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Oxylipins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans*

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Summary

Secreted, hormone-like lipogenic molecules, called oxylipins, mediate the balance of asexual to sexual spore ratio in *Aspergillus nidulans*. Oxylipin production in this fungus is dependent on developmental regulation of three conserved fatty acid oxygenases, PpoA, PpoB and PpoC. Here, we show that in addition to altering spore ratios, loss of *ppo* genes affect natural product biosynthesis and seed colonization. *ΔppoA;ΔppoC* and *ΔppoA;ΔppoB;ΔppoC* mutants were unable to produce the mycotoxin sterigmatocystin (ST) *in vitro* or *in planta* but in contrast overproduced the antibiotic penicillin (PN). These findings were correlated with decreased expression of genes involved in ST biosynthesis and increased expression of a PN biosynthetic gene, thus suggesting that oxylipin species regulate secondary metabolites at the transcriptional level. Additionally, the *ΔppoA;ΔppoC* and the *ΔppoA;ΔppoB;ΔppoC* mutants were defective in colonization of peanut seeds as reflected by a decrease in conidiation and production of the seed degradative enzyme lipase. These results indicate that oxylipin production is important for host colonization and mycotoxin production and may provide a promising target for future control strategies.

Introduction

Oxylipins constitute a family of oxidized polyenoic fatty acids that exhibit vital biological functions as signals of intra- and intercellular communication in animals, plants and fungi. In mammals, fatty acid-derived oxylipins (e.g. prostaglandins and leukotrienes) regulate inflammation and other homeostatic responses through an autocrine-paracrine sensing system (Funk, 2001). In plants, oxylipins mainly synthesized from linolenic acid regulate the expression of host defence genes against pathogen and pests and are central to most wound-mediated plant responses (Blee, 2002; Farmer *et al.*, 2003; Shah, 2005). Additionally, plant oxylipins play a pivotal role in the formation of phytohormones and in senescence (Feussner and Wasternack, 2002; Wasternack and Hause, 2002). In the prokaryotic kingdom, several species of lipogenic diffusible molecules regulate a variety of responses such as bioluminescence, virulence, and biofilm formation in a density-dependent manner through the quorum-sensing mechanism (Davies *et al.*, 1998; Fuqua *et al.*, 2001; Schauder and Bassler, 2001; Wang *et al.*, 2004). In fungi and fungal-like organisms, oxylipin production is ubiquitous and plays a role in life cycle control, notably in sexual and asexual development for a number of fungal genera (Kock *et al.*, 2003; Noverr *et al.*, 2003), including *Aspergillus* spp. (Calvo *et al.*, 1999; Tsitsigiannis *et al.*, 2004a), *Alternaria tomato* (Hyeon, 1976), *Septoria fructicola* (Katayama and Marumo, 1978) and *Neurospora crassa* (Nukima *et al.*, 1981; Roeder *et al.*, 1982).

The structural similarity of many of these oxylipins has given rise to a hypothesis that they are important molecules in cross kingdom communication (Tsitsigiannis *et al.*, 2004a). Evidence for such oxylipin-mediated signalling has been derived from the *Aspergillus*/seed pathosystem. *Aspergillus* spp. are notorious pathogens that cause tremendous yield losses through seed maceration and contamination of seed tissues with the mycotoxin aflatoxin (AF), the most potent natural carcinogen known (Payne and Brown, 1998; Hicks *et al.*, 2002). *In vitro* studies showed that linoleic acid and its two plant oxylipin products 9*S*- and 13*S*-hydroperoxy linoleic acid (9*S*-HPODE and 13*S*-HPODE) play a significant role on differentiation processes in *A. nidulans, A. flavus* and *A. parasiticus* (Calvo *et al.*, 1999). Whereas all of the 18 C polyunsaturated fatty acids promoted sporulation in all three species, 9*S*-HPODE stimulated and 13*S*-HPODE inhibited mycotoxin production (Burow *et al.*, 1997; Calvo *et al.*, 1999). These data led to the proposal that plant oxylipins affected *Aspergillus* developmental processes due to their mimicry of native *Aspergillus* 18 C oxylipins collectively called ‘psi

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factor' (Champe *et al*., 1987; Champe and el-Zayat, 1989; Mazur *et al*., 1991).

Although the sporulation properties of psi factor were described nearly 20 years ago, only recently have genetic studies confirmed their role in spore development. The biosynthetic pathway of psi factor was elucidated by the characterization of three distinct *A. nidulans* oxylipin biosynthetic enzymes, PpoA (Tsitsigiannis *et al*., 2004b), PpoB (Tsitsigiannis *et al*., 2005a) and PpoC (Tsitsigiannis *et al*., 2004a) that show similarity to mammalian prostaglandin synthases (Tsitsigiannis *et al*., 2005b) and are conserved in the kingdom fungi. *ppoA* encodes a fatty acid oxygenase required for biosynthesis of the linoleic acid-derived psi factor component psiBα and *ppoC* and *ppoB* both encode fatty acid oxygenases responsible for the formation of the oleic acid-derived oxylipin psiBβ. The characterization of *ppo* mutant strains led to the conclusion that oxylipins provide a fitness mechanism to *A. nidulans* by temporally balancing ascospore (meiospore) to conidia (mitospore) development (Tsitsigiannis *et al*., 2004a); these findings complemented prior physiological studies (Champe *et al*., 1987; Champe and el-Zayat, 1989; Mazur *et al*., 1991; Calvo *et al*., 1999). Additional studies in the human pathogen *A. fumigatus* showed that *ppo* genes are involved in the virulence machinery of this fungus as was demonstrated by an enhancement in the development of pulmonary aspergillosis by *ppo* mutants (Tsitsigiannis *et al*., 2005b).

The objective of this study was to examine whether loss of oxylipins alters virulence mechanisms of *A. nidulans* when it invades seed tissues. This model system has been successfully used to identify virulence attributes of *A. flavus* and *A. parasiticus*, which are the prevalent field plant pathogens of the genus but genetically recalcitrant (Dean and Timberlake, 1989; Hicks *et al*., 2002). Previous *in planta* studies showed that oleate desaturase mutants of *A. nidulans* and *A. parasiticus,* abnormal in oxylipin biosynthesis, are impaired in their ability to colonize corn seeds (Wilson *et al*., 2004). Here, we show that the *ΔppoA;ΔppoC* and the *ΔppoA;ΔppoB;ΔppoC A. nidulans* oxylipin mutants are defective in colonization of peanut seeds, an observation that was correlated with a decrease in the production of the degradative enzyme lipase. Additionally, we showed that these two mutants were unable to produce the mycotoxin sterigmatocystin (ST; the penultimate precursor to AF) *in vitro* or *in planta* and this blockage was mediated by a pronounced reduction or absence of the transcription factor *aflR* that regulates the ST cluster (Brown *et al*., 1996). Ppo mutations also caused changes in the production of other secondary metabolites of *A. nidulans*. This is the first demonstration that fungal oxylipins jointly regulate spore development, natural product biosynthesis and virulence in the *Aspergillus/*seed pathosystem.

Results

ppo *genes are required for ST biosynthesis in* A. nidulans

Considering that plant oxylipins affect ST biosynthesis (Burow *et al*., 1997), we thought it possible that *Δppo* mutants would be altered in ST production. As shown in Fig. 1, deletion of *ppoB* led to precocious production of ST and significantly higher levels of mycotoxin over time compared with the wild type. *ΔppoC* and *ΔppoA* mutants also showed a different temporal pattern of ST production compared with wild type, with a *ΔppoC* mutant producing more and a *ΔppoA* mutant producing less ST at day 6 than the wild type (Fig. 1A). After 8 days, *ΔppoA* and *ΔppoC* mutants did not show any critical changes in ST levels compared with wild type although the *ΔppoB* mutant still overproduced ST (data not shown). Neither the double *ΔppoA*;*ΔppoC* nor the triple *ΔppoA*;*ΔppoB*;*ΔppoC* mutant was able to produce any detectable ST even after 8 days of cultivation. Additionally, based on the thin-layer chromatography (TLC) profile, the production of several other unknown secondary metabolites was also altered in these mutants (Fig. 1B). Complementation of *ppoB* in the *ΔppoB* strain and *ppoC* in the *ΔppoA;ΔppoC* strain returned ST production to levels similar to wild type (data not shown).

In order to determine levels of ST produced *in planta*, *Δppo* strains were grown on peanut seeds and solvent extracts of infected seed examined by TLC. As shown in Fig. 2A, the single mutants produced similar to slightly higher levels of ST compared with the wild type 6 days after inoculation. However, the double and triple mutants did not produce ST when grown on peanut seeds (Fig. 2A), similar to the result from culture extracts (Fig. 1). Studies with the triple mutant grown on corn seeds showed that it is also defective in producing ST on this host (Fig. 2B). These results indicated a requirement of the *ppo* gene/gene products for ST production *in vitro* and *in planta*.

Transcriptional regulation of secondary metabolism genes in ppo mutants

Previous studies had shown that plant oxylipins regulated ST and AF biosynthesis at a transcriptional level (Burow *et al*., 1997). We therefore tested whether *ppo* mutants would show altered expression of ST genes and/or genes known to transcriptionally activate ST genes. As shown in Fig. 3A, both *aflR* (a transcription factor required for ST biosynthetic gene expression) (Fernandes *et al*., 1998) and *stcU* (a ST biosynthetic gene) (Brown *et al*., 1996) transcript accumulation was significantly higher in *ΔppoB* compared with wild type and at very low levels or absent in the double and triple mutant strains. These results correlated with product formation (Fig. 1). However, expression of *laeA*, a methyltransferase required for *aflR*

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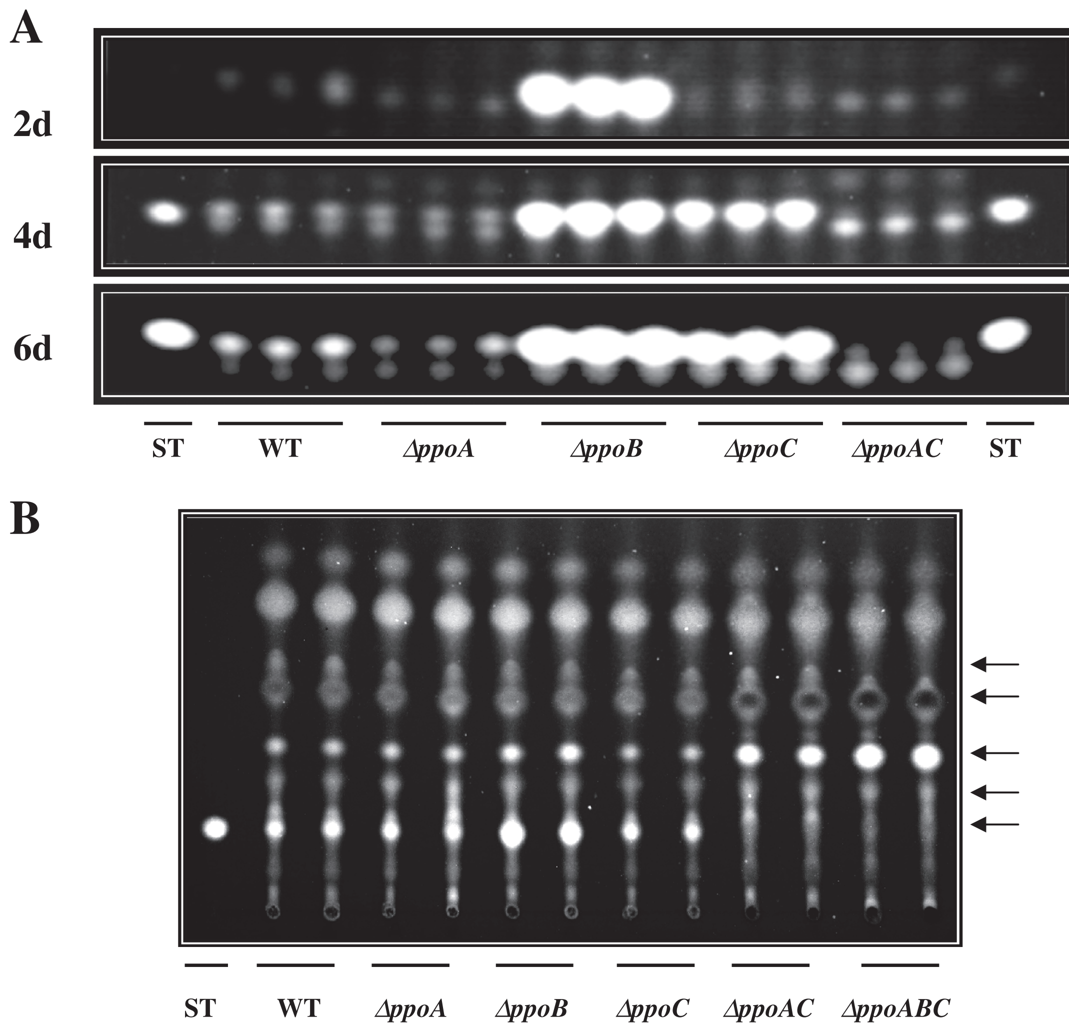

Fig. 1. A. *ppo* mutations differentially affect ST production in *A. nidulans*. TLC analysis of chloroform extracts of wild type (WT) and Δ*ppo* mutants after 2, 4 and 6 days of cultivation on solid GMM. The experiment was performed in triplicate.
B. *ppo* regulation of secondary metabolite production in *A. nidulans*. TLC analysis of chloroform extracts of WT and Δ*ppo* mutants after 8 days of cultivation on solid GMM. The experiment was performed in duplicate. Arrows indicate secondary metabolites that are differentially produced in Δ*ppo* mutants.
ST, sterigmatocystin standard. WT (RDIT9.32), Δ*ppoA* (RDIT12.9), Δ*ppoB* (RDIT59.1), Δ*ppoC* (RDIT58.12), Δ*ppoAC* (RDIT54.7), Δ*ppoABC* (RDIT62.3).

expression (Bok and Keller, 2004), was not significantly affected in the Δ*ppo* mutants (Fig. 3A), suggesting that the mode of action of the oxylipin pathway is not through LaeA but directly on *aflR* or effectors of *aflR* expression. In support of this, fusion of the *aflR* open reading frame (ORF) to an inducible promoter (*alcA*) remediated ST production in the Δ*ppoA;*Δ*ppoC* and triple *ppo* mutants (Fig. 3C).

Because solvent extracts of the *ppo* mutants suggested that production of other metabolites in addition to ST were affected in these mutants (Fig. 1B), we examined the possible effect of *ppo* deletions on penicillin (PN) biosynthetic gene expression and product formation. In contrast to *aflR* and *stcU* expression, *ipnA* (a PN biosynthetic gene) (Tilburn *et al*., 1995) expression was greatly increased in double and triple mutant strains (Fig. 3A). PN synthesis of Δ*ppo* mutants was examined using a *Micrococcus luteus* sensitivity bioassay (Bok and Keller, 2004). Culture filtrates from Δ*ppoB*, Δ*ppoA;*Δ*ppoC* and Δ*ppoA;*Δ*ppoB;*Δ*ppoC* strains showed an increased production of PN (Fig. 3B).

Altered ST production by ΔodeA *strains is attributable to psiBβ accumulation*

Previous studies showed that disruption of the *A. nidulans odeA* gene, encoding a Δ9-oleate desaturase, results in a strain unable to produce linoleic acid but instead accumulates large amount of oleic acid and oleic acid-derived oxylipins called psiBβ (Calvo *et al*., 2001). Additionally,

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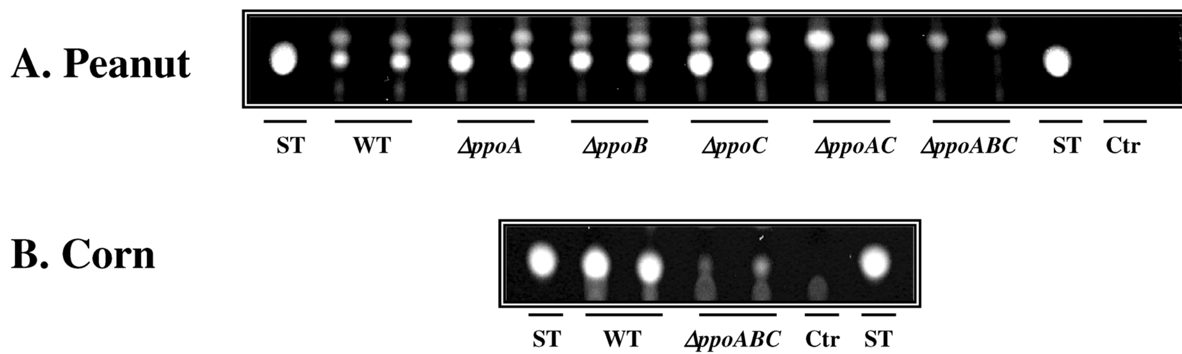

Fig. 2. Ppo proteins are required for ST production *in planta*.
A. TLC analysis of solvent extracts of WT and Δ*ppo* mutants after growing on peanut seeds for 6 days. The experiment was performed in duplicate. Ctr, non-infected peanut seed.
B. TLC analysis of organic extracts of WT and Δ*ppoA*;Δ*ppoB*;Δ*ppoC* mutant (RDIT62.3) after growing on corn seeds for 6 days. The experiment was performed in duplicate.
Ctr, non-infected corn seed; ST, sterigmatocystin standard. WT (RDIT9.32), Δ*ppoA* (RDIT12.9), Δ*ppoB* (RDIT59.1), Δ*ppoC* (RDIT58.12), Δ*ppoAC* (RDIT54.7), Δ*ppoABC* (RDIT62.3).

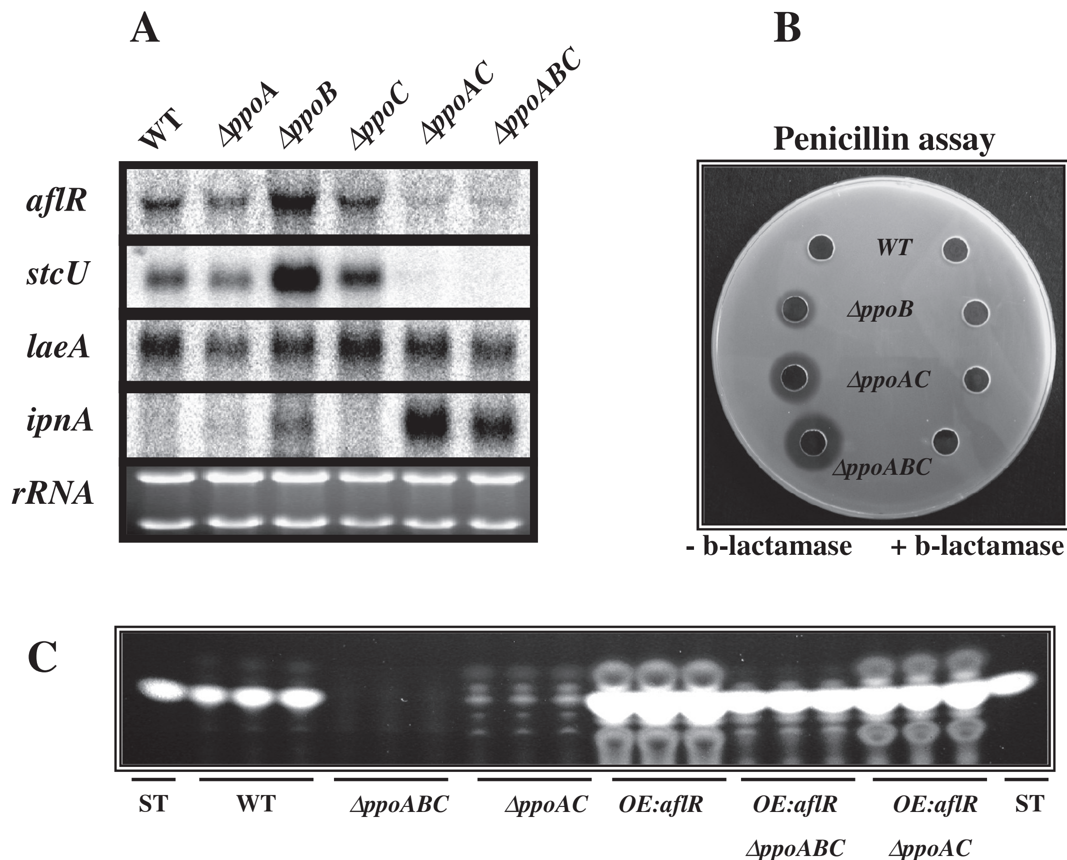

Fig. 3. A. Transcriptional regulation of secondary metabolite biosynthetic and regulatory genes in *ppo* mutant strains. Cultures of *A. nidulans* WT and Δ*ppo* strains were grown at 37°C in stationary liquid GMM for 72 h and analysed for the expression profile of *aflR*, *stcU*, *laeA* and *ipnA* genes. Equal loading of total RNA (20 mg) is depicted by ethidium bromide staining of the rRNA.
B. *ppo* genes negatively regulate the PN production in *A. nidulans*. For each strain, 6 ml of culture extracts were lyophilized, and resuspended in 1 ml of distilled water. One hundred microlitre samples, with or without 6 U of β-lactamase, were placed in 10 mm diameter wells of *M. luteus* plates to evaluate PN inhibition zones.
C. Overexpression of *aflR* restores ST production in *A. nidulans* Δ*ppoA*;Δ*ppoC* and Δ*ppoA*;Δ*ppoB*;Δ*ppoC* mutants. TLC analysis of chloroform extracts of *alcA*(p) fusion strains after 3 days of cultivation in liquid GMM amended with 30 mM cyclopentanone. The experiments were performed in triplicate.
ST, sterigmatocystin standard. WT (RDIT9.32), Δ*ppoA* (RDIT12.9), Δ*ppoB* (RDIT59.1), Δ*ppoC* (RDIT58.12), Δ*ppoAC* (RDIT54.7), Δ*ppoABC* (RDIT62.3). *OE:aflR* (RDIT103.5), *OE:aflR; ΔppoABC* (RDIT94.2), *OE:aflR; ΔppoAC* (RDIT94.4).

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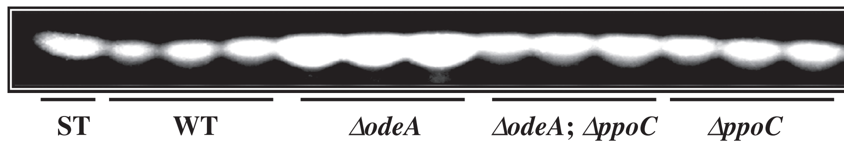
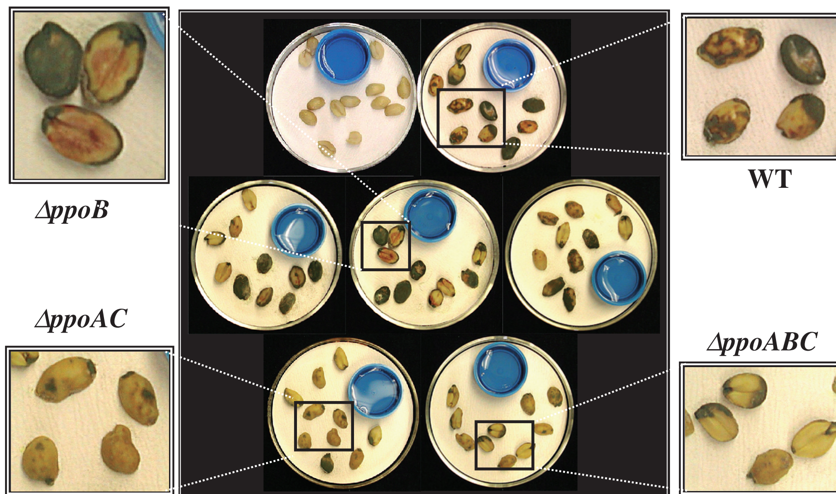


Fig. 4. Introduction of *ΔppoC* allele in the ST overproducing *ΔodeA* strain restores ST production in *A. nidulans*. ST, sterigmatocystin standard. WT (RDIT9.32), *ΔppoC* (RDIT58.12), *ΔodeA* (RRAW5.2), *ΔppoABC* (RDIT62.3), *ΔodeA;ΔppoC* (RDIT89.28).

Maggio-Hall *et al.* (2005) recently found that the *ΔodeA* mutant produces at least twice as much ST as the wild type. PpoC is involved in the production of oleic acid-derived oxylipins and its expression is significantly upregulated in *ΔodeA* (Tsitsigiannis *et al.*, 2004a). Therefore, to test whether the effect of a *ΔodeA* mutation on ST production was due to increased psiBβ accumulation, we created a *ΔodeA*;*ΔppoC* double mutant strain, predicted to be impaired in the ability to produce oleic acid-derived oxylipins. Deletion of *ppoC* in the *ΔodeA* mutant returned ST production to wild type levels (Fig. 4), suggesting that the psiBβ oxylipins may play a role in stimulating ST biosynthesis.

ΔppoA;ΔppoC *and the triple* ppo *mutant strains are impaired in the colonization of peanut seeds*

To dissect the role of Ppo proteins in *Aspergillus*/seed interaction and pathogenesis, we examined the ability of *A. nidulans Δppo* strains to colonize peanut seeds (Fig. 5).



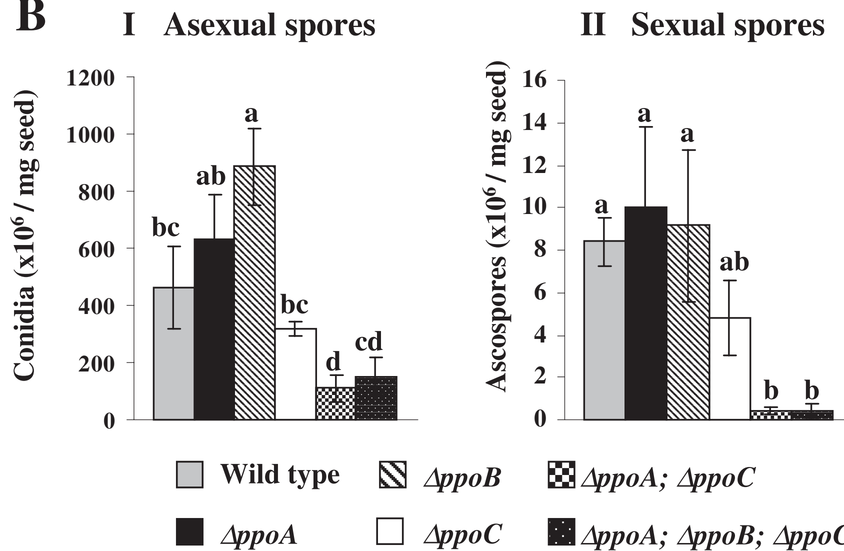


Fig. 5. *Aspergillus nidulans ΔppoA;ΔppoC* and *ΔppoA;ΔppoB;ΔppoC* mutants are impaired in the colonization of peanut seeds.
A. Infected peanut cotyledons (cultivar Florunner) were placed in a glass Petri dish containing filter paper saturated with sterile distilled water and a water reservoir to keep the humidity high, and incubated in the dark at 37°C for 6 days. Top (left to right): Control-mock inoculated seeds, WT (RDIT9.32). Middle (left to right): *ΔppoA* (RDIT12.9), *ΔppoB* (RDIT59.1), *ΔppoC* (RDIT58.12). Bottom (left to right): *ΔppoAC* (RDIT54.7), *ΔppoABC* (RDIT62.3).
B. Conidia (I) and ascospore (II) production were assessed from peanut cotyledons infected with the different *Δppo* mutants and incubated in the dark at 37°C for 6 days. Values are the mean of three replicates and error bars represent standard errors. Columns with different letters represent values that are statistically significantly ($P < 0.05$).

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Table 1. Lipase activity of Δ*ppo* mutants.

Strain	Depth of medium clearing (mm)	
Wild type	13.8 ± 0.29	a[a]
Δ*ppoA*	13.7 ± 0.15	a
Δ*ppoB*	17.8 ± 0.32	b
Δ*ppoC*	12.3 ± 0.30	c
Δ*ppoA;*Δ*ppoC*	11.7 ± 0.58	c
Δ*ppoA;*Δ*ppoB;*Δ*ppoC*	11.5 ± 0.50	c

a. Different letters represent statistically different values ($P < 0.05$).

Colonization was assessed in terms of visual symptoms (Fig. 5A) as well as conidial and ascospore production on seeds (Fig. 5B). In contrast to the wild type, the Δ*ppoA;*Δ*ppoC* and Δ*ppoA;*Δ*ppoB;*Δ*ppoC* strains showed a reduced ability to colonize peanut seeds (Fig. 5A); in addition, asexual and sexual sporulation of both of these two mutants was significantly reduced compared with the wild type ($P \leq 0.05$) (Fig. 5B). Visual inspection suggested a slight increase in seed colonization by the Δ*ppoB* strain as reflected by increased conidia production on seed compared with the wild type ($P \leq 0.05$). No difference was noted in either macroscopic colonization or spore production by Δ*ppoA* and Δ*ppoC* mutants compared with the wild type on peanut seeds (Fig. 5B).

To further investigate which mechanisms of seed colonization might be defective in *A. nidulans* Δ*ppo* mutants, we assessed enzymatic activity for a suite of degradative enzymes associated with seed maceration. *Aspergillus* and other pathogenic fungi secrete the hydrolytic enzymes esterase and lipase implicated in seed rot (Smart *et al*., 1990; Berto *et al*., 1999; Yu *et al*., 2003). As shown in Table 1, the Δ*ppoB* mutant possesses a more pronounced lipase activity than the wild type, whereas all strains possessing a Δ*ppoC* allele showed lower lipase activity. For the Δ*ppoB* strain, the increase in enzymatic activity was correlated to an increase in lipase transcript levels (AN7046.2 – data not shown). A colorimetric assay for non-specific esterase activity yielded similar results to the lipase activity for all the Δ*ppo* mutants (data not shown).

Discussion

Natural product biosynthesis is often associated with the advent of sporulation, cellular development and virulence in filamentous fungi (Calvo *et al*., 2002; Yu and Keller, 2005). These developmental processes reflect the need to access multiple nutrients and to optimize cellular morphology and metabolic differentiation for effective competition in complex environments. Based on our studies, we hypothesize that oxylipins act as signals that co-ordinate these processes in *A. nidulans.* This hypothesis is supported by previous observations that the oxylipins balance the ratio of sexual to asexual spores (Tsitsigiannis *et al*., 2004a; 2005a) and by our current studies demonstrating altered secondary metabolite profiles and virulence in *A. nidulans* oxylipin mutants.

Oxylipin mediated regulation of secondary metabolism in A. nidulans

Analysis of the Δ*ppo* mutants grown on media or on live seeds demonstrated significant alteration in the profile and timing of secondary metabolite production (Figs 1–3). Additional studies support a global role for *ppo* genes in natural product biosynthesis. For example, disruption of a *ppo* orthologue in *Fusarium sporotrichioides* impaired T2 toxin production (McDonald *et al*., 2004) and studies of the plant pathogenic fungus *Cercospora zeae-maydis* showed that the *lds* gene (a *ppo* homologue) is upregulated under conditions that favour the production of the mycotoxin cercosporin in this fungus (Shim and Dunkle, 2002). Additionally, long-chain unsaturated fatty acid mutants with oxylipin defects are altered in ST and AF production at the level of gene regulation (Maggio-Hall *et al*., 2005).

In an attempt to further understand the role of oxylipins in ST production we introduced the Δ*ppoC* allele (Tsitsigiannis *et al*., 2004a) into the oleate desaturase mutant Δ*odeA* (Calvo *et al*., 2001) that overproduces ST. This overproduction of ST in the Δ*odeA* strain was speculated to be due to increased beta-oxidation (Maggio-Hall *et al*., 2005) and the current work suggests that increased production of oleic acid-derived oxylipins contributes to this phenotype. Considering that Δ*odeA* mutants in *A. parasiticus* and *A. flavus* overproduce AF (Wilson *et al*., 2004; Maggio-Hall *et al*., 2005) and that these fungi contain *ppo* homologues, we suggest a conserved role for oxylipin stimulation of AF in these species.

Earlier studies had shown that Ppo/oxylipin regulation of sporulation processes was at the transcriptional level (Tsitsigiannis *et al*., 2004a; 2005a). This also appeared to be true of ST and PN regulation; *aflR* and *stcU* gene transcripts were reduced or eliminated in Δ*ppoA;*Δ*ppoC* and Δ*ppoA;*Δ*ppoB;*Δ*ppoC* mutant strains and elevated in the Δ*ppoB* strain, concomitant with the respective absence of or overproduction of ST. This was also reflected in the increased levels of a PN biosynthetic gene transcript (*ipnA*) in double and triple *ppo* mutant strains (Fig. 3). Additionally, overexpression of the *aflR* allele in Δ*ppo* mutants overcame repression of ST biosynthesis, further reinforcing the hypothesis that oxylipin regulation is transcriptional (Fig. 3C).

The suppression of *aflR* expression in the double and triple *ppo* mutant strains offers hints as to the signalling pathways mediating oxylipin signalling. Deletion of the

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ST regulator *laeA* or increased protein kinase A (PkaA) activity both repress *aflR* expression (Shimizu and Keller, 2001; Bok and Keller, 2004). Normal transcription of *laeA* in *ppo* mutant strains suggests that *aflR* regulation by PpoA and PpoC products is not mediated by LaeA. However, we postulate that oxylipin signalling may be PkaA-mediated. In support of this hypothesis, we note that the inverse regulation of ST and PN in the double and triple mutants was reminiscent of the opposite regulation of these two metabolites observed in a heterotrimeric G protein mutant ($FadA^{G42R}$) of *A. nidulans* (Tag *et al*. 2000). The constitutively activated Gα-subunit $FadA^{G42R}$ suppresses *aflR* expression, but enhances gene expression levels for *ipnA*. This suppression of *aflR* in $FadA^{G42R}$ is mediated by PkaA (Shimizu and Keller, 2001).

The significance of G protein signalling pathways in natural product biosynthesis, sporulation and virulence reveals that environmental ligands must be important in initiating these cascades, presumably through G-protein coupled receptors (GPCRs) or similar cell surface proteins (Yu and Keller, 2005). Psi factor (Champe *et al*., 1987; Champe and el-Zayat, 1989) is one of the first extracellular oxylipin signals described to regulate the sporulation and secondary metabolite synthesis. Current studies in our laboratory suggest a model where the different oxylipin products generated by Ppo oxygenases are secreted and function as ligands activating specific GPCR signalling cascades in *Aspergillus* and other fungi (M. Brodhagen *et al*., unpubl. data).

Role of oxylipin signals in Aspergillus *seed colonization*

Lipid-rich seeds are the agricultural commodities most affected by AF contamination (Hicks *et al*., 2002). There has been a lack of studies investigating the mechanisms controlling plant resistance to necrotrophic seed pathogens and the host contributions towards regulation of sporulation and mycotoxin production. In this work, we used *A. nidulans ppo* mutants to further explore the role of oxylipins in the plant/seed interaction. In contrast to their *in vitro* phenotypes (Tsitsigiannis *et al*., 2004a), the individual *ppoA* and *ppoC* mutant strains did not show significant spore or ST production alterations when grown on seeds. One possible explanation for this difference is that seed oxylipins can restore the asexual and sexual sporulation or ST defects in these strains (Fig. 5). In contrast, the Δ*ppoB* strain exhibited hyperconidiation both *in vitro* and on seeds (Tsitsigiannis *et al*., 2005a). The phenotype of the Δ*ppoA;*Δ*ppoC* double mutant and the triple *ppo* mutant on seed differed from that on agar plates in that these strains overproduced ascospores *in vitro* but not on seed. However, in other respects these two mutants showed similar phenotypes on seed and *in vitro*, producing very few conidia (Fig. 5B) and no-to-little detectable ST (Fig. 2). These observations support a requirement of the *ppo* genes and/or their products collectively for successful colonization and mycotoxin development. These results may help explain why *A. nidulans* and *A. parasiticus odeA* mutants, altered in oxylipin production, are impaired in the ability to colonize peanut and corn seed (Wilson *et al*., 2004). We suggest that changes in the oxylipin profile, herein achieved via *odeA* or *ppo* mutations, leads to a malfunctional signalling system in the fungal cell, resulting in an inability to regulate the myriad processes required for pathogenicity.

Based on visual observations, Δ*ppoB* appeared more aggressive in tissue maceration compared with wild type whereas the opposite was observed for the double and triple *ppo* mutants (Fig. 5A). Quantification of this difference was not, however, feasible. As one indicator of maceration potential, we assessed overall lipase and esterase activity of these mutants. Lipases are thought to play a role in virulence during fungal infections by assisting in cell penetration by allowing fungal catabolism of host lipids (Smart *et al*., 1990; Commenil *et al*., 1995; Berto *et al*., 1999). The increased lipase activity of the Δ*ppoB* mutant and decreased activity of the double and triple *ppo* mutants may indicate lipase activity contributes to pathogenesis through tissue degradation in these strains (Table 1).

Previous studies have shown exogenous application of oxylipins regulating mycotoxin production (Burow *et al*., 1997), but to our knowledge this represents the first work to genetically demonstrate a connection between native fungal oxylipins and mycotoxigenesis. We moreover expand the regulatory repertoire of oxylipins in *A. nidulans* to secreted enzymes and demonstrate their overall potential importance in seed colonization. Our findings represent an advance in understanding of the complex regulation and connection between *ppo* gene products, sporulation and secondary metabolite production and contribute to the broader elucidation of the signalling network by which fungal secondary metabolites are produced. The manipulation of the different *ppo* genes in filamentous fungi may provide improved strains with increased production of pharmaceuticals or the elimination of fungal toxins. Increased knowledge of oxylipin signalling may permit the design of novel control strategies, to reduce the survival and spread of seed-colonizing Aspergilli or other fungi. The conserved presence of *ppo* genes in fungal genomes (Tsitsigiannis *et al*., 2005a) coupled with conserved lipid stimulation of sporulation in several fungi (Katayama and Marumo, 1978; Nukima *et al*., 1981; Calvo *et al*., 1999; Klose *et al*., 2004) suggests that oxylipin signalling is ubiquitous in the fungal kingdom. Thus, the results pre-

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Table 2. *Aspergillus nidulans* strains used in this study.

Fungal strain	Genotype	Source
RDIT9.32	*veA*[a]	Tsitsigiannis *et al.* (2004b)
RDIT12.9	*metG1; ΔppoA::metG; veA*	Tsitsigiannis *et al.* (2004b)
RDIT59.1	*pyroA4; ΔppoB::pyroA; veA*	Tsitsigiannis *et al.* (2005a)
RDIT58.12	*ΔppoC::trpC; veA; trpC801*	Tsitsigiannis *et al.* (2004a)
RDIT54.7	*ΔppoC::trpC; metG1; ΔppoA::metG; veA; trpC801*	Tsitsigiannis *et al.* (2004a)
RDIT62.3	*ΔppoB::pyro; ΔppoC::trpC; pyroA4; metG1; ΔppoA::metG; veA; trpC801*	Tsitsigiannis *et al.* (2005a)
RDIT54.13	*ΔppoC::trpC; pyroA4; metG1; ΔppoA::metG; veA; trpC801*	Tsitsigiannis *et al.* (2005a)
RRAW5.2	*argB2; ΔodeA::argB; veA*	R.A. Wilson
RJH077	*yA2; metG1; veA1; alcA(p)::aflR::trpC*	J. Hicks
RDIT55.7	*pyroA4; veA; trpC801*	Tsitsigiannis *et al.* (2004a)
RDIT62.15	*biA1; ΔppoB::pyro; ΔppoC::trpC; pyroA4; metG1; ΔppoA::metG; veA; trpC801*	This study
RDIT103.5	*yA2; veA; alcA(p)::aflR::trpC*	This study
RDIT94.2	*yA2; ΔppoB::pyro; ΔppoC::trpC; pyroA4; metG1; ΔppoA::metG; veA; trpC801; veA; alcA(p)::aflR::trpC*	This study
RDIT94.4	*yA2; ΔppoC::trpC; metG1; ΔppoA::metG; veA; trpC801; veA; alcA(p)::aflR::trpC*	This study
RAMC29.24	*biA1; argB2; ΔodeA::argB; veA*	Calvo *et al.* (2001)
RDIT89.28	*ΔppoC::trpC; argB2; ΔodeA::argB; veA; trpC801*	This study

a. *veA* indicates the wild-type *veA+* allele.

sented herein should have broad implications for fungal pathogenesis.

Experimental procedures

Fungal strains and growth conditions

A list of the strains generated for this study is shown in Table 2. All strains were grown at 37°C, maintained on glucose minimal medium (GMM; Käfer, 1977) and stored as glycerol stocks. Appropriate supplements corresponding to the auxotrophic markers were added to the medium as required. The *Δppo* isogenic strains were generated as previously described (Tsitsigiannis *et al.*, 2004a, b; 2005a, b). RDIT103.5 [*alcA*(*p*)*:aflR*] is a recombinant strain of the cross between RDIT55.7 and RJH077, RDIT94.2 [*alcA*(*p*)*:aflR; ΔppoA;ΔppoB;ΔppoC*] and RDIT94.4 [*alcA*(*p*)*:aflR; ΔppoA;ΔppoC*] are recombinant strains of the cross between RDIT62.15 and RJH077, and RDIT89.28 (*ΔodeA;ΔppoC*) is a recombinant strain of the cross between RDIT54.13 and RAMC29.24. All strains used for infection studies and secondary metabolite analysis were prototrophic.

Gene expression analysis

Cultures for RNA extraction were grown by inoculating 30 ml of liquid GMM with 1×10^6 spores ml^{-1} of the appropriate strain and incubating at 37°C for 72 h without shaking. Total RNA was extracted from lyophilized mycelia using TRIzol reagent (Invitrogen) according to manufacturer's recommendations. Approximately 20 μg of total RNA was separated on a 1.2% agarose/1.5% formaldehyde gel and transferred to a Hybond-XL membrane (Amersham Pharmacia Biotech). RNA was probed with a radiolabelled 0.7 kb SacII–KpnI fragment from pRB7 containing the *stcU* coding region (Hicks, 1997), a 1.3 kb EcoRV–XhoI fragment from pJW19 containing the *aflR* coding region (Bok and Keller, 2004), a 3 kb HindIII fragment from pJW45.4 containing the *laeA* coding region (Bok and Keller, 2004) and a 1.1 kb EcoRI–HindIII fragment from pUCHH(458) containing the *ipnA* coding region (Tilburn *et al.*, 1995).

Peanut infection studies

Peanut seeds (cultivar Florunner) from the same field and year were selected for approximately similar size (between 0.4 and 0.6 g). Seeds were shelled, the cotyledons separated, and the embryos removed. Prior to inoculation, cotyledons were surface-sterilized by immersion in 0.05% sodium hypochloride for 3 min, followed by a wash with sterile water, a brief immersion in 70% ethanol, and two final washes with sterile water. For inoculation, 30 cotyledons were immersed in 30 ml of sterile distilled water containing 10^6 spores ml^{-1} of the appropriate strains for 30 min, with continuous shaking on a horizontal shaker at 80 rpm. The infected peanut seeds were separated into groups of 10, and placed in glass Petri dishes atop water-saturated filter paper containing a water reservoir to keep the humidity high. Seeds were incubated in the dark at 37°C for 6 days. At harvest, seeds were collected in 50 ml Falcon tubes, weighed, and vortexed for one minute to release spores in 5 ml of sterile water supplemented with 0.01% Tween 80. An aliquot of this spore suspension was used to count using a haemocytometer. Treatments without the addition of *Aspergillus* spores were used as non-infected control seeds. Three replicates of 10 seeds each were used for each strain. Spore data were statistically compared by analysis of variance (ANOVA) using Fisher's Least Significant Difference (LSD) with the Statistical Analysis System (SAS Institute, Cary, NC).

Mycotoxin analysis

Experiments generating data for Figs 1 and 4 were performed on plates containing 30 ml of solid GMM containing 1.5% agar. Each plate was overlaid with 5 ml of cool melted GMM (0.7% agar) containing 10^6 conidia of the appropriate strain. Cultures were incubated in continuous dark at 37°C.

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The experiments were performed with three replicates. Three cores of 12.5 mm diameter from each replicate were removed from each plate at the appropriate time interval. The agar cores were collected in a 15 ml tube and homogenized for 1 min in 3 ml of sterile water. ST was extracted by adding 3 ml of chloroform. Samples were vortexed vigorously for 1 min and allowed to stand for 5 min at room temperature. This procedure was repeated twice followed by centrifugation for 10 min at 2000 rpm to remove residual aqueous material and separate the organic phase. The extracts were allowed to dry and then resuspended in 100 μl of chloroform before 10 μl of each extract was fractionated on a silica gel TLC plate using a toluene-ethyl acetate-acetic acid [80:10:10 (v/v/vo)] or a hexane : ethyl acetate [40:10 (v/v)] solvent systems. The TLC plates were sprayed with aluminium chloride (15% in 95% ethanol) to enhance ST fluorescence, baked for 10 min at 80°C and exposed to long-wave (365 nm) UV light (Stack and Rodricks, 1971).

Cultures for ST quantification of *alcA*(p) fusion strains (shown in Fig. 3C) were generated by inoculating 50 ml GMM liquid medium with 1×10^6 spores ml^{-1} for each strain and shaking for 24 h at 300 rpm at 37°C. Cultures were then amended with 30 mM cyclopentanone (to induce the *alcA* promoter; Waring *et al*., 1989) and continued to grow for an additional 48 h. The experiments were performed with three replicates. ST was extracted by adding 50 ml of chloroform. Samples were vortexed vigorously for 1 min and allowed to stand for 2 h at room temperature. Then, we followed the same procedure as above.

For the mycotoxin analysis *in planta*, peanut seeds were infected with the different *A. nidulans* Δ*ppo* strains as previously described. Treatments without the addition of *Aspergillus* spores were used as non-infected control seeds. Six days after infection, cotyledons were collected in 50 ml Falcon tubes with the addition of 5 ml of 0.01% Tween 80 (v/v in water) and vortexed vigorously for 1 min. Five millilitres of acetone was added to the samples followed by shaking for 10 min at 150 rpm. Samples were allowed to stand for 5 min at room temperature. Then, 5 ml of chloroform was added to the samples and they were shaken for 10 min at 150 rpm. Samples allowed to stand for an additional 10 min at room temperature, vortexed briefly and centrifuged for 10 min at 3000 rpm to collect the organic lower phase. Samples were further dried out completely at room temperature under the fume hood. The presence of abundant seed lipids in the samples hampered the clear observation of ST on TLC plates, so a second extraction-purification was carried out as follows. Samples were resuspended in 5 ml of 0.1 M NaCl methanol : water [55:45 (v/v)] and 2.5 ml of hexane and vortexed vigorously at high speed for 1 min. Samples were centrifuged at 2000 rpm for 5 min. The hexane layer was collected and the fatty acid interphase layer was discarded. The remaining aqueous phase was washed with 2.5 ml hexane. The hexane extracts were combined, allowed to dry and then resuspended in 500 μl of hexane before 10 μl of each extract was separated on a silica gel TLC plate using a hexane : ethyl acetate [40:10 (v/v)] solvent system (Lopez *et al*., 1998).

Penicillin assay

For the assessment of PN production, we performed a slight modification of previously described methods (Brakhage *et al*., 1992; Bok and Keller, 2004). *M. luteus* ATCC 9341 was cultivated in TBS medium (17 g of Bacto Tryptone, 3 g of Bacto Soytone, 5 g of NaCl, 2.5 g of K2HPO4, and 2.5 g of glucose in a one L total volume) at 37°C at 200 rpm to reach an OD of 1.0. Three millilitres of *M. luteus* culture (OD = 1.0) was mixed with 40 ml of TSA medium (15 g of Bacto Tryptone, 5 g of Bacto Soytone, 5 g of NaCl, and 10 g of agar per litre) and poured into 150 mm diameter plates to solidify. Fifteen millilitre cultures of the corresponding *A. nidulans* strains were grown in GMM with shaking (250 rpm) for 72 h at 37°C. For each strain, 6 ml was removed, lyophilized and resuspended in 1 ml of distilled water. One hundred microlitre samples, with or without 6 U of β-lactamase (a PN degrading enzyme), were placed in 10 mm diameter wells of the *M. luteus* plates. Plates were placed for 2 h at 4°C and then incubated overnight at 37°C to evaluate PN inhibition zones. All experiments were duplicated.

Enzymatic activities

The lipase activity was assayed using the deep agar diffusion method (Paterson and Bridge, 1994). The medium was composed of 5 g of mycological peptone, 3 g of yeast extract and 10 g of agar for 1 l of medium. The medium was autoclaved for 10 min and when the agar was cooled to approximately 60°C, filter sterilized tributyrin (Glyceryl Tributyrate: a bitter oily triglyceride of butyric acid) was added to give a final concentration of 0.1% (v/v). Hot agar and the tributyrin were mixed together in a blender twice on low setting for 1 min. The blended mixture was then dispensed into sterile test tubes (15 ml per tube) and chilled rapidly. Medium was overlaid with equal amounts of conidia (~10^4 conidia per tube) and incubated at 37°C for 5 days. A positive reaction was the clearing of the opaque medium (Paterson and Bridge, 1994).

The fatty acid esterase activity was assessed on Tween 80 medium (Paterson and Bridge, 1994). The medium contained 10 g of mycological peptone, 5 g of NaCl, 0.1 g of $CaCl_2.2H_20$, 25 mg of bromocresol purple and 15 g of agar for 1 l of medium. The pH was adjusted to 5.4 and dispensed into 90 ml aliquots. The Tween solution was prepared as a 10% (v/v) aqueous Tween 80 solution by slowly adding 10 ml of Tween 80 to 90 ml of warmed (60–70°C) distilled water. Agar and Tween solution were sterilized by autoclaving for 10 min. When the media were cooled to 65–70°C, 10 ml of Tween solution was added to each 90 ml basal medium and were mixed. The completed medium was dispensed into 5 cm diameter Petri dishes (10 ml per plate). Plates were single point inoculated centrally with ~10^3 conidia and incubated for 7 days at 37°C. A positive reaction was identified colorimetrically by blue-purple of the medium (Paterson and Bridge, 1994).

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