

Induction of a Complete Secondary-Product Pathway in a Cultured Lichen Fungus

CHICITA F. CULBERSON AND DANIELE ARMALEO

Department of Botany, Duke University, Durham, North Carolina 27706

Accepted for publication October 21, 1991

CULBERSON, C. F., AND ARMALEO, D. 1992. Induction of a complete secondary-product pathway in a cultured lichen fungus. *Experimental Mycology* 16, 52-63. Experimental studies on the secondary metabolism characteristic of lichens have been impeded by the slow growth of the fungi and by the inconsistent results of many attempts to induce the pathways in the fungi isolated from their photosynthetic partners. In the present study, a lichen-specific secondary pathway was consistently induced in a lichen fungus (*Cladonia grayi*) grown in the absence of the alga. The depside (4-*O*-demethylsphaerophorin) and two depsidones (grayanic and 4-*O*-demethylgrayanic acids) found in the natural lichen began to accumulate a few days after the transfer of lightly fragmented mycelia from liquid to solid medium. Induction was enhanced on drier substrates and was correlated with the proliferation of aerial hyphae, where the major product (grayanic acid) accumulated in extracellular patches visible by fluorescence microscopy. The time course was analyzed by quantitative high-performance liquid chromatography of extracts from small cultures grown on nylon filters. Induction was rapid in view of the slow growth of the fungus, and secondary productivity was comparable to that of some nonlichen fungi. These results confirm that the alga is not needed for catalysis in lichen depside and depsidone biosynthesis and suggest instead that the characteristic secondary metabolism of natural lichen is linked to their aerial habit of growth. © 1992 Academic Press, Inc.

INDEX DESCRIPTORS: Secondary metabolism; polyketides; lichen fungi; *Cladonia grayi*; grayanic acid; depsides; depsidones; aerial hyphae; photobiont; hyphal differentiation.

Lichens produce hundreds of unique extracellular secondary products of which many belong to classes of compounds rarely found in other organisms (Elix *et al.*, 1984). Their chemical structures and occurrences in thousands of species have been extensively studied using natural material (C. F. Culberson *et al.*, 1977; C. F. Culberson and Elix, 1990). Some lichen products have biological activity of interest in medicine and agriculture and many are used in phylogenetic reconstructions for lichen fungi (W. L. Culberson, 1986; Lawrey, 1984; Sankawa *et al.*, 1982; Umezawa *et al.*, 1983). Despite extensive knowledge of the structures and distributions of the compounds, the regulation and biological significance of secondary metabolism remains unclear, in part because of complications arising from the lichen's dual nature. Lichens are stable, structurally organized,

symbiotic associations between fungi (mycobionts) and green algae or cyanobacteria (photobionts). The partners can be cultured separately, but the natural equilibrium between the combined symbionts is not easily maintained under laboratory conditions. For some, however, symbiotic associations have been successfully established or maintained *in vitro* (reviews: Ahmadjian, 1980; Ahmadjian and Jacobs, 1985; Bubrick, 1988).

While the controls and genetics of secondary metabolism in nonlichen fungi are extensively studied (reviews: Drew and Demain, 1977; Garraway and Evans, 1984; Martín and Liras, 1989), the literature offers no clear clues to critical factors affecting analogous pathways in isolated lichen fungi. The many efforts to detect secondary products in cultures of lichen fungi have yielded inconsistent results. A few isolates

have spontaneously produced these compounds, some even copiously (Leuckert *et al.*, 1990), but more often the cultured fungus either lacks secondary products or synthesizes compounds not found in the natural lichen (Ahmadjian and Culberson, unpublished results; Fox and Huneck, 1969; Hamada and Ueno, 1987). In contrast to the largely negative results for the fungus cultured alone, many isolates consistently biosynthesize characteristic secondary products when they are artificially lichenized or even simply grown in the presence of their normal photobiont (e.g., C. F. Culberson and Ahmadjian, 1980; Ahmadjian and Jacobs, 1985; Kon *et al.*, 1990; Yamamoto *et al.*, 1985). This frequent correlation of lichenization or coculture with the production of lichen-specific secondary compounds has reinforced the idea that both partners play essential roles in secondary metabolism (C. F. Culberson and Ahmadjian, 1980; Hamada, 1988). However, the definition of these roles has remained elusive mostly because the slow growth of lichen fungi has impeded experimental studies.

By combining two technologies, a culture method on microfilters over solid media (Mulvany, 1969; Oliver *et al.*, 1989) and quantitative high-performance liquid chromatography, the present study shows that secondary metabolism can be induced and followed in submilligram cultures of an isolated lichen fungus. The fungus was *Cladonia grayi*, from a common lichen producing characteristic lichen-specific polyketide derivatives. The experiments define critical factors affecting the induction of this typical secondary pathway in culture, the hyphal sites and patterns of secondary-product accumulation, and the biosynthetic capacity of the mycobiont.

MATERIALS AND METHODS

Strains, Media, and Reagents

The fungus used in this work was a single-spore isolate (30-197#8) of *Cladonia*

grayi Merr. *ex* Sandst. (North Carolina: Mitchell Co., Roan Mountain, W. L. Culberson 19971, DUKE). The culture medium was a modified Lilly and Barnett (LB)¹ medium with proline (2 g/liter) instead of asparagine and half (10 g/liter) the usual concentration of glucose (Ahmadjian, 1967). Solid media containing 1.5% Bactoagar (Difco) or agarose (Bio-Rad) were delivered (5-ml aliquots in 5-cm plastic Petri dishes) by pipet to increase uniformity. Excess moisture was removed by leaving the covered plates in the reverse-flow sterile hood for 24 h ("moist" plates) or for approximately 3 days ("semi-day" plates). After drying and during the course of each experiment, plates were inverted and sealed with Parafilm. Mycelia for the induction experiments came from stock cultures, typically in 50-ml glass tubes containing 10 ml of medium and 10 g of acid-washed beads (Thomas Scientific, 0.5 mm diam.). The LB liquid medium was changed every 2-3 months, and mycelia were fragmented by rapid vortexing (5-10 s) once a month (Armaleo, 1991). In the preparation of HPLC samples, all solvents and reagents were HPLC-grade. Plastics were used sparingly and were prewashed, nylon filters with methanol and microfuge tubes with acetone. Substitution of electrophoresis-grade agarose for agar in the solid medium improved HPLC analysis of trace amounts of secondary compounds by reducing background peaks. Blank filters from LB Petri plates served as controls for residual medium-derived artifacts.

Transfer of Mycelia to Solid Medium

The nylon filters (Fisher, Magna Nylon 66, 20 μ m pore-size) to receive liquid-grown mycelia were washed five times in

¹ Abbreviations used: LB, Lilly and Barnett medium; GR, grayanic acid; DMGR, 4-*O*-demethylgrayanic acid; DMSPH, 4-*O*-demethylsphaerophorin.

methanol and dried, and the weights (Cahn 25 microbalance) were penciled along the margins. The filters were autoclaved and all subsequent manipulations were performed under sterile conditions in a reverse-flow hood. Immediately before transfer onto filters, a liquid culture (typically 2–4 weeks after the last medium change) was diluted with fresh medium to 1–2 mg mycelium (dry weight)/ml. Equal-volume samples (0.5 or 1.0 ml) were withdrawn from the stirred hyphal suspension with an automatic pipet fitted with tips cut off to enlarge their opening to about 3 mm. Each sample was delivered onto a separate nylon filter placed under suction on a fritted glass surface padded with filter paper (Whatman No. 1). To confine the mycelia to uniform areas on the nylon filters, a tube of appropriate size was centered on the filter until all of the liquid had passed through. Most experiments used filters 13 mm in diameter, with 0.1–0.5 mg mycelia seeded through inverted 1.5-ml polypropylene microfuge tubes with lids and bottoms cut off. Larger filters (25 mm in diameter) used with larger amounts of mycelia (1–3 mg) were seeded through 17 × 100-mm polypropylene test tubes. The rim of the tube pressed against the padded filter provided an efficient seal so that each filter received a well-defined circular mat of mycelia covering all but 2–3 mm at the margin. Each inoculated filter was placed immediately onto solid medium (typically one per plate) and incubated at room temperature (25°C).

Filter Harvest, Extraction, and HPLC

Sample Preparation

Filters were removed from agar media at each time point and stored immediately at –100°C for analysis after the last time point. Upon removal from the freezer, filters from the same time point were generally extracted together in a 10-ml beaker with 0.5- to 1-ml portions of 90% methanol–water (four times) and methanol (two

times), each extraction for ca. 2 min at room temperature. The extracted filters and attached fungal mats, which remained intact, were used for the determination of biomass (see below). The six extracts were pooled and evaporated under a stream of nitrogen, at reduced pressure, with gentle heat (ca. 40°C). The residue was taken up in acetone (washing five times with 0.1 ml) and transferred quantitatively to a 1.5-ml microfuge tube. After evaporating the solvent, the solid on the wall of the microfuge tube was concentrated to the bottom with ca. 100 µl of acetone, vortexing and spinning the solution, and evaporating the solvent. For early time-point samples (through 16 days), the use of acetone to transfer the residue from the original methanol/water extract to the microfuge tube gave the cleaner sample needed to quantify the small amounts of secondary products. At the longer time points, however, an acetone-insoluble residue began to accumulate, trapping a fraction of the secondary products. To quantify the trapped fraction, this residue was dissolved in 90% methanol. Alternatively, to bypass the acetone-insoluble precipitate at the longer time points, the residue from the initial 90% methanol extract was transferred to a microfuge tube and concentrated to the bottom using only 90% methanol. In either case, the final residues were quantitatively resuspended in measured volumes of methanol immediately before HPLC.

Identification of Secondary Products

The isocratic HPLC method for the initial identification of compounds and the first time-course experiment used a Beckman (Gold Star) Ultrasphere ODS 5-µm (4.6 × 250 mm) column, a methanol–water–acetic acid solvent (82:18:1.6, v/v/v) flowing at 1.0 ml/min, and detection at 270 nm. For compounds in extracts of filter cultures, capacity factors (k') and retention index values relative to methyl ketone con-

trols (*I*) were compared to an extensive database for lichen products, including the secondary products of natural *C. grayi* and its related chemotypes in the *C. chlorophaea* complex (C. F. Culberson *et al.*, 1985; C. F. Culberson and Elix, 1990). Final identifications were based on direct TLC and HPLC comparisons with authentic samples (Table 1): natural GR and 4-*O*-demethylgrayanic (DMGR) acids from *C. grayi* and *C. anitae* (W. L. Culberson *et al.*, 1982), respectively, and synthetic 4-*O*-demethylsphaerophorin (DMSPH) (Elix and Wardlaw, 1987). Thin-layer chromatography (TLC) was on Merck Silica Gel F-254 plates, in three solvent systems (Solvents A, B, and C) by methods standardized for lichen products (C. F. Culberson and Elix, 1990). DMSPH, tentatively identified (by HPLC) as a trace component of an acetone extract of freshly collected natural *C. grayi* (350 mg) (North Carolina: Orange Co., near Chapel Hill, *Armaleo*, 1991, DUKE), was confirmed as follows: After precipitation of most of the GR from the crude extract, preparative TLC (Solvent C) enrichment of trace components comigrating with authentic DMSPH, and elution from the spot further separated by 2D-TLC (Solvent C, then B), the purity and identity of the isolated DMSPH were confirmed by HPLC.

HPLC Quantification of Secondary Products

A gradient HPLC method, providing additional confirmations of identifications and improved quantifications of the secondary products, used a Beckman C8 (4.6 × 250 mm; 5- μ m) column with Solvent I (70% water/methanol + 1% *ortho*-phosphoric acid) and Solvent II (methanol). The 20-min linear gradient from 35 to 10% Solvent I efficiently separated all of the secondary products (Fig. 1). Between runs, a 2-min gradient to 2% Solvent I (held for 5 min) cleaned the column. Flow was 1.0 ml/min throughout, and detection was at 270 nm. The sample was dissolved in sufficient methanol,

containing dodecanophenone (0.1 mg/ml; Aldrich Chemical Co.) as an internal standard, to give on-scale peaks with 5- μ l injections in all analyses so that the limits of solubility of the secondary products in methanol were never approached. Peak broadening, observed in chromatograms of longer time-point samples, could be virtually eliminated by adding 1% trifluoroacetic acid to the injection solvent. Quantification by HPLC-peak area used standard solutions of pure GR and DMSPH. The response factors relating HPLC-peak area to nanomoles of compound are not measurably different for depsidones like GR and DMGR that differ only by 4-*O*-methylation (unpublished result), but those for GR and DMSPH (570,000 and 858,000 area units/nmol, respectively) reflected the stronger absorbance of depsides compared to depsidones at the detection wavelength.

Biomass Determination

Mycelia on each filter increased in density and height during the experiments, but hyphae did not extend horizontally beyond the original boundary of the seeded patch and only to a limited extent penetrated through the filter into the agar. The biomass and secondary-product measurements involved only the material within and above the filter. After the methanol/water extraction of secondary products, the filter cultures were dried overnight at room temperature in a vacuum desiccator over P₂O₅ and

TABLE 1
Representative Chromatographic Data

Compound ^a	TLC (<i>R_f</i> × 100) in solvents			HPLC <i>k'</i> / <i>I</i>
	A ^b	B	C	
DMGR	21	49	20	2.24/1014
DMSPH	35	59	32	4.47/1168
GR	37	63	50	6.27/1259

^a Chemical structures are in Fig. 2.

^b Norstictic acid and atranorin, standard TLC controls for lichen products, ran at 36 and 82 in Solvent A, at 30 and 78 in Solvent B, and at 29 and 87 in Solvent C.

weighed. Subtracting the original weight of the empty filter gave the biomass of the culture after extraction. Since the methanol/water-soluble but acetone-insoluble residue (possibly carbohydrate) described above constituted a sizable fraction (11–13%) of the biomass after Day 16, this weight (measured on a dried aliquot of each methanol-water extract) was added to the values determined from the weights on the filters. The accuracy of the biomass estimate improves as the number or the size of filters per time point increases. Using additional filters for each time point also averages slight variations in conditions (e.g., dryness) between different plates. Further, the extra filters allowed removal of any that showed contamination during the long course of the experiments.

Fluorescence Microscopy

Diluted samples from suspensions of fragmented mycelia were spread directly onto LB plates and incubated at room temperature. (Fluorescence microscopy on filter cultures was impeded by the high background generated by the filter itself). Observations at less than 250 \times enlargement were made without a coverglass on colonies on small agar sections removed aseptically from the plates and placed onto slides immediately before microscopy. Due to uv-radiation damage, the same colony could not be followed over time. When higher resolution was needed, a single colony was excised from the plate and freed of most agar under a dissecting microscope. It was then transferred onto a coverglass held under the dissecting microscope by two strips of double-stick tape placed over the right and left edges of the glass. The colony was fragmented with forceps, and individual fragments were carefully dragged across the coverglass so as to stretch out aerial hyphae. Manipulations were prompt after removal of the colony from the plate, since drying out impeded the sliding of hyphae. The coverglass and attached tape

were then inverted onto a microscope slide with the specimen adhering to the underside of the coverglass. The tape fastened the coverglass to the slide, allowing observations under oil, and formed a chamber between the two glass surfaces to accommodate the often clumpy specimen. A Leitz Dialux 20 fluorescence microscope with a camera attachment was used to photograph (Kodak TriXpan 400 black-and-white film) samples under long-wave uv light.

RESULTS

Identification of Secondary Products

Three characteristic, biogenetically related lichen products were identified in induced cultures of the isolated *C. grayi* mycobiont, the depside DMSPH and two depsidones DMGR and GR (Figs. 1 and 2). The natural lichen accumulates high amounts of GR and small amounts of DMGR (C. F. Culberson *et al.*, 1985). The previously unreported trace of DMSPH was demonstrated by preparative TLC enrichment of the minor compounds followed by two-dimensional TLC and HPLC confirmation. Time-course experiments described below showed the order and speed of induction of these secondary products.

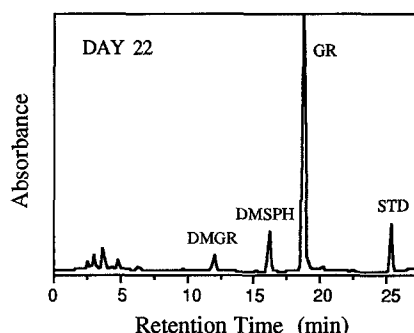


FIG. 1. Sample HPLC (gradient method) of the extract from a 22-day culture of the *C. grayi* fungus grown on a nylon filter. The chromatogram represents 1/40th of the total extract. For the identification and quantitation of secondary products, see Materials and Methods. STD is the internal standard.

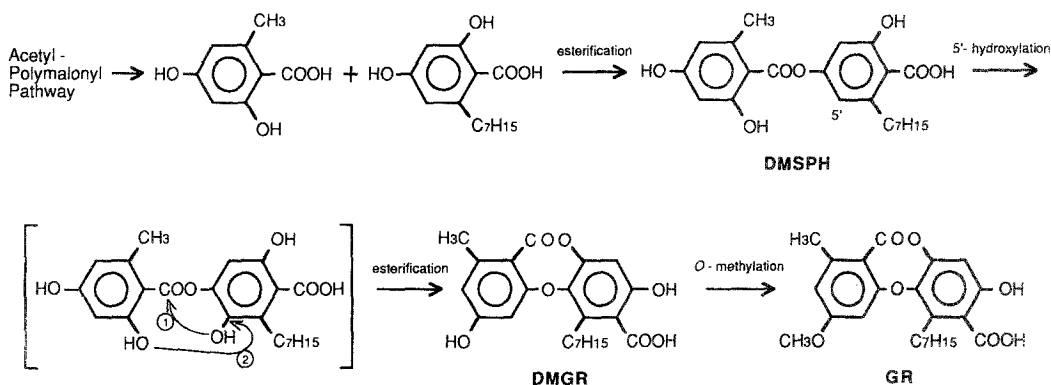


FIG. 2. A possible biosynthetic pathway to GR (adapted from Elix *et al.*, 1987). The two reactions summarized within the brackets are (1) an acyl migration and (2) a Smiles rearrangement. For further explanation, see Discussion.

Induction of Secondary Metabolism and the Effect of Water Loss

Mycelia of *C. grayi* grown in liquid LB medium for weeks or months produced no significant amounts of DMSPH, DMGR, and GR. Upon transfer from liquid to solid LB medium, the cultures made secondary products that were detectable by 1 week and increased rapidly by 3 weeks (Figs. 3A and 3B). In this and similar induction experiments, the three identified components of the pathway showed distinct patterns of change. DMSPH accumulated most rapidly in the earliest phases, initially often surpassing GR (Fig. 3B). Later, however, while GR continued to accumulate rapidly over a long period of time, DMSPH

reached a plateau. DMGR, always present in small amounts, kept increasing (Fig. 3A).

Since induction followed the transfer from liquid to solid medium, we tested the effect of different degrees of hydration. Filter cultures were placed on regular ("moist") agar plates and on others that had been predried so that the thickness of the medium had been reduced by about half ("semi-dry"). During the first 20 days (Figs. 3B and 3C), secondary-product biosynthesis was induced much more rapidly in the semi-dry cultures. To confirm that the observed effect was due to decreased water content and not to the higher concentration of medium components in the semi-dry plates, the time course of early induction was analyzed in a separate experiment

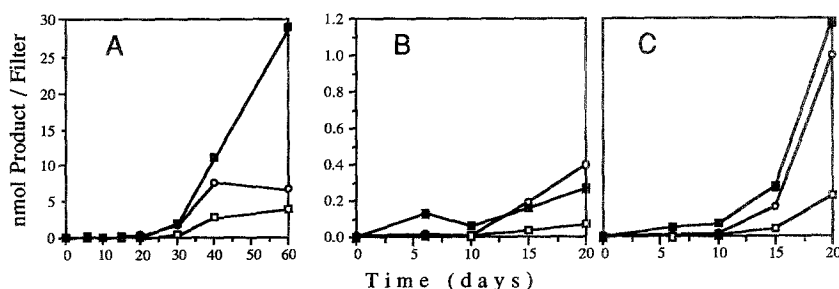


FIG. 3. Time course of secondary product synthesis in filter-cultures on regular ("moist") and on partially dried ("semi-dry") LB agar medium. Symbols represent the amounts of DMSPH (○), DMGR (□), and GR (■) per filter-culture. For each time-point, the combined extracts from three to five cultures on 13-mm filters were analyzed by HPLC (isocratic method). (A) Sixty days on "moist" LB agar. (B) The first 20 days on "moist" LB agar. (C) Twenty days on "semi-dry" LB agar. The scale for nmol product/filter is the same in panels B and C.

comparing filter cultures grown on identical LB media containing either 1.5 or 3% agarose. The concentrations of all components were the same except for the higher agarose and the consequently lower free water in the 3% medium. By Day 20 the 3% cultures contained, per milligram biomass, 2.5 \times the amount of grayanic acid found in the 1.5% controls. Clearly, the level of secondary-product induction in this isolate was inversely correlated with the amount of available water.

Relation of Biomass to the Timing and Rate of Biosynthesis

Seeding mycelia on individually pre-weighed filters allowed measurement of biomass for the samples analyzed chemically. Even with the limited availability of the slow-growing mycobiont, the small size of the samples needed for each microculture permitted replicates for each time point. Growth, after an initial lag, accelerated and then slowed (Fig. 4). The acceler-

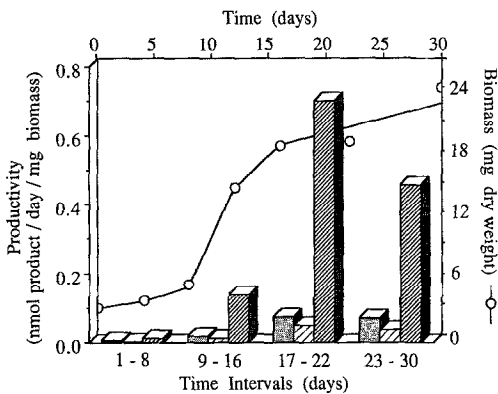


FIG. 4. Daily rate of secondary-product synthesis by the lichen fungus per milligram of biomass (bar graph) superimposed on the growth curve (line graph). The biomass at each time-point is the average of two 25-mm filter-cultures. The secondary products per filter-culture (averaged from two filters) were quantified by HPLC (gradient method). The time intervals used for calculation of daily productivity are indicated below the lower abscissa. Bars represent the nanomoles product per filter made during interval/number of days in interval/average biomass per filter in interval. Bar markings: \square DMSPH, \square DMGR, \square GR.

ated-growth phase between Days 8 and 16 was correlated with the proliferation of aerial hyphae emerging from an agglutinated hyphal base resting on the filter. The growth curve measured for this mycobiont on the solid substrate resembles those of other lichen mycobionts measured in liquid media (Ahmadjian, 1961; Lawrey, 1984). Many nonlichen fungi display similar growth curves but with vastly shortened time scales. Most nonlichen secondary metabolism is studied in liquid culture; mycophenolic acid production by *Penicillium brevicompactum*, however, has been analyzed in sufficient detail on solid media (Bartman *et al.*, 1981) to provide an appropriate comparison for our results. The accelerated-growth phase between Days 8 and 16 in our fungus spanned Days 2 to 3 in the nonlichen fungus. The lichen fungus resembled *P. brevicompactum* (and many other nonlichen fungi in liquid culture) by showing a significant increase in secondary products during the growth spurt, the rate of accumulation per unit of biomass peaking just after growth slowed, and then declining. Also the maximum secondary productivity (0.7 nmol product/day/mg biomass) of our slow-growing lichen fungus was comparable to that of the faster-growing *P. brevicompactum*.

Hyphal Sites and Pattern of Secondary-Product Accumulation

uv-Irradiated GR and DMSPH crystals emit a blue fluorescence that allows patterns of localization of these compounds on hyphae to be followed microscopically during induction (Fig. 5). Mycelial fragments were seeded at low density directly on agar or agarose plates, and colonies were observed periodically by fluorescence microscopy. The earliest signs of accumulation were small patches of blue fluorescence on the surface of aerial hyphae about 250 μ m or more from the tip (Fig. 5A). During induction, the patches increased in size and intensity from the apical region (mostly de-

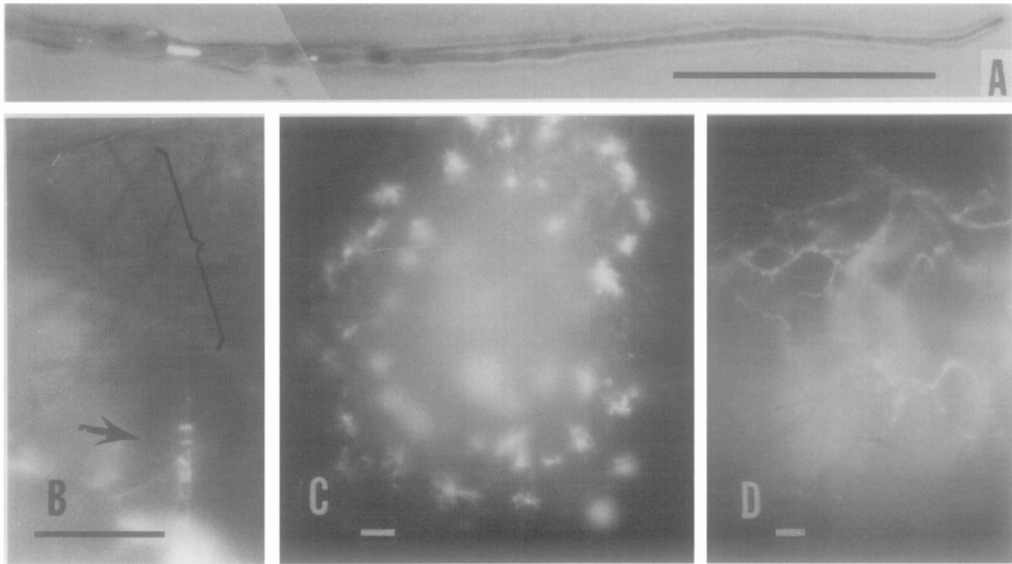


FIG. 5. Fluorescence microscopy of the cultured lichen fungus. Bars represent 100 μm . (A) Aerial hypha showing new deposits of secondary product 260–350 μm from the tip. (B) Two weeks into the induction the apical region of an active aerial hypha shows no deposits (bracket). In the basal region, the more distal and smaller deposits appear banded (arrow), while larger ones predominate closer to the base emerging from the body of the colony (out of the plane of focus). Also in the surrounding hyphae, most deposits are near the base (out of the plane of focus). (C) Top overview of the same colony as in (B), showing the largest deposits at the sites of emergence of aerial hyphae. (D) After 4 weeks, the aerial mycelium is almost completely covered with secondary product. In (A) the hypha was stretched under a coverglass, and no coverglass was used in B–D (see Materials and Methods).

void of patches) to the base of individual aerial hyphae (Fig. 5B), a polarity consistent with a model proposed by Moss (1984). Often the sites of accumulation displayed characteristic banding patterns (Fig. 5B). However, not all aerial hyphae were affected during induction, many lacking patches even when growing near active hyphae. Many aerial hyphae both branched and coalesced in bundles as growth progressed, and fluorescent patches became particularly conspicuous where such aerial bundles emerged from the central agglutinated mass of the colony (Fig. 5C). Eventually, the entire aerial mycelium became covered with fluorescent accretions, obliterating most traces of discontinuity and of hyphal tip-to-base gradations (Fig. 5D). In general, the density of the accretions tended to be the highest in the aerial regions around the top of the dome-shaped colony, and decrease toward the agar surface. Blue

patches could not be seen at the lower edges of the colony, where hyphae had penetrated the medium.

When acetone or methanol was swept through a colony under a coverglass, the blue fluorescent patches disappeared quickly, leaving a uniform background of surface autofluorescence typical of hyphal walls, as seen after removal of secondary products or in uninduced mycelia. From their fluorescence, kinetics of accumulation, and solubility in acetone or methanol, we believe that the blue patches represent extracellular accretions of secondary products, mostly grayanic acid.

Effects of Other Variables

The results reported here for cultures on nylon filters over an agar medium also apply to mycelia growing directly on agar. For experiments, the nylon-filter method is

more versatile and sensitive. In one experiment using Durapore (Millipore Corp.) instead of nylon filters, growth and production of secondary compounds although initiated were not sustained. This is probably due to the fact that hyphae cannot easily penetrate the Durapore matrix (Oliver *et al.*, 1989), whereas they can pass through the nylon mesh. The mycelia for all of our experiments were fragmented (Ahmadjian, 1987; Armaleo, 1991) when transferred to solid media. However, differences in the degree and timing of the preplating fragmentation probably contributed to variations in the length of the initial lag phase observed in different experiments. Work in progress using a variety of lichen mycobionts grown on media other than LB indicates that the choice of medium, as well as the genetic makeup of the fungus, can have quantitative and qualitative effects on induction. Given the proper match of medium and genotype, however, the results outlined here apply to a diversity of lichen mycobionts.

DISCUSSION

Induction of Secondary Metabolism and Aerial Growth on Solid Substrates

We have found that a complete secondary-product pathway is consistently induced in an isolated lichen mycobiont when hyphal suspensions are transferred from a liquid to a solid medium. The three polyketide-derived compounds identified in the cultures are all found in the natural lichen. In the isolated mycobiont, accumulation begins after a lag of several days and is correlated with the growth of an aerial mycelium, where the end-product of the pathway (grayanic acid) is deposited. Induction is enhanced on progressively drier substrates, suggesting that decreased water is a critical activating factor (review: Jennings, 1984), probably in conjunction with exposure to air. The same correlation between differentiation of aerial hyphae and

the biosynthesis of specific polyketide-derived secondary metabolites was reported for the nonlichen fungi *Penicillium brevicompactum* with mycophenolic acid (Bartman *et al.*, 1981) and *P. patulum* with 6-methylsalicylic acid (Peace *et al.*, 1981). The overlap of maximum productivity and the end of the aerial-growth spurt conforms to the view that links secondary metabolism to conditions of reduced growth (Bu'Lock, 1975; Moss, 1984). However, the localization of compounds on the aerial mycelium suggests that on solid media not only growth limitation but also specific biochemical consequences of dryness and air trigger secondary metabolism as a component of aerial differentiation.

In the few studies reporting characteristic lichen depsides and depsidones from isolated lichen mycobionts and two nonlichen fungi, the cultures were almost invariably grown on solid media (Komiya and Shibata, 1969; Umezawa *et al.*, 1983; Ejiri and Shibata, 1975; Yamamoto *et al.*, 1976; Hamada and Ueno, 1987; Leuckert *et al.*, 1990). The secondary products characteristic of lichen fungi seem more stringently linked to aerial growth than are the compounds commonly associated with nonlichen fungi and so extensively studied in liquid media. The parallels between our lichen mycobiont and the two penicillia, however, suggest an aspect of secondary metabolism shared by lichen and nonlichen fungi that is best studied on solid media. Conditions promoting aerial growth are especially relevant for lichen fungi where adaptations for life above a substrate, exposed for years to light and air and without protection from water loss, are more likely to be expressed than in liquid culture. Indeed, the critical importance of drying for establishing and maintaining the lichen symbiosis has been amply verified in artificial lichenizations.

Patterns of Accumulation and Biogenesis of Grayanic Acid

Grayanic acid is progressively and unevenly deposited in an amorphous matrix

on the surface of aerial hyphae. The discontinuities in the deposition are best observed during the first 3 weeks of induction. Some hyphae appear to be inactive whereas others accumulate secondary product in defined but irregular areas, as if the enzymes responsible for grayanic acid synthesis and/or translocation were fixed in localized patches, perhaps within the cell wall. The fluorescent accretions do not reflect any obvious underlying cell structure, but increase in size and intensity toward the hyphal base, perhaps a consequence of the larger build-up of the pathway's machinery or of decreased NADPH (Moss, 1984) in the older vs. the younger regions of the hypha. Eventually, as the metabolic pathway accelerates and aerial growth slows, the entire aerial mycelium becomes covered with secondary product. The localization of grayanic acid suggests that the aerial hyphae in the culture are equivalent to the medullary hyphae inside the natural lichen thallus where most of the characteristic secondary products accumulate.

The joint occurrence of the depside DMSPH with DMGR and GR in the mycobiont cultures and in the natural lichen is consistent with two theories that see depsides giving rise to depsidones either by oxidative coupling (Erdtman and Wachtmeister, 1957) or by a route illustrated in Fig. 2 for the biosynthesis of GR (Elix *et al.*, 1987). According to the latter proposal, acetyl-polymalonyl-derived phenolic acids (here orsellinic acid and sphaerophorol carboxylic acid) are esterified to form a depside (DMSPH). 5'-Hydroxylation followed by acyl migration and a Smiles rearrangement yields a diphenyl ether, which cyclizes to a depsidone (DMGR) by intramolecular esterification. *O*-Methylation of DMGR would give the depsidone GR characteristic of the natural lichen. During induction, the proportion of depside (DMSPH) to depsidones (DMGR and GR) is initially high and decreases with time, a pattern consistent with the above proposals. The pathway steps leading to DMSPH

are activated first. With age, the subsequent steps gain efficiency and convert almost all the DMSPH to depsidones. Eventually the proportions should match those in the natural lichen, which contains only traces of DMSPH and large amounts of GR.

Factors Affecting Productivity and Detection

Besides growth on solid substrates, other factors contribute to the production and detection of secondary products in cultures of lichen fungi. As the mycobionts generally do not conidiate in culture, suspensions of lightly fragmented mycelia are sown on filter or agar surfaces to provide large numbers of synchronous growth initials amplifying aerial development and secondary productivity. While fragmentation does not induce the characteristic compounds in submerged culture, mycobionts transferred to solid media without fragmentation remain agglutinated and make only small amounts of secondary products. Coupling the manipulation of small amounts of mycelia on filters with current microanalytical methods (TLC, HPLC, and fluorescence microscopy) provides the versatility and sensitivity needed for experiments with the slow-growing lichen fungi. Most workers have used various microchemical analyses on extracts of milligram (or less) quantities of the fungus, and only few have attempted to extract and purify characteristic lichen products (Komiya and Shibata, 1969; Ejiri and Shibata, 1975; Hamada and Ueno, 1987). The earlier methods (e.g., paper chromatography and microcrystal tests) were not sufficiently discriminating or sensitive for such small cultures. The composition of the solid medium and the genetic variability of spores from natural lichens also affect the production of secondary compounds in cultured mycobionts.

The Roles of the Symbionts in Secondary Metabolism

The ability of our culture to complete the

pathway to grayanic acid indicates that the biosynthetic capacity for this depsidone lies entirely within the mycobiont. Work in progress in our laboratory shows that other solid-media cultures of related and unrelated mycobionts produce a variety of depsides and depsidones. These results indicate that the alga is not required for catalysis at any step, and confirm the reports discussed earlier in which occasional productivity was described in isolated mycobionts but could not be tied to any specific conditions of culture. Regulatory effects of the alga on secondary metabolism might still be found, and may perhaps contribute to the synthesis of compounds seen in artificially lichenized or cocultured symbionts. However, the use in those experiments of dispersed inocula deposited onto solid substrates (e.g., mica, soil, or agar) seems a more likely explanation of the observed productivity.

ACKNOWLEDGMENTS

We thank John A. Elix for a sample of 4-*O*-demethylsphaerophorin, Stephen Johnston and Bruce R. Nicklas for the use of fluorescence microscopes, Anita Johnson and Gerda Vergara for technical assistance, and William Louis Culberson for helpful discussions. This study was supported in part by Grants BSR-8806659 from the National Science Foundation and ARIG-882134 from the North Carolina Biotechnology Center.

REFERENCES

- AHMADJIAN, V. 1961. Studies on lichenized fungi. *Bryologist* 64: 168-179.
- AHMADJIAN, V. 1967. *The Lichen Symbiosis*. Blaisdell, Waltham, MA.
- AHMADJIAN, W. 1980. Separation and artificial synthesis of lichens. In *Cellular Interactions in Symbiosis and Parasitism* (C. B. Cook, P. W. Pappas, and E. D. Rudolph, Eds.), Ohio State University Biosciences Colloquia, No. 5, pp. 3-29.
- AHMADJIAN, V. 1987. Laboratory culture of lichens and lichen symbionts. In *Proceedings of Symposium on Tissue Culture of Lichen and Bryophyte*, pp. 1-10. Nippon Paint Co., 19-17 Ikedanaka-machi, Neyagawa, 572, Japan.
- AHMADJIAN, V., AND JACOBS, J. B. 1985. Artificial re-establishment of lichens. IV. Comparison between natural and synthetic thalli of *Usnea strigosa*. *Lichenologist* 17: 149-165.
- ARMALEO, D. 1991. Experimental microbiology of lichens: Mycelial fragmentation, a novel growth chamber, and the origins of thallus differentiation. *Symbiosis* 11: 163-178.
- BARTMAN, C. D., DOERFLER, D. L., BIRD, B. A., REMALEY, A. T., PEACE, J. N., AND CAMPBELL, I. M. 1981. Mycophenolic acid production by *Penicillium brevicompactum* on solid media. *Appl. Environ. Microbiol.* 41: 729-736.
- BUBRICK, P. 1988. Methods for cultivating lichens and isolated bionts. In *Handbook of Lichenology* (M. Galun, Ed.), Vol. 3, pp. 127-138. Academic Press, London.
- BULLOCK, J. D. 1975. Secondary metabolism in fungi and its relationship to growth and development. In *The Filamentous Fungi* (J. E. Smith and D. R. Berry, Eds.), Vol. 1, pp. 33-58. Edward Arnold, London.
- CULBERSON, C. F., AND AHMADJIAN, V. 1980. Artificial reestablishment of lichens. II. Secondary products of resynthesized *Cladonia cristatella* and *Lecanora chrysoleuca*. *Mycologia* 72: 90-109.
- CULBERSON, C. F., CULBERSON, W. L., AND JOHNSON, A. 1977. *Second Supplement to "Chemical and Botanical Guide to Lichen Products."* American Bryological and Lichenological Society. St. Louis, MO.
- CULBERSON, C. F., CULBERSON, W. L., AND JOHNSON, A. 1985. Orcinol-type depsides and depsidones in the lichens of the *Cladonia chlorophaea* group (Ascomycotina, Cladoniaceae). *Bryologist* 88: 380-387.
- CULBERSON, C. F., AND ELIX, J. A. 1990. Lichen substances. In *Methods in Plant Biochemistry* (J. B. Harborne, Ed.), Vol. 1, pp. 509-535. Academic Press, London.
- CULBERSON, W. L. 1986. Chemistry and sibling speciation in the lichen-forming fungi: Ecological and biological considerations. *Bryologist* 89: 123-131.
- CULBERSON, W. L., CULBERSON, C. F., AND JOHNSON, A. 1982. A new endemic red-fruited *Cladonia* from the North Carolina coast. *Mycologia* 74: 662-667.
- DREW, S. W., AND DEMAIN, A. L. 1977. Effect of primary metabolites on secondary metabolism. In *Annual Review of Microbiology* (M. P. Starr, J. L. Ingraham, and A. Balows, Eds.), Vol. 31, pp. 343-356. Annual Reviews Inc., Palo Alto, CA.
- EJIRI, H., AND SHIBATA, S. 1975. Squamatic acid from the mycobiont of *Cladonia crispata* (Ach.) Flot. *Phytochemistry* 14: 2505.
- ELIX, J. A., JENIE, U. A., AND PARKER, J. L. 1987. A novel synthesis of the lichen depsidones divaronic acid and stenosporonic acid, and the biosynthetic implications. *Aust. J. Chem.* 40: 1451-1466.
- ELIX, J. A., AND WARDLAW, J. H. 1987. Synthesis of

- lichen *para*-sphaerophorol depsides. *Aust. J. Chem.* 40: 425-429.
- ELIX, J. A., WHITTON, A. A., AND SARGENT, M. V. 1984. Recent progress in the chemistry of lichen substances. *Progr. Chem. Org. Nat. Prod.* 45: 103-234.
- ERDTMAN, H., AND WACHTMEISTER, C. A. 1957. Phenoldehydrogenation as a biosynthetic reaction. In *Festschrift Arthur Stoll*, pp. 144-165. Sandox Ltd., Basel.
- FOX, C. H., AND HUNECK, S. 1969. The formation of roccellic acid, eugenitol, eugenetin, and rupicolon by the mycobiont *Lecanora rupicola*. *Phytochemistry* 8: 1301-1304.
- GARRAWAY, M. O., AND EVANS, R. C. 1984. *Fungal Nutrition and Physiology*. Wiley, New York.
- HAMADA, N. 1988. Depside from isolated lichen mycobiont. II. *Lichenologist* 20: 294-295.
- HAMADA, N., AND UENO, T. 1987. Depside from an isolated lichen mycobiont. *Agric. Biol. Chem.* 51: 1705-1706.
- JENNINGS, D. H. 1984. Water flow through mycelia. In *The Ecology and Physiology of the Fungal Mycelium. Symposium of the British Mycological Society*. (D. H. Jennings and A. D. M. Rayner, Eds.), pp. 143-164. Cambridge Univ. Press, Cambridge.
- KOMIYA, T., AND SHIBATA, S. 1969. Formation of lichen substances by mycobionts of lichens. Isolation of (+)-usnic acid and salazinic acid from mycobionts of *Ramalina* spp. *Chem. Pharm. Bull.* 17: 1305-1306.
- KON, Y., KASHIWADANI, H., AND KUROKAWA, S. 1990. Induction of lichen thalli of *Usnea confusa* Asah. ssp. *kitamiensis* (Asah.) Asah. *in vitro*. *J. Japan. Bot.* 65: 26-33.
- LAWREY, J. D. 1984. *Biology of Lichenized Fungi*. Praeger, New York.
- LEUCKERT, C., AHMADJIAN, V., CULBERSON, C. F., AND JOHNSON, A. 1990. Xanthones and depsidones of the lichen *Lecanora dispersa* in nature and of its mycobiont in culture. *Mycologia* 82: 370-378.
- MARTÍN, J. F., AND LIRAS, P. 1989. Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. In *Annual Review of Microbiology* (L. N. Ornston, A. Balows, and E. P. Greenberg, Eds.), Vol. 43, pp. 173-206. Annual Reviews, Inc., Palo Alto, CA.
- MOSS, M. O. 1984. The mycelial habit and secondary metabolite production. In *The Ecology and Physiology of the Fungal Mycelium. Symposium of the British Mycological Society*. (D. H. Jennings and A. D. M. Rayner, Eds.), pp. 127-142. Cambridge Univ. Press, Cambridge.
- MULVANY, J. G. 1969. Membrane filter techniques in microbiology. In *Methods in Microbiology* (J. R. Norris and D. W. Ribbons, Eds.), Vol. 1, pp. 205-253. Academic Press, London.
- OLIVER, E., CRITTENDEN, P. D., BECKETT, A., AND BROWN, D. H. 1989. Growth of lichen-forming fungi on membrane filters. *Lichenologist* 21: 387-392.
- PEACE, J. N., BARTMAN, C. D., DOERFLER, D. L., AND CAMPBELL, I. M. 1981. 6-Methylsalicylic acid production in solid cultures of *Penicillium patulum* occurs only when an aerial mycelium is present. *Appl. Environ. Microbiol.* 41: 1407-1412.
- SANKAWA, U., SHIBUYA, M., EBIZUKA, Y., NOGUCHI, H., KINOSHITA, T., AND IITAKA, Y. 1982. Depside as potent inhibitor of prostaglandin biosynthesis: A new active site model for fatty acid cyclooxygenase. *Prostaglandins* 24: 21-34.
- UMEZAWA, K., MURAMATSU, S., ISHIZUKA, M., SAWA, T., TAKEUCHI, T., AND MATSUSHIMA, T. 1983. Inhibition of histidine decarboxylase and tumour promoter-induced arachidonic acid release by lecanoric acid analogues. *Biochem. Biophys. Res. Commun.* 110: 733-739.
- YAMAMOTO, Y., MITZUGUCHI, R., AND YAMADA, Y. 1985. Tissue culture of *Usnea rubescens* and *Ramalina yasudae* and production of usnic acid in their cultures. *Agric. Biol. Chem.* 49: 3347-3348.
- YAMAMOTO, Y., NISHIMURA, K., AND KIRIYAMA, N. 1976. Studies on the metabolic products of *Aspergillus terreus*. I. Metabolites of the strain IFO *Chem. Pharm. Bull.* 24: 1853-1859.