

Characterization of the *Aspergillus parasiticus* Δ^{12} -desaturase gene: a role for lipid metabolism in the *Aspergillus*–seed interaction

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In the mycotoxigenic oilseed pathogens *Aspergillus flavus* and *Aspergillus parasiticus* and the model filamentous fungus *Aspergillus nidulans*, unsaturated fatty acids and their derivatives act as important developmental signals that affect asexual conidiospore, sexual ascospore and/or sclerotial development. To dissect the relationship between lipid metabolism and fungal development, an *A. parasiticus* Δ^{12} -desaturase mutant that was unable to convert oleic acid to linoleic acid and was thus impaired in polyunsaturated fatty acid biosynthesis was generated. The Δ^{12} -desaturase mutant demonstrates delayed spore germination, a twofold reduction in growth, a reduced level of conidiation and complete loss of sclerotial development, compared to the wild-type. Host colonization is impaired, as reflected by a decrease in conidial production on live peanut and corn seed by the mutant compared to the wild-type. Similarly, the previously isolated *A. nidulans* Δ^{12} -desaturase mutant has reduced colonization capabilities compared to the wild-type. Therefore, desaturation mutants display a key requisite that affords a genetic solution to oilseed crop contamination by mycotoxigenic *Aspergillus* species: a reduction in the production of conidia, the infectious particle of the pathogenic aspergilli.

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INTRODUCTION

In agriculture, colonization of oilseed crops by the mycotoxigenic pathogens *Aspergillus flavus* and *Aspergillus parasiticus* is a major health and economic concern. Each season, asexual spores called conidia are sources of inoculum for the colonization of crops (Payne, 1992). Two other reproductive structures also play a role in the fungal life cycle. In some species of *Aspergillus*, sexual ascospores formed in cleistothecia can potentially act as inoculum, whereas mitotic *Aspergillus* species, such as *A. flavus* and *A. parasiticus*, do not produce ascospores and have no known sexual stage, but do produce overwintering structures, sclerotia, which are postulated to be derived from vestigial cleistothecia (Geiser *et al.*, 1996). Recent studies have suggested possible roles for seed- and fungal-derived unsaturated fatty acids in affecting the development of these reproductive structures (Burow *et al.*, 1997; Calvo *et al.*, 1999, 2001). Specifically, linoleic and linolenic acids have been shown to induce asexual

sporulation in *Aspergillus* species (Calvo *et al.*, 1999). Sporulation is also induced by hydroperoxy fatty acid derivatives of linoleic and linolenic acids (Calvo *et al.*, 1999), which are produced by seed lipoxygenases (LOX) during the plant's defensive response to *Aspergillus* infections (Burow *et al.*, 2000; Wilson *et al.*, 2001). *Aspergillus* species also produce endogenous hydroxy fatty acids from oleic acid, as well as linoleic and linolenic acids, collectively called psi factor, that regulate spore production (Mazur *et al.*, 1990, 1991; Calvo *et al.*, 2001; Tsitsigiannis *et al.*, 2004; H. W. Gardner & N. P. Keller, unpublished results).

Oleic and linoleic acids are the most common unsaturated fatty acid components of *Aspergillus* and oil seeds (Bewley & Black, 1985; Evans *et al.*, 1986; Calvo *et al.*, 2001). Due to the requirement for oleic acid, linoleic acid and linolenic acid in generating psi factor and LOX products, our investigations have been aimed at understanding the connections between fatty acid metabolism and fungal development and pathogenesis in this host–parasite interaction. In particular, our goals are to generate and characterize *Aspergillus* mutants that are unable to synthesize polysaturated fatty acids. We previously disrupted the Δ^{12} -oleic acid desaturase gene, *odeA* (Calvo *et al.*, 2001), in the saprophyte *Aspergillus*

Abbreviations: FAME, fatty acid methyl ester; LOX, lipoxygenase.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AF528822.

nidulans and showed how this mutation leads to almost total loss of polyunsaturated fatty acids and altered conidial and ascospore development. Here, we describe how disrupting an *odeA* orthologue, *ApodeA*, in the oilseed pathogen *A. parasiticus* similarly affects fatty acid biosynthesis and results in delayed spore germination, reduced growth and impaired developmental processes in this agronomically important plant pathogen. In addition, this mutant is also severely compromised in its capacity to colonize host corn and peanut seed, as determined by reduced conidiation on seed.

METHODS

Fungal strains and growth conditions. *A. nidulans* and *A. parasiticus* strains used in this study are listed in Table 1. Cultures of both species were grown on *A. nidulans* glucose minimal medium (GMM) (Calvo *et al.*, 2001) unless otherwise indicated, at 29 °C. Cultures were grown either in the dark or in continuous white light by using an incubator equipped with a General Electric 15 W broad-spectrum fluorescent light bulb (F15T12CW) positioned 50 cm from the agar surface.

Molecular biology techniques. DNA was extracted from lyophilized mycelium by using standard techniques. For Southern blot analysis, 10 µg *Pst*I restriction-digested genomic DNA was separated by electrophoresis in a 1% agarose gel and transferred by capillary action to a Hybond membrane (Amersham Biosciences).

Total RNA was extracted from mycelia by using Trizol (Life Technologies). Aliquots of RNA (10 µg) were separated on a 1.2% agarose/1.5% formaldehyde gel. RNA was transferred to a Hybond membrane (Amersham Biosciences) by capillary action. *fasA* gene expression was analysed by using a 0.5 kbp PCR-amplified fragment of *A. nidulans fasA* (GenBank accession no. U75347), which was generated with the primers *fasAF* (5'-GGATTCACAGCGG-3') and *fasAR* (5'-GGGAGCACGGAGAG-3') from genomic DNA of strain RDIT9.32.

DNA fragments to be used as probes were radiolabelled with ³²P by using the random primer method (Sambrook *et al.*, 1989). Following prehybridization and addition of the probe, membranes were hybridized overnight at 60 °C and washed with increasing stringency up to 0.1 × SSC, 0.1% SDS at 60 °C.

Identification, sequencing and disruption of the Δ^{12} -oleic acid desaturase-encoding gene, *odeA*, from *A. parasiticus*. The *odeA* gene was sequenced by using previously described techniques and software (Calvo *et al.*, 2001).

The cosmid pAMC8, containing the *A. parasiticus odeA* gene, was

identified by heterologous hybridization of an *A. parasiticus* genomic library with a Δ^{12} -desaturase-encoding gene from *Candida albicans*, as described by Calvo *et al.* (2001). The sequence of the *odeA* gene has been deposited in GenBank under accession no. AF528822.

The *A. parasiticus odeA* disruption vector pAMC37 was created by introducing a *pyrG* coding-sequence cassette into the ORF of *odeA*. Firstly, plasmid pBZ5 (kindly provided by Dr J. E. Linz, Department of Food Sciences and Human Nutrition, Michigan State University, East Lansing, MI, USA) was digested to release a 2.8 kbp *Eco*RI–*Hind*III fragment containing the *pyrG* gene marker from *A. parasiticus*. The fragment was ligated into pBluescript KS (Stratagene) that had been previously digested with *Eco*RI and *Hind*III, generating the plasmid pAMC34. A 4 kbp fragment corresponding to the 5' flanking region of *odeA* was amplified from genomic DNA by PCR with proof-reading *Pfu* polymerase (Stratagene) and the primers *ApodeAF1* (5'-GCTGTGAAGCTTCTCCGCAG-3') and *ApodeAR1* (5'-GGTCCG-AAGCTTGCTATATCTGG-3'). Thermocycler conditions were 2 min at 94 °C, followed by 30 cycles of 96 °C denaturing (1 min), 48 °C annealing (1 min) and 72 °C extension (4 min 30 s). Each of these two primers incorporates a new *Hind*III site, which were used to ligate the digested PCR product into the *Hind*III site of pAMC34. Next, a 6 kbp *Eco*RI fragment corresponding to the 3' end of the gene was released from pAMC8 and incorporated into the vector at the *Eco*RI site adjacent to the *pyrG* cassette, to give pAMC37. Therefore, pAMC37 represents 11 kbp of the gene and flanking regions of *odeA*, but has 1047 bp of the coding region removed and replaced by *pyrG*. Transformation of the *A. parasiticus pyrG*⁻ strain CS10 with pAMC37 was performed by using previously described techniques (Skory *et al.*, 1992) and transformants were initially selected based on their ability to grow on GMM lacking the supplements (uracil and uridine) that are required by the *pyrG*⁻ parent.

Physiological studies. Germination studies were performed by inoculating 10⁶ spores of each strain into 25 ml liquid GMM and shaking at 29 °C. Colony diameters were measured by point-inoculating 5 µl water containing 10⁶ spores of either strain onto the centre of plates containing 25 ml GMM agar, followed by growth at 29 °C for 5 days. Conidial production studies were performed on plates containing 25 ml GMM agar. For each plate, a 5 ml top layer of cool but molten agar that contained 10⁶ spores of the appropriate strain was added. For each strain, there was a minimum of four replicate plates. Strains were grown in continuous light or dark for 72 h (unless otherwise stated) at 29 °C. A core of 15 mm was removed from the plates at the appropriate time interval and homogenized in 2 ml 0.01% Tween 80 in water to release the spores. Spores were counted on a haemocytometer.

To study the effect of the Δ *odeA* mutation on sclerotial production in *A. parasiticus*, strains were grown on coconut agar medium (CAM) (Trail *et al.*, 1995) and GMM agar. To 30 ml CAM was added a 5 ml top layer of cool but molten agar that contained 10⁶ spores of the appropriate strains. For GMM agar, 5 µl water containing 10⁶ spores of

Table 1. Fungal strains used in this study

Strain	Species	Genotype	Source
RRAW5.2	<i>A. nidulans</i>	<i>argB2</i> ; Δ <i>odeA</i> :: <i>argB</i> ; <i>veA</i>	Wilson <i>et al.</i> (2004)
RDIT9.32	<i>A. nidulans</i>	Prototroph	D. Tsitsigiannis, Department of Plant Pathology, University of Wisconsin, USA
ATCC 36537	<i>A. parasiticus</i>	<i>wA</i> ; <i>ver1</i>	American Type Culture Collection
CS10	<i>A. parasiticus</i>	<i>wA</i> ; <i>ver1</i> ; <i>pyrG</i> ⁻	Skory <i>et al.</i> (1992)
TAMC37.41	<i>A. parasiticus</i>	<i>wA</i> ; <i>ver1</i> ; <i>pyrG</i> ⁻ ; Δ <i>odeA</i> :: <i>pyrG</i>	This study

either strain was point-inoculated onto the centre of each plate. Plates were incubated in the dark at 29 °C for 14 days for CAM and 7 days for GMM agar. After this time, plates were sprayed with ethanol to flatten conidia and total numbers of sclerotia per plate were counted by using a dissecting scope. Both CAM and GMM agar experiments were carried out with four replicates.

Colonization studies. Seeds of the near-isogenic Sunrunner and SunOleic97R peanut lines were kindly provided by Dr C. Holbrook (USDA, GA, USA). Sunrunner has 50% oleic acid and 30% linoleic acid content, whilst SunOleic97R is a high oleic acid line that has 80% oleic acid and approximately 2% linoleic acid content. Prior to infection by *A. parasiticus* or *A. nidulans* strains, the seeds were shelled, the cotyledons separated and the embryo removed. Seeds from both peanut lines were weighed so that all the peanuts used were between 0.4 and 0.6 g in weight. For dead-seed experiments, the cotyledons were autoclaved in a liquid cycle for 30 min. For living-seed experiments, cotyledons were surface-sterilized by immersion in 10% Clorox bleach for 1 min, followed by immersion in sterile distilled water for 1 min. For *A. parasiticus*, the seeds were inoculated as follows: for each strain, four peanut cotyledons were placed in a 50 ml Falcon tube and to this was added 500 µl sterile distilled water containing 10⁶ spores of the appropriate strain. Each tube was vortexed for 1 min, the caps were loosened and the samples were placed in the dark at 29 °C for 4 days. These experiments were performed with four replicates. Simultaneously, GMM agar plates were inoculated, using the top-layer method described above, with the same spore suspensions that were used to infect the peanut seed (at a rate of 10⁶ spores per plate, four replicates for each) and placed in the incubator beside the infected seed. After 4 days, spores on the plates were counted as described above. Spores from the infected seed were released by adding 5 ml Tween 80/water to each Falcon tube, vortexing for 1 min and using an aliquot of this for counting using a haemocytometer.

A. nidulans does not readily infect peanut seed and so the inoculation protocol was modified as follows. One cotyledon was immersed in 1 ml sterile distilled water containing 10⁶ spores of the appropriate strain for 30 min, with vigorous vortexing every 10 min. This was repeated six times for each experiment. After 30 min, the infected peanut seeds were placed in a glass Petri dish containing water-saturated filter paper and a water reservoir and left in the dark at 29 °C for 4 days (in the case of dead seed) and 6 days (in the case of live seed). Plates of GMM agar that were inoculated with the same strains were placed in the incubator simultaneously and counted at the same time points as the infected seed. Spores were released from the seed by using Tween 80/water and vortexing for 1 min. Four cotyledons were used for counting per treatment.

Corn seeds were surface-sterilized as described above. Four seeds were placed in one 50 ml Falcon tube and inoculated as described above for *A. parasiticus*. Tubes were placed at 29 °C under dark conditions for 4 days. Each experiment was performed with four replicates.

In all seed-inoculation experiments, Koch's postulates were determined after spore-counting to verify the identity of the *Aspergillus* strains infecting the seed.

Fatty acid analysis. Fatty acid methyl esters were generated from *Aspergillus* mycelium for GC analysis as follows. After growth in GMM for 72 h under conditions of constant light, mycelia were lyophilized and ground into fine powder and the lipids were extracted three times in chloroform:methanol (2:1) following the method of Bligh & Dyer (1959). Heptadecanoic acid (17:0; 0.64 ng) was added as an internal standard. The samples were dried down, resuspended in 1 ml 5% HCl in 90% methanol and placed at 95 °C for 30 min to generate fatty acid methyl esters (FAMES). The methylating reaction was quenched with water and the FAMES were extracted three

times in hexane. The samples were concentrated and a 1 µl aliquot of the hexane layer was examined by GC. Identification of peaks was achieved by comparison of sample retention times to those of palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid standards (FAME-GC mix; Sigma).

Statistical analysis. Spore data and colony diameters were evaluated by analysis of variance (ANOVA) using the Statistical Analysis system (SAS Institute, Cary, NC, USA).

RESULTS

Isolation and characterization of the *A. parasiticus odeA* gene

Cosmid pAMC8, which hybridized to the *C. albicans odeA* gene, was identified from the *A. parasiticus* genomic library. Sequencing of hybridizing regions of pAMC8 and BLAST analysis of these regions revealed the presence of a putative Δ^{12} -desaturase gene (GenBank accession no. AF528822). Further comparison of the *A. parasiticus* genomic DNA sequence to the cDNA sequence obtained by RT-PCR (accession no. AY267349) showed that the *A. parasiticus odeA* gene contained one intron and encoded a predicted protein of 466 aa. The OdeA protein contained the conserved histidine clusters that are common to Δ^{12} -desaturases and demonstrated 99 and 86% sequence similarity to the *A. flavus* (AY280867) and *A. nidulans* (AF528822) OdeA proteins, respectively, at the amino acid level.

Disruption of the *A. parasiticus odeA* gene

The Δ^{12} -oleic acid desaturase gene of *A. parasiticus* was disrupted by homologous recombination as depicted in Fig. 1a. A rapid screen of 96 *pyrG*⁺ transformants for integration of *pyrG* at the *odeA* locus was employed. Undigested genomic DNA from these transformants was run out on an agarose gel and probed with the 0.7 kbp *Bam*HI–*Eco*RI internal fragment of *odeA*, shown hatched in Fig. 1a. One transformant, TAMC37.41, did not hybridize to the *odeA* probe (Fig. 1b, lane 2), indicating removal of the *odeA* coding region from the genome. Single-copy integration of *pyrG* at the *odeA* locus in TAMC37.41 was subsequently confirmed by Southern blot analysis following *Pst*I restriction digestion, using the full-length *odeA* coding region as a probe, in addition to DNA sequencing and PCR analysis (data not shown).

Fatty acid profile of the *A. parasiticus* Δ *odeA* mutant

To examine the effect of the Δ *odeA* mutation on fatty acid biosynthesis, mycelial fatty acid content of the *A. parasiticus* Δ *odeA* strain was compared to that of the wild-type. Table 2 shows the total amount of fatty acids produced by the mutant and wild-type, as well as the proportion of each fatty acid detected in the FAME mixture. Two major observations were evident from this table. Firstly, the Δ *odeA* mutant accumulated approximately four times more

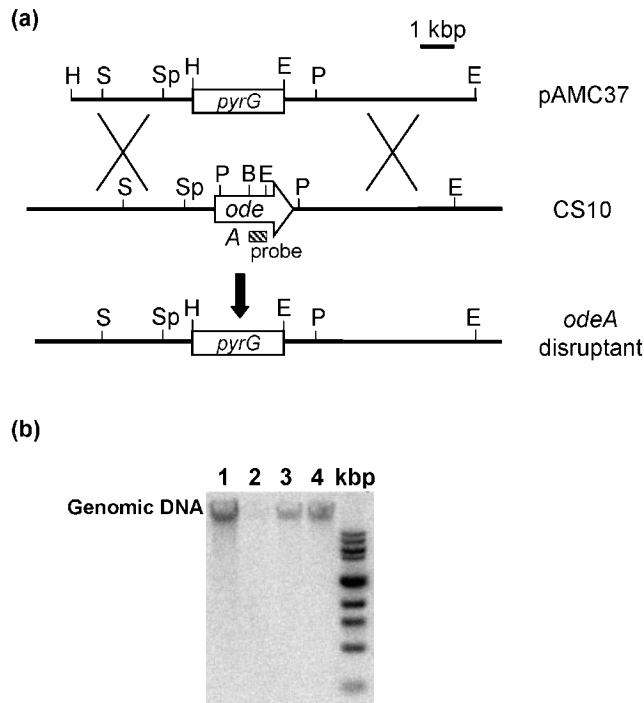


Fig. 1. Disruption of the *odeA* locus by *pyrG*. (a) The *pyrG* gene replaces *odeA* in the genome. The 0.7 kbp *Bam*HI–*Eco*RI internal fragment is shown as a hatched box. (b) Identification of *odeA* disruptants. Undigested genomic DNA from *odeA* disruptant strains will not hybridize to radiolabelled *Bam*HI–*Eco*RI internal fragment. Lane 1, DNA from wild-type; lane 2, DNA from TAMC37.41, later confirmed as the Δ *odeA* strain; lanes 3 and 4, DNA from two other *pyrG*⁺ strains with intact *odeA* genes.

oleic acid than the wild-type, but only trace amounts of linoleic and linolenic acids. Secondly, total fatty acid production in the Δ *odeA* mutant was increased dramatically by almost fivefold over that of the wild-type. This increase in total fatty acid production correlated with elevated expression of *fasA*, which encodes the α -subunit of the fatty acid synthase, as shown in Fig. 2.

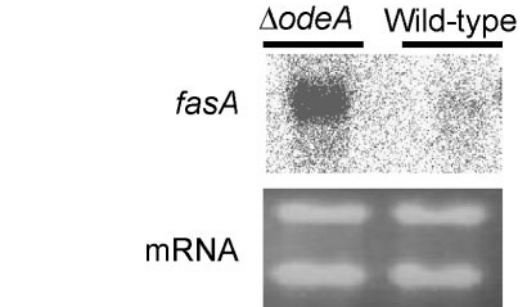


Fig. 2. Gene expression of *A. parasiticus fasA*, encoding the α -subunit of fatty acid synthase, is upregulated in Δ *odeA* compared to the wild-type.

Δ ¹²-desaturase mutation reduced rates of conidial spore germination and radial colony growth

Germination of Δ *ApodeA* conidiospores was delayed, compared to the wild-type. Spores of the wild-type required 9 h growth in liquid GMM medium to achieve 100% germination rates, whereas Δ *ApodeA* required 14 h. Although germination was delayed by 5 h in Δ *ApodeA*, we still observed 100% germination at this time, indicating that deletion of the *odeA* gene does not affect spore viability. Δ *ApodeA* growth was similarly reduced, compared to the wild-type. After 5 days, the mean colony diameter was 43.88 ± 0.13 mm for wild-type *A. parasiticus* and 21.98 ± 0.55 mm for Δ *ApodeA* ($n=4$), indicating a 50% reduction in Δ *ApodeA* growth, compared to the wild-type. These differences in colony diameter are significant ($P \leq 0.05$).

Δ ¹²-desaturase mutation reduced *A. parasiticus* conidial production and inhibited sclerotial development

Conidial production of *A. parasiticus* Δ *odeA* and wild-type strains after 3 days growth at 29 °C in conditions of continuous light and 4 days growth in conditions of continuous darkness is shown in Fig. 3. The *A. parasiticus* Δ *odeA*

Table 2. Fatty acid composition of mycelia of *A. parasiticus* wild-type (Wt) and Δ *odeA*

Analysis was carried out on mycelia grown for 72 h in liquid GMM under conditions of shaking and continuous light. Composition is given as ng FAME (μ g lyophilized weight of mycelia)⁻¹. 16:0, Palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. Values are means \pm SD of three replicates.

Sample	Total FAME	Content of individual FAMES (%):					
		16:0	16:1	18:0	18:1	18:2	18:3
Wt	17.1 \pm 0.35	18.45 \pm 0.22	0.32 \pm 0.01	13.63 \pm 0.37	23.5 \pm 0.57	40.98 \pm 0.88	1.1 \pm 0.01
Δ <i>odeA</i>	82.1 \pm 0.85	7.3 \pm 0.1	0.5 \pm 0.01	2.93 \pm 0.01	86.23 \pm 0.98	0.12 \pm 0.01	0.16 \pm 0.02

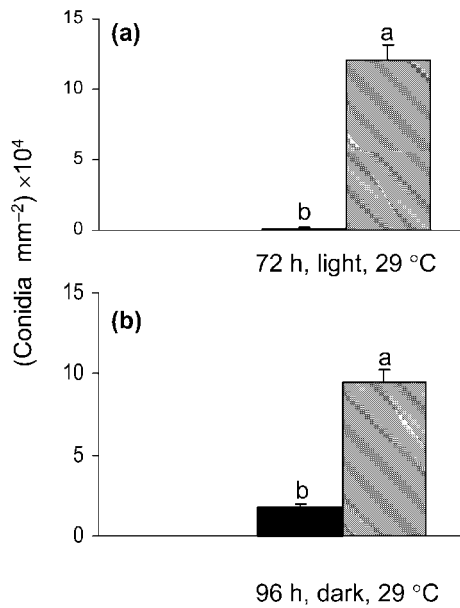


Fig. 3. Conidial production of the *A. parasiticus* $\Delta odeA$ mutant (filled column) and wild-type (hatched column). Values are means of four replicates. Columns with the same letter are not significantly different at a significance level of 0.05.

mutant was significantly reduced in conidial development, compared to the wild-type strain, under both conditions.

Sclerotial development was also affected in the $\Delta odeA$ strain. On GMM agar plates, wild-type *A. parasiticus* produced a mean of 21.5 ± 1.3 sclerotia per plate ($n=4$), whereas $\Delta odeA$ produced no sclerotia. Sclerotia on GMM agar plates were counted after 7 days and these differences are significant ($P \leq 0.05$). On CAM plates, wild-type *A. parasiticus* produced a mean of 3.5 sclerotia per plate ($n=4$), whereas $\Delta odeA$ produced no sclerotia. Sclerotia on CAM plates were counted after 14 days, but even after 1 month, $\Delta odeA$ did not produce sclerotia (data not shown). Sclerotial mean values for CAM plates were taken from four out of a total of 15 plates per strain. On all CAM plates, the wild-type produced between two and six sclerotia, whereas $\Delta odeA$ produced zero. Low sclerotial production by this *A. parasiticus* parental strain has been reported previously (Trail *et al.*, 1995). By comparing results from the two growth media, it can be seen that the $\Delta odeA$ mutation abolishes production of sclerotia and this effect is medium-independent.

$\Delta odeA$ mutant strains were impaired in the colonization of peanut seed

We assayed for the ability of *A. parasiticus* and *A. nidulans* $\Delta odeA$ strains, termed $\Delta ApodeA$ and $\Delta AnodeA$, respectively, and the corresponding wild-type controls, to colonize two isogenic lines of peanut seed that differed only in oleic acid : linoleic acid ratios. Colonization was assessed in

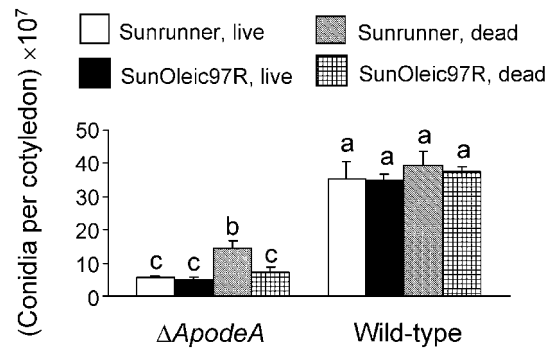


Fig. 4. Colonization of peanut seed by *A. parasiticus* $\Delta odeA$ and wild-type strains. Strains were inoculated onto Sunrunner and SunOleic97R peanut-seed lines and incubated at 29 °C in the dark for 96 h. Dead and living seeds were used. Values are means of four replicates. Columns with the same letter are not significantly different at a significance level of 0.05.

terms of conidial production on seed. Sunrunner has typical peanut seed oil content, containing 50 % oleic acid and 30 % linoleic acid [see Norden *et al.* (1985) and references therein]. SunOleic97R has increased oleic acid (80 %) and reduced linoleic acid (2–5 %) contents (Gorbet & Knauff, 2000; Andersen & Gorbet, 2002). These differences are due to recessive mutations of *odeA* orthologues in SunOleic97R (Knauff *et al.*, 1993; Jung *et al.*, 2000). Both live and dead seeds were used to assess the interactions between host seed and the fungus.

Wild-type *A. parasiticus* produced significantly more conidia ($P \leq 0.05$) on all peanut seeds than did $\Delta ApodeA$. Wild-type *A. parasiticus* showed no significant differences ($P \leq 0.05$) in conidiation on any peanut seed, live or dead (Fig. 4), after 96 h growth. In control-plate growth tests, wild-type produced significantly more conidia than did $\Delta ApodeA$ (Fig. 3b, $P \leq 0.05$) on GMM under dark conditions at 29 °C for 96 h.

Similar to *A. parasiticus*, wild-type *A. nidulans* produced more conidia on live seed than did the $\Delta AnodeA$ mutant. However, there were considerable differences in colonization between the two species. The *A. nidulans* strains produced markedly more conidia on dead seed than on live seed (Fig. 5a). On dead seed, $\Delta AnodeA$ and wild-type had the same rate of conidiation ($P \leq 0.05$). Both strains produced significantly more conidia ($P \leq 0.05$) on dead SunOleic97R than dead Sunrunner. On live seeds, conidial production of *A. nidulans* wild-type did not significantly differ on Sunrunner or SunOleic97R seeds. For $\Delta AnodeA$, conidial production on live SunOleic97R and Sunrunner was reduced significantly ($P \leq 0.05$), compared to wild-type, with the greatest reduction seen on SunOleic97R. Interestingly, on control-growth test plates, *A. nidulans* $\Delta AnodeA$ produced significantly more conidia ($P \leq 0.05$) than wild-type on GMM under dark conditions at 29 °C for 96 and 144 h (Fig. 5b).

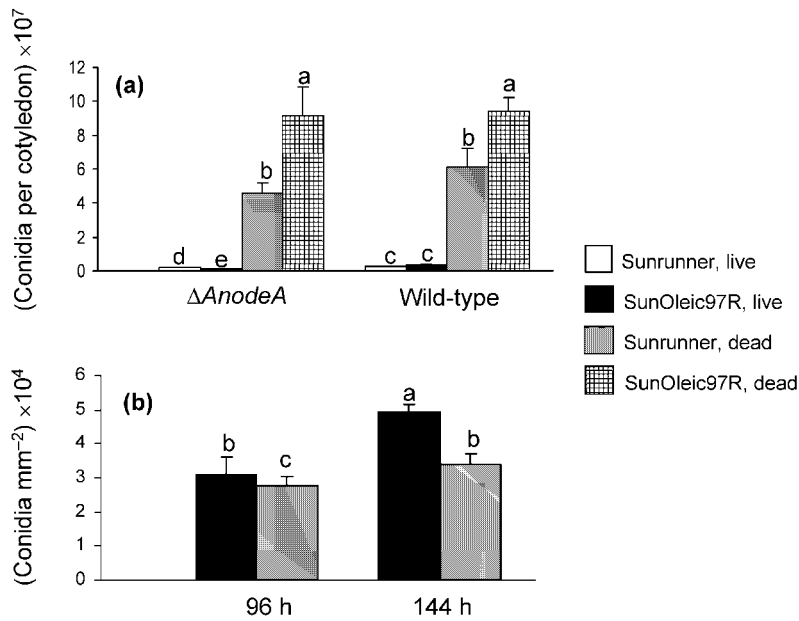


Fig. 5. Colonization of peanut seed by *A. nidulans* $\Delta odeA$ and wild-type strains. (a) Cultures of *A. nidulans* $\Delta odeA$ and wild-type strains were inoculated onto dead and live Sunrunner and SunOleic97R peanut-seed lines. Strains were incubated for 96 h (for dead seed) or 144 h (for live seed) in the dark at 29 °C. Values are means of four replicates. (b) Cultures of $\Delta odeA$ (hatched column) and wild-type (filled column) strains were grown in the dark on GMM at 29 °C for 96 and 144 h as a control for the colonization studies. Values are means of four replicates. Columns with the same letter are not significantly different at a significance level of 0.05.

A. parasiticus $\Delta odeA$ mutant was impaired in colonization of corn seed

We also assayed for the ability of *A. parasiticus* wild-type and $\Delta odeA$ strains to produce conidia on corn-seed lines. We chose Asgrow 404, a commercially available line that is susceptible to mycotoxin contamination in the field, and Tex6, a line with some resistance to mycotoxin production (Hamblin & White, 2000). On dead seed, both strains had the same rates of conidiation and there was no significant difference ($P \leq 0.05$) between rates of conidiation on either corn-seed line (data not shown). However, on live seed, the *A. parasiticus* $\Delta odeA$ strain produced significantly fewer conidia ($P \leq 0.05$) than the wild-type on both seed lines (Fig. 6). Interestingly, both strains produced fewer conidia ($P \leq 0.05$) on Asgrow 404 seed lines than on Tex6 lines. Corn seed was not infected with *A. nidulans* strains.

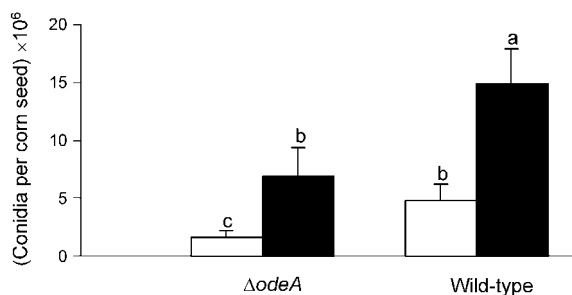


Fig. 6. Colonization of corn seed by *A. parasiticus* $\Delta odeA$ and wild-type strains. Strains were inoculated onto Asgrow 404 (empty column) and Tex6 (filled column) lines and incubated for 96 h in the dark at 29 °C. Values are means of four replicates. Columns with the same letter are not significantly different at a significance level of 0.05.

DISCUSSION

One potential area for the control of oilseed crop infection by mycotoxigenic *Aspergillus* species is to reduce production of the infectious particle, the conidium. Here, we studied the ability of *Aspergillus* desaturase mutants to colonize and produce conidia on seed, partly to investigate the suitability of focusing on fatty acid metabolism as a means to reduce spore production.

The *odeA* gene, which encodes a Δ^{12} -oleic acid desaturase that is involved in converting oleic acid to linoleic acid, was identified and disrupted in *A. parasiticus*. Mycelia of the resulting $\Delta odeA$ mutant ($\Delta ApodeA$) had a similar fatty acid content to the *A. nidulans* $\Delta odeA$ mutant ($\Delta AnodeA$; Calvo *et al.*, 2001), with dramatically increased oleic acid content and reduced contents of linoleic and linolenic acids (Table 2). In addition, total fatty acid content accrued in $\Delta ApodeA$ to a level five times greater than that seen for wild-type. Calvo *et al.* (2001) reported a similar increase in the total fatty acid content of $\Delta AnodeA$ and attributed it to a lack of polyunsaturated fatty acid inhibition of *fasA* gene expression, leading to upregulation of fatty acid biosynthesis. The increase in fatty acid content in $\Delta ApodeA$ (Table 2), coupled with increased *fasA* gene expression (Fig. 2), suggests that the feedback inhibition mechanism is conserved in *A. parasiticus*.

Linoleic acid is a major constituent of fungal lipid and commonly comprises 30–50% of the total fatty acid content in mycelia and conidia of aspergilli (Table 2; Singh & Sood, 1973; Sood & Singh, 1973; Rambo & Bean, 1974; Budinska *et al.*, 1981; Evans *et al.*, 1986; Chattopadhyay *et al.*, 1987; Calvo *et al.*, 2001). Several studies have shown that various unsaturated fatty acids affect *Aspergillus* developmental processes (Mazur *et al.*, 1990, 1991; Calvo *et al.*, 1999), whilst ratios of unsaturated:saturated fatty

acids are important for *Aspergillus* and *Mucor* development (Calvo *et al.*, 1999; Khunyoshiyeng *et al.*, 2002). In *A. nidulans*, specific individual fatty acids are also required at different stages of development. For example, Evans *et al.* (1986) reported that mycelial linolenic acid concentration was prominent in 1-day-old cultures, but declined to trace levels in cultures older than 3 days. The Δ ApodeA mutant was altered in all these parameters of fatty acid metabolism, displaying reduced production of linoleic and linolenic acids and altered ratios of unsaturated:saturated fatty acids. It was consequently defective in rates of spore germination, colony growth and development. In a parallel study, a Δ odeA mutation that was generated in an *O*-methylsterigmatocystin-accumulating strain of *A. parasiticus*, SRRC 2043, also resulted in altered conidiation and sclerotial development (Chang *et al.*, 2004). This supports our conclusion that the reduction in conidiation and abolishment of sclerotial production seen for Δ ApodeA compared to the wild-type is due solely to aberrant polyunsaturated fatty acid metabolism and serves to emphasize the genetic connection between fatty acid metabolism and fungal development.

The role of fatty acid metabolism in the plant–fungal interaction was studied by using two peanut lines and two corn lines. Because of an increasing interest in developing peanut seed with higher oleic and lower linoleic acid content, due to the value of monounsaturated fatty acids in the diet (Massaro *et al.*, 1999), and considering the importance of fatty acids in *Aspergillus* development, we investigated whether *Aspergillus* species differentially colonized two near-isogenic commercial peanut lines with altered fatty acid content. One line is SunOleic97R, which contains high amounts of oleic acid (80%), but reduced amounts (2–5%) of linoleic acid and undetectable amounts of linolenic acid. Another is Sunrunner, which contains 50% oleic acid and 30% linoleic acid (Holbrook *et al.*, 2000). For the corn lines, we chose Asgrow 404 and Tex6, lines that exhibit some susceptibility and resistance to aflatoxin contamination, respectively, in the field (Hamblin & White, 2000). Conidiation of wild-type *A. parasiticus* and *A. nidulans* on live peanut seed did not differ between these two lines, indicating that the reduced amount of linoleic acid in SunOleic97R is still sufficient to induce and support conidiation. In contrast, the Δ odeA mutants of *A. parasiticus* and *A. nidulans* exhibited a significant reduction in conidiation on live peanut seed (Figs 4 and 5), indicating a direct or indirect role for the *odeA* gene in fungal development on peanut seed. On corn seed, the *A. parasiticus* Δ odeA strain also produced fewer conidia on both corn lines, compared to the wild-type (Fig. 6). Wild-type *A. parasiticus* did not produce significantly different amounts of conidia on dead Asgrow 404, live Tex6 and dead Tex6 seed (data not shown). However, conidial production of wild-type *A. parasiticus* was reduced significantly on live Asgrow 404 ($P \leq 0.05$), suggesting an inhibitory effect of this line that is not seen for Tex6. This observation is interesting as it contrasts with the situation

observed in the field, where the Asgrow 404 line is more susceptible to aflatoxin contamination, whereas Tex6 has some resistance (Hamblin & White, 2000). Nonetheless, reducing fungal colonization and sporulation would be a prerequisite for curbing aflatoxin contamination of crops. Taken together, these results suggest that whilst altering the fatty acid content of oil seeds might not reduce *Aspergillus* conidiation and probable spread of the pathogen, inactivation of the fungal *odeA* gene may do so. Therefore, a promising strategy for controlling contamination could be the selective targeting and inactivation of fungal Δ^{12} -desaturases by fungicides.

Wild-type *A. parasiticus* did not produce different amounts of conidia on either live or dead peanut lines, suggesting that the plant defensive response of live seed is not effective to reduce the development of this fungus. In contrast, all *A. nidulans* strains produced more conidia on dead seed than on live seed, even when growth on live seed was prolonged. This indicates the effectiveness of the plant defensive response against *A. nidulans*, which is not a pathogen of peanut in nature.

This study shows that pathogenic differences exist between *A. nidulans* and *A. parasiticus* and that loss-of-function desaturase mutants of both species are impaired in colonization abilities. One future course of our study will be an in-depth analysis of the interactions that are involved in the colonization of oilseed crops by *A. nidulans* and *A. parasiticus* and the closely related *A. flavus*. By using available genomic and expressed sequence tag resources of *A. nidulans* and *A. flavus*, coupled with microarray technology, subtractive libraries and proteomics, one could further dissect the *Aspergillus*–seed interaction to identify the molecular bases for differences in colonization by saprophytic, pathogenic and mutant aspergilli as new targets for reducing the infestation of oilseed crops by these fungi.

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