Evidence from Mycelial Studies for Differences in the Sterol Biosynthetic Pathway of *Rhizoctonia solani* and *Phytophthora cinnamomi*

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Phytophthora cinnamomi, a member of the Pythiaceae, does not synthesize sterols. Small amounts of squalene, but no squalene epoxide or sterol, were isolated from the dried mycelium of this fungus after growth in sterol-free medium. The dried mycelium of *Rhizoctonia solani*, a sterol-synthesizing fungus grown under the same conditions, contained small amounts of squalene and squalene epoxide and large amounts of ergosterol. When the two organisms were grown in the presence of [^{14}C]acetate, only labelled geraniol, farnesol and squalene were recovered from the *P. cinnamomi* mycelium, whereas labelled geraniol, farnesol, squalene, squalene epoxide and ergosterol were recovered from the *R. solani* mycelium. Similar results were obtained when the organisms were incubated in the presence of [2- ^{14}C]mevalonate; in this case, labelled lanosterol was also detected in the *R. solani* mycelium. Both organisms, when incubated in the presence of unlabelled squalene, squalene epoxide or lanosterol, incorporated these compounds into their mycelia; however, only the *R. solani* mycelium was able to convert these substrates into products further along the sterol pathway. It appears that squalene is the terminal compound in the sterol biosynthetic pathway of *P. cinnamomi*.

Fungi are generally considered to be sterol producers. Most fungi make ergosterol as their major sterol component, and many also produce other sterols as minor components (Bergmann, 1953; Weete, 1973, 1974). However, one family in the Phycmycetes, the Pythiaceae, does not produce any sterol (Elliott *et al.*, 1964; Hendrix, 1966; McCorkindale *et al.*, 1969; Schlösser *et al.*, 1969). There have been reports that certain early sections of the sterol biosynthetic pathway are present in members of this family. Hendrix (1966) reported the recovery of label from [^{14}C]acetate in the non-saponifiable-lipid fraction (but not sterol) of *Pythium aphanidermatum* and *Phytophthora palmivora*. Schlösser *et al.* (1969) were able to detect squalene in extracts of four *Pythium* species by means of g.l.c. The conversion of [^{14}C]mevalonate into labelled farnesol by *Phytophthora cactorum* was demonstrated by Richards & Hemming (1972). Hendrix (1974) reported that *Pythium periplocum*, *Pythium sylvaticum* and *Phytophthora parasitica* var. *nicotianae* actively synthesize labelled isoprenoid compounds from [^{14}C]mevalonate. The purpose of the present investigation has been to determine the extent of the sterol biosynthetic pathway in *Phytophthora cinnamomi*, a member of the Pythiaceae, and to compare the pathway present in this organism with that present in a sterol-synthesizing fungus, *Rhizoctonia solani*.

Materials and Methods

Chemicals

Sterols and squalene were from Sigma Chemical Co., St. Louis, MO, U.S.A.; farnesol was from Schwarz-Mann, Orangeburg, NY, U.S.A.; and geraniol was from K and K Laboratories, Plainview, NY, U.S.A. Sodium [2- ^{14}C]acetate (2.0 mCi/mmol) was from New England Nuclear, Boston, MA, U.S.A., and DL-[2- ^{14}C]mevalonic acid lactone (17.5 mCi/mmol) was from Amersham/Searle Corp., Arlington Heights, IL, U.S.A. The labelled mevalonic acid lactone was used without conversion into the acid form. n-Hexane was redistilled and the fraction boiling at 67.2–69.2°C collected. Low-boiling light petroleum was redistilled and the 30–60°C-boiling-point fraction collected. All other chemicals were reagent grade.

Organisms

The non-sterol-synthesizing fungus, *Phytophthora cinnamomi* Rands, no. 150 (Dr. G. A. Zentmyer's isolate no. SB 216-1 from avocado, University of California, Riverside, CA, U.S.A.) was a gift from Dr. J. W. Hendrix, University of Kentucky, Lexington, KY, U.S.A. *Rhizoctonia solani* Kühn, Illinois strain, was selected as the sterol-synthesizing fungus

for comparative experiments because of its prolific mycelial production, its lack of spore formation, and our previous experience with it. (Although a perfect stage is known for this organism, it generally does not occur under usual cultural conditions.)

Media

Potato dextrose agar (Difco Laboratories, Detroit, MI, U.S.A.) was used as the maintenance medium throughout this investigation. All experimental cultures were grown in glucose asparagine (GA) liquid medium of the following composition: D-glucose, 20g; L-asparagine, 2g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g; thiamine hydrochloride, 2mg; 1M- KH_2PO_4 , 6.5ml; 1M- K_2HPO_4 , 3.5ml; salt solution I, 1.0ml; salt solution II, 1.0ml; water, 1 litre. The pH before autoclaving was about 6.4, and after autoclaving, about 6.3. Salt solution I contained per 100ml: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 440mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 40mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 600mg; NaCl, 260mg; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 180mg. Salt solution II contained per 100ml: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 500mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 275mg. The medium was dispensed in 125ml quantities into loosely capped 500ml Erlenmeyer flasks and autoclaved. Supplements were added as indicated in specific experiments.

Cultivation and harvesting of organisms

The fungi were grown on agar medium at 26°C for 3–4 days or until the new cultures were well established, and then either incubated at room temperature (about 22.5°C) or wrapped in aluminium foil and stored at 6°C. Cultures maintained at room temperature were subcultured approximately once monthly; those maintained at 6°C were subcultured once every 6 months. The ergosterol production potential of *R. solani* cultures stored in the dark at 6°C and subcultured infrequently remained high. When subcultures of this organism were stored at room temperature (exposed to room fluorescent lighting for about 12h/day), total ergosterol production decreased and remained low on repeated subculture. Subcultures of the low-producing organism were used in the majority of experiments in this investigation.

Fungi maintained on solid medium were adapted to liquid-culture conditions by the following technique. A small block of established culture was minced in a sterile Erlenmeyer flask containing GA medium and incubated on a reciprocal shaker [6.35cm (2.5in) stroke, 94 strokes/min] at 26°C for 2–4 days or until well grown. The resultant culture was homogenized in a sterile Waring Blender for 30s, and 1.0–5.0ml samples (depending on the density of the homogenate) were delivered into the appropriate number of flasks of fresh medium. For the rapid-growing organism, *R. solani*, inoculum density

was equivalent to 2.5–6.5mg of dry wt./flask; inoculum density for *P. cinnamomi*, the slow-growing organism, ranged from 5 to 15mg dry wt./flask. Cultures were incubated on a reciprocal shaker until harvest. Flasks were removed from the shaker once each day and the contents swirled to dislodge any mycelia adhering to the flask walls. This procedure gave maximum production of mycelia in the desirable form of spherical pellets.

Cultures were harvested by suction filtration on to Whatman no. 1 filter paper in a Buchner funnel. Retained mycelium was washed liberally with water or fresh GA medium and suction-filtered to damp dryness. When dry mycelium was the desired material for an experiment, the damp-dry mycelium was transferred to an aluminium-foil boat and dried overnight in a vacuum oven at 70°C. Dried mycelium was ground to a fine powder and stored under N_2 in a screw-capped vial at room temperature in the dark. For experiments requiring intact mycelium, the damp-dry mycelium was resuspended in appropriate medium or buffer and treated as specified in the particular experiment.

Lipid-extraction procedures

For the recovery of total lipids, 1g of ground dried mycelium was subjected to 3 × 1h stirring extractions with a total of 100ml of chloroform/methanol (2:1, v/v). The extracts were collected by filtration, combined, washed with 3 × 0.2 vol. of 1% (w/v) NaHCO_3 , dried over anhydrous Na_2SO_4 , evaporated to dryness in a tared round-bottom flask *in vacuo* and weighed: 1g of dried mycelium usually yielded about 60mg of total lipids. Extracts were stored under N_2 at room temperature in the dark.

For the extraction of non-saponifiable lipids from mycelia, 1g of ground dried mycelium was saponified with 30ml of 20% (w/v) KOH in 70% (v/v) methanol. For the recovery of non-saponifiable lipids from fluids, such as culture filtrates, 20ml of 30% (w/v) KOH in absolute methanol was added for every 10ml of fluid. The mixture was allowed to reflux under N_2 over a 90°C-water bath for 45min. The saponified mixture was subjected to three shaking extractions (200 strokes each) with a total of 100ml of either the light petroleum or chloroform.

Lipid-fractionation procedures

Silicic acid (100 mesh powder; Mallinckrodt Chemical Works, St. Louis, MO, U.S.A.) was boiled in 6M-HCl for about 2h, washed exhaustively with water, rinsed with methanol, oven-dried, sieved and activated in a 100°C oven for 1h. Activated silicic acid (100–200 mesh, 3.5g) was slowly poured into a column (40cm × 0.5cm internal diam.) containing reagent-grade chloroform, accompanied by tapping

Scheme 1. Protocol for the fractionation of total fungal lipids on silicic acid columns
For details, see the Materials and Methods section.

of the column to effect proper packing. Equilibration with chloroform was continued until the column bed was fully changed from translucent to transparent. Deactivated columns were effectively re-activated several times by clearing the column of remaining
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lipids with absolute methanol, followed by a flush with acetone, and then equilibration with chloroform or n-hexane.
Total lipids were fractionated by a modification of the scheme of Dittmer & Wells (1969). An outline

Table 1. *Typical retention times of relevant neutral lipid compounds on gas chromatographic columns*
For details, see the Materials and Methods section.

Compound	Retention time (min)				
	3% OV-1 column		3% OV-17 column		
	150	230	120	180	275
Geraniol			2.9		
Farnesol	2.0			2.2	
Squalene		3.0			2.5
Squalene epoxide		3.9			
Ergosterol		6.8			8.3
Lanosterol		9.0			10.4

Table 2. *Relative intensities of important peaks in the mass spectra of relevant authentic and suspected compounds*
 m/e , Mass charge; M^+ , molecular ion. For details, see the Materials and Methods section.

m/e	Relative intensity of peak (%)					
	Authentic squalene	<i>R. solani</i> 'squalene'	Combined* 'squalene'	Synthesized squalene epoxide	Authentic ergosterol	<i>R. solani</i> 'ergosterol'
40				94.3		
41	98.5	97.9	93.1		43.2	62.3
42				94.5		
43					37.9	52.6
55	99.3	54.6	86.2	100	70.9	93.9
67	99.5	67.4	98.7	99.9	33.1	46.3
68	100	61.5	95.5	99.7		
69	98.2	98.9	96.7	99.7	100	100
71				96.3		
79				98.3		
81	99.3	100	98.4	98.3	56.8	70.0
93	99.5	68.4	99.5	95.4		
95	99.9	99.8	100	94.4		
107	99.2	49.3	75.6	92.6		
109	99.7	46.4	71.9	91.2		
121	99.9	54.7	85.9	89.7		
135				93.8		
137	100	59.7	93.9			
143					54.8	74.7
145					37.0	50.3
157					44.6	63.4
253					32.0	40.1
363					32.5	40.4
396 (M^+)					8.1	10.3
410 (M^+)	6.2	2.4	3.1			
426 (M^+)				1.1		

* Combined suspected compounds from *Phytophthora cinnamomi*, *Pythium ultimum* and *P. graminicola*.

of the procedure is included in the overall fractionation protocol shown in Scheme 1. Unless otherwise specified, all fractions were evaporated to dryness in tared round-bottom flasks *in vacuo* and weighed. G.l.c. analysis of the complex lipid fraction failed to show the presence of any compounds relevant to the sterol biosynthetic pathway.

For the fractionation of non-saponifiable lipids, both the preliminary column-chromatographic separation of simple lipids from complex lipids and the

procedure for removal of fatty acids were eliminated, since saponifiable lipids were generally retained in the aqueous phase during extraction with organic solvents.

G.l.c.

Gas chromatographic analyses were carried out by means of an F and M model 609 flame ionization gas chromatograph fitted with a model 400 oven.

Silanized glass columns [183 cm (6 ft)] were packed with either 3% (w/w) OV-1 on Gas-Chrom Q (60–80 mesh) or 3% (w/w) OV-17 on Gas-Chrom Q (60–80 mesh) (Applied Science Laboratories, The Anspeck Co., Ann Arbor, MI, U.S.A.). Gas flow rates were: H₂, 35 ml/min; O₂, 220 ml/min; He, 80 ml/min. Both injector port and detector temperatures were maintained at 280°C. Columns were generally operated isothermally, the temperature depending on the compound(s) suspected in the sample. Under these conditions, all compounds of interest were readily detectable without derivatization, and the retention times indicated in Table 1 could be expected.

Mass spectrometry

Mass-spectral analyses were performed either on a Varian-MAT model CH-7 mass spectrometer operated in conjunction with a Varian Aerograph series 1700 gas chromatograph or on a Varian-MAT model CH-5 mass spectrometer. During the gas chromatography-mass spectrometry procedure, a glass column [183 cm (6 ft)] packed with 3% (w/w) OV-17 on Gas-Chrom Q (100–120 mesh) (Applied Science Laboratories) was utilized for separation of sample components before introduction of components into the spectrometer. The model CH-5 spectrometer was utilized for direct probe analysis of halogenated compounds such as squalene hexachloride. Mass-spectral data were processed and plotted by computer. The relative intensities of the most important peaks in the mass spectra of relevant authentic and suspected compounds are listed in Table 2.

T.l.c.

Plastic sheets commercially pre-coated with silica gel (no. 13179 silica gel without fluorescent indicator; Eastman-Kodak Co., Rochester, NY, U.S.A.) were used for t.l.c. analysis of sterols, squalene and terpenoid alcohols. Chromatograms were developed in Brinkmann Sandwich Chambers with heptane/benzene (9:1, v/v) for the separation of squalene and squalene epoxide from other lipid components, and hexane/diethyl ether (7:3, v/v) or benzene/acetone (20:1, v/v) for the separation of sterols, geraniol and farnesol. Where indicated, lipid-containing spots were detected by spraying with 20% (w/v) phosphomolybdic acid in ethanol and heating in a 100°C oven for a few minutes. Chromatograms were analysed by cutting into appropriate strips and scanning with the radiochromatogram scanner, or by scraping spots either into scintillation vials for counting of radioactivity or into test tubes for subsequent elution with light petroleum and analysis as above by g.l.c.

Radioactivity measurements

The radioactive content of samples was measured by liquid-scintillation counting in a Packard Series 3000 Tri-Carb liquid-scintillation spectrometer system using Bray's (1960) fluid as the scintillation cocktail. Throughout the investigation, the minimum value for significant radioactivity in a counted sample was 74 d.p.m (equivalent to twice the background value); values below that were considered not significant.

T.l.c. strips were scanned with a Packard model 7201 radiochromatogram scanner. The quenching gas was a mixture of 98.3% helium and 1.7% butane.

Incubation of mycelia in the presence of sterol precursors

In experiments involving unlabelled precursors, samples (3 g wet wt.) of 3-day-old *R. solani* or *P. cinnamomi* mycelium were added to flasks containing fresh medium to which had been added 50 mg of Tween 80 plus either 0.2 ml of acetone (control) or 0.2 ml of acetone containing approx. 2.5 mg of squalene, squalene epoxide or lanosterol (final concn. about 20 µg/ml). The flasks were incubated on a reciprocal shaker at 26°C for 3 h, after which the mycelium was harvested by suction filtration, washed, dried *in vacuo*, ground and subjected to saponification. Non-saponifiable lipids were extracted with light petroleum, washed, dried and analysed by g.l.c. and t.l.c.

To study the fate of radioactive precursors, flasks of GA medium were inoculated with *R. solani* or *P. cinnamomi* and incubated on a reciprocal shaker at 26°C. After 2 or 3 days, either 0.26 µmol of [2-¹⁴C]-mevalonic acid (17.5 mCi/mmol) or 25 µmol of sodium [2-¹⁴C]acetate (2.0 mCi/mmol) were added per flask. In some experiments, 12 µmol of unlabelled lanosterol were added per flask at the same time as the [¹⁴C]acetate. Incubation was continued for 30 min to 3 h for acetate or 24–77 h for mevalonate, after which the mycelium was harvested as usual. Neutral lipid extracts of the ground dried mycelium were fractionated on silicic acid columns (see under 'Lipid-fractionation procedures'), and the fractions analysed by g.l.c. and t.l.c. Culture filtrates, washes and all steps during the extractions and fractionations of the mycelia were monitored for radioactivity.

Squalene 2,3-oxide synthesis

Squalene 2,3-oxide (squalene epoxide) was synthesized by the procedure of Nadeau & Hanzlik (1969) for use as a reference compound. The crude epoxide was purified by fractional elution with hexane from slightly deactivated silicic acid columns. The epoxide so purified contained a very small amount of squalene (about 1%). Mass spectrometry was used to verify the structure of the epoxide (Table 2).

Squalene hexachloride synthesis

The hexachloride of squalene was synthesized by the general procedure of Tchen (1963) to determine the radiochemical purity of [^{14}C] squalene. Direct-probe mass-spectral analysis was used to verify the structure of the compound.

Results

Growth studies

Since both non-sterol-producing and sterol-producing fungi readily incorporate exogenously supplied sterols into their mycelia (Hendrix, 1970, 1975a,b; Weete, 1974), it was important to ensure that all unsupplemented media used during the present investigation were free of these compounds. No squalene or sterol was detected by means of gas chromatography in either the potato/dextrose/agar

medium used for maintenance of stock cultures or the unsupplemented glucose/asparagine liquid medium used for experimental cultures. Further, no squalene or sterol was detected in any of the culture filtrates of the organisms grown in unsupplemented media in this study.

Growth-curve data from a typical experiment designed to compare squalene, squalene epoxide and ergosterol production during the growth of *R. solani* and *P. cinnamomi* are in Figs. 1 and 2 respectively. The growth curves of both fungi, based on mycelial dry wt./flask, are typically sigmoid, though the rates of growth differed. *R. solani*, a fairly rapid grower, entered stationary phase on day 4 when inoculum equivalent to 5 mg dry wt./125 ml of medium was used. With the same quantity of inoculum, *P. cinnamomi* grew much more slowly, barely entering late-exponential-growth phase by day 6. In other experiments, when inocula equivalent to 2.5 mg dry

Fig. 1. Squalene, squalene epoxide and ergosterol contents and mycelial dry weight during the growth of *R. solani* $\blacktriangle$, Squalene (—, $\mu\text{g/g}$ dry wt.; ----, total $\mu\text{g/flask}$); $\blacksquare$, squalene epoxide (—, $\mu\text{g/g}$ dry wt.; ----, total $\mu\text{g/flask}$); $\bullet$, ergosterol (—, mg/g dry wt.; ----, total mg/flask); $\circ$, mycelial dry wt.

Fig. 2. Squalene content and mycelial dry weight during the growth of *P. cinnamomi*
 ●, Squalene (—, µg/g dry wt.; ---, total µg/flask);
 ○, mycelial dry wt.

wt. for *R. solani* and 15mg dry wt. for *P. cinnamomi* were used, both organisms entered stationary phase by day 6.

Squalene, squalene epoxide and ergosterol synthesis during growth

Detectable quantities of squalene were produced throughout the growth of *R. solani* and *P. cinnamomi* (Figs. 1 and 2 respectively). The notable similarity for the two organisms was the time of peak production of the compound. On the basis of mycelial dry weight (Figs. 1 and 2), peak squalene production occurred at day 1 when both organisms were just entering exponential-growth phase.

R. solani produced detectable quantities of squalene epoxide and large quantities of ergosterol throughout its growth (Fig. 1). On the basis of mycelial dry weight, peak production of squalene epoxide occurred at day 1, thus coinciding with the squalene peak-production time. Peak production of ergosterol by *R. solani* generally occurred during mid-exponential-growth phase (day 2), a day later than that of either squalene or squalene epoxide. This pattern of ergosterol production was consistent for all *R. solani* cultures, whether maintained under conditions of low or high ergosterol production potential. At peak-production time, ergosterol accounted for about 0.35% of the mycelial dry weight in the low-producing organism and about 1.2% in the high-producing organism. On the basis of a mycelial total lipid content

of about 6%, ergosterol comprised about 6% of the total lipid in the mycelium of the low-producing organism and about 20% in the high-producing organism.

No squalene epoxide, ergosterol or any other sterol was detected at any stage during the growth of *P. cinnamomi* (Fig. 2).

Recovery of specific sterol-pathway compounds from mycelia incubated in unsupplemented medium

Squalene was recovered from the mycelia of both the sterol-synthesizing fungus, *R. solani*, and the non-sterol-synthesizing fungus, *P. cinnamomi* (Table 3). In each case, mass-spectral analysis was used to verify the identification of the compound as squalene (Table 2).

The limit of detection of both squalene epoxide and ergosterol by g.l.c. was 5ng per injected sample or, on the basis of the extraction of 1 g of dried mycelium, 2.5µg or 0.00025% of the mycelial dry weight. At this degree of detection, squalene epoxide and ergosterol were found in the mycelium of *R. solani*, but not in the mycelium of *P. cinnamomi* (Table 3).

Recovery of specific sterol-pathway compounds from mycelia incubated in the presence of unlabelled precursors

R. solani mycelium incubated in unsupplemented medium (control) contained its usual complement of squalene, squalene epoxide, lanosterol and ergosterol (Table 4). The mycelium of the non-sterol-producing fungus, *P. cinnamomi*, incubated in unsupplemented medium contained only squalene.

When *R. solani* mycelium was incubated in medium containing squalene, squalene epoxide or lanosterol, not only was the amount of the added precursor in the mycelium increased over that in the control mycelium, but also the amounts of the subsequent compounds in the pathway were increased. *P. cinnamomi* mycelium incubated under the same conditions had an increased content of added precursor, but there was no evidence of compounds formed further along the pathway.

Recovery of specific sterol-pathway compounds from mycelia incubated in the presence of sodium [2-¹⁴C]-acetate

When 2-day-old *R. solani* and *P. cinnamomi* mycelia were incubated for 30min in the presence of sodium [2-¹⁴C]acetate, both the total radioactivity taken up by *P. cinnamomi* (per g dry wt. of mycelium) and the radioactivity incorporated into the neutral lipid fraction of this organism were less than for *R. solani* (Table 5). This occurred in spite of the fact that the quantities of mycelia in the flasks were similar for

Table 3. *Recovery of specific lipids from dried mycelia*
Mycelial age was 3–4 days. N.D., none detected.

Organism	Squalene ($\mu\text{g/g}$ dry wt.)	Squalene epoxide ($\mu\text{g/g}$ dry wt.)	Ergosterol (mg/g dry wt.)
<i>R. solani</i>	6.9	3.3	9.4*
<i>P. cinnamomi</i>	4.7	N.D.	N.D.

* This value is the average for *R. solani* cultures maintained under conditions of high ergosterol production potential; the average of all day 3–4 values for *R. solani* throughout the investigation was 3.7 mg/g dry wt.

Table 4. *Conversion of squalene, squalene epoxide and lanosterol by R. solani and P. cinnamomi mycelia*
Approx. 2.5 mg of precursor was added per flask. N.D., None detected; N.Q., present but not quantified.

Precursor	Compounds analysed in mycelium	Content ($\mu\text{g}/\text{flask}$)	
		<i>R. solani</i>	<i>P. cinnamomi</i>
None	Squalene	2.1	1.1
	Squalene epoxide	30	N.D.
	Lanosterol	N.Q.	N.D.
	Ergosterol	1067	N.D.
Squalene	Squalene	156	184
	Squalene epoxide	107	N.D.
	Lanosterol	N.Q.	N.D.
	Ergosterol	1132	N.D.
Squalene epoxide	Squalene	5.3*	4.3*
	Squalene epoxide	76	91
	Lanosterol	N.Q.	N.D.
	Ergosterol	1128	N.D.
Lanosterol	Squalene	1.9	1.1
	Squalene epoxide	23	N.D.
	Lanosterol	207	268
	Ergosterol	1230	N.D.

* Exogenously supplied squalene epoxide contained a small amount of contaminating squalene.

Table 5. *Distribution of radioactivity in silicic acid-column fractions and sterol-pathway compounds of R. solani and P. cinnamomi mycelia incubated in the presence of sodium [2- ^{14}C]acetate*
Total radioactivity in dried mycelium was calculated by summing the individual radioactivities of the saponified mycelial residue, the bicarbonate wash and the neutral lipid extract. N.S., Not significant.

Fraction or compound	<i>R. solani</i>		<i>P. cinnamomi</i>	
	($10^{-3} \times \text{d.p.m.}/\text{g}$ dry wt.)	(mg/g dry wt.)	($10^{-3} \times \text{d.p.m.}/\text{g}$ dry wt.)	(mg/g dry wt.)
Dried mycelium	35400	1000	20400	1000
Neutral lipid extract	18800	34.3	2310	21.8
Hexane	6960	9.3	184	2.6
Squalene	5.6		2.7	
15% (v/v) Benzene/hexane	7670	14.1	1710	11.6
Squalene epoxide	2.8		N.S.	
5% Diethyl ether/hexane	1020	2.6	129	2.3
Geraniol	33.1		14.0	
Farnesol	41.8		20.1	
Ergosterol	502		N.S.	
15% Diethyl ether/hexane	730	1.4	65.4	1.5
Methanol	1930	5.0	123	3.3

both organisms and the incorporation conditions comparable. In addition, the distribution of label among the various silicic acid-column fractions differed markedly for the two organisms. For example, there was a 38-fold difference in the incorporation of radioactivity into components of the hexane fractions of the two organisms, but only an 8-fold difference in the radioactive contents of the fractions eluted with 5% (v/v) diethyl ether/hexane.

T.l.c. separation of the hexane fractions of both organisms yielded squalene and a number of unidentified components. Definitive proof that the squalene in each case was significantly labelled, and had therefore been derived from the labelled acetate, was obtained by preparing the hexachloride of the compound and crystallizing it to constant specific radioactivity. Gas chromatographic comparison of the experimentally prepared hexachloride with authentic squalene hexachloride verified the identity of the squalene isolated from the hexane fraction of each organism. In both organisms the label in the squalene accounted for only a small portion of the total radioactivity in the hexane fractions.

Similarly, both fungi produced significantly labelled geraniol and farnesol, presumably as pyrophosphates, from the labelled acetate. These compounds were identified in the fraction of each organism eluted with 5% (v/v) diethyl ether/hexane (Table 5), one fraction earlier than expected. This change in elution pattern occurred whenever the hexane used in the elution solvents contained components in a higher boiling range than 67.2–69.2°C, or whenever the silicic acid in the column bed had not been fully re-activated.

R. solani synthesized labelled squalene epoxide, found in the 15% (v/v) benzene/hexane fraction of this organism, and labelled ergosterol, the major labelled component of the 5% (v/v) diethyl ether/hexane fraction. *P. cinnamomi*, however, did not produce either squalene epoxide or ergosterol from labelled acetate.

A comparable experiment involving labelled acetate was modified by using 3-day-old *R. solani* and *P. cinnamomi* mycelia, incubating the former for 30 min and the latter for 3 h. Under these conditions, the quantity of label appearing in the neutral lipid extract of the *P. cinnamomi* mycelium was similar to that in the corresponding extract of the *R. solani* mycelium. However, the total radioactivity taken up by *P. cinnamomi* (per g. dry wt. of mycelium) was much more than that for *R. solani*.

When the neutral lipids of both organisms in this experiment were fractionated on silicic acid columns, the hexane fraction of *P. cinnamomi* contained 8-fold more radioactivity than did the corresponding *R. solani* fraction. However, there was close similarity in radioactive content in the corresponding 15% benzene/hexane, 5% diethyl ether/hexane, 15% diethyl ether/hexane and methanol fractions of the

two organisms. Nevertheless, when the corresponding fractions of both organisms were compared by scanning radiochromatography, there were large differences in the number, size and distribution of peaks of radioactivity. Only those peaks representing geraniol, farnesol, squalene, squalene epoxide and ergosterol were positively identified by both t.l.c. and g.l.c. The first three compounds were present in the appropriate fractions of both organisms; squalene epoxide and ergosterol were present in *R. solani* fractions but not in *P. cinnamomi* fractions.

In several experiments unlabelled lanosterol was added to *R. solani* cultures (12 µmol per flask) at the same time as the labelled acetate in an attempt to cause accumulation of squalene and perhaps enhance the possibility of labelling the squalene. In no case did the addition of lanosterol result in an accumulation of squalene; instead, the uptake and distribution of label by these cultures was much the same as for those without added lanosterol.

Recovery of specific sterol-pathway compounds from mycelia incubated in the presence of [2-¹⁴C]mevalonic acid

When labelled mevalonic acid was added to the medium at the time of inoculation of *R. solani* and *P. cinnamomi* cultures, both the total radioactivity taken up by *P. cinnamomi* (per g dry wt. of mycelium) and the radioactivity incorporated into the neutral lipid extract of this organism were less than for *R. solani*. In addition, when the neutral lipid extracts of both organisms were fractionated on silicic acid columns, there were marked differences in the quantities of radioactivity distributed in the corresponding fractions of the two organisms.

T.l.c. separations of the corresponding fractions of the two organisms provided results similar to those obtained when the mycelia were incubated in the presence of labelled acetate; that is, both organisms contained labelled geraniol, farnesol and squalene. However, only *R. solani*, and not *P. cinnamomi*, synthesized labelled squalene epoxide, lanosterol and ergosterol from labelled mevalonic acid.

Discussion

Generally a close relationship exists between the growth cycle of an organism and its various biosynthetic pathways. Optimum production of both squalene and squalene epoxide by *R. solani* occurred just as the organism was entering exponential-growth phase (Fig. 1). This was generally a day earlier than the peak-production time for ergosterol. The failure of squalene synthesis to remain at peak production throughout the remainder of the growth cycle probably reflects the inability of the synthetic process to keep up with the rapid utilization of squalene for ergosterol production. Therefore the squalene

content dropped to a constant lower value for as long as ergosterol was being produced. The peak-production time in *P. cinnamomi* also occurred just as the organism was entering exponential-growth phase (Fig. 2); a drop in squalene production occurred shortly thereafter. In this case, however, there was no ergosterol synthesis occurring that would use up the squalene as it was produced. Instead, the failure of squalene synthesis to remain at peak production may indicate that a squalene-degradation process became operational at some time during the peak-production period.

A question of special relevance in the current investigation was whether squalene is indeed synthesized by *P. cinnamomi*, a member of the Pythiaceae. The recovery of label in the squalene of both *R. solani* and *P. cinnamomi* incubated in the presence of either labelled acetate or labelled mevalonate indicates that the squalene was, in fact, produced biosynthetically by both organisms. There were no apparent differences in the components involved in the central (mevalonate-to-squalene) portion of the pathway in

the two organisms, and probably none in the components involved in the early (acetate-to-mevalonate) portion of the pathway since labelled acetate yielded the same labelled intermediates as labelled mevalonate.

The enhanced amounts of squalene epoxide and ergosterol in the *R. solani* mycelium incubated in the presence of unlabelled squalene over those in the control mycelium suggests that at least a portion of the squalene taken up by this organism was converted into squalene epoxide, and a portion of the latter compound into ergosterol (Table 4). Similarly, the enhanced ergosterol in the *R. solani* mycelium incubated in the presence of either unlabelled squalene epoxide or unlabelled lanosterol suggests that at least a portion of either compound taken up by this organism was converted into ergosterol. Although all three unlabelled compounds were taken up by the *P. cinnamomi* mycelium, there was no evidence of conversion of any of the compounds into intermediates that would normally occur later in the pathway of a sterol-synthesizing organism.

Scheme 2. Sterol biosynthetic pathway in *R. solani* and *P. cinnamomi*
For details of determining the extent of the pathway in the two organisms, refer to the text.

Moreover, incubation of intact mycelia of the two organisms in the presence of either labelled acetate (Table 5) or labelled mevalonate resulted in the recovery of label in squalene epoxide, lanosterol and ergosterol in the case of *R. solani* mycelium; these three compounds were not detected by any means in *P. cinnamomi* mycelium incubated under the same conditions. The difference in the ability of the two organisms to convert squalene into squalene epoxide is the first indication of a difference in components of the sterol pathway in sterol-producing and non-sterol-producing fungi.

Thus it appears that *R. solani* possesses the full sterol biosynthetic pathway from acetate to ergosterol, but that squalene is the terminal compound in the sterol biosynthetic pathway of *P. cinnamomi* (Scheme 2). Apparently, *P. cinnamomi* is unable to carry out the reactions involving the epoxidation of squalene, the cyclization of squalene epoxide, and the conversion of lanosterol into ergosterol.

Schlösser *et al.* (1969) reported the detection of squalene by g.l.c. in each of four species of *Pythium*. However, their attempt to demonstrate the incorporation of label from [¹⁴C]acetate into the squalene of *P. graminicola* was unsuccessful. This may have occurred because the organism was incubated in medium that contained sodium acetate of high concentration, but low specific radioactivity. Assuming that the amount of squalene present in their organism was similar to that of either organism in the present study (4.7–6.9 µg/g dry wt.), calculation of the final specific radioactivity, after dilution with carrier squalene and conversion into the hexachloride, indicates about 10 d.p.m./mg of their hexachloride, a non-significant and essentially non-detectable amount.

Richards & Hemming's (1972) inability to demonstrate the presence of squalene in *Phytophthora cactorum* 21168 may have been due to the age of their cultures at the time label was added and at the time of harvest, and/or to the relatively insensitive t.l.c. technique. In the present investigation, it was found that the labelled precursor must be added early enough in the growth cycle to be incorporated into the compound in question during maximum synthesis of the compound; further, the labelled mycelia should be harvested reasonably soon after this period of high production. In addition, it was found that compounds such as squalene were detectable at 2 ng per injected sample by means of g.l.c.; t.l.c., on the other hand, required at least 100-fold higher concentrations for colour-detection procedures.

There is some evidence that the pythiaceae fungi are able to alter exogenously supplied sterols. Several investigators (Hendrix *et al.*, 1970; Elliott & Knights, 1974; Gain, 1972; Hendrix, 1970, 1974, 1975a,b) have reported that some of the sterol taken up by these fungi is esterified and some is converted into more polar metabolites. More recently, Knights & Elliott

(1976) have demonstrated the ability of *Phytophthora cactorum* to alter a portion of the double-bond structure of the sterol molecule itself. They conclude that the organism slowly converts Δ^7 -sterols into $\Delta^{5,7}$ -sterols and rapidly converts the latter into Δ^5 -sterols. It is possible therefore that the pythiaceae fungi are able to carry out some of the interconversion reactions between lanosterol and ergosterol, but not the full conversion of lanosterol into ergosterol.

In the present study, there were large differences in the number, size and distribution of labelled peaks in the radiochromatographic scans of corresponding neutral lipid fractions from *R. solani* and *P. cinnamomi* mycelia incubated in the presence of [¹⁴C]acetate. Moreover, a major portion of the ¹⁴C from both acetate and mevalonate in *P. cinnamomi* appeared in the 15% (v/v) benzene/hexane fraction, which contained no detectable compounds relevant to the sterol biosynthetic pathway (Table 5). Identification of the labelled components of these fractions would provide additional insight into the nature of the various pathways involving these precursors. Some progress in this direction has been reported from Hemming's laboratory. Stone & Hemming (1967) identified squalene, ergosterol, ubiquinone-10, and a number of hexahydronols as metabolites of labelled mevalonate in *Aspergillus fumigatus*, a sterol-synthesizing fungus. Richards & Hemming (1972), using *Phytophthora cactorum* 21168, a non-sterol-synthesizing fungus, demonstrated the conversion of labelled mevalonate into farnesol, geranylgeraniol, ubiquinones-9 and -10, and several dolichols. Continued study in this area would probably point up additional differences between the sterol-synthesizing and the non-sterol-synthesizing fungi, and might provide information regarding the evolutionary development of these groups of fungi.

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