10 μm.

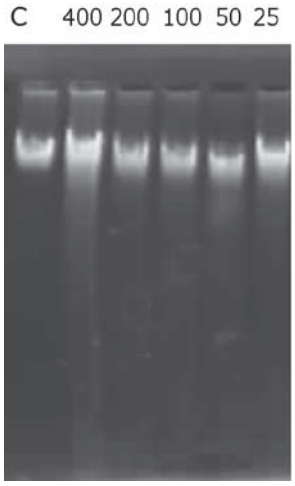

FIG. 6 - The genome DNA (each 10 μl) agarose identification. The germ tube was treated with 25-400 μM farnesol or not for 2 h. The genome DNA was extracted by CTAB and identified in 2% agarose 120 mV for 2 h.

DISCUSSION

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 different cellular functions, including development and apoptosis (Edwards and Ericsson, 1999). This study showed that the addition of exterior farnesol arrested the hyphae growth in liquid medium which kept coincident with the effect of farnesol on *C. dubliniensis, A. nidulans* and *Saccharomyces cerevisi*ae (Hornby *et al*., 2003; Machida *et al*., 1998; 1999; Jabra-Rizk *et al*., 2006). Some slight differences were observed between the effects of farnesol on *A. nidulans* and *P. expansum*. Such that farnesol can induces the conversion of hyphal growth direction in *P. expansum*, but not in *A. nidulans* (Semighini *et al*., 2008). Monomeric GTPases or cell wall stress signalling pathways could involved in morphogenesis alter according to previous reports (Park and Bi, 2007).

Over 90% nuclear condensation (only 5% in control), DNA fragmentation and the integrity of plasma membranes approve that the hyphae appear apoptosis after treated with farnesol. Apoptosis-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 RAF, sphingoid bases and α-Tomatine) that trigger this response (Chen and Dickman, 2005; Cheng *et al*., 2003; Ito *et al*., 2007; Mousavi and Robson 2004; Leiter *et al*., 2005; Semighini *et al*., 2006). Farnesol acts as a signal molecule that affects liquid synthetic, protein synthesis and degradation (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. Farnesol may induce *P. expansum* apoptosis by inhibiting Ras and Ras-related G proteins farnesylation.

Unlike *A. nidulans*, the present study found farnesol changed the hyphal growth style. In *C. parapsilosis*, there was obvious polarization of sterols to the tip of budding cells, and bud necks after being treated with farnesol (Rossignol *et al*., 2007). Farnesol affected sterol synthesis at the transcriptional level, which suggested that farnesol may affect membrane structure and permeability. Several genes involved in GPI anchor biosynthesis decreased expression following exposure to farnesol, correlating with the changes in the localization of the lipid raft (Rossignol *et al*., 2007). This report showed, in *P. expansum*, farnesol may affect sterol metabolism, membrane permeability and the lipid raft to disrupt the polarity of hyphae. This polarization disrupted by farnesol may be the reason of hyphal growth conversion.

In *A. niger*, decreased conidiation and growth rates are associated with increased levels of cAMP (Saudohar *et al*., 2002),

TABLE 2 - DNA fragmentation of hyphae of *Penicillium expansum* induced by farnesol

Farnesol (μM)	Control	50	100	200
Positive (%)	2	62	83	90

Germinated hyphae were treated with farnesol for 2 h, then fixed and stained with TUNEL. Slides viewed by fluorescent microscope; 200 nuclei per sample were examined and the results represent the average of two independent experiments, with three replicates per experiment.

and addition of cAMP can moderate intracellular amounts of cAMP which dramatically decreased by farnesol, whereas conidia formation is again blocked by adding higher cAMP concentration (Lorek *et al*., 2008). The result showed that cAMP plays a crucial role in spore formation and colony morphogenesis. In *P. expansum*, regulating fungal morphogenesis via farnesol through cAMP may be one reason for it, but specific differences between species in their regulatory networks should also be taken into account.

Acknowledgments

Thank Prof. Yunjiang Cheng and Rui Guan for helpful discussions. This work was supported by the grants from the National Natural Science Foundation of China (NNSFC-30771507)

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