De novo* Synthesis of Alternariol in Conidia of *Alternaria alternata

By PER HÄGGBLOM

Department of Physiological Botany, Uppsala University, Box 540, S-751 21 Uppsala, Sweden

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Alternaria alternata synthesizes the polyketides alternariol (AOH) and alternariol monomethyl ether (AME) during the stationary growth phase when it is grown in the dark. In the present study it is reported that these polyketides are also found in the conidia of this fungus as well as the vegetative hyphae. Young conidia were able to synthesize AOH *de novo* as shown by the incorporation into AOH of [³H]acetate. Since AOH synthesis in the conidia showed no need for *de novo* synthesis of proteins, it is proposed that hyphal cytoplasm that is able to synthesize AOH is translocated into the developing conidia.

INTRODUCTION

Synthesis of secondary metabolites by filamentous fungi, especially the Deuteromycetes, is considered to occur during the stationary growth phase in batch culture (Bu'Lock, 1980). Secondary or special metabolites such as mycotoxins are found in the fungal mycelium although Wicklow & Shotwell (1982) found aflatoxin in conidia of *Aspergillus flavus* and *A. parasiticus*.

Various species within the genus *Alternaria* produce large numbers of secondary metabolic products often with toxic properties against various cell types (King & Schade, 1984). Most of the toxic substances from *Alternaria* belong to the dibenzopyrone or polyketide biosynthetic groups (Stinson, 1985). *Alternaria alternata* is a mould with worldwide distribution and is a common saprophyte on many kinds of plants, foodstuffs and other substrates. When grown in the dark *A. alternata* synthesizes the polyketides alternariol (AOH) and alternariol monomethyl ether (AME) during the stationary growth phase (Häggblom & Unestam, 1979; Häggblom & Niehaus, 1986; Söderhäll *et al.*, 1978).

The objective of this study was (i) to investigate whether conidia from *A. alternata* contain the polyketides AOH and AME and (ii) if AOH and AME can be synthesized in these asexual spores.

METHODS

Alternaria alternata (Fr.) Keissler was originally obtained from Dr K. Hult, Royal Institute of Technology, Stockholm. The fungus was maintained as dehydrated conidia in dry sterilized silica gel (Kieselgel 60, Merck, 70–230 mesh) at –20 °C (Smith & Onions, 1983). Cultures were started on 2% malt agar in 9 cm plastic Petri dishes by sprinkling on silica gel (with conidia) using a sterile pasteur pipette.

Sporulation was induced by the method of Shahin & Shepard (1979) except that water was not added to the sucrose/CaCO₃ plates (S-medium). Conidia were produced within 24 h of incubation on the S-medium and their age was determined by the time of harvest. Conidial preparations were made at 4 °C or on ice. Conidia were scraped off the plates, suspended in 0.05% Tween 80, filtered through cheese cloth, and centrifuged at 500 r.p.m. Resuspension followed by centrifugation was repeated three times. The conidia were counted in a haemocytometer. (On average $1.9 \pm 0.25 \times 10^6$ spores were produced per plate after 3 d incubation in the dark.)

De novo synthesis of AOH and AME was measured by the incorporation into the polyketides of [³H]acetic acid (Amersham; specific activity 746 GBq mmol⁻¹; sodium salt). The incorporation medium consisted of (g l⁻¹): KH₂PO₄, 1; KCl, 0.25; NaCl, 0.25; yeast extract, 1; glucose, 5; MgSO₄·7H₂O, 0.5; ZnSO₄·7H₂O, 0.01;

Abbreviations: AOH, alternariol; AME, alternariol monomethyl ether.

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01. The pH of the medium was adjusted to 5.5 with dilute NaOH. [^3H]Acetate (1.85 MBq; 25 μmol) was added to 1 ml of medium containing 1.9×10^6 conidia. This suspension was incubated for 4 h on a shaker (150 r.p.m.) at 20 °C in the dark. The incubation medium was separated from the conidia by centrifugation at 2000 g for 5 min. Before extraction the conidia were washed with 0.05% Tween 80 (3 $\times$ 2 ml) and centrifuged. Each conidial portion was then extracted with 500 μl ethyl acetate for 24 h. AOH and AME in the conidia were determined according to Häggblom & Niehaus (1986). Samples of the extracts were purified by thin-layer chromatography (TLC) (Silica Gel 60, non-indicating) using (1) toluene/dioxane/acetic acid (95:25:4, by vol.) and (2) methanol/2 M-HCl (5:1, v/v).

Radioactivity with the same R_f as standards of AOH or AME was eluted with ethyl acetate from the TLC plate developed in system 1, and the extract was concentrated and spotted on a second plate together with standards of AOH and AME, and developed in system 2. Radioactive polyketides were eluted as before. After evaporation of the solvent polyketides were dissolved in methanol (300 μl) and 10 ml scintillation cocktail was added (Lipoluma, Lumac). Radioactivity was counted and corrected for quenching in a LKB 1217 scintillation counter.

Protein was labelled by incubating conidia in [^{35}S]methionine (22 TBq mmol $^{-1}$; Amersham) in incorporation medium (30 kBq ml $^{-1}$) for 2.5 h. The method of McLeod & Horgen (1979) was used for extraction of protein. The precipitated material was filtered off on Whatman GF/A filters, and after washing with cold 5% (w/v) trichloroacetic acid the filters were dried and radioactivity was counted. Cycloheximide [100 μg (ml incorporation medium) $^{-1}$] was used to inhibit protein synthesis. The conidia were incubated in medium with the inhibitor 30 min before addition of labelled methionine. Experiments were repeated two or three times using different conidial preparations.

RESULTS AND DISCUSSION

After filtration and centrifugation the conidial suspension was free from filaments and conidiophores. Table 1 shows the concentrations of AOH and AME in conidia of different age. In 1-d-old conidia neither AOH nor AME could be detected. A rapid increase in conidial polyketides was found from day 1 and in 12-d-old conidia the concentration was reduced. Similar concentrations of polyketides were found in conidia and hyphae (data not shown). Incorporation of [^3H]acetate into AOH and AME was most rapid during day 3 and 4 and by day 12 only low incorporation could be detected. These results represent the first report of *de novo* synthesis of secondary metabolites in conidia of a Deuteromycete.

It is suggested that hyphal cytoplasm that has the ability to synthesize AOH and AME is translocated into the developing conidia (porospores) by the enteroblastic tretric conidiogenesis that occurs in *Alternaria* (Hawksworth *et al.*, 1983).

Polyketide synthesis in the conidia shows no need for *de novo* protein synthesis as the addition of cycloheximide did not affect incorporation of label into AOH although incorporation into protein was greatly inhibited (data not shown). It therefore appears likely that the enzyme(s) involved in the biosynthesis are transported from the hyphal cytoplasm during conidial development. Thus, in *A. alternata*, the biosynthesis of the polyketides AOH and AME not only takes place in stationary cultures of the organism but also in newly developed conidia. It is not known whether this is a unique characteristic of *Alternaria*; other fungi with similar conidial development may possess the same characteristics.

Table 1. Concentrations of AOH and AME and incorporation of [^3H]acetate into AOH in conidia of *A. alternata*

Age of conidia (d)	$10^{-6} \times$ No. of conidia per plate	AOH concn (μg per 10^6 conidia)	AME concn (μg per 10^6 conidia)	[^3H]Acetate incorporation (d.p.m. per 10^6 conidia) into:*	
				AOH	AME
1	1.7	ND	ND	190 $\pm$ 60	104 $\pm$ 37
3	1.6	2.90 $\pm$ 0.40	0.16 $\pm$ 0.03	1425 $\pm$ 352	1112 $\pm$ 260
4	2.2	2.44 $\pm$ 0.42	0.21 $\pm$ 0.04	2299 $\pm$ 110	1427 $\pm$ 196
7	2.1	1.11 $\pm$ 0.11	0.19 $\pm$ 0.06	870 $\pm$ 258	539 $\pm$ 162
12	2.1	0.68 $\pm$ 0.16	0.40 $\pm$ 0.07	415 $\pm$ 101	278 $\pm$ 64

ND, Not detected.

* Results are means $\pm$ SEM of four to eight experiments.

The presence of high concentrations of AOH and AME in conidia of *A. alternata* could be of importance for its saprophytic behaviour as AME particularly is strongly necrotic for plant tissues (Bemmann, 1986; Stinson, 1985).

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