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STIMULATION OF ACCUMULATION OF TERPENOIDS BY CELL SUSPENSIONS OF *LAVANDULA ANGUSTIFOLIA* FOLLOWING PRE-TREATMENT OF PARENT CALLUS

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Key Word Index — *Lavandula angustifolia*; Labiatae; tissue culture; wick-feeding technique; monoterpenoids; sesquiterpenoids; redox enzymes of sesquiterpenoid biosynthesis.

Abstract—Lines of callus of *Lavandula angustifolia* have been established that contained up to 20% of the levels of mono- and sesquiterpenoids found in foliage but in contrast, negligible quantities of these lower terpenoids accumulated in derived cell suspensions. Pretreatment of callus by pulse-feeding of mevalonate had little effect on terpenoid accumulation but now the derived cell suspensions stored monoterpenoids at levels *ca* 10^3-fold those of controls and these accumulations could be magnified a further 10^2-fold by use of a two-phase culture medium. Cell-free extracts of both productive and non-accumulating lines of callus converted [^{14}C]isopentenyl pyrophosphate into predominently 2*E*,6*E*- and 2*Z*,6*E*-farnesols together with the sesquiterpenoids humulene and caryophyllene; the pattern of predominating monoterpenoids characteristic of the productive callus lines was not observed. Preincubation of the extracts with NADPH or $NADP^+$ increased the accumulations of the sesquiterpenoids by up to 20-fold. Reasons for these unexpected patterns of products are discussed.

INTRODUCTION

Callus and cell suspensions of *Lavandula angustifolia* Mill., subsp. angustifolia (≡ *L. officinalis* Chaix. ≡ *L. vera* DC. ≡ *L. vulgaris* Lam.) have previously been found neither to accumulate detectable levels of the lower terpenoids or their conjugates such as β-glucosides nor to secrete them into the culture medium [1–6]. We now report the establishment of callus lines that exhibited acceptable accumulations of such terpenoids and record application of a novel pulse-feeding technique that had proved efficacious in enhancing biosynthesis of alkaloids in callus of another species [7].

RESULTS AND DISCUSSION

Callus that had been maintained for eight years (over 200 passages) was obtained from Dr D. G. Watson (University of Strathclyde). This was further sub-cultured under the original regime (line A) or with a different auxin (line B) and a third clone (line C) was set up using explants of stem of the original parent of line A.

After 10 passages, lines A–C were all friable and comprised largely homogeneous tissue lacking differentiation save for scattered tracheid-formation: all were blue in colour owing to the presence of unusual phenylpropanoids [4]. These lines accumulated 0.01–0.05% fr. wt of the lower terpenoids, corresponding at maximum to 20% of the levels in flowerheads and foliage of the parent plant. Such assays required extractions to be carried out under controlled conditions at near neutral pH as linalool and its derivatives (significant components) easily rearrange during isolation [8, 9]. The pattern of terpenoids accumulated in the calli (Table 1) resembled that in the flowerheads of the parent [10]. When last assayed (at passage 60), line A had produced negligible quantities ($< 10^{-6}$% fr. wt, if any) of terpenoids; either habituation had subsequently occurred or transportation between laboratories had triggered shock-induced onset of secondary metabolism [11–13]. Fine cell suspensions (clumps of up to 10^2 cells) derived from the productive callus lines did not yield significant levels of terpenoids and the recoveries did not improve when a second phase such as the lipophilic triglyceride Miglyol 812® or n-hexadecane were added to the culture medium as a trap [14–16].

Recently we have discovered that flooding a rapidly growing callus of *Ceanothus americanus* with a cocktail of amino acids by means of a novel wick-feeding technique resulted in the efficient accumulation of the immediate precursors of alkaloids that did not occur in the unperturbed callus [7]. Consequently, we applied the technique to terpenoid synthesis in *L. angustifolia* callus using mevalonate (MVA) as the possible stimulator. As before,

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Table 1. Accumulation of lower terpenoids in callus and parent tissue of *L. angustifolia*

Compound	Parent*		Callus line A†
	Flowers	Foliage	
Total (% wt/wet wt)	1.69	0.34	0.05
Linalool	28.9	—	20
Linaloyl acetate	21.3	—	50
Lavandulyl acetate	5.2	—	—
1,8-Cineol	11.2	25.0	—
Borneol	8.5	—	5
α-Terpineol	7.7	—	5
Ocimene	0.4	10.3	2
Nerol	—	29.7	—
Limonene	0.7	5.6	13
Caryophyllene	2.3	2.9	5
Others‡	13.8	26.5	0.1

* Terpenoids as percentage of total extract.

† Terpenoids as percentage of total extract: analyses (GC-MS) only accurate to ± 2 to 5% (actual value) owing to presence of wax and resin in extract. Culture lines B and C yielded similar analyses.

‡ Geraniol, allo-ocimene, α- and β-pinenes, oct-1-en-3-yl-acetate, myrcene humulene, caryophyllene oxide, unidentified sesquiterpenoid hydrocarbons and alcohols.

the treated callus was supported on a cellulose cylinder that stood in liquid medium and the precursor was injected into the base of the 'wick' thus ensuring efficient pulse-feeding with the callus bathed in the medium by capillary flow [7]. The results, using callus line C, are shown in Table 2. Wick-feeding at periods over the entire cell cycle had negligible effect on terpenoid yield. In contrast, cell suspensions prepared from callus that had been pre-treated (at periods in the latter half of the cell cycle) with MVA gave a 10^3-fold increase in the levels of the lower terpenoids compared with the controls and this increase was further magnified 10^2-fold by use of a sink. These stimulated suspensions did not produce the full pattern of products as exhibited by the parent callus but predominantly (> 90%) accumulated the 'early' monoterpenoids linalool, ocimene and limonene; the yields of these were, at maximum, *ca.* 10% of those total terpenoids from flowering heads of the plant. One explanation of the pattern of results is that enhanced formation of terpenoids in callus treated with MVA was counteracted by extensive turnover whereas such degradative or modification processes were less important in cell suspensions. In addition the removal of toxic metabolites possible with the two-phase system could allow the accumulations to be maximised. Our results in conjunction with the previous studies of alkaloids suggest that pre-treatment of callus with a suitable biosynthetic precursor using the wick-feeding technique may be useful for achieving worthwhile yields of secondary metabolites.

Activities in cell-free extracts of callus lines A–C as monitored by incorporation of [1 – ^{14}C]-isopentenyl pyrophosphate (IPP) into the lower terpenoids are shown in Table 3. Extracts from the MVA-treated callus of line C gave a similar pattern of products as controls but with a two-fold (6.2% incorporation) increase in total uptake. The main point of interest is that the predominant products were farnesols, humulene and caryophyllene, all sesquiterpenoids, in contrast with the predominance of monoterpenoids as accumulated products of the parent callus. Thus sesquiterpenoids which comprise only 5% of the lower terpenoids in the callus make up 99% of the same fraction from the cell-free extracts derived from the same callus material.

Studies on zonal centrifugation of cell homogenates supported the view that a true sesquiterpenoid pathway rather than a truncated steroid pathway has been characterized. Thus, over 80% of the FPP-synthetase activity occurred in the plastid fraction and this activity was eight- to 10-fold greater than the levels in the microsomal S_{105}-fractions that are considered [17] to be the sites of steroid biosynthesis *in vivo*.

The consequences for lower terpenoid biosynthesis of pre-incubation of the extracts with various additives are given in Table 4. Such treatment with NAD^+ increased the total incorporation into terpenoids some three-fold over that in controls, but did not significantly affect the distribution of tracer into the various products. In contrast, treatment with $NADP^+$ or NADPH which had

Table 2. Accumulation of lower terpenoids in callus and cell suspensions of *L. angustifolia* that had been pre-treated with MVA

Culture type (ex Line C)	Treatment/conditions	10^6.yield (%)‡	Main products
Callus	—	1500	as Table 1
Cell suspension*	—; 1-phase	*ca* 1	ocimene; α-pinene
Cell suspension	—; 2-phase	*ca* 1	ocimene; α-pinene
Callus	MVA	1200	as Table 1
Cell suspension	MVA†; 1-phase	1800	linalool; ocimene
Cell suspension	MVA†; 2-phase	170000	linalool; ocimene limonene

* All suspensions were of the fine-cell type (1 to 100 cell aggregates).

† Cell suspensions prepared from callus that had been treated with MVA.

‡ % wt/wet wt.

Table 3. Terpenoid formation in cell-free extracts from callus lines of *L. angustifolia* as monitored by interconversions of [^{14}C]IPP

Products	Culture line* A	B	C
Protein conc (mg cm^{-3})	0.5	0.4	0.7
Incorporation (%)	15.1	11.0	3.0
C_5 compounds†	2	1	2
2*E*,2*Z*-Farnesols‡	80	74	63
Humulene	12	15	8
Isohumulene§	2	1	0
Caryophyllene	1	1	2
Ocimene/myrcene	0	1	15
β-Pinene/limonene	0	< 1	1

* % Incorporation of [^{14}C]IPP. All values are averages of four experiments. SE ± 10% estimated.

† Isopentenol and 3,3-dimethylallyl alcohol derived from IPP.

‡ *E*:*Z* ratio typically *ca* 20:1.

§ Tentative identification.

little effect on the total incorporation of tracer gave a different distribution of products that also carried over when the two coenzymes were used in conjunction. Now, much less farnesol was formed but there were increased yields of the cyclic sesquiterpenoids humulene and caryophyllene and the levels of the monoterpenoids were enhanced. The increase in the levels of the sesquiterpenoids by up to 20-fold may result from facilitation of the redox conversion of 2*E*,6*E*-FPP into its 2*Z*,6*E*-isomer required for cyclisation, but the effects of monoterpenoid synthetases are mysterious. There is little precedent for these unexpected effects [18, 19].

EXPERIMENTAL

Culture methods. Callus line A was established at Glasgow using previously detailed conditions [4]. It was further sub-cultured on M&S medium [20] modified by the addition of asparagine (100 mg l^{-1}) and by the replacement of IAA with NAA (2 mg l^{-1}), with sucrose content 30 g l^{-1}. The callus was supported on 0.8–1.2% agar (Oxoid No. 1; concn varied to ensure that solid medium prepared with different batches of agar allowed the callus to bed down). Subculture was for 2 or 3 week periods. Line B was established at UCL from line A with the NAA replaced by 2,4-D (2 mg l^{-1}): now the passage time was 12–16 days. Line C was generated from explants of stem tissue from the plant species used (8 years previously) to establish line A using the original method [4], save that the initiation medium contained 2,4-D (2 mg l^{-1}) rather than coconut milk and this was weaned after 3 sub-cultures and the callus maintained on NAA (2 mg l^{-1}); the culture period was 14–16 days. Lines A–C were all maintained on the same diurnal regime of 16 hr at 30° with 1500 lux (Thorn 'white' tubes, λ_{max} 580 nm) followed by 8 hr at 25° in darkness. Cell suspensions were established on M&S liquid medium with the additives as above at 27° in continuous light (λ_{max} 580 nm; 450 lux) in an orbital incubator (120 rpm). Miglyol 812© and n-hexadecane (100 ml l^{-1}) were added before autoclaving. Mevalonate (100 mM) was injected into cellulose cylinders (Sorbarods®) using the technique for wick-feeding of callus that we have previously described in detail [7].

Extraction and identification. Leaves and flowerheads (*ca* 2 g) were extracted by microsteam distillation from a phthalate buffer (pH 7.0; 0.3 M). The worked-up (hexane) extract was subjected to GC-MS using a Kratos MS25 spectrometer linked to a Pye series 204 chromatograph with SE-30 (20% v/v on Chromosorb W: 100–200 mesh; 3 m × 0.80 mm) at 60–160° programmed at

Table 4. Effect of coenzymes on terpenoid synthesis from [^{14}C]IPP in cell-free extracts from callus of *L. angustifolia*

	% Incorporation† Control*	NAD^+	NADPH§	$NADP^+$	$NADPH^+$ NAD^+
Incorporation‡	6.5	19.3	4.5	5.2	20.4
2*E*,2*Z*-Farnesols	82	89	48	36	61
Humulene	12	4	3	30	1
Isohumulene‖	2	3	1	1	1
Caryophyllene	1	1	19	13	21
Ocimene/myrcene	0	0	17	13	10
β-Pinene/limonene	1	0	10	6	4

* All experiments used culture line A. Lines B and C showed similar trends (in less extended studies). ATP had no effect; 2,4-D caused two-fold increase in incorporation but no alteration in percentage distribution from controls.

† All additives at 2 mM. (max. effects at approx this concentration).

‡ Total incorporation; individual compounds as a percentage of this value. All results are mean of four independent experiments. SE ± 10% (estimated).

§ Similar results when NADPH-generating system employed.

‖ Tentative identification.

6° min^{-1} with helium 0.5 l hr^{-1} as carrier. Spectra were assigned using a terpenoid data base provided by Mr J. Janes (BBA Ltd, London, U.K.).

Similarly prepared hexane extracts of callus led to difficulties owing to damage to the capillary column by waxes present at high level in the cultures and for these an alternative method of extraction was employed. The callus (10 g) was frozen (liq. N_2) and ground-up under hexane or Et_2O (100 ml) containing phytol (2% v/v) as marker. The triturate was allowed to warm to room temp. shaken for 8 hr; filtered; the solvent reduced in volume to *ca* 10 ml and passed through Al_2O_3 with hexane–EtOAc (7:3 v/v). The frs containing the lower terpenoids were reduced to small volume (50 μl) by evapn in a stream of N_2 at $-78°$ for analysis.

Volatiles from cell suspensions (one phase systems) were collected by equiping the neck of the culture flask (250 ml) with a sintered-glass filter containing activated charcoal (200 mesh; 14 g) which was washed with Et_2O after the culture cycle. The efficiency of collection was calibrated by the addition of limonene, linaloyl acetate and caryophyllene to the medium as markers.

In addition to GC-MS, the products were identified by comparison with authentic standards on TLC (silica gel 60; 200 μm) at 4° with 4 solvent systems: (i) *n*-C_6H_{14}–EtOAc (17:3); (ii) *n*-C_6H_{14}–EtOAc (9:1); (iii) C_6H_6–EtOAc (7:3); and (iv) $CHCl_3$–EtOH (99:1). Visualization was with phosphomolybdic acid–EtOH (1:19) or vanillin–conc H_2SO_4–EtOH (1:1:48) followed by heating at 80° or 110°, respectively, for 5 min.

Products from the experiments with cell-free extracts were identified by the above TLC correlations and by a micro-method of finger-printing (Banthorpe, D. V. and Yusif, A., unpublished data) whereby the components separated on the chromatograms were eluted with C_6H_6 and heated (70–150°) in sealed capillary tubes with Al_2O_3, P_2O_5 and HCl (*ca* 10 mg) to yield TLC-patterns of unidentified products that could be compared with those patterns resulting from similar treatment of authentic standards.

Cell-free extracts. All glassware was silanized with 1,1,1-trichloroethylsilane. Callus (200 g; optimally in the stationary phase) was frozen (liq. N_2) and ground-up at 4° with an extraction buffer (150 ml) consisting of Na_2HPO_4–NaH_2PO_4 (0.2 M; pH 7.0) containing sucrose (0.25 M), sodium metabisulphite (5 mM), ascorbic acid (5 mM) and dithioerythritol (1 mM). During this procedure polyvinylpyrrolidine beads (200 g; Polyclar-AT) and Amberlite XAD-4 resin (100 g) were added portionwise (over 5 min) to remove phenolics and endogenous terpenoids. The XAD-4 had previously been extensively washed with Me_2CO followed by dil HCl and was stored in the dark under H_2O at 4°. The extract was filtered through cheesecloth (4 layers) and centrifuged (10 000 *g*; 30 min) to yield a supernatant that was used for incubations. Greater sedimentation rates, e.g. 27 000 *g*; cf. [21]; led to less active preparations. The protein content was determined by Bradford's method [22].

The enzymic extract (1 ml) was incubated with [^{14}C]isopentenyl pyrophosphate (1 μCi) in the extraction buffer (5 ml) together with additives (Table 4): the latter showed optimum effects at *ca* 2 mM. Incubations were in stoppered tubes with a layer of hexane (1 ml: added to entrap volatiles) at 27° for 3 hr to the plateau region. All assays were carried out in triplicate and boiled enzyme controls were performed. After incubation the reaction was quenched with aq. NaOH (10 μl; 10 M), the hexane layer was removed and the aq. residue was re-extracted with hexane (3 × 5 ml): the aq. layer was then adjusted to pH 9.0 with Na_2CO_3–$NaHCO_3$ buffer (0.1 M) and the soln was reincubated (27°; 50 mins) with alkaline phosphate and apyrase (1 mg each; ex Sigma) to cleave any phosphate or pyrophosphate esters present. After quenching, the reaction mixture was re-extracted with hexane (3 × 5 ml) and the combined organic fractions reduced (to *ca* 100 μl) for assay. Cofactors were added to the incubation medium and equilibrated (30 min) before the addition of the ^{14}C-labelled precursor. In a few experiments a NADPH-generating system comprising NADP, glucose-6-phosphate, glucose-6-dehydrogenase and $MgCl_2$ was employed [19].

Miscellaneous. Fractional centrifugation (4°) of the cell-free extract yielded plastid (1000 *g*; 3 min), mitochondrial (15 000 *g*; 15 min), ribosomal (105 000 *g*; 2 hr) and supernatant (S_{105}) fractions. Products were assayed for tracer by elution from TLC plates and LSC using Optiscint-O (ex LKB, Croydon, UK) as scintillant. Sufficient disintegrations (4×10^4) were accumulated to ensure that 2σ was $\pm$ 1%. Some products in low yield were characterized on the TLC plates by autoradiography (exposure time 2 months at 30°).

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