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Deletion of the Δ12-oleic acid desaturase gene of a nonaflatoxigenic *Aspergillus parasiticus* field isolate affects conidiation and sclerotial development

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ABSTRACT

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Aims: To investigate how linoleic acid affects conidial production and sclerotial development in a strictly mitotic *Aspergillus parasiticus* field isolate as related to improving biocompetitivity of atoxigenic *Aspergillus* species.

Methods and Results: We disrupted *A. parasiticus* Δ12-oleic acid desaturase gene (*odeA*) responsible for the conversion of oleic acid to linoleic acid. We examined conidiation and sclerotial development of SRRC 2043 and three isogenic mutant strains deleted for the *odeA* gene (ΔodeA), either with or without supplementing linoleic acid, on one complex potato dextrose agar (PDA) medium and on two defined media: nitrate-containing Czapek agar (CZ) and Cove's ammonium medium (CVN). The ΔodeA mutants produced less conidia than the parental strain on all media. Linoleic acid supplementation (as sodium linoleate at 0·3 and 1·2 mg ml^{-1}) restored the ΔodeA conidial production comparable to or exceeding the unsupplemented parental level, and the effect was medium dependent, with the highest increase on CVN and the least on PDA. SRRC 2043 and the ΔodeA mutants were unable to produce sclerotia on CVN. On unsupplemented PDA and CZ, ΔodeA sclerotial mass was comparable to that of SRRC 2043, but sclerotial number increased significantly to two- to threefold. Supplementing linoleic acid to media, in general, tended to decrease wild type and ΔodeA sclerotial mass and sclerotial number.

Conclusions: Linoleic acid stimulates conidial production but has an inhibitory effect on sclerotial development. The relationship between the two processes in *A. parasiticus* is complex and affected by multiple factors, such as fatty acid composition and nitrogen source.

Significance and Impact of Study: Conditions that promote sclerotial development differ from those required to promote maximum conidial production. Manipulation of content and availability of linoleic acid at different fungal growth phases might optimize conidial and sclerotial production hence increasing the efficacy of biocompetitive *Aspergillus* species.

Keywords: Δ12-oleic acid desaturase gene, *Aspergillus parasiticus*, biocompetition, conidia, linoleic acid, sclerotia.

INTRODUCTION

Oil-rich crops such as corn, peanuts, cottonseed and tree nuts are often infested before harvest by *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi produce carcinogenic aflatoxins, which are strictly regulated both for animal feed and food for human consumption because of health concerns (Jelinek *et al.* 1989; Van Egmond 1995). Limited uses of contaminated agricultural commodities greatly reduce the profitability of farmers. One method for the reduction of aflatoxin contamination is through field application of atoxigenic *Aspergillus* strains to displace toxigenic strains (Cotty and Bayman 1993; Dorner *et al.* 1999). A major

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breakthrough in recent years is the US Environmental Protection Agency's approval of large-scale commercial use of the biopesticide *A. flavus* AF36 in Arizona and South Texas to reduce aflatoxin in cottonseed (Cleveland *et al.* 2003). Application of experimental use permit (EUP) for field testing a biopesticide containing *A. flavus* NRRL 21882 in Alabama, Florida and Georgia has also been filed (75624-EUP-R). The efficacy and success of the biocompetitive method lie in the fitness of applied biopesticides in the field. Fungal fitness can be defined in terms of its ability to reproduce quickly, to disseminate widely, to colonize the host plants efficiently, and to survive harsh environments, such as heat, cold, arid soil conditions and drastic temperature changes (Pringle and Taylor 2002).

Both *A. flavus* and *A. parasiticus* are strictly mitotic and undergo asexual reproduction only (Geiser *et al.* 1996). Asexual spores called conidia, dispersed by wind, water and insects, are the main disseminating mechanisms for fungal propagation, and several generations of conidia can be produced in a single growing season. Besides, some isolates of *A. flavus* and *A. parasiticus* produce specialized propagules called sclerotia (Bennett *et al.* 1979; Wicklow and Shotwell 1983), postulated to be derived from vestigial cleistothecia (Malloch and Cain 1972), the latter woven from specialized hyphae and containing thousands of ascospores (Yager 1992). Sclerotia are pigmented, compacted aggregates of hyphae that are capable of remaining dormant for long periods to resist unfavourable environmental conditions (Wicklow *et al.* 1993). Production of large numbers of conidia and sclerotia thus is desirable for greater flexibility in dispersal and survival during suboptimal environmental conditions and also to improve and sustain the carryover effect of an applied atoxigenic biocompetitive *Aspergillus* in reducing aflatoxins from one year to the next. Understanding external factors that influence *Aspergillus* development is key to achieving overproduction of conidia and sclerotia in a potential biocontrol strain.

Linoleic acid is a major constituent of fungal lipid in conidia and mycelia and typically comprises 30–50% of the total fatty acid mass of *A. flavus* and *A. nidulans* isolates (Sood and Singh 1973; Rambo and Bean 1974; Wilson *et al.* 2004). It is also a major fatty acid of oil seed crops susceptible to aflatoxin contamination (Burow *et al.* 1997; Mellon *et al.* 2000; Wilson *et al.* 2001). Several lines of evidence suggest that the 18-carbon polyunsaturated fatty acids, linoleic and linolenic acid, and their derivatives are important sporogenic effectors of several *Aspergillus* species, including *A. flavus* and *A. parasiticus* (Burow *et al.* 1997; Calvo *et al.* 1999, 2001; Tsitsigiannis *et al.* 2004). Studies have also suggested possible roles of unsaturated fatty acids in affecting the development of sclerotia (Calvo *et al.* 1999).

In this study, we assessed the effects of linoleic acid on *A. parasiticus* development by construction of isogenic Δ12-oleic acid desaturase mutants. We also examined how the production of conidia and sclerotia was influenced by available nitrogen sources in complex and defined media.

MATERALS AND METHODS

Fungal strains, media and culture conditions

The *A. parasiticus* strains used in this study were SRRC 2043, a field isolate that produces abundant sclerotia and is defective in aflatoxin biosynthesis (Yu *et al.* 1998), and its isogenic derivatives. The recipient strain used in transformation experiments was RHN1, a NiaD (nitrate reductase) mutant of SRRC 2043. Three independent isolated ΔodeA mutants (the *odeA* gene encoding the Δ12-oleic acid desaturase was disrupted) generated in this study were used in conidial and sclerotial production study. Cultures were maintained on potato dextrose agar (PDA) plates (Difco Laboratories, Detroit, MI, USA). Conidia were produced on V8 agar medium [50 ml l^{-1} V8 juice, a commercial beverage (Campbell Soup Company, Camden, NJ, USA) consisting of eight vegetable juices, 20 g l^{-1} agar, adjusted to pH 5·2 prior to autoclaving].

Construction of the *odeA* replacement vector

The *odeA* gene disruption vector was constructed as follows: A 0·8-kb 5′ untranslated region (UTR) and a 0·4-kb 3′ UTR of the *odeA* gene were obtained by PCR from *A. parasiticus* SU-1 genomic DNA. The primer sets used were GCGATTCGTCACGGGAGGA and TAGATGTGCTTCTGCAGGCC (*Pst*I is underlined), and TAATCTGCAGTCGAGTGGCCACGATTTGGA (*Pst*I is underlined) and AGATGTCTAGATATTCGGGC (*Xba*I is underlined). The 0·8-kb *Sph*I–*Pst*I fragment and the 0·4-kb *Pst*I–*Xba*I fragment were cloned sequentially into pUC18 by standard recombinant techniques. Then, the ends of the 6·7-kb *Xba*I fragment containing the *A. parasiticus niaD* gene (Chang *et al.* 1996) was filled and ligated into the filled *Pst*I site of the above construct to give podeDV. Before transformation, the disruption vector was linearized with *Sph*I and *Xba*I to release the portion derived from pUC18. Replacement of the *odeA* gene in the *A. parasiticus* RHN1 genome was carried out by the *niaD*-based polyethylene glycol-$CaCl_2$ mediated fungal transformation protocol (Horng *et al.* 1990). Czapek solution agar (CZ; Difco) supplemented with KCl (45 g l^{-1}) was used as the protoplast regeneration medium.

Confirmation of the disruption of the *odeA* gene

PCR with gene-specific oligoprimer pairs were used to confirm the deletion of *odeA* in the recipient genome. The

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primer pairs were as follows: Pair 1, ode5′: GCGATTCG TCACGGGAGG and niaDXV1: CTGTTTCGGACTC TCTTCTG; pair 2, ode1600: CGACCTGAACGAATTG ATGG and ode2050: GGGTCGGTGTGCTGGAGGTA; and pair 3, niaDInt2: CATTGGGTGTGTCGCAGCA and ode3′: CACTATCCAGACCCAGATCC.

Conidial and sclerotial production

For production of conidia and sclerotia, agar plates containing 30 ml of each of the following were used: (i) complex PDA medium, (ii) Cove's minimal salt medium (Cove 1976) with ammonium sulfate (0·66 g l^{-1}) as the sole nitrogen source, and (iii) Czapek Solution Agar (Difco), a defined medium with nitrate as the sole nitrogen source. Media used were either supplemented with sodium linoleate (Sigma-Aldrich, St. Louis, MO, USA) at 0·3 or 1·2 mg ml^{-1} or without supplementation. An aliquot of spore suspension containing approximately 10^3 conidia was seeded at the center of each agar plates (Falcon no. 351209, 100 × 15 mm style; Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA). Cultures were grown in the dark at 30°C for 2 weeks.

Determination of conidial and sclerotial numbers and sclerotial dry weight

At the end of the 2-week incubation, conidia were washed off the agar plates using 100% ethanol to reduce human exposure. Conidia were counted on a haemocytometer. Sclerotia on the washed agar plates were scraped off the surface with a surgical scalpel and collected in microcentrifuge tubes. The sclerotia were dried at 37°C for a week before weight was determined. The sclerotia were counted manually. Plates in duplicate from three isogenic ΔodeA mutants were used for conidal and sclerotial determinations.

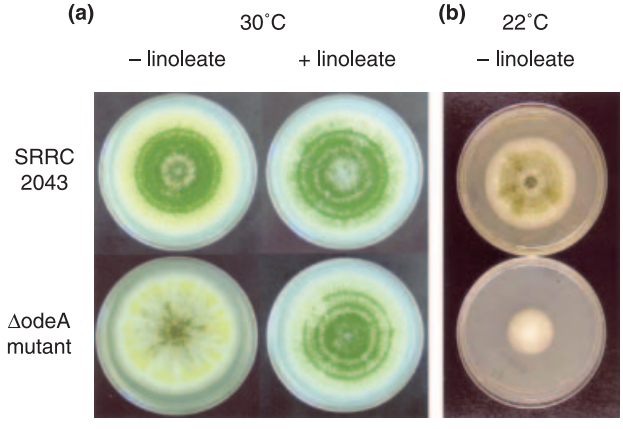

Fig. 1 Representative growth morphology of *Aspergillus parasiticus* SRRC 2043 and isogenic ΔodeA mutants at (a) 30°C and (b) 22°C on PDA plates without or with 1 mM linoleic acid supplementation

RESULTS

Generation of *A. parasiticus odeA*-deleted mutants

Fifty-four transformants were obtained on CZ regeneration plates. All transformants were transferred onto PDA plates and examined for morphological changes. Six of the transformants showed a severe reduction in sporulation (Fig. 1a) at 30°C, and they grew much slower at 22°C than SRRC 2043 (Fig. 1b). PCR analysis confirmed that the *odeA* gene of these morphological variants was deleted via double-crossover recombination (Fig. 2a). Briefly, regions

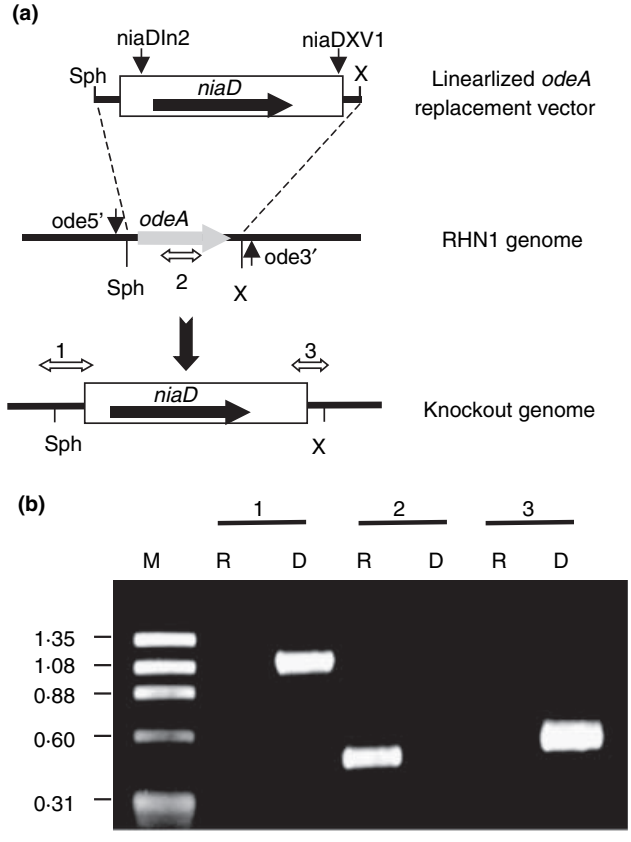

Fig. 2 Confirmation of *odeA* deletion by PCR. (a) Schematic diagram depicting replacement of the *odeA* gene by *niaD* via a double crossover recombination. 'X' indicates a *Xba*I site. (b) Representative PCR profiles obtained from genomic DNA of the transformation recipient RHN1 and *odeA*-deleted mutants. The regions amplified by the primer set 1: ode5′/niaDIn2; set 2: ode1600/ode2050; and set 3: niaDXV1/ode3′ are shown in panel A. The molecular size marks are *Hin*dIII-digested 8 and *Hae*III-digested Nx174 DNA fragments. R = RHN1; D = *odeA*-deleted mutant

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from the *niaD* selectable marker to a 5′ sequence and a 3′ sequence beyond the recombination sites but not the *odeA* coding region were present in the genome of the variants. In contrast, the genome of the transformation recipient RHN1 gave the opposite PCR results (Fig. 2b). Three independent *odeA*-deleted mutants (nos 3, 13 and 37; ΔodeA) were used to determine how lack of the *odeA* gene affects conidial production and sclerotial development of *A. parasiticus* on growth media containing different nitrogen sources.

Conidial and sclerotial production of SRRC 2043 and ΔodeA on unsupplemented media

Conidiation of SRRC 2043 and ΔodeA was affected by medium composition and nitrogen sources (Fig. 3a). For SRRC 2043, complex PDA medium induced the largest amount of conidia, conidial production was the least on ammonium-containing CVN, while nitrate-containing CZ produced an intermediate number of conidia. Conidiation of ΔodeA also was greatest on PDA, but in contrast to SRRC 2043, nitrate-containing CZ medium produced the least amount of conidia, while CVN was intermediate in its effect. However, compared with SRRC 2043, ΔodeA had a dramatic decrease in conidiation on all the unsupplemented media (Fig. 3a), producing 76, 46 and 88% less conidia on PDA, CVN and CZ, respectively.

Sclerotial production of SRRC 2043 and ΔodeA, as determined by sclerotial number and sclerotial mass, was, like conidiation, affected differently by medium composition. However, sclerotial production was abolished on ammonium-containing CVN. For SRRC 2043, sclerotial number and sclerotial mass were less on PDA and more on nitrate-containing CZ. Sclerotial production on CZ was 1·6-fold more than on PDA. For ΔodeA, sclerotial number and mass also were more on CZ than on PDA. Interestingly, sclerotial number, compared with SRRC 2043, was increased to 2·6-fold and 1·9-fold on PDA and CZ, respectively (Fig. 3b), while sclerotial mass remained the same as SRRC 2043 on PDA and CZ (Fig. 3c).

Linoleic acid supplementation on conidial production of SRRC 2043 and ΔodeA

Figure 3a shows the effect of supplementing the different media with increasing concentrations of linoleic acid on SRRC 2043 and ΔodeA conidiation. For SRRC 2043, conidiation was increased with increasing concentrations of linoleic acid on PDA. Conidiation increased on CVN at 0·3 mg ml^{-1} but did not increase further at 1·2 mg ml^{-1} supplementation. Conidiation decreased with increasing linoleic acid concentration on CZ. At 1·2 mg ml^{-1} supplementation, SRRC 2043 had a 69% increase, a 140% increase, and a 27% decrease on PDA, CVN and CZ, respectively, compared with unsupplemented media.

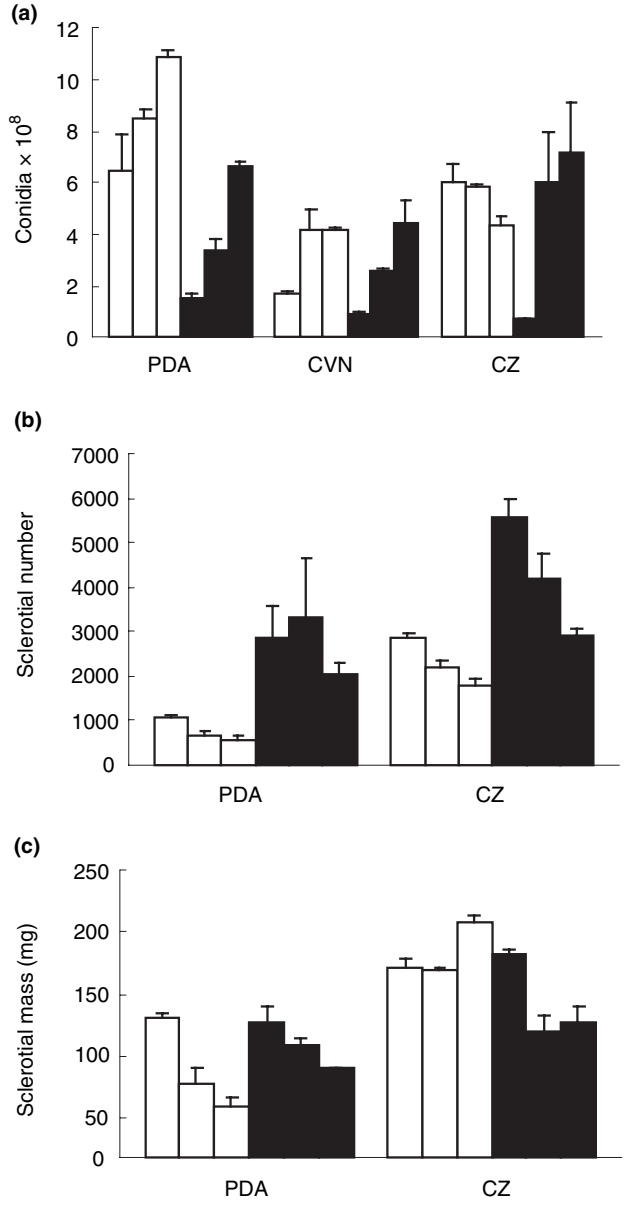

Fig. 3 Conidial production and sclerotial development of SRRC 2043 (open column) and ΔodeA (closed column) on media without or with linoleic acid supplementation. For each strain, three columns are shown representing, from left to right, none, 0·3 and 1·2 mg ml^{-1} sodium linoleate supplementation of the media. (a) conidial production, (b) number of sclerotia, and (c) sclerotial mass produced by SRR 2043 and ΔodeA. Values of ΔodeA are from three isogenic mutants determined in duplicate

Conidiation of ΔodeA was increased on all media after linoleic acid supplementation, compared with unsupplemented media (Fig. 3a). However, different concentrations

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of linoleic acid were required for ΔodeA to attain the equivalent of SRRC 2043 conidiation on unsupplemented media. PDA required 1·2 mg ml^{-1}, but only 0·3 mg ml^{-1} on CVN and CZ was required to achieve the SRRC 2043 unsupplemented conidiation level. At 1·2 mg ml^{-1} supplementation, ΔodeA produced a comparable level of conidiation on PDA, 150% more on CVN, and 20% more on CZ compared with unsupplemented SRRC 2043 on the same media.

Linoleic acid supplementation on sclerotial production of SRRC 2043 and ΔodeA

Linoleic acid supplementation affected sclerotial numbers of SRRC 2043 and ΔodeA. An increase in linoleic acid supplementation generally decreased sclerotial numbers of SRRC 2043 and ΔodeA on PDA and CZ except for a slight increase for ΔodeA at 0·3 mg ml^{-1} supplementation on PDA (Fig. 3b).

An increase in linoleic acid supplementation decreased the sclerotial mass of SRRC 2043 on PDA, with SRRC 2043 producing 40% and 55% less sclerotial mass at 0·3 and 1·2 mg ml^{-1} supplementation, respectively (Fig. 3c). In contrast, SRRC 2043 produced comparable and 20% more sclerotial mass on CZ at 0·3 and 1·2 mg ml^{-1} supplementation, respectively, compared with no supplementation.

ΔodeA consistently produced decreased sclerotial mass on linoleic acid supplemented PDA and CZ media (Fig. 3c). The decrease at 1·2 mg ml^{-1} compared with unsupplemented media was in the range of 25–30% on PDA and CZ. Linoleic acid supplementation decreased the sclerotial mass produced by ΔodeA to below the SRRC 2043 level regardless of growth media (Fig. 3c). Therefore, linoleic acid or a linoleic acid derivative, appears to inhibit sclerotial development in both strains.

DISCUSSION

The *odeA* gene encodes the Δ12-linoleic acid desaturase responsible for the conversion of oleic acid ($C_{18:1}$) to linoleic acid ($C_{18:2}$). Deletion of the *odeA* gene has been shown to result in depletion of linoleic acid and linolenic acid ($C_{18:3}$) in *A. nidulans* (Calvo *et al.* 2001) and a white-spored *A. parasiticus* mutant (Wilson R.A., Calvo A.M., Chang P.-K. and Keller N.P., unpublished data), hence changing fatty acid composition. However, it appears that lack of linoleic acid production resulting from the *odeA* gene deletion does not adversely affect ΔodeA mycelial growth and expansion at 30°C, but affects growth at suboptimal temperatures (Fig. 1). Linoleic acid is a major constituent of mycelial and conidial fatty acid composition in *Aspergillus* species and other fungi (Sood and Singh 1973; Rambo and Bean 1974; Budinska *et al.* 1981; Evans *et al.* 1986; Chattopadhyay *et al.* 1987; Goodrich-Tanrikulu *et al.* 1998), and thus might be critical in maintaining fungal cell membrane fluidity for normal biological functions during adaptation to lower growth temperatures.

In addition to poor growth at 22°C, the ΔodeA mutants displayed significant changes in development at 30°C. They demonstrated reduced conidial production, a significant increase in sclerotial number and the production of smaller sclerotia than SRRC 2043. Supplementing ΔodeA with increasing linoleic acid reversed the trend in conidial and sclerotial production. The level and availability of linoleic acid thus might be a major determinant for the two developmental processes. Morphological studies of *A. flavus* have shown that it consists of two distinct groups based on sclerotial size, called S and L (Bayman and Cotty 1992). The S-type *A. flavus* tends to produce sparse conidia and a greater number of small sclerotia, while the L-type *A. flavus* tends to produce a great number of conidia but a few large sclerotia. The morphological changes from SRRC 2043 to ΔodeA, which is reminiscent of the above observation, suggest an interlinked but inverse relationship between conidial production and sclerotial development.

The sporogenic effect of linoleic acid has been reported for other fungi, such as *Alternaria tomato* (Hyeon 1976), *Sclerotinia fructicola* (Katayama and Marumo 1978), and *Neurospora crassa* (Nukima *et al.* 1981). Calvo *et al.* (1999) showed that linoleic acid and hydroperoxylinoleic acid derivatives induce conidiation in *A. flavus*, probably by mimicking the action of endogenous hydroxylated fatty acids which are similar to the *A. nidulans* precocious sexual inducer (PSI) factor (Champe and El-Zayat 1989). Changes in fatty acid composition also affect development of asexual conidia and sexual ascospores in *A. nidulans* (Champe and El-Zayat 1989; Calvo *et al.* 2001; Wilson *et al.* 2004) and sexual ascospore development in *N. crassa* (Goodrich-Tanrikulu *et al.* 1998). Treatment of *A. nidulans* with concentrations of linoleic acid similar to those used in our study resulted in an increase of conidia and decrease in ascospore production (Calvo *et al.* 1999). Our results are reminiscent of this and further support the hypothesis that sclerotia are derived from cleistotheica, the bodies containing ascospores (Malloch and Cain 1972). *Aspergillus parasiticus* is a strictly mitotic (asexual) species. Deletion of the *odeA* gene likely affects fatty acid composition and the ratio of saturated to unsaturated fatty acids of ΔodeA mycelia and, in turn, alters the timing and frequency of appearance of sclerotial initials and maturation of sclerotia (Littley and Rahe 1992; Willetts and Bullock 1992). Loss of *odeA* would also alter the composition of PSI factor, which we predict would affect the conidia to sclerotia ratio. Recently Tsitsigiannis *et al.* (2004) demonstrated that deletion of an *A. nidulans* dioxygenase, PpoA, required for linoleic PSI factor production increased conidial production but decreased

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ascospore production much in the same manner as linoleic acid supplementation did (Calvo *et al.* 1999). As we have found a putative orthologue of the *ppoA* gene in *A. flavus* and *A. parasiticus* (Wilson R.A., Chang P.-K. and Keller N.P., unpublished data), we speculate that loss of linoleic PSI factor synthesis in the *A. parasiticus* Δ*odeA* strain could also be contributing to the altered conidial/sclerotial phenotype. We do not know how the significant reduction in sclerotial size and possible changes in fatty acid composition will affect the germination and survivability of sclerotia in the field.

The relationship between fungal development, medium composition and fatty acid metabolism is multifactoral and complex. It was demonstrated from this study that conditions promoting sclerotial development for improving overwintering survivability and seasonal carryover of a strain in the field are not the same as those required to promote maximum conidial production and dispersal during growing seasons. Nonetheless, further studies on genetic links between fatty acid metabolism, conidiation, and sclerotial development could result in strategies that allow for the manipulation of linoleic acid production at different fungal growth phases, thus optimizing conidial and sclerotial production and increasing the efficacy of biocontrol *Aspergillus* species.

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