Inhibition of *Fusarium graminearum* growth and development by farnesol

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**Abstract**

The isoprenoid farnesol was previously shown to induce morphological features characteristic of apoptosis in the filamentous fungus *Aspergillus nidulans*. This study demonstrates that under similar liquid media growth conditions, farnesol also triggers apoptosis in the plant pathogenic fungus *Fusarium graminearum*. However, unlike *A. nidulans*, *F. graminearum* spores treated with farnesol exhibited altered germination patterns and most (>60%) lysed upon prolonged exposure. Given the economic importance of *F. graminearum* as a pathogen of small grains, this study proposes that farnesol may have potential value as an antifungal compound.

**Introduction**

The plant pathogen *Fusarium graminearum* (teleomorph Gibberella zeae) is the causative agent of *Fusarium* head blight (FHB) or ‘scab’ on wheat and other cereals. The pathogen not only significantly reduces yield but also produces mycotoxins that make the grain unsuitable for processing or consumption by livestock (McMullen *et al*., 1997). Plant cultivars highly resistant to FHB or tolerant to the mycotoxins are not currently available and the use of fungicides for controlling the disease is not cost-effective (McMullen *et al*., 1997; Goswami & Kistler, 2004). For this reason, FHB has emerged as a plant disease with significant impact on the U.S. and worldwide agriculture during the last decade (Goswami & Kistler, 2004; Windels, 2000).

*Fusarium* spp. are also the causative agents of superficial and systemic infections in humans, collectively referred to as fusariosis. Recently, an outbreak of *Fusarium* keratitis, a corneal infection that can result in vision loss, was reported in contact lens users (Centers for Disease Control and Prevention, 2006). Fusarioses are difficult to treat and the systemic forms are often fatal, primarily because the pathogen possesses intrinsic resistance to the modern arsenal of antifungal agents (Lionakis & Kontoyiannis, 2004). Amphotericin B has shown good *in vitro* activity against some *Fusarium* isolates (Pujol *et al*., 1997) but limited *in vivo* activity. However, because of the lack of other therapeutic options, amphotericin B is the current option of choice for clinical treatment of fusariosis (Lionakis & Kontoyiannis, 2004).

The dimorphic fungus *Candida albicans* secretes farnesol, which acts as a quorum-sensing molecule that prevents the yeast to mycelium conversion (Hornby *et al*., 2001). The authors recently reported that externally added farnesol triggers morphological features characteristic of apoptosis in the filamentous fungus *Aspergillus nidulans* (Semighini *et al*., 2006). Both mitochondria and reactive oxygen species (ROS) were found to participate in farnesol-induced apoptosis. Additionally, growth and development of *A. nidulans* were impaired in a farnesol-dependent manner. To determine whether the effects of farnesol are unique to *A. nidulans* or apply more generally to other filamentous fungi, its activity was tested against *F. graminearum*. This study reports that extracellular farnesol can trigger apoptosis and impair the development of *F. graminearum*. Furthermore, exposure to farnesol adversely affected the germination of macroconidia, thereby suggesting that farnesol may have potential as an antifungal drug against *F. graminearum*.
Materials and methods

Strains and growth conditions

Fusarium graminearum strain PH-1 and yeast extract man- nitol agar (YMA) (0.4% sucrose, 0.4% yeast extract, 0.4% malt extract and 1.5% agar when necessary) media were used in this work. Macroconidia were harvested in sterile distilled water immediately before use.

Trans-trans farnesol (Sigma, St Louis, MO) was added at the indicated concentrations from a 4.5 M stock solution prepared fresh in methanol before each experiment. The original flask containing farnesol was de-gassed with nitrogen gas each time after being opened in order to avoid degradation by oxygen. Oligomycin (Sigma) was added to 10 μM from a 50 mM stock solution prepared in dimethyl sulfoxide. N-Acetyl-cysteine (NAC; Sigma) was added to 5 mM from a 1 M stock solution prepared in water.

Apopotic markers

PH-1 macroconidia were germinated on coverslips in liquid YMA medium at 28 °C for 6 h followed by 2 h of treatment with farnesol at 28 °C. For oligomycin treatment, PH-1 was grown at 28 °C in YMA for 2 h, treated with 10 μM oligomycin for 4 h, and then treated with 300 μM farnesol for an additional 2 h. Samples were processed for nuclear staining, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay, ROS detection and Evan’s Blue staining as described previously (Semighini et al., 2006). Slides were viewed using an Olympus BX51 fluorescent microscope and individual images were captured with a Photometrics CoolSnap HQ CCD camera.

Developmental assays

Different assays were used to assess the formation of macroconidia in the presence of farnesol. First, PH-1 was inoculated onto solid YMA containing 25 μM farnesol or left untreated. The plates were then sealed with parafilm and incubated at 28 °C for 4 days. Second, 25–100 μM farnesol was added to the lid of YMA plates freshly inoculated with PH-1. The plates were then sealed with parafilm and incubated at 28 °C for 4 days. For both assays, agar blocks (3 mm in diameter) were excised from the resulting colonies, transferred to tubes containing 1 mL of water, and vortexed for 30 s. The number of macroconidia was counted using a hematocytometer under a light microscope.

Spore germination

PH-1 macroconidia were inoculated on YMA liquid medium in the presence of 300 μM farnesol or left untreated. The inocula were immediately transferred to 100mm × 100 mm Petri dishes containing five coverslips arrayed on their bottom. Each dish was sealed with parafilm and incubated at 28 °C for 6 h. At hourly intervals after 2 h, the coverslips were fixed and stained with Hoechst 33258, and then visualized with the Olympus BX51 fluorescent microscope. To test the viability of macroconidia germinated in the presence of farnesol, the same initial inoculum of PH-1 was incubated in liquid YMA containing 0 or 300 μM farnesol at 28 °C for 6 h. The germlings were washed twice with fresh YMA, diluted and plated into YMA, and incubated at 28 °C for 4 days.

Results

Farnesol induces apoptosis in F. graminearum

Growing F. graminearum hyphae were treated with a range of farnesol concentrations (100–300 μM) for 2 h (Fig. 1a). A dose-dependent increase in the percentage of hyphae with condensed nuclei was observed, such that 83.6% (±5.3) of nuclei were condensed following treatment with 300 μM farnesol (Fig. 1a). Notably, a corresponding fraction of hyphae displayed TUNEL positive staining (Fig. 1b, c) after similar treatment with farnesol. These results suggest that farnesol induces programmed cell death via apoptosis in F. graminearum. To test the role of ROS in this process, hyphae treated with 300 mM farnesol for 2 h were stained with 2′,7′-dichlorodihydrofluorescein diacetate (DCF) to assess ROS production. Under these conditions around 70% of hyphae displayed positive staining, compared with 8% of untreated controls (Fig. 1b). Moreover, hyphae grown in the presence of the antioxidant NAC and then treated with 300 μM of farnesol exhibited a reduced percentage of chromosome condensation (Fig. 1a), indicating that ROS production is an important factor underlying farnesol-induced apoptosis. Finally, the role of mitochondria in farnesol-induced apoptosis was tested using oligomycin, a specific inhibitor of mitochondrial F0F1-ATPase. Pretreatment with oligomycin reduced nuclear condensation caused by subsequent treatment with farnesol to 28.6% (Fig. 1d), demonstrating that normal mitochondrial function is essential for farnesol-induced apoptosis.

Farnesol impairs development of F. graminearum

The present study tested the effect of farnesol on F. graminearum growth in solid media. Fusarium graminearum hyphae are able to grow on YMA plates supplemented with low concentrations of farnesol. However, under these conditions, the development of macroconidia was significantly impaired. For example, strain PH-1 inoculated onto YMA plates containing as little as 25 μM of farnesol produced only 3 macroconidia mL⁻¹ whereas the control plates produced 1.6 × 10⁵ macroconidia mL⁻¹. Interestingly, vaporized
farnesol (boiling point = 149 °C in 4 mm Hg vapor) also impaired macroconidiation. Addition of 100 μM of farnesol to the lid of the plates instead of the media reduced macroconidia production in half when compared with control plates.

**Inhibition of macroconidia germination and viability**

While characterizing the effects of farnesol on *F. graminearum*, a novel effect not observed in *A. nidulans* was noted. Farnesol appeared to inhibit the germination of *F. graminearum* macroconidia. To address this possibility, macroconidia from strain PH-1 spores were germinated in the presence of 0 and 300 μM farnesol, and samples were collected after 2, 3, 4, 5 and 6 h for the analysis of germ tube emergence (Fig. 2a). Although germ tube emergence marks the culmination of the germination process, it was used as a convenient marker for germination in these experiments. *Fusarium graminearum* macroconidia typically display a bipolar germination pattern in which one germ tube emerges from each apical cell of the macroconidium (Harris, 2005). Macroconidia germinated in control conditions (i.e. without farnesol) began to form germ tubes after 2 h. By 6 h, ~90% of macroconidia possessed germ tubes, and only 9% had more than two germ tubes per spore (Fig. 2a). As expected, germ tubes emerged from apical cells (Fig. 3a).

Farnesol treatment affected the germination of macroconidia in two ways. First, the spores showed a delay in germ tube formation and even after 6 h, 44% of them failed to germinate (Fig. 2a). In addition, 60.5% (± 7.1) of the nongerminated macroconidia underwent cell lysis (Fig. 3b). Second, for those macroconidia that did germinate in the presence of farnesol, there was a significant increase in the number of germ tubes per macroconidium. For...
example, after 6 h 30% of treated macroconidia possessed more than two germ tubes, which usually emerged from nonapical cells (Fig. 3c, arrowheads). Interestingly, macroconidia with multiple germ tubes also contained condensed nuclei (Fig. 3c, arrow), and many of them (74% ± 5.0) ultimately lysed after 6 h (Fig. 3d).

The vital dye Evan’s Blue was used to verify that germinating macroconidia lysed in the presence of farnesol. Accordingly, 62.5% (±5.5) of spores germinated in the presence of 300 μM farnesol for 6 h exhibited positive Evan’s Blue staining (Fig. 2b, right panel), compared with 0% of untreated spores (Fig. 2b, left panel). Finally, to confirm that farnesol treatment reduced spore viability, macroconidia incubated in the presence of 300 μM farnesol for 6 h were washed and plated onto YMA plates (Fig. 2c). Only 11% of treated spores were able to form a colony (compared with 100% of untreated control). Taken together these observations show that in the presence of farnesol, macroconidia either did not germinate or germinated but lost their viability.

Fig. 2. Farnesol inhibits germination of macroconidia and decreases *Fusarium graminearum* viability. (a) The number of germ tubes per spore was determined after macroconidia germination in the presence of zero (control) or 300 μM farnesol for 2, 3, 4, 5 and 6 h. For each sample, 200 hyphae were examined in three independent experiments. (b) Farnesol treatment induces lysis of hyphal cells. Macroconidia were germinated on coverslips in YMA for 6 h at 28 °C in the presence of zero (left panel) or 300 μM farnesol (right panel). Hyphae were stained with Evan’s Blue (1%) for 5 min followed by two washes with phosphate-buffered saline. Slides were examined under bright-field illumination from an Olympus BX51 fluorescent microscope. Darkened hyphal regions correspond to lysed cells stained with Evan’s Blue. Scale bars, 10 μM. (c) Farnesol decreases *F. graminearum* viability and inhibits macroconidia formation. Macroconidia grown in the presence of zero (left panel) or 300 μM farnesol (right panel) for 6 h were washed, diluted and plated into YMA plates. Note the pale color of colonies on the right panel compared with the red color conferred by the presence of macroconidia on the left panel.
Discussion

Farnesol is generated within cells by enzymatic dephosphorylation of farnesyl pyrophosphate, which is part of the highly conserved sterol biosynthetic pathway. Components derived from this pathway often act as signaling molecules that affect different cellular functions, including development and apoptosis (Edwards & Ericsson, 1999). The present study demonstrates that the effects of farnesol on *F. graminearum* resemble those that were previously characterized in *A. nidulans* (Semighini et al., 2006). Treatment of *F. graminearum* with farnesol in liquid media triggered morphological features characteristic of apoptosis such as nuclear condensation, DNA fragmentation and production of ROS in a mitochondrial-dependent manner. In addition, similar effects were also found in solid media, conditions where farnesol inhibits the growth and development of *A. nidulans* and *F. graminearum*. The fact that the effects of farnesol differ between liquid and solid media can be explained by the incubation conditions. For example, plates were incubated for an extended period (3 days, compared with 2 h of incubation in liquid media), when prolonged exposure of farnesol to oxygen may lead to loss of its activity. Nevertheless, in both the conditions farnesol was able to inhibit the growth and development of the fungal pathogen *F. graminearum*.

To address the specificity of farnesol as an inducer of fungal apoptosis, a series of experiments that test the effects of natural and synthetic farnesol analogs has been initiated (Shchepin et al., 2003). Preliminary results suggest that cyclization of the carbon backbone or the incorporation of oxygen atoms reduces levels of chromosome condensation in *F. graminearum* by over 50% (C. Semighini and S. Harris, unpublished results). These effects are consistent with the high specificity of farnesol as a quorum-sensing agent in *C. albicans* (Shchepin et al., 2003).

Unlike *A. nidulans*, the present study found that farnesol affects the germination of *F. graminearum* macroconidia. The observed increase in the number of germ tubes per macroconidia suggests that the localization or activity of monomeric GTPases involved in morphogenesis or cell wall stress signaling pathways could be altered (Park & Bi, 2007). On the other hand, these effects could also be specific features associated with apoptosis in germinating *Fusarium*.
macroconidia. Other compounds that trigger fungal apoptosis also disrupt membrane organization and promote aberrant hyphal morphology (Leiter et al., 2005; Hagen et al., 2007), raising the possibility that a common pathway might mediate these morphological responses.

The ability of *F. graminearum* macroconidia to adhere and germinate on host surfaces presumably plays a crucial role in FHB (Harris, 2005). Moreover, the ability to form macroconidia likely facilitates dissemination and the establishment of human fusariosis (Dignani & Anaissie, 2004). The observation that both the formation and germination of macroconidia are impaired in the presence of farnesol suggests that this isoprenoid or its derivatives may have value as topical antifungal agents that limit infection of plants or humans by *Fusarium* species.

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**References**


