Phytochemistry, Vol. 27, No. 8, pp. 2567–2580, 1988.
Printed in Great Britain.

0031–9422/88 $3.00+0.00

CO-ORDINATED INHIBITION OF SQUALENE SYNTHETASE AND INDUCTION OF ENZYMES OF SESQUITERPENOID PHYTOALEXIN BIOSYNTHESIS IN CULTURES OF *NICOTIANA TABACUM**

DAVID R. THRELFALL and IAN M. WHITEHEAD

Department of Plant Biology and Genetics, University of Hull, Hull HU6 7RX, U.K.

(*Received* 4 *February*, 1988)

IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Nicotiana tabacum*; Solanaceae; cell suspension culture; cell-free-synthesis; sesquiterpenoid phytoalexins; elicitation; inhibition of metabolism; inhibition of squalene synthetase; FPP-carbocyclase.

Abstract—Treatment of log-phase cell suspension cultures of *Nicotiana tabacum* with the biotic elicitor cellulase completely inhibited cellular growth within a few hours and caused a rapid accumulation of the sesquiterpenoid phytoalexins capsidiol and debneyol. Acetosyringone (an unrelated phenolic compound) was also produced by the elicited cultures. Capsidiol levels fell to low concentrations after the accumulation period had ceased whereas the level of debneyol was maintained and that of acetosyringone was bi-phasic. Growth of the elicited cultures resumed after 24 hr and paralleled that of control cultures. When capsidiol or debneyol were supplied to unelicited cultures they were rapidly catabolized to low levels. Elicitor treatment inhibited the catabolism of capsidiol within three hr but did not affect the metabolism of debneyol. A cell-free preparation obtained from cells 12 hr after elicitation efficiently catalysed the formation of two uncharacterised sesquiterpenoids (one of which [unknown I] may be an eremophilane hydrocarbon) when incubated with either [1-^{14}C]IPP or [1-$^{3}H_2$]FPP as substrate. In the presence of NADPH and O_2, the cell-free system catalysed the formation of capsidiol and debneyol with the amount of unknown **I** being proportionately reduced. None of the above biosynthetic capabilities were present in cell-free extracts from unelicited cells. The total amount of radioactivity incorporated into capsidiol, debneyol and the two unknowns provided an indirect estimate of the activity of FPP-carbocyclase, the enzyme catalysing the first committed step in the biosynthesis of these compounds. The time course of the rate of synthesis of capsidiol and debneyol in cell-free preparations showed that the synthesis of the phytoalexins was detectable within two hours after elicitation of the cultures with cellulase. Thereafter, the rate of synthesis of both phytoalexins rose rapidly with the maximum rate for each phytoalexin preceding the maximum level of its accumulation. The synthesis of unknowns **I** and **II** was detectable at all time points after elicitation. Squalene synthetase (EC 2.5.1.21) activity underwent a rapid decline over the first six hr period of elicitation and remained low until about 48 hr. The apparent K_m values (FPP as substrate) for squalene synthetase from unelicited cultures and for capsidiol, debneyol, unknown **I** and unknown **II** biosynthesis in elicited cultures were 10, 2, 12, 10 and 10 μm respectively.The rapid inhibition of squalene synthetase in elicited cultures may operate to channel FPP away from sterol biosynthesis (which is required for cellular growth) and towards the FPP-carbocyclase involved in the biosynthesis of the two sesquiterpenoid phytoalexins or may be part of a more general response of plant cells to treatment with elicitors. The levels of debneyol which accumulated in the cultures in response to cellulase were lower than those of capsidiol. These differences are thought to be due to the inhibitory effect of cellulase on the catabolism of capsidiol but not that of debneyol. The results indicate that a co-ordinated sequence of changes involving the regulation of several enzymic activities occurs in tobacco cultures upon elicitation. Studies on the regulation of sterol biosynthesis by elicitor treatment may have wider implications for other eukaryotic systems.

INTRODUCTION

Carbocyclic sesquiterpenoid phytoalexins are accumulated in suspended callus and/or cell suspension cultures of pepper, potato, Thorn Apple and tobacco which have been treated with a suitable biotic (eg. fungal spores [1], fungal cell wall fractions [2–4], cellulase [4–6]) or abiotic (metal ions [3]) elicitor. The very rapid accumulation of these phytoalexins in elicitor-treated cell suspension culture of solanaceous plants means that these systems, which between them produce examples from all three biosynthetic pathways (Scheme 1) are ideal for studying the regulation of the biosynthesis of these compounds. Recent biosynthetic studies with both potato [7, 8] and sweet pepper cell suspension cultures [DRT, unpublished results] have been hampered by the gradual loss of the ability of the cultures to respond to elicitation and accumulate phytoalexins. It is now clear that tobacco

Abbreviations: FPP, (2*E*,6*E*)-farnesyl pyrophosphate; IPP, isopentenyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, (3*R*)-mevalonic acid.

*This paper is dedicated to the memory of Professor Tony Swain, formerly Honorary Professor of this department, who died so tragically on 25 September 1987. Tony was a true scholar, an ardent scientist, a good companion and a staunch friend. He will always be remembered with warmth and affection by his many friends at Hull.

[2, 3, 6] and Thorn Apple [3] cultures continue to accumulate sesquiterpenoid phytoalexins in response to elicitation even after frequent sub-culture (in the case of our tobacco cultures, more than 300 times over six years). These two systems are therefore emminently suitable for the study of the enzymes concerned with the biosynthesis of vetispirane and eremophilane phytoalexins (Scheme 1).

We have restricted our studies to the elucidation and regulation of the post-FPP reactions of sesquiterpenoid phytoalexin biosynthesis since this area represents the unique part of the biosynthetic pathway(s). Others are investigating the regulation of the acetate to FPP part of the pathway at the level of HMG-CoA reductase [2, 9, 10].

Previous work from this laboratory has shown that a cell-free system which catalyses the incorporation of radiolabel from [1-^{14}C]IPP or [1-$^{3}H_2$]FPP into the vetispirane lubimin can be prepared from potato tuber tissue which has been treated with spores of *Phytophthora infestans*, an elicitor preparation of the same fungus or arachidonic acid [11]. Feeding experiments with [2-^{14}C]MVA (at saturating concentrations for sterol biosynthesis) have provided good evidence that the incorporation of FPP into sesquiterpenoid phytoalexins in potato cell suspension cultures inoculated with zoospores of *P. infestans* is accompanied by a concomitant inhibition of its incorporation into sterols at the level of squalene synthetase [7, 8].

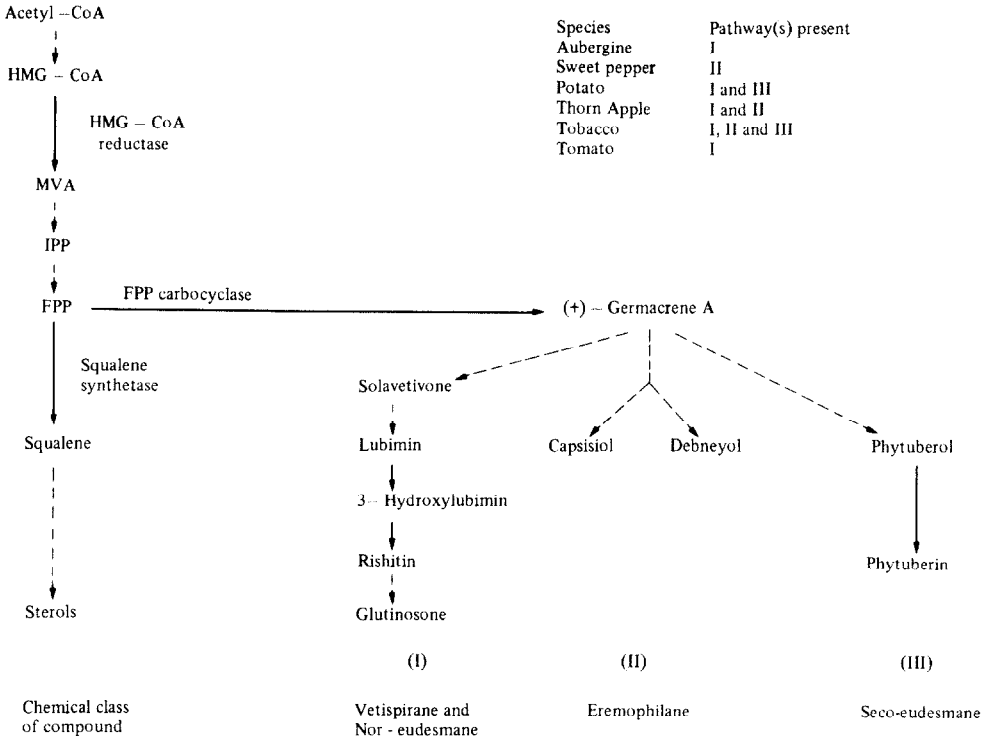
Any study of the regulatory processes involved in the accumulation of phytoalexins should include an assessment of the importance of turnover. In elicited cell suspension cultures of tobacco [2, 3], potato [8] and pepper [DRT, unpublished results] the rapid accumulation of phytoalexins is followed by an almost equally rapid disappearance. Several biotransformations of sesquiterpenoid phytoalexins supplied exogenously to healthy cultures have been reported [12–14], although there is little known about the metabolism of endogenously produced sesquiterpenoid phytoalexins [15].

In this paper we report on the elicitation, accumulation and metabolism of two of the eremophilane class of phytoalexins in cell suspension cultures of tobacco (*Nicotiana tabacum* var. White Burley) in response to elicitation with cellulase. We also describe for the first time, the successful preparation of a cell-free system capable of catalysing the formation of this class of sesquiterpenoid phytoalexin. In addition to this we have shown that the onset of phytoalexin biosynthesis is accompanied by the inhibition of squalene synthetase activity.

RESULTS

Activity of elicitors

The following compounds (final concentrations in the media are indicated in parenthesis) did not elicit the formation of any sesquiterpenoid phytoalexins when added to five-day-old cell suspension cultures of tobacco for 16 hr: arachidonic acid (25 μM); *Phytophthora infestans* sonicate (1 ml/flask); pectinase (Ex. *Aspergillus niger*) (1 mg/flask); $CuSO_4$, $NiSO_4$, $Pb(NO_3)_2$ and $NaAuCl_4$ (0.1–10 mM). Only cellulase (Ex. *Trichoderma viride*) was found to be positive (0.025–1 mg/100 ml).



Scheme 1. Pathways of sesquiterpenoid phytoalexin biosynthesis and their distribution in some economically important solanaceous plants.

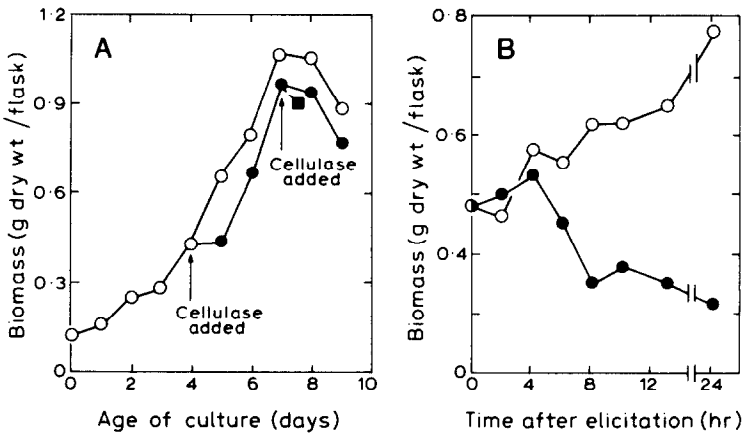


Fig. 1. Effect of cellulase on the growth of tobacco cell suspension cultures. A. General effects. B. Detailed effect on biomass production after the first cellulase treatment. ○-○, Untreated; ●-●, first cellulase treatment; ●---■, second cellulase treatment.

Growth of control and cellulase-elicited cultures

The growth curves of both control and cellulase-elicited cultures are shown in Fig. 1. The control cultures showed a typical sigmoidal increase in dry weight with a growth index (GI, final dry wt/initial dry wt) of 9. The growth of the cellulase-elicited cultures was completely inhibited by the addition of the elicitor for *ca* 24 hr. After this time the growth of the cultures resumed and paralled that of the control cultures although never achieving the same maximum biomass.

The treatment of the tobacco cultures with cellulase did not cause any apparent cell death. As shown in Fig. 1 the treated cultures grew well after the initial inhibition and vital staining with fluorescein diacetate of cellulase-treated cells indicated 100% viability at 24 and 48 hr after treatment.

The cellulase-treated cultures rapidly changed colour from very pale brown to a deeper yellow-brown within two hr of treatment. This colour then faded over the next few hours but the cultures were still darker than the controls even after five days. The compounds responsible for this colouration were located mainly in the culture medium and are probably phenolic in nature.

Re-treatment of an elicited culture after four days with a further aliquot of cellulase (see Fig. 1A) did not re-elicit the formation of any secondary products. However, the cultures rapidly became yellow-brown and then faded as before. We did not test to see if elicitor-treated cultures recover the ability to produce secondary products upon sub-culture.

Accumulation of phytoalexins and other compounds in response to cellulase treatment

A typical time course for the accumulation of the sesquiterpenoid phytoalexins capsidiol and debneyol in the cultures after treatment with cellulase is shown in Fig. 2A. Although these two compounds are biogenically closely related, and may be derived from the same hydrocarbon precursor (see Scheme 2 and [6]) there are several interesting differences between the time courses. Capsidiol accumulated rapidly to about three times the level of debneyol. However, after the accumulation phase had ceased, capsidiol was rapidly metabolised to very low levels whereas the levels of debneyol fell only slowly. By 72 hr neither phytoalexin could be detected in the cultures. The debneyol levels quoted in Fig. 2A probably include small amounts of 7-*epi*-debneyol (*ca* 9%) which co-chromatographs with debneyol under the conditions employed [6].

The time course for the accumulation of acetosyringone after treatment with cellulase is shown in Fig. 3. The accumulation of acetosyringone in cellulase-treated tobacco cultures has not been reported before. The compound accumulated in two phases, the first coinciding with that of capsidiol and the second after 48 hr.

None of the above mentioned compounds could be detected in untreated control cultures, in cultures treated with autoclaved cellulase or in resuspended freeze/thaw-killed cells treated with cellulase. The addition of a substrate amount for sterol synthesis of MVA to the elicited cultures had no effect on the levels of phytoalexins accumulated in the culture.

Fungitoxicity assays

Acetosyringone was found to be inactive when tested for fungitoxicity by the mycelial growth assay method described in [16] against four fungal species using capsidiol as a control (Table 1).

Studies on the metabolism of phytoalexins by the cultures

The time-course for the accumulation of phytoalexins in the cellulase-elicited tobacco cultures indicated that capsidiol was rapidly lost after the accumulation phase had ceased whereas the level of debneyol was maintained (see Fig. 2A). These differences were examined by comparing the ability of both unelicited and elicited cultures to catabolise the two sesquiterpenoid phytoalexins.

In the first experiment, the relevant compound (1 mg capsidiol or 0.5 mg debneyol/culture) in 0.5 ml ethanol was added to unelicited six-day-old cultures. After the stated times, the cultures were harvested and analysed as described in the Experimental. The results show that

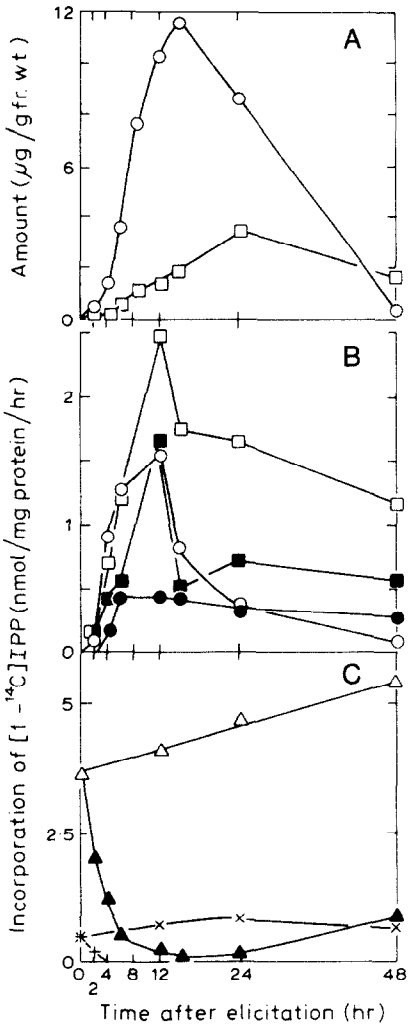


Fig. 2. Time course of changes in the rate of formation of squalene, capsidiol, debneyol and other terpenoids from [1-^{14}C]IPP in five-day-old tobacco cell suspension cultures. A. Amounts of capsidiol and debneyol isolated from culture filtrates of elicited cultures. B. Rate of formation of capsidiol, debneyol, unknown I and unknown II from [1-^{14}C]IPP by cell-free systems from elicited cultures. C. Rate of formation of squalene and farnesol from [1-^{14}C]IPP by cell-free systems from elicited and unelicited cultures. The cell-free systems were prepared by method B. Each assay was performed in the presence of an NADPH-generating system. ○-○ Capsidiol; □-□, debneyol; ●-●, unknown I; ■-■, unknown II; △-△, squalene (unelicited); ▲-▲, squalene (elicited); ×-×, farnesol (unelicited); +—+, farnesol (elicited).

both capsidiol (Fig. 4A) and debneyol (data not shown) are rapidly catabolized by unelicited cultures. The unelicited cultures converted part of the capsidiol provided into 3-*O*-acetylcapsidiol. However, the total level of sesquiterpenoid material (i.e. capsidiol + 3-*O*-acetylcapsidiol) fell with time, indicating that the acetate was itself metabolized. The possiblity that the acetate was an intermediate on the catabolic pathway of capsidiol in this

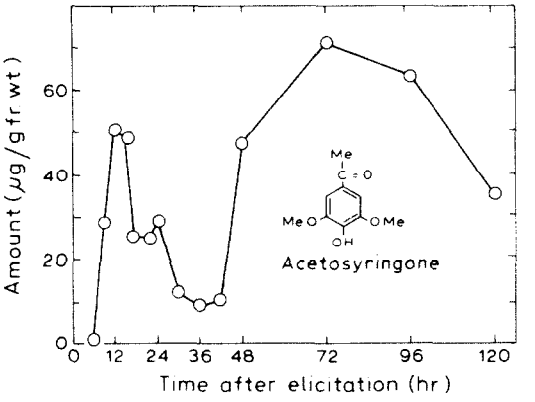


Fig. 3. Time course showing the levels of acetosyringone in the culture medium after treatment of four-day-old tobacco cell suspension cultures with cellulase.

system was tested by feeding some of the 3-acetate (synthesized chemically from capsidiol, see Experimental) to unelicited cultures as above. The results indicated that the acetate was converted back (in part) to capsidiol although the total amount of sesquiterpenoid material decreased with time as before. It thus appears that the two compounds (capsidiol and the 3-acetate) are in some form of equilibrium and that one or even both compounds are catabolised by the unelicited cultures (see Discussion). No catabolites of debneyol were detected. The possibility that glycosylation could account for the observed loss in recoverable capsidiol or debneyol (by ether extration) was investigated by treating ether-extracted media samples from the feeding experiments with β-glucosidase. However, no aglycone (capsidiol or debneyol) was recoverable after this treatment (see Experimental). As in the elicited system, only 10% of the total sesquiterpenoid material was located in the cells from the cultures.

It is apparent from a comparison of the results of the time-course of phytoalexin accumulation (Fig. 2A) and the metabolic studies with unelicited cultures (Fig. 4A)

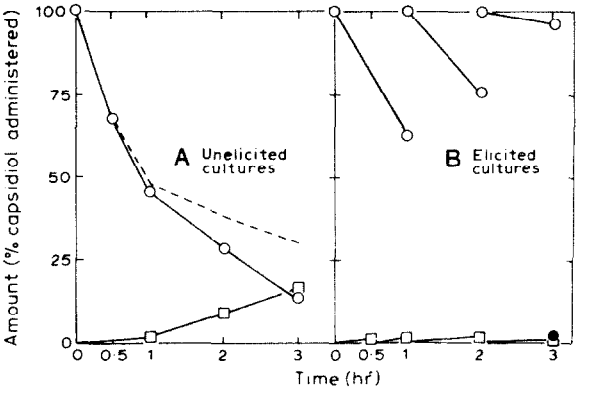
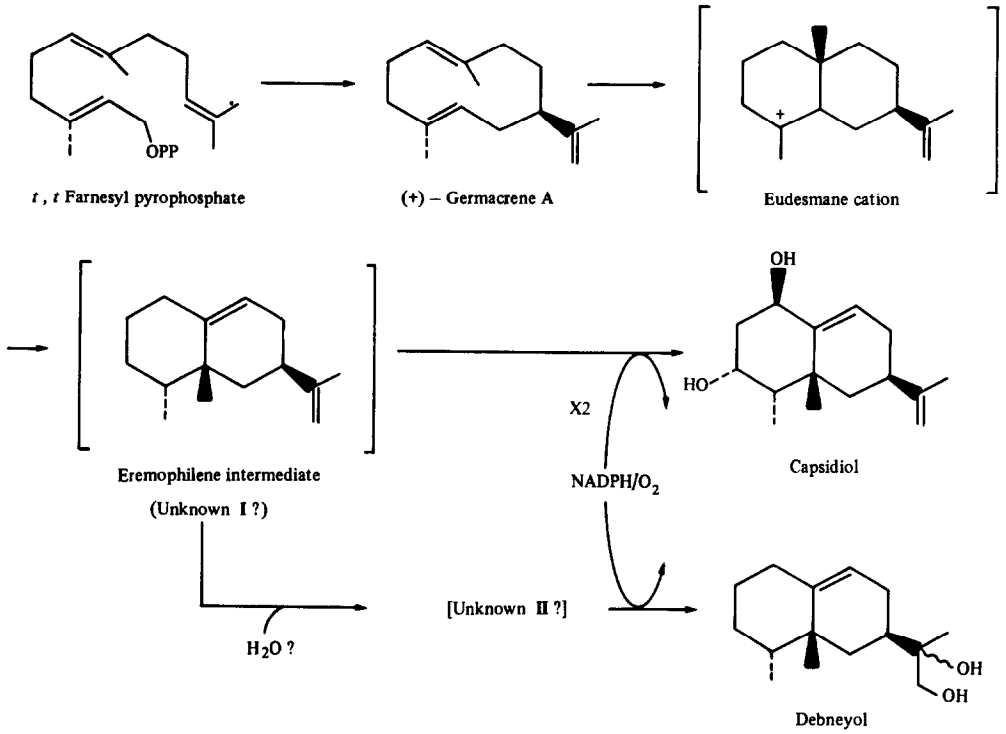


Fig. 4. Loss of exogenously supplied capsidiol (1 mg/culture) from the medium of five-day-old unelicited (A) and elicited (B) tobacco cell suspension cultures. ○-○, Capsidiol; □-□, 3-O-acetylcapsidiol; -----, capsidiol plus 3-O-acetylcapsidiol; ●, level of capsidiol in control (elicited) culture.



Scheme 2. Possible route for the biosynthesis of capsidiol and debneyol from farnesyl pyrophosphate in tobacco.

Table 1. Assay of mycelial growth inhibition of fungi by capsidiol and acetosyringone

	Capsidiol ED_{50} (μM)*	Inhibition of mycelial growth (%)					
		Capsidiol (mM)			Acetosyringone (mM)		
		0.01	0.1	1.0	0.01	0.1	1.0
Cladosporium herbarum	20	37	91	100	6	11	19
Rhizoctonia solani	40	12	41	100	2	6	28
Trichoderma viride	240	13	23	82	−9	9	14
Botrytis cinerea	260	8	13	100	−7	3	57

*Values taken from ref. [16].

that the elicited cultures could only accumulate substantial levels of either capsidiol or debneyol if there is either (i) massive *de novo* synthesis of the phytoalexins or (ii) a rapid inhibition of the activity catabolizing these two compounds at the onset of phytoalexin synthesis. These possibilities were investigated by feeding either capsidiol or debneyol to cellulase-elicited cultures for short periods at various times after elicitation. The results (Fig. 4B) show clearly that the rate of metabolism of capsidiol by cellulase-elicited cultures starts to decrease after one hour and reaches zero between two and three hr after elicitation, which just precedes the time at which capsidiol first starts to accumulate. The ability of the cultures to accumulate relatively high levels of capsidiol is thus facilitated by the rapid inhibition of the catabolism of this compound. However, debneyol was still catabolized in the elicited system (data not shown) and this may explain why relatively low levels of this compound accumulate. A control experiment demonstrated that the formation of free capsidiol from the 3-*O*-acetylcapsidiol was catalysed by the cells and not by a contaminating enzyme activity in the cellulase preparation used to elicit the cultures. This showed that the inhibition of capsidiol catabolism by cellulase treatment was due to an effect of cellulase on the cells and was not due to the hydrolysis of a possible catabolic intermediate, i.e. the 3-acetate.

Preparation and properties of cell-free systems capable of the biosynthesis of squalene, capsidol and debneyol from either [1-^{14}C]*IPP or* [1-$^{3}H_2$]*FPP*

In a preliminary investigation, cell-free systems prepared from unelicited and cellulase-elicited tobacco cells by the method used for the successful demonstration of the biosynthesis of lubimin from IPP by cell-free extracts of elicitor treated potato tuber tissue [11] were incubated with 0.2 μCi [1-^{14}C]IPP (55 μCi/μmol) in the presence or absence of an NADPH-generating system for 90 min.

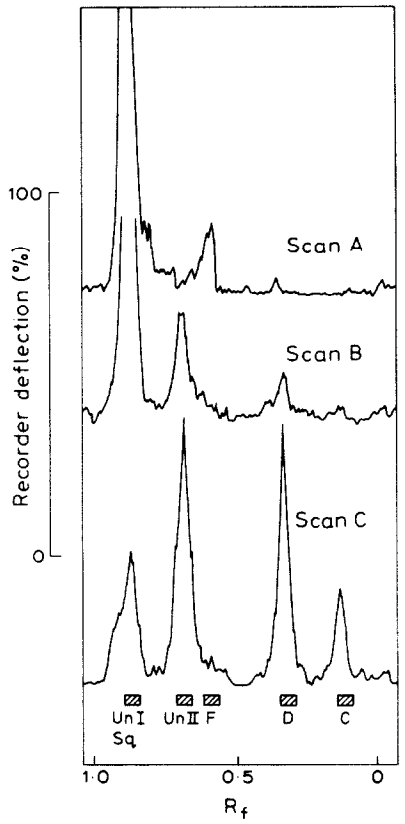


Fig. 5. Radioscans of a thin-layer chromatogram (developed with solvent A) of the chloroform-soluble lipids isolated after incubation with [1--^{14}C]IPP of cell-free preparations from either an unelicited tobacco culture in the presence of an NADPH-generating system (Scan A) or from an elicited tobacco culture in the absence (scan B) or presence (scan C) of an NADPH-generating system. The cell-free preparations were obtained by method A. The elicited culture had been treated with cellulase for nine hr. TLC in solvent C confirmed that no radioactive squalene was present in either sample obtained with the cell-free preparation from the elicited culture. C, capsidiol; D, debneyol; F, farnesol; Sq, squalene; Un I, unknown I; Un II, unknown II.

The incubations were terminated by the addition of a chloroform–methanol mixture and the chloroform-soluble compounds isolated, assayed for radioactivity and examined by radio-TLC (Fig 5).

Each of the cell-free incubations incorporated some 75% of the radioactivity administered in the form of [1-^{14}C]IPP into chloroform-soluble compounds. The NADPH-deficient incubation of the cell-free system from unelicited cells, in keeping with the requirement of squalene synthesis for this co-factor, contained very little radioactivity and was not examined further.

The chloroform extract from the NADPH-containing incubation of the cell-free system prepared from unelicited cells gave two peaks on radio-TLC (Fig 5A). These co-chromatographed with squalene (major peak) and farnesol respectively in all TLC systems tested (see next section).

The chloroform extract from the NADPH-containing incubation of the cell-free extract from elicited cells gave four major peaks on radio-TLC (system A), three of which were co-incident with squalene (this area contained unknown **I**, see below), capsidiol and debneyol respectively whilst the fourth compound (unknown **II**) ran ahead of farnesol (Fig. 5C). The greater part of the radioactivity co-incident with squalene in system A ran with an unknown compound (Unknown **I**) when the extract was subjected to TLC in system C and the peaks located by radio-scanning as above (unknown **I** R_f 0.61; squalene R_f 0.38); the remainder co-migrated with squalene. The extract from the NADPH-deficient incubation contained the same amount of radioactivity as the extract from the NADPH-containing incubation and gave two major peaks corresponding to unknowns **I** and **II** and two minor peaks which were co-incident with capsidiol and debneyol (Fig. 5B).

Radio-GLC of the radioactive material from under the two peaks corresponding to capsidiol and debneyol showed that at least 89 and 67% respectively of the activity eluted from the TLC plate was associated with these two phytoalexins. This was further confirmed by radio-TLC and radio-GLC of the acetates of the phytoalexins and of the isopropylidene derivative of debneyol (see Experimental).

A similiar set of results to those just described above was obtained when the [1-^{14}C]IPP was replaced by 1 μCi [1-^{3}H]FPP (40 μCi/μmol)(see later). The most notable difference was that there was a proportionately greater incorporation of radioactivity into unknowns **I** and **II** and farnesol.

The above results demonstrated that cell-free preparations of unelicited cells were capable of supporting the NADPH-dependent synthesis of squalene and that the NADPH-supplemented cell-free preparations from elicited cells, in addition to very small amounts of squalene, produced at least four compounds unique to this (elicited) system, two of which were identified as capsidiol and debneyol. Unknowns **I** and **II** were assumed to be sesquiterpenoid hydrocarbons (probably carbocylic) or possibly in the case of **II**, a hydrated (carbocylic) sesquiterpenoid, since they were both formed from FPP in the absence of molecular oxygen and NADPH (see Table 2).

Cell-free synthesis of capsidiol and debneyol. In subsequent experiments it was shown that the presence of a high concentration of mercaptoethanol (other thiol reagents not tested) and of either soluble PVP or insoluble PVP in the extraction buffer were essential to the preparation of a biosynthetically active cell-free system (data not shown). The inclusion of a gel filtration step led to an increase in the rates of terpenoid synthesis in the preparations containing soluble PVP (Table 2), almost certainly due to the replacement of the extraction buffer containing a high concentration of EDTA by the incubation buffer. This step however, had no effect on the activity of the cell-free system prepared with insoluble PVP and double-strength column/incubation buffer (pH 7.5) (Table 2). This latter finding established that the preparations contained only small pools of endogenous terpenoid precursors and that the rate of synthesis of capsidiol and debneyol was unaffected by the small amounts of these phytoalexins present in the cell-free preparations from elicited cells.

The demonstration of the synthesis of both capsidiol and debneyol from either IPP or FPP in the cell-free preparations described above was dependent upon the presence of Mg^{2+}, an NADPH-generating system (or

Table 2. Biosynthetic properties of cell-free systems prepared from elicited tobacco cells by four different methods

Method of preparation of cell-free system and assay conditions	Rate of formation of product (nmol product/mg protein/hr)			
	Unknown I*	Unknown II*	Debneyol	Capsidiol
Soluble PVP				
†IPP/NADPH§	0.08	0.03	0.06	0.03
Soluble PVP, gel filtration				
IPP	0.87	0.34	0.06	0.04
IPP/NADPH	0.07	0.37	0.54	0.73
IPP/NADPH/N_2	0.84	0.38	0.18	0.09
‡FPP	36.89	19.24	0.87	0.50
FPP/NADPH	24.60	21.40	13.10	4.65
Insoluble PVP				
IPP	1.00	0.40	0.53	0.17
IPP/NADPH	0.11	0.52	1.35	1.26
IPP/NADPH/N_2	0.72	0.30	0.09	0.07
Insoluble PVP, gel filtration				
IPP	1.52	0.21	0.05	0.02
IPP/NADPH	0.16	0.83	0.91	1.53
FPP	18.60	45.20	1.03	0.27
FPP/NADPH	4.78	44.09	18.70	6.89

*Rate of formation calculated on the assumption that the compounds are sesquiterpenoids.
†3.6 μM [1-^{14}C]IPP.
‡25 μM [1-$^{3}H_2$]FPP.
§NADPH generating system.
The cell-free systems were prepared from the cells of a five-day-old culture which had been incubated with cellulase for 12 hr. The methods of preparation of the four systems and the details of the assay conditions are described in Experimental.

NADPH) and molecular oxygen (data not shown). The efficacy of other divalent ions and of other reduced coenzymes was not tested.

Fractionation of the cell-free preparation by differential centrifugation established that the phytoalexin-synthesising capability was restricted to the post-20 000 *g* supernatant and required the presence of both the membranes (microsomes) and the soluble portion of the fraction for full activity.

The rate of synthesis of the major radioactive components remained linear for at least 45 min and was approximately proportional to the protein concentration over the range 0.2 to 0.6 mg protein/ml incubation mixture. The apparent K_m's for the synthesis of capsidiol, debneyol and unknowns **I** and **II** are given in Table 3.

Cell-free synthesis of squalene. This could be demonstrated in cell-free preparations of unelicited tobacco cells prepared by any of the procedures described in the previous section (Table 2). As expected squalene synthesis from either [1-^{14}C]IPP or [1-^{3}H]FPP was Mg^{2+} and NADPH-dependent. In all the incubation systems tested farnesol was produced due to the presence of phosphatases. The squalene and farnesol were characterised by radio-TLC in two adsorptive systems and on Ag^{+}–silica gel. In addition the radiochemical purity of the labelled squalene was confirmed by purification of a mixture of either ^{14}C- or ^{3}H-squalene plus unlabelled squalene via its thiourea adduct (see Experimental). The apparent K_m for the synthesis of squalene was 10 μm. The optimal concentration of FPP for squalene synthesis was *ca* 25 μM. Above this concentration there was a progressive inhibition of squalene synthesis and a concomitant increase in the amount of farnesol produced. The addition of bovine serum albumin and potassium fluoride, two inhibitors of phosphatase activity, reduced the yield of both squalene and farnesol. Squalene 2,3-epoxide and cycloartenol were not produced under the assay conditions employed for this study.

Time course of the changes of squalene synthetase activity and of the rate of synthesis of capsidiol and debneyol in elicited cultures

This was studied with 4500 *g* cell-free systems prepared from five to seven-day-old cultures by the insoluble PVP procedure. The first study (Fig. 2) used [1-^{14}C]IPP at a substrate limiting concentration (3.6 μM) whereas the second (Table 3) employed [1-^{3}H]FPP at a range of concentrations (2.5–37.5 μM). The results from both studies were complementary and showed that the synthesis of phytoalexins was detectable within two hr after elicitation of the cultures with cellulase. Thereafter, the rate of synthesis of both phytoalexins rose rapidly with the maximum rate for each phytoalexin preceding the maximum level of its accumulation. The rate of synthesis of capsidiol and its levels in the cultures then declined rapidly while those of debneyol declined only slightly (Fig. 2A & B and Table 3). The synthesis of squalene showed a marked decline over the first six hr period after elicitation. Thereafter, the level of detectable squalene synthetase activity remained low until about 48 hr when the cultures started to recover the ability to synthesize

Table 3. Squalene synthetase activity and rate of formation of unknowns I and II, capsidiol and debneyol in cell-free systems prepared from unelicited and elicited tobacco cells

Enzyme activity or compound	Rate of incorporation of [1-$^3H_2$]-FPP (25μM) (nmol/mg protein/hr) Time after elicitation (hr) 0	6	12	24	Apparent K_m* (μM)	Experimentally obtainable V_{max}* (nmol/mg protein/hr)	FPP§ (μM)
Squalene synthetase	26.3 (7.9)†	1.4	3.1 (0.8)	2.6	10	28	38
Farnesol	5.0 (0.6)	3.5	2.9 (0.8)	7.5	—	—	—
Unknown **I**	—	12.9	31.6 (0.5)‡	8.8	10‡	32	25
Unknown **II**	—	5.5	15.1 (2.0)‡	5.9	10‡	15	25
Capsidiol	—	5.7	3.0 (3.1)	0.8	2	4	5
Debneyol	—	8.4	10.7(4.1)	6.4	12	11	13
Total	31.3 (8.5)	37.4	66.4 (11.3)	32.0	—		

Values take into account the loss of one ^3H atom in the formation of squalene.

*Taken from the kinetic data (not shown) obtained with cell-free preparations from 0 hr unelicited (squalene synthetase activity) and 12 hr elicited cultures.

†Values in parenthesis from incubations containing a sub-optimal concentration (2.5 μM) of [1-$^3H_2$]FPP.

‡The rate of incorporation of [1-$^3H_2$]FPP into unknown **I** remained unchanged until after the concentration of FPP exceeded 5 μM. The K_m values for compounds **I** and **II** were estimated from the rates observed in NADPH-deficient incubations.

§FPP inhibitory at higher concentrations.

Cell-free systems prepared by Method B (without gel filtration) from the cells of five-day-old cultures which had been incubated with cellulase for the times shown (elicited cells). The incubation mixture contained 25 μM [1-$^3H_2$]FPP and the NADPH generating system. Although not shown here very similar rates were observed when the NADPH-generating system was replaced by 1 mM NADPH. In the case of the 0 hr unelicited and 12 hr elicited preparations the rates were determined over the concentration range 2.5–37.5 μM [1-$^3H_2$]FPP.

squalene (Fig. 2C). The synthesis of unknowns **I** and **II** was detectable at all time points after elicitation of the cells.

Nature of the inhibition of squalene synthetase activity in elicited cultures

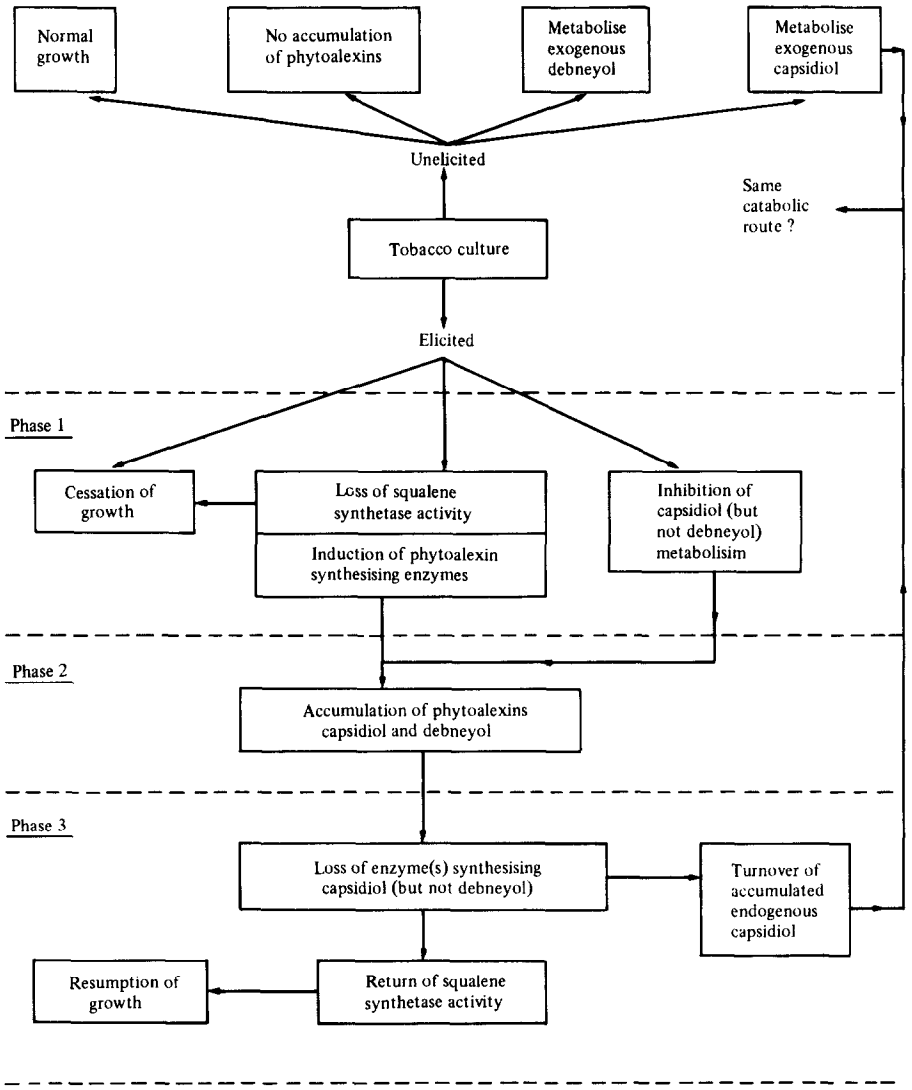
The rapid inhibition of squalene synthesis in the elicited cultures was not due to inhibition by low-M_r compounds (e.g. phytoalexins) or macromolecules produced by the cells in response to elicitation, since an increase in the rate of squalene synthesis relative to those of phytoalexin synthesis could not be demonstrated in cell-free extracts which had been passed through a PD-10 column (Table 2) and squalene synthesis in cell-free systems from unelicited cultures was unaffected upon admixture of the cell-free system with one prepared from an elicited culture (data not shown). Furthermore, washed microsomes, isolated from elicited cells, contained very little detectable squalene synthetase activity (data not shown).

Precursor-product relationships between unknowns **I** *and* **II** *and capsidiol and debneyol*

The total radioactivity in the chloroform extracts from incubations of cell-free preparations from the elicited system was very similar in both the presence and absence of NADPH and /or molecular oxygen. These results, and the observation that the proportion of unknown **I** relative to those of capsidiol and debneyol decreased when both NADPH and molecular oxygen were present (see Fig. 5 and Table 2), suggested that unknown **I** might be a precursor of either one or both phytoalexins. The amount of unknown **II** was unaffected by the presence or absence of NADPH and molecular oxygen. However, this does not preclude the possibility that unknown **II** is a hydrated product of unknown **I** and thus a precursor of debneyol (see Scheme 2). Pulse-chase experiments using [1-^{14}C]IPP as the pulse and IPP (in the presence of NADPH and molecular oxygen) as the chase failed to provide any clear cut evidence for the involvement of the two unknowns in phytoalexin biosynthesis as did experiments when labelled samples of the unknowns (isolated from previous incubations) were incubated with the cell-free system in the presence of NADPH and molecular oxygen. The latter experiments however, were hampered by the volatility and hydrophobic nature of the two unknowns.

DISCUSSION

The results presented in this paper show that a co-ordinated, rapid and transient sequence of events takes place in *Nicotiana tabacum* cell suspension cultures after treatment with the biotic elicitor cellulase. The events occur in three distinct phases (Scheme 3). Firstly, within six hr, there is a rapid inhibition of both squalene synthetase and the activity required for the catabolism of



Scheme 3. Schematic representation of the events which take place in tobacco cell suspension cultures after treatment with cellulase.

capsidiol but not apparently that of debneyol. Concomitantly, the cultures cease to grow and the enzyme activities required for the biosynthesis of the two sesquiterpenoid phytoalexins appear (probably by *de novo* synthesis). The cultures also produce some material(s) (probably phenolic) which colours the cultures yellow.

In the second phase, the two phytoalexins accumulate rapidly along with the phenolic compound acetosyringone and both squalene synthetase and cellular growth remain inhibited. The third phase starts when the enzymic activities synthesising the two phytoalexins start to decrease. Those needed for the synthesis of capsidiol fall rapidly and shortly after, the level of capsidiol in the culture starts to fall probably as a result of a derepression of the activity effecting its catabolism. Conversely, the enzymic activities needed for the synthesis of debneyol remain high although there is no change in the amount of this phytoalexin in the culture. These results suggest that a steady-state of synthesis and turnover exists for debneyol during this phase. Towards the end of this phase, the activity of squalene synthetase starts to increase and cellular growth resumes.

The time course of capsidiol accumulation reported here is very similiar to that found in tobacco cell suspension cultures treated with a biotic elicitor preparation prepared from *Phytophthora megasperma* f. sp. *glycinea* [2]. However, neither acetosyringone nor debneyol were detected in the *P. megasperma*-elicited cultures.

Previous work in this laboratory with cell suspension cultures of potato elicited with zoospores of *P. infestans* had provided good evidence that, concomitant with the onset of sesquiterpenoid phytoalexin accumulation, sterol biosynthesis is inhibited at the level of squalene synthetase [7, 8]. The results presented in this paper have extended these observations by measuring *in vitro* squalene synthetase and capsidiol and debneyol synthesizing activity in preparations from both unelicited and elicited

tobacco cultures and indicate that there is a temporal correlation between the activity of squalene synthetase, cellular growth and phytoalexin synthesis/accumulation in elicited cultures. They have also established that, as expected, the synthesis of the phytoalexins from IPP takes place in the same compartments of the cell (microsomes and cytosol) as those involved in the synthesis of squalene.

The kinetic data presented in this paper suggests that the inhibition of squalene synthetase would be a prerequiste for the efficient and rapid accumulation of sesquiterpenoid phytoalexins if the endogenous FPP levels are limiting. Previous work with potato provided evidence that FPP levels are indeed limiting for sterol biosynthesis in unelicited cell suspension cultures [7, 8]. Recently, it has been shown that the levels of HMG-CoA reductase activity rapidly and transiently increase in response to treatment of tobacco cell suspension cultures with the *P. glycinea* elicitor [2]. However, the increased levels of HMG-CoA reductase, and by implication FPP levels, were barely sufficient to account for the levels of capsidiol which accumulated in the cultures. Whilst the extractable level of HMG-CoA reductase may be less that the actual *in vivo* level, the data indicate that the FPP could still be limiting in this elicited system.

A direct consequence of the marked reduction of squalene synthetase activity would be the inhibition of cellular growth. There are several reports of the inhibition of growth in cell suspension cultures of other species treated with elicitors in which the products accumulated are non-terpenoid in nature [17, 18]. Although these studies did not attempt to establish the reasons behind the inhibition, it is possible that the effect is in part due to the inhibition of squalene synthetase, although other factors may play a role. These observations suggest that the inhibition of cellular growth/squalene synthetase might be a general phenomenon which occurs in plant cells in response to elicitation rather than forming part of a specific mechanism designed to facilitate sesquiterpenoid phytoalexin biosynthesis in solanaceous species.

It is not clear yet whether the reduced activity of squalene synthetase is due to enzyme inactivation or the rapid turnover of existing enzyme. However, on the basis of the results from the gel-filtration and admixture experiments and from work with washed microsomes it seems unlikely that either phytoalexin or plant synthesized macro-molecular material(s) is involved in reducing the activity of the enzyme.

To date, it has not been possible to obtain any firm evidence that either unknown **I** or unknown **II** is an intermediate on the biosynthetic pathway from FPP to capsidiol and/or debneyol, apart from the demonstration that the level of unknown **I** is increased when capsidiol and debneyol formation is inhibited either by the omission of NADPH from the incubation mixtures or by performing the incubations in the absence of molecular oxygen. However, both compounds are most probably sesquiterpenoid and are only formed in elicited systems. It is therefore reasonable to suppose that either one or both unknowns may be a precursor for either one or both of the phytoalexins. Furthermore, the much greater mobility of unknown **I** (R_f 0.61) relative to squalene (R_f 0.38), farnesene (R_f 0.46, a common metabolite of FPP), germacrene A (R_f 0.36, a proposed product of the FPP-carbocyclase involved in the formation of capsidiol and debneyol (see[19] and Scheme 2) and unknown **II** (R_f 0.00) on adsorptive TLC with petrol as solvent (system C) suggests that unknown **I** may be the eremophilane hydrocarbon postulated to be involved in the biosynthesis of capsidiol and debneyol (Scheme 2). In turn, since unknown **II** is immobile in system C and its synthesis does not require molecular oxygen or NADPH, it could be a side-chain hydrated derivative of the eremophilane hydrocarbon and thus a precursor of debneyol (Scheme 2). This is further supported by the observation that the changes in the rate of formation of this compound from [1-^{14}C]IPP in elicited cultures mimic those of debneyol. The total amount of radioactivity incorporated into capsidiol, debneyol and possibly the two unknowns provides an indirect estimate of the activity of FPP-carbocyclase, the enzyme catalysing the first committed step in the biosynthesis of these compounds (see Scheme 2).

Reports on the ability of plant cell suspension cultures to catabolise and/or transform (including acetylation, glycosylation and hydroxylation) secondary products are widespread [13, 14, 19, 20]. Cell suspension cultures of solanaceous plants have been shown to metabolize and/or transform exogenously supplied sesquiterpenoids. In this context, capsidiol was converted to 13-hydroxycapsidiol by sweet pepper cultures [12]. However, only *cis*-9, 10-dihydrocapsenone has been identified as a possible catabolite of endogenously formed capsidiol in unelicited sweet pepper cultures [15]. In the studies on the catabolism of both capsidiol and debneyol reported here, only 3-*O*-acetyl-capsidiol was isolated as a metabolite. This compound has recently been isolated from TMV inoculated leaves of *Nicotiana undulata* along with capsidiol and 17 other related sesquiterpenoids [21]. This *in planta* result suggests that 3-*O*-acetylcapsidiol is a natural metabolite of capsidiol. However, from our results, it is not possible to decide whether this compound is an actual catabolite of capsidiol or is formed non-specifically. The fact that only the 3-hydroxy position of the eremophilane skeleton was acetylated (other hydroxy positions available are 1- in capsidiol and 11- and 12- in debneyol) suggests that a specific 3-*O*-acetyltransferase (with acetyl CoA as the donor) might be involved. If the reverse reaction were catalysed by a non-specific esterase (such a system of a specific acetyl transferase/unspecific esterase is thought to be involved in the biosynthesis of the alkaloid vindoline in cell suspension cultures of *Catharanthus roseus* [22]) the formation of each compound may not be in a true form of equilibrium as the results suggest at first sight. Furthermore, the observation that cellulase-elicitation of the tobacco cultures inhibited the catabolism of capsidiol but not debneyol suggests that the two compounds may be catabolized by different routes and also may in part explain why higher levels of capsidiol are able to accumulate in the elicited cultures. If an allowance is made for some residual synthesis of capsidiol at late points in the time course then the rate of loss at these times is comparable to that in the unelicited cultures. This suggests that the inhibitory effect of cellulase on capsidiol catabolism is only present during the first two phases of the response to elicitation.

The work presented in this paper also reports for the first time, the accumulation of acetosyringone in tobacco cultures in response to treatment with cellulase. Acetosyringone and α-hydroxyacetosyringone occur in exudates of wounded but metabolically active plant cells [23]. Studies have shown that these two compounds specifically activate *Agrobacterium tumefaciens* virulence gene ex-

pression and probably enable this organism to recognise susceptible cells in nature [23]. In the light of these findings, it was perhaps not surprising to find that this compound possessed no antifungal activity. It seems likely that, in addition to eliciting sesquiterpenoid phytoalexin accumulation, the cellulase causes some damage to the cells which mimics wounding and thus induces the formation of acetosyringone. The reasons behind the biphasic accumulation of this compound in these cultures are unclear.

Future work with the cell-free system will focus on identifying unknowns **I** and **II** and characterizing the individual enzymes involved in the biosynthesis of both capsidiol and debneyol. In addition to this, we intend to examine the regulation of squalene synthetase activity in response to elicitation in both solanaceous and non-solanaceous plant species.

EXPERIMENTAL

^1H and ^{13}C NMR were recorded at 270.05 and 67.9 MHz respectively. All TLC was performed on 0.5 mm plates using the following systems: A–D on rhodamine 6G-impregnated silica gel G with EtOAc–cyclohexane (1:1), EtOAc–*i*-PrOH (9:1), petrol (40–60°) and EtOAc–C_6H_6 (3:22) respectively; E on silica gel H with *n*-PrOH–NH_3–H_2O (6:3:1) and F on 10% $AgNO_3$ –impregnated silica gel G with MeOH–toluene (1:19). Compounds were detected by UV_{254} light (systems A–D), and by spraying with either vanillin–H_2SO_4 reagent (plus heating) (systems A–D [non-impregnated plates], H and F) or with Ammolybdate–$FeCl_3$ reagent (system F).

Radiochemicals and chemicals. [1-^{14}C]IPP (55 mCi/mmol) and NaB^3H_4 (10Ci/mmol) were purchased from the Radiochemical Centre, Amersham. *t,t*-[2,4,12-^{14}C] FPP (64 μCi/mmol) was a gift from Dr P. A. Brindle. Farnesol (mixture of isomers) and acetosyringone (3′,5′-dimethoxy-4′-hydroxy-acetophenone) were purchased from Aldrich Chemical Co., Gillingham, Dorset; the latter compound was purified by recrystallization (× 2) from Et_2O and TLC (solvent A) before use. Capsidiol and debneyol were available from previous work in our laboratory [6, 15]. Germacrene A and farnesene were obtained from *Senecio* (*Rugelia*) *nudicaulis* by a method similiar to that described in [24]. Arachidonic acid, cellulase (Ex. *Trichoderma viride*) and pectinase (Ex. *Aspergillus niger*) were purchased from Sigma Chemical Co., Poole, Dorset. *Phytophthora infestans* sonicate was prepared by the method of [11]. All reagents were Analar or best grade available. Solvents were redistilled before use.

Chemical synthesis of t,t-[1-$^3H_2$] *farnesyl pyrophosphate*

(a) *Isolation of* t,t-*farnesol from a mixture of farnesol isomers of commercial origin.* In a previous study [25], *t,t*-farnesol was isolated in g amounts from a commercial sample of farnesol by CC followed by fractional crystallization of the diphenylurethane derivatives of the *t,t*- and *c,t*-farnesols present in the CC-fractions enriched in the *t,t*-isomer. In our hands however, the derivatization, crystallization and alkaline hydrolysis steps used in ref. [25] were difficult to perform. We therefore, resorted to an alternative procedure in which the 4-nitrobenzoyl derivatives were prepared and the fractional crystallization from MeOH was performed at −30°. The protocol followed was very similar to that described in ref. [25]. The main modifications made were that: all chromatographic steps were omitted, equimol amounts of alcohol and the acid chloride (in our case 4-nitrobenzoyl chloride) were used, the derivatisation and saponification times were changed from 4 and 6 hr respectively to 70 min and overnight at room temp. and the crystallization steps (× 4) were carried out from 100 ml vols of MeOH and at −30°. The overall yield of GC and ^1H NMR pure *t,t*-farnesol from 20 mmol commercial farnesol containing 7.8 mmol *t,t*-farnesol was 2.1 mmol.

(b) *Synthesis of* t,t-[1-$^3H_2$] *farnesol.* *t,t*-Farnesol (30 mg) in 1.2 ml cyclohexane was stirred with 0.3 g activated MnO_2 [26] for 10 min. The mixture was then centrifuged and 0.9 ml of the supernatant taken to dryness under a stream of N_2. The residue, *t,t*-farnesal (*ca* 20 mg), was taken up in 1 ml *i*-PrOH and added to 100 mCi NaB^3H_4 (10 Ci/mmol). After 2 hr, 1 ml 0.05% NH_4OH was added and 30 min later the mixture was partitioned (× 3) against 2–3 vols Et_2O. The Et_2O extracts were bulked, backwashed, taken to dryness and subjected to prep TLC (system A, R_f 0.48). The ^3H-farnesol (*ca* 43 mCi) was recovered from the plate with Et_2O and stored in C_6H_6 until required. Radio-GC (conditions as [11], except col. temp. 140°) showed that at least 88% of the ^3H was associated with the *t,t*-isomer (R_t 3.8 min) with the remainder being distributed between the *c,t*-, *t,c*- (both R_t 3.3 min) and *c,c*- (R_t 2.7 min) farnesols formed from the *t,t*-farnesol in the course of the oxidation and reduction steps just described.

(c) *Synthesis of* t,t-[1-$^3H_2$]*FPP.* A mixture of 0.4 mmol unlabelled farnesol and 16 mCi [1-$^3H_2$] farnesol was phosphorylated by the method of [27] as described by [28]. The ^3H-labelled FPP, FMP, FTP and F-tetra-P formed in the reaction were isolated from the petrol-extracted reaction mixture by adsorption onto, and desorption from, Amberlite XAD-2 resin then separated from each other by CC on DEAE-cellulose [28]. The [1-$^3H_2$]FPP- containing fractions from the DEAE-cellulose CC were pooled and the [1-$^3H_2$]FPP recovered by the use of Amberlite XAD-2 resin as above [27]. The [1-$^3H_2$]FPP-containing methanolic eluant from the resin was reduced in vol. to 10 ml with gradual replacement of the MeOH by the addition of 0.01 M NH_3. The resultant ammoniacal solution of [1-$^3H_2$]FPP was then made up to 25 ml by the addition of 0.2 M Tris–HCl, pH 8.0, and stored at −70° as small aliquots. The overall yield of *t,t*-[1-$^3H_2$]FPP (40 mCi/mol, *ca* 90% *t,t*-isomer) based on radioactivity was 25%. All steps in this synthesis were monitored by TLC (system E: FMP, R_f 0.56; FPP, 0.44; FTP, 0.23; F-tetra-P, 0.09).

Synthesis of 3-*O*-*acetylcapsidiol.* This compound was prepared by partially acetylating capsidiol. 100 mg of capsidiol was stirred with 330 μl pyridine and 250 μl Ac_2O in 5 ml C_6H_6 for 40 hr at room temp. The reaction was stopped by the addition of 1 ml EtOH and 500 μl 2 M HCl. After the addition of 5 ml H_2O the mixture was partitioned into Et_2O (4 × equal vol.). The Et_2O extracts were bulked, and after removing the solvent *in vacuo* the residue was separated by TLC (system A) to give 45 mg unreacted capsidiol (R_f 0.12), 26.9 mg 1-*O*-acetylcapsidiol (R_f 0.42), 29.9 mg 3-*O*-acetylcapsidiol (R_f 0.47) and 5 mg 1,3-di-*O*-acetylcapsidiol (R_f 0.68). The 3-*O*-acetylcapsidiol fraction was further purified by re-TLC (system A). The purified product gave a single peak on GC and had the following spectral characteristics. ^1H NMR: 4.38 (*t*, H-1e); 5.63 (*dt*, H-3a). [Capsidiol: 4.36 (*dd*, H-1e), 4.60 (*dt*, H-3a); 1,3-di-*O*-acetyl capsidiol: 5.42 (*dd*,H-1e), 5.56 (*dd*, H-3a)]. GC conditions as described [11]. The RR_t values [Me stearate (R_t 4 min) = 1] were as follows: Capsidiol 2.32; 1-*O*-acetylcapsidiol 2.85; 3-*O*-acetylcapsidiol 2.61 and 1,3-di-*O*-acetylcapsidiol 2.24

Plant material. Cell suspension cultures of *Nicotiana tabacum* L. var. White Burley were grown as described previously [6]. The cultures used in the present study were standardized by transfering 10 ml of a 7-day-old culture to fresh media with a sterile pipette every 7 days. The cultures were elicited by the addition of 750 μg of cellulase (made up in 1 ml sterile H_2O; this

concentration had been established as optimal, in the range 0.025–5 mg/flask, for the elicitation of the phytoalexins capsidiol and debneyol; unpublished results) on day 6 of the growth cycle (late log phase) for material required for the cell-free work or as otherwise indicated.

Extraction, characterisation and estimation of phytoalexins and acetosyringone from culture media and cells. Cultures were harvested by filtration through Miracloth. Cell material was extracted by the method of ref. [8]. The culture filtrate was extracted with Et_2O (2 × equal vol.) and after bulking the two Et_2O extracts the solvent was removed *in vacuo*. Extracts from both cell material and culture filtrates were analysed by GC as described previously [6, 11] except that the column temp. was lowered to 150° in order to resolve the acetosyringone and debneyol. The *RR*, values [Me stearate (13.7 min) = 1] were as follows: debneyol 1.60; acetosyringone 1.75; capsidiol 2.07. The levels of capsidiol, debneyol and acetosyringone were estimated by comparison with stock solutions of known concentration. The extracts were not analysed for the presence of 7-*epi*-debneyol, 1-hydroxydebneyol or 8-hydroxydebneyol which we have recently isolated in small quantities from ether extracts of large-scale tobacco cultures elicited with cellulase (see ref. [6]). The acetosyringone obtained from the elicited cultures was characterised by MS, IR and ^1H and ^{13}C NMR and was identical in these respects and by TLC (systems A and B) and GC (above) to an authentic sample purchased from Aldrich.

Examination of media and cells for the presence of sesquiterpenoid glycosides. Capsidiol (2 mg) or debneyol (1 mg) in 0.5 ml EtOH were added to two 5-day-old cultures for 5 hr. The cultures, after harvesting as above, were treated as follows. Half of the cells from the cultures (*ca* 30 g) were homogenised with 70 ml H_2O in a blendor and after filtration through Miracloth, the filtrate was extracted with Et_2O. The culture filtrates from the two cultures of each were combined and also extracted with Et_2O. After the removal of traces of Et_2O (*in vacuo*) from both samples 45U of β-glucosidase (Ex. Almonds, Sigma) was added to each and the samples incubated at 40° for 2 hr in a shaking water bath. After this time both samples were re-extracted with Et_2O and analysed for the presence of either capsidiol or debneyol as previously described. Mild acid hydrolysis (0.5 M HCl final concentration, 60° for 2 hr) was not attempted on the samples since a control experiment showed capsidiol to be unstable under these conditions.

Examination of commercial cellulase for esterase and β-glucosidase activity

The relatively crude commercial preparation of cellulase used in the work described in this paper was examined for other enzymic activities to ensure that some of results attributed to the cell cultures were not spurious.

(a) *Esterase.* 750 μg cellulase and 1 mg 3-*O*-acetylcapsidiol were incubated together for 5 hr in 120 ml fresh tobacco culture media at 25°. Et_2O extraction of the media and GC analysis showed a quantitative recovery of the substrate.

(b) *β-Glucosidase.* 750 μg cellulase and 0.5 mg 2-nitrophenyl-β-D-glucopyranoside (Koch-light) were incubated together in 5 ml fresh culture media for 2 hr at 25°. The addition of alkali after this time failed to reveal the presence of any 2-nitrophenol.

Assay of squalene synthetase activity and phytoalexin synthesis in cell-free systems

(a) *Preparation of cell-free system.* The cells from 120 ml of either an unelicited culture or a cellulase-elicited culture (see above) were harvested by filtration through Miracloth and the retained cells washed with 300 ml H_2O. The filtrate and washings were bulked and analysed for phytoalexins as described above. The washed cells were weighed and typically 10 g taken for the preparation of a cell-free system by one of the following two methods: *Method A (soluble-PVP, gel filtration).* This method was based on the one used by ref. [11] to demonstrate the biosynthesis of lubimin from [1-^{14}C]IPP by cell-free preparations of potato tuber tissue inoculated with an elicitor preparation from *Phytophthora infestans.* The 10 g sample of cells along with 2 g of acid-washed sand was transfered to a pre-cooled (−10°) pestle and mortar and ground for 30 sec in 5 ml ice-cold 0.1 M K-Pi buffer, pH 8.0, containing 0.5 M sucrose, 8% soluble-PVP [M_r(avg.) 44 000, BDH], 10 mM EDTA and 10 mM 2-mercaptoethanol. The homogenate was filtered through Miracloth and the filtrate centrifuged at 4500 *g* for 15 min at 0°. A 2 ml aliquot of the 4500 *g* supernatant was applied to a prepacked disposable column containing Sephadex G-25 M (PD-10, Pharmacia) which had been pre-equilibrated with ice-cold 0.05 M K-Pi buffer, pH 7.5, containing 0.5 M sucrose, 1 mM EDTA and 1 mM 2-mercaptoethanol (running buffer). The column was eluted according to the manufactures instructions to give 3.5 ml of cell-free preparation containing 0.2–1.0 mg protein/ml. Protein concentration was determined by the method of [29] with BSA (Sigma, fraction V) as the standard. *Method B (insoluble-PVP, ± gel filtration).* This method was similar to Method A except that the cells were homogenized with 2 g acid-washed sand, 1 g insoluble PVP (Sigma) and 5 ml of double-strength PD-10 running buffer (see method A). In most experiments the gel-filtration step was omitted. The protein content of the preparation fell within the same range as that given for method A.

(b) *Incubation of cell-free preparation.* The standard incubation mixture (total vol. 1 ml) was made up in a 13 × 120 mm thick-walled test tube and consisted of 0.5 ml cell-free preparation (0.1–0.5 mg protein) and 0.5 ml PD-10 running buffer pH 7.5 (see above), containing an NADPH-generating system (0.25 mM NADP, 3.2 mM glucose-6-phosphate, 0.8 units glucose-6-phosphate dehydrogenase) and 10 mM $MgCl_2$ (added last to avoid pptn). After a 5 min pre-incubation of these components in a shaking water bath at 30°, the reaction was started by the addition of either 4 μl [1-^{14}C]IPP (0.2 μCi; final concentration 3.6 μM) or 10 μl [1-$^3H_2$]FPP (1 μCi; final concn 25 μM) and the incubation continued for 15, 30 or 45 min. The incubation was terminated by the addition of 5 ml $CHCl_3$–MeOH (1:2) to the reaction mixture.

(c) *Extraction of cell-free incubation mixtures.* The $CHCl_3$–MeOH-treated reaction mixture was left to stand for 30 min at room temp., after which time it was centrifuged at 600 *g* for 10 min. The supernatant was transfered to a clean thick-walled test tube (13 × 120 mm), and diluted with 5 ml H_2O. The mixture was centrifuged at 600 *g* for 10 min and the aq. MeOH phase removed by means of a Pasteur pipette attached to a vacuum line. The $CHCl_3$ phase was then overlaid with 5 ml H_2O and after centrifugation and removal of the aq. phase was transferred to a small screw-topped vial. After careful evapn of the solvent at low temp. under a stream of N_2, the $CHCl_3$-soluble extract was taken up in 100 μl Me_2CO containing 100 μg each of capsidiol, debneyol, farnesol and squalene, 10 μl of which was assayed for radioactivity. The remainder, unless analysed by TLC immediately, was stored at −20°.

(d) *Standard procedure for the analysis of ^{14}C- and ^{3}H-labelled compounds produced in cell-free incubations.* A 10 or 20 μl aliquot of the $CHCl_3$-soluble extract in Me_2CO was subjected to TLC (system A). The developed plate was scanned to check that the radioactivity was co-incident with squalene/unknown I (R_f 0.75), farnesol (R_f 0.48), debneyol (R_f 0.30) and capsidiol (R_f

0.12) and then the areas of gel containing these compounds and unknown **II** (R_f 0.56) were transfered directly into scintillation fluid and assayed for radioactivity [8]. To determine the distribution of radioactivity between squalene and unknown **I**, a 10 μl aliquot from the original sample was subjected to TLC (system B) and the areas of gel containing these compounds (unknown **I**, R_f 0.61; squalene, 0.38) assayed for radioactivity as above. If the resolution between unknown **II** and farnesol was incomplete in the first TLC system, a further 10 μl aliquot was TLC'd in another system (system D, double-developed). The radioactivity content of the fractions recovered from TLC were used to calculate the squalene synthetase activity and rate of synthesis of unknowns **I** and **II**, debneyol and capsidiol in the cell-free preparations. No allowance was made for the presence of the small amounts of radio-labelled impurity in the capsidiol and debneyol fractions recovered from the TLC (see below).

Characterisation of radio-labelled compounds produced by cell-free systems.

(i) *Capsidiol and debneyol.* The unused portions of the extracts from the incubation mixtures containing cell-free preparations from elicited cells, [1-^{14}C]IPP and NADPH were combined. After the addition of authentic unlabelled capsidiol and debneyol (2 mg each, as marker and carrier material) the sample was run on TLC (system A) and the areas of the plate which co-chromatographed with the capsidiol (R_f 0.12) and debneyol (R_f 0.30) markers were eluted (the peaks of radioactivity were also coincident with the phytoalexin markers in system B). Radio-GC analysis of each of the samples indicated that at least 89 and 67% of the radioactivity eluted from the plates was associated with capsidiol and debneyol respectively. (Conditions for the radio-GC were identical to those used previously [11]. Calibration of the apparatus with both [^{14}C]Me palmitate and [15-^{3}H] lubimin [30] indicated that 92–95% of the portion of the sample diverted from the FID was collectable by the method employed. The quoted recoveries are corrected for this value.) The remainder of the capsidiol sample and half of the debneyol sample were acetylated overnight using Ac_2O–pyridine. The products were analysed by TLC (system A) and radio-GC as above. Both 1,3-di-*O*-acetyl-capsidiol (R_f 0.68) and 12-*O*-acetyl-debneyol (R_f 0.54) migrated as single areas of radioactivity which were coincident with authentic markers of the two acetates. The radio-GC analysis indicated that at least 64 and 60% of the radioactivity eluted from the plates was associated with the two products respectively. (The low recovery of these purified compounds from the radio-GC apparatus may be due to their increased volatility).

The second half of the debneyol fraction was treated with dry acidified Me_2CO for 1 hr at room temp. Radio-TLC analysis (system A) gave two peaks of radioactivity in a ratio of *ca* 5:1. The higher (and major) peak at R_f 0.71 corresponded to 11,12-*O*-isopropylidenedebneyol and was coincident with an authentic marker whilst the lower area at R_f 0.30 represented unreacted debneyol [6]. The isopropylidene derivative was not analysed by radio-GC due to its volatile nature.

(ii) *Squalene and farnesol.* The unused portions of the extracts from the incubation mixtures containing cell-free preparations from unelicited cells, NADPH and either [1-^{14}C]IPP or [1-$^{3}H_2$]FPP were combined on the basis of their isotope content and subjected to prep. TLC (system A). The radioactive areas of the plate which co-chromatographed with the carrier/marker squalene and farnesol, and which between them accounted for all the radioactivity applied to the plate, were eluted. Radio-TLC analysis of each of the two bands in systems C (squalene R_f 0.38; farnesol, 0.00) and F (squalene R_f 0.21; *t,t*-farnesol, 0.01; *c,t*/*t,c*/*c,c*-farnesol 0.09–0.12) established that the radioactivity in each band co-chromatographed with the carrier/marker compounds. Additional proof as to the radiochemical purity of the squalene was provided by the demonstration that the specific activity of a mixture of labelled (^{14}C or ^{3}H) squalene and unlabelled squalene was unchanged after purification (× 2) via its thiourea adduct [31].

Properties of unknowns **I** *and* **II.** The only measured properties of either unknown **I** or **II** were the R_f values in various TLC systems (quoted in the text). In addition to this, the loss of radioactively labelled material which occured during routine handling indicates that both unknowns are volatile.

Acknowledgements—The authors wish to thank Dr D. F. Ewing and Mr A. D. Roberts of the Department of Chemistry, for obtaining and interpreting the NMR data and for assistance with GC-MS respectively, Mrs M. Huffee for technical assistance and the AFRC for financial support.

REFERENCES

1. Brindle, P. A., Kuhn, P. J. and Threlfall, D. R. (1983) *Phytochemistry* **22**, 2719.
2. Chappell, J. and Nable, R. (1987) *Plant Physiol.* **85**, 469.
3. Threlfall, D. R. and Whitehead, I. M. (1988) *Biochem. Soc. Trans.* **16**, 71.
4. Brooks, C. J. W., Watson, D. G. and Freer, I M. (1986) *Phytochemistry* **25**, 1089.
5. Watson, D. G., Rycroft, D. S., Freer, I. M. and Brooks, C. J. W. (1985) *Phytochemistry* **24**, 2195.
6. Whitehead, I. M., Ewing, D. F. and Threlfall, D. R. (1988) *Phytochemistry* **27**, 1365.
7. Brindle, P. A., Kuhn, P. J. and Threlfall, D. R. (1986) in *Secondary Metabolism in Plants* (Morris, P. *et al.*, eds), p. 117. Cambridge University Press.
8. Brindle, P. A., Kuhn, P. J. and Threlfall, D. R. (1988) *Phytochemistry* **27**, 133.
9. Obâ, K., Kondo, K., Doke, N. and Uritani, I. (1985) *Plant Cell Physiol.* **26**, 873.
10. Stermer, B. A and Bostock, R. M. (1987) *Plant Physiol.* **84**, 404.
11. Coolbear, T. and Threlfall, D. R. (1985) *Phytochemistry* **24**, 1963.
12. Ward, E. W. B., Stoessl, A. and Stothers, J. B. (1977) *Phytochemistry* **16**, 2024.
13. Zacharius, R. M. and Kalan, E. B. (1984) *Plant Cell Reports* **3**, 189.
14. Zacharius, R. M., Kalan, E. B. and Kimoto, W. I. *Plant Cell Reports* **4**, 1.
15. Whitehead, I. M., Threlfall, D. R. and Ewing, D. F. (1987) *Phytochemistry* **26**, 1367.
16. Ward, E. W. B., Unwin, C. H. and Stoessl, A. (1974) *Can J. Botany* **52**, 2481.
17. Hargreaves J. A. and Selby, C. (1978) *Phytochemistry* **17**, 1099.
18. Funk, C., Gügler, K. and Brodelius, P. (1987) *Phytochemistry* **26**, 401.
19. Dixon, R. A., Dey, P. M. and Lamb, C. J. (1983) in *Advances in Enzymology and Related Areas of Molecular Biology*, (Meister, A., ed.) p.1. Wiley, New York.

Note added to proof: Since submission of this manuscript, Dr J. Chappell (Kentucky University, U.S.A.) has communicated to us that he obtained similar results with respect to squalene synthetase inhibition and FPP carbocyclase (sesquiterpene synthase) induction in *Nicotiana tabacum* cell suspension cultures elicited with a *Phytophthora parasitica* elicitor preparation.

20. Furuya, T., Ushiyama, M., Asada, Y. and Yoshikawa, T. (1987) *Phytochemistry* **6**, 2983.
21. Uegaki, R., Kubo, S. and Fujimori, T. (1988) *Phytochemistry* **27**, 365.
22. Fahn, L., Lauβermair, W., Deus-Neumann, E. and Stöckigt, J. (1985) *Plant Cell Reports* **4**, 337.
23. Stachel, S. E., Messens, E., Montagu, M. V. Zambryski, P. (1985) *Nature* **18**, 624.
24. Bohlmann, F., Gupta, R. K., Jakupovic, J., King, R. M. and Robinson, H. (1982) *Phytochemistry* **21**, 1665.
25. Cane, D. E., Iyenger, R. and Shiao, M-S. (1981) *J. Am. Chem. Soc.* **103**, 914.
26. Pratt, E. F. and Castle, J. F. (1961) *J. Org. Chem.* **26**, 2973.
27. Cramer, F. and Bohm, W. (1959) *Angew. Chem.* **71**, 775.
28. Cornforth, R. H. and Popják, G. (1969) *Methods Enzymol.* **15**, 359.
29. Lowry, O. H., Rosebrough, N. J., Fair, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
30. Whitehead, I. M., Ewing, D. F. and Threlfall, D. R. (1988) *J. Chem. Soc., Perkin II* (in press).
31. Nicolaides, N. and Loves, F. (1954) *J. Am. Chem. Soc.* **76**, 2596.