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MONOTERPENE METABOLISM IN CULTURES OF *ROSA* SPECIES

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Key Word Index—*Rosa damascena*; Rosaceae; damask rose; tissue culture; enzymes; biosynthesis; oxidative degradation; monoterpenes; geraniol; nerol.

Abstract—Callus and suspension cultures of *Rosa damascena* maintained under ranges of conditions that were predicted on the basis of current hypotheses to favour secondary metabolism accumulated negligible amounts of monoterpenes. However, enzymes that converted mevalonate and isopentenyl pyrophosphate into geraniol and nerol could be extracted from the apparently inactive callus with activities up to 100-fold greater than those from the parent plant, and these activities were optimum in cultures that were slow growing or were in the stationary phase. Callus and suspension cultures both rapidly metabolized exogenously supplied monoterpenes via oxidative pathways. Thus non-accumulation may be due to degradation of nascent products formed endogenously. Similar results were obtained from less detailed studies on *R. gallica*, *R. canina* and *R.* cv. Paul's Scarlet.

INTRODUCTION

The accumulation of monoterpenes by tissue cultures of oil-producing higher plants has not commonly been reported [1, 2] and several of the extant claims have failed to prove *de novo* synthesis (rather than carry-over from the explant) or have involved inadequate characterization. However, valid examples have been found and the achievement of such secondary metabolism may depend on a fortuitous choice of physiological state for the explant coupled with fairly strictly defined culture conditions.

A good model system for evaluating the factors that control terpenoid biosynthesis and accumulation (two quite independent properties, although not often so distinguished) in culture is *Rosa damascena* L. or its cultivars. The petals of this species not only yield valuable commercial oils, but ones comprising as their main terpenoid components the parents of the class—geraniol and nerol [3]. The biosynthesis of the other main component of the oil, 2-phenylethanol, will be considered in a forthcoming publication. Callus of this species, indeed of *Rosa* species in general, may also be a favourable test case as no specific internal structures or organs appear to be associated with the occurrence of oil in the petals, storage taking place in modified epithelial cells [4]. Consequently, accumulation of the oil in culture may not require extensive cytodifferentiation or organogenesis. We here report a detailed study on cultures of *R. damascena* cv. Trigentpetalla. There are good reasons, cf. [5], for expecting callus rather than suspension cultures preferentially to exhibit secondary metabolism, and most of our work has involved the former material. Our present report extends a preliminary study which showed that callus of this species, whilst not accumulating terpenoids, nevertheless yielded cell-free extracts that contained high levels of the enzymes catalysing the steps from MVA to geraniol and nerol [6]. Cultures of *R. damascena* have often been established [7 and refs therein] but the only relevant phytochemical report is a claim that a Russian cultivar yielded callus that accumulated geraniol and linalool [8]: however, it is not clear whether *de novo* synthesis was unambiguously demonstrated in this work, or whether carry-over occurred. Cultures of *R.* cv. Paul's Scarlet did not produce lower terpenoids [9].

RESULTS AND DISCUSSION

Terpene formation by cultures of Rosa damascena

Several callus lines from stem, leaf and petal of *R. damascena* at different stages of growth (young to mature plants and flowerheads) were established on both the Nash and Davies medium that is especially suitable for *Rosa* species and on Murashige and Skoog's medium that (unlike the former) does not contain 2,4-D. (There is some evidence that 2,4-D inhibits secondary metabolism in culture [10].) Friable pale yellow calli were typically obtained, but about 10% showed greening with chlorophyll contents (up to 5% of that of the parent) typical of such adventitiously coloured material [11, 12]. These cultures mainly comprised parenchymous tissue with little differentiation, although the older lines had lignified cell walls and some tracheid formation. Little other morphological change occurred over 5 years in culture (up to 40 passages) and chromosomal analysis of several lines for up to 2 years (no analyses were made for older cultures) showed no change from that of the explant ($2n = 56$, for both callus and suspensions).

Callus lines were assayed (CG-MS) for monoterpenes and other lower terpenoids after 1–24 weeks in sub-

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Abbreviations: MVA, mevalonate; IPA, isopentenyl alcohol; DMAA, 3,3-dimethylallyl alcohol; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate.

culture with isothermal conditions in constant illumination (28°; 200–1000 lux) and under cycles of diurnal thermoperiodicity (12–16 hr day; 12–28°) and in particular under the six 'standard' conditions A–F outlined in Table 1 (legend). In addition, randomly selected cultures were vented to remove accumulations of CO_2 periodically. Under most of these conditions, cultures were easily induced to turn green (up to 20% chlorophyll content of the parent) by addition of raffinose (in place of sucrose) to the media and by the use of a low 2,4-D to kinetin ratio [13]. Both these sets of modified culture lines were also assayed for the accumulation of lower terpenoids, as was the supporting media for selected culture lines. The last analyses were carried out to determine whether biosynthesized products were excreted by the calli. Suspension cultures were also maintained under conditions varied as above (A–F), and the cell pellets and the liquid media assayed after from 1 to 5 passages of 1–6 weeks in culture.

A few callus lines, apparently randomly scattered as to cultural conditions, contained *ca* 10^{-4}% wt/wet wt of citronellol and linalool (via GC-MS), but most showed no detectable ($< 10^{-7}$%) accumulation of monoterpenes. The positive results were at levels *ca* 0.01% that in mature petals (no monoterpenes occurred in leaves or stem) and were some 10^3-fold greater than any likely carry-over from the explants, as shown by controls. No monoterpenes could be detected in any suspension culture or were excreted into any media. These almost negligible accumulations may result from isolated centres of biosynthetic activity within favoured calli, or may derive from a more widespread synthetic ability that is usually effectively vitiated by lack of compartmented storage accommodation and so leads to metabolic turnover (see later). There was no apparent correlation between the stage of growth of the explant and the occurrence of the low levels of terpene accumulation.

This failure to exhibit (to any appreciable extent) the presumed chemical totipotency of the cultures suggested a systematic attempt to induce secondary metabolism using three plausible hypotheses as guidelines. These are that secondary metabolism in plants, and perhaps in cultures, is triggered or favoured by: (a) the ready availability of an excess of acetyl coenzyme-A, ATP and NADPH at the biosynthetic sites [14, 15]; (b) slow growth or stationary conditions [16, 17]; or (c) stressed conditions [18]. The last two, in particular, are obviously interdependent. Consequently, for randomly selected callus lines, various inhibitors were added to the media at a range of non-toxic concentrations and specific culture conditions were adopted. Thus for situation (a), mitochondrial inhibitors (ethidium bromide, chloramphenicol, KCN, ferulic acid), Krebs cycle inhibitors (malonate, fluoroacetate, oligomycin), anaerobic conditions, enhanced levels of sucrose and addition of glucose or pyruvate were used. For (b), inhibitors of protein synthesis (cycloheximide, actinomycin D) were added and low (5–20% of usual) levels of C, P and N sources, variations in 2,4-D:kinetin ratio, and low temperatures (10–25°) were applied. And for (c), temperature variation (0–35°), addition and application of $HgCl_2$, partial necrosis by freezing and/or heat treatments were tested.

No treatment elicited detectable effects on any culture line. Similar negative results have been obtained for *Jasminum officinale* and *Gardenia jasminoides* [19] and induction of terpene accumulation by this approach seems unlikely or at least difficult to attain.

Ability of callus to synthesize monoterpenes

Despite the above negative results, cell-free extracts of culture lines maintained under the six different sets of conditions of illumination and diurnal variation of heat and light (A–F) were all found to contain prenyltransferase (EC 2.5.1.1; catalyses IPP → GPP) and an isomerizing system (perhaps redox) for the interconversion of geraniol and nerol (Table 1). Little difference was found under the various culture conditions or whether the line was derived from an explant of stem, leaf or petal of varying maturity. Small amounts of 2(*E*)-farnesol were also detected; and the table also records less detailed analyses of callus from three other *Rosa* species.

The extractable enzyme activities gave low (maximum *ca* 2.5%) incorporations of substrate, but these comparable with the best results usually obtainable with such crude cell-free extracts from higher plants [20], and the crucial point is that the levels were 50- to 120-fold greater than the activities assayed in extracts of the parent petal, stem or leaf tissue (see legend to Table 1). These results confirm and extend the previous results obtained from a slow growing line (passage time 6 weeks) maintained

Table 1. Enzymes for terpenoid synthesis extracted from callus of *Rosa* species

Species*	Conditions†	% Incorporation (IPP)‡ G	N	F
R. damascena	A	2.0	0.4	0.1
R. damascena	B	0.5	0.4	0.1
R. damascena	C	0.7	0.5	0.0
R. damascena	D	1.9	0.5	0.1
R. damascena	E	0.5	0.2	0.0
R. damascena	F	0.3	0.1	0.0
R. canina	A	0.6	0.5	0.0
R. gallica	A	0.5	0.3	0.0
R. cv. Paul's Scarlet	A	0.5	0.1	0.0

*Callus (from stem explant) assayed after three passages on Nash and Davies medium, as culture entered stationary phase (6–12 weeks, variously).

†A, Natural illumination (south-facing room); *ca* 400 lux maximum, 28°. B, Constant illumination (Thorn 'White' tube, λ_{max} *ca* 580 nm), *ca* 600 lux, 28°. C, Periodic cycle: 'White' light *ca* 1500 lux for 16 hr, 30°; dark for 8 hr, 25°. D, Constant illumination (Thorn 'Growlux' tube, λ_{max} 660 nm), *ca* 600 lux, 28°. E, Natural illumination; as A; 37°. F, Natural (summer) room temperature and illumination; 1500–2500 lux maximum, 10–20°.

‡% Incorporation of IPP (assay conditions as in Experimental) into geraniol, nerol and 2*E*-farnesol.

N.B. Extraction and assay (both carried out under the same conditions as used above) of soluble enzymes from parent *R. damascena* yielded total incorporations of *ca* 0.21% (leaf and stem) and 0.05% (petals) of IPP into lower terpenoids. Only geraniol, nerol and 2*E*-farnesol were detected in ratios close to those typically found from assay of callus (e.g. 1:0.7:0.05). The parent tissue assayed was at the same state of development as that used for explants in culture, but there was little variation (± 20%) of biosynthetic ability with age of the material.

under a single set of conditions [6]. As previously discussed [6], the high activities in extracts from cultures relative to those from the parent plants may not be an intrinsic property of the former, but may well reflect the ease of breakage of the cell walls and also the lower levels of phenolics in the former material.

The extracts also converted MVA into C_5 and C_{10} terpenols but with efficiencies only 20% of the conversions of IPP. Nevertheless, this demonstrated that the enzymes for the stages of monoterpene synthesis from MVA onwards were present in the cultures. Typical profiles for incorporation of MVA and IPP into terpenoids in extracts from callus at different stages of a growth cycle are in Table 2. For all incubations studied—with both environmental and media variations—the cultures were relatively slow growing with a passage time of 6–8 weeks (compared with 2–3 weeks for most herbaceous species under comparable conditions). The variation shown in Table 2 with the maximum enzymic activity attained as the culture entered the stationary phase was found in all examples studied. This supports hypothesis (b), previous section, that secondary metabolism may intervene when cell division either slows or ceases and primary metabolism is reduced to background levels, although the extractable enzymic activity fell off rapidly when the stationary phase had been achieved. It is noteworthy that after 16 weeks in sub-culture the callus which possessed no detectable enzymic activity was not dead. Although no doubt largely necrotic, it rapidly regenerated when sub-cultured. These results imply that conditions of maximum growth and the use of rapidly growing cultures (such as are especially used for studies with cell suspensions) may be quite inappropriate for secondary metabolism to occur.

Cell-free extracts were also prepared from a series of calli that had been treated with potential inducers or inhibitors (at concentrations that did not effectively reduce growth) for 6 weeks in culture. The relative incorporations were: control 1.0 (conditions A, Table 1: total incorporation 2.5%); green cultures 0.9; KCN-treated 0.9: ethidium bromide or chloramphenicol-treated, anaerobic conditions (under N_2) 0.0. All the mitochondrial inhibitors except KCN destroyed the enzyme activities. If the last additive penetrated the callus, this may indicate that a KCN-insensitive respiratory pathway, such as is well established in certain higher plants, occurs in these cultures. The implications of these inhibition studies are being further pursued. Suspension cultures maintained under the variety of conditions as used for callus and of different passage times contained < 1%, if any, of the levels of the terpenoid-biosynthesizing enzymes obtained from callus.

Table 2. Enzymes for terpenoid synthesis from callus of varying ages (ex. *R. damascena*)

Age*	MVA† C_5§	MVA† C_{10}§(%)	IPP‡ C_5	IPP‡ C_{10} (%)
1	0.05	0.04	—	0.05
2	0.09	0.11	—	—
4	0.23	0.33	—	0.9
6	0.25	0.43	30	1.8
8	0.08	0.44	32	2.0
10	0.07	0.13	—	1.6
12	0.05	0.03	—	1.0
16	0.01	0.00	—	0.03

*Age of callus (weeks) after sub-culture. Conditions: M & S medium; 16 hr at 28°/400 lux; 8 hr at 16°, dark.

† % Incorporation of 3*R*-MVA into C_5 alcohols (IPA, DMAA) and C_{10} alcohols (geraniol and nerol); assay via cell-free systems as in the Experimental section. S.e. ± 0.03, actual value. Total recovery of radioactivity 60–90%.

‡Incorporation of IPP as for MVA.

§IPA and DMAA not separated. Geraniol:nerol *ca* 2:1.

N.B. In this set of experiments, the stationary phase was entered at 7–8 weeks after subculture.

It could be argued that the prenyltransferase activity here demonstrated to be extracted from callus is not involved in direct synthesis of mono- and sesquiterpenes *in vivo* but is part of the pathway to steroids (which must be ubiquitous in the cultures) that has been isolated as an artefact. This view is, however, not supported, cf. discussion in ref. [5], by: (a) our findings that prenyltransferase activity as demonstrated here can only be detected in callus derived from plants that accumulate the lower terpenoids as essential oils [5]; and (b) the lack of such activity in extracts from suspension cultures not only of the various callus lines previously studied [5] but of the *Rosa* species here investigated. These cell suspensions certainly have the capacity to synthesize and accumulate steroids, and surely any truncation of the steroids pathway in callus (to yield enzyme systems that synthesize mono- and sesquiterpenes) would have similarly occurred here. In our opinion, this latter set of observations indicates a fundamental difference in terpenoid metabolism between callus and suspension cultures of the *Rosa* (and other) species that we have investigated (cf. [5]). It is certainly possible to truncate the steroid pathway. Cell-free extracts that can sustain the synthesis of the lower terpenoids geraniol and farnesol (as their pyrophosphates) from C_5 and C_6 precursors can be prepared from plants [21 and refs therein] and liver [22]. These sources do not accumulate the lower terpenoids and the artefactual isolation of the early steps of the steroid pathway seems likely. It is significant that these extracts do not produce nerol, a compound not on the steroid pathway but which we have found in our *Rosa* extracts. It is also significant that it has not been possible to isolate a purified fraction, let alone a crude extract, that synthesizes essentially only GPP. In all examples, extracts that produce GPP also predominantly form the farnesyl ester and (at least in liver and probably in plants) a single enzyme is thought to catalyse formation of both compounds in sequence [22]. In contrast, a route to geraniol that is not involved in the steroid pathway seems to occur in higher plants, as geranyl pyrophosphate synthetases that do not form farnesyl compounds or higher terpenoids have been characterized [23 and refs therein]. For these reasons, we consider that a true geranyl pyrophosphate synthetase system has been isolated from our *Rosa* cultures. The small percentage of farnesol may come from a concomitantly extracted farnesyl pyrophosphate synthetase—and it is noteworthy that farnesol does indeed occur at low levels (1–2%) in the essential oil of *R. damascena* and most other species of *Rosa*.

Oxidative degradation of terpenoids in culture

Terpenes may not accumulate in culture, despite the presence of the appropriate enzyme systems, because storage structures (glands, ducts, trichomes) are not formed in the relatively undifferentiated callus and so nascent products are not compartmented and are subject to further metabolism. Indeed, rapid turnover of monoterpenes in petals of *Rosa* species (to form unidentified products) is known [24]. An obvious pathway of metabolism is via epoxidation, followed by hydration and cleavage and such metabolism of a variety of types of compounds both naturally occurring (including terpenes) and xenobiotic, has been demonstrated in plants, in their extracts and similarly from cultures [25–27 and refs therein]. Table 3 records the metabolic fate of ^{14}C-labelled substrates injected into callus or incubated with suspension cultures. The epoxides of IPA, DMAA, geraniol and nerol (ratios in Table 3) and the polyols derived therefrom by hydration were detected and assayed: the balance of tracer was recovered in unreacted substrate, unidentified extractable (oxidation?) products and water-solubles and the last group comprised 60–70% of the recovered tracer in the incubation period 0.5–3 hr. Conversions were low, but this is to be expected with exogenously supplied substrates that may have difficulty in access to the reactive sites, and with reactive polyols that are presumably rapidly further degraded to smaller water-soluble, as yet unidentified, metabolites. The latter process could account for the lack of recovery of epoxides or polyols after the initial hour or so of incubation (Table 3). These results demonstrate the existence of enzyme systems in callus and suspensions that are capable of rapidly oxidizing and degrading the exogenously supplied terpenoids or their immediate precursors. The inertness of citronellol (no $\Delta^{2,3}$ bond) is noteworthy.

Cell-free extracts from callus could be prepared that converted IPP (or IPA with *ca* 50% less efficiency) into its epoxide, diol and triol in yields of 2–6%, with the ratio of the three classes of compounds *ca* 1:4:1. Addition of 1,2-epoxy-*n*-octane, a known inhibitor for hepatic epoxide hydratase [28], eliminated formation of the diols and triols, but also reduced the incorporation into the epoxides by 60%. A detailed study revealed that Cu^{2+} inhibited these oxidation processes, but addition of NADPH over a range of concentrations had no effect [29]. The epoxides of IPA and DMA were converted into diols and triols in yields of up to 7% by these extracts; but geraniol, nerol or citronellol and their oxides were not metabolized. These oxidation processes co-occurred in cell-free extracts that were assayed for terpenoid formation. Although these latter assays were not at optimal conditions for oxidative processes, studies using the inhibitor 1,2-epoxy-*n*-octane suggests that their occurrence generally reduced the incorporations into terpenes by up to 2-fold and so the figures in Table 1 probably underestimate the true levels of activity for terpene biosynthesis.

The above work clearly demonstrates that callus of *R. damascena* (maintained under a wide range of conditions) that did not accumulate terpenoids nevertheless yielded cell-free extracts that possessed the ability to synthesize the lower members of this class. As such callus and also cell suspensions were capable of very rapidly oxidatively degrading certain exogenously supplied ubiquitous hemi- and monoterpenes, it is possible that the lack of accumulation in callus is a consequence of a lack of storage structures or of general subcellular organization that results in oxidation of these nascent products in a manner precluded in the parent plants. Similar results (in less detail) have been obtained with cultures *R. canina* L., *R. gallica* cv. *versicolora* and *R.* cv. Paul's Scarlet, and may be general for oil-producing plants.

EXPERIMENTAL

Culture conditions. Explants (0.5 × 0.1 cm) of leaf, stem and petal tissue from both young and mature specimens of *Rosa* species were cultured in Nash and Davies medium [30] modified by the inclusion of glutamate (600 mg l^{-1}); $NaNO_3$ (200 mg l^{-1}) and EDTA (3:1, w.r.t. Fe^{3+} to prevent precipitation of ferric phosphate), or Murashige and Skoog's medium [31], both made up in 0.7–1.0% w/w Oxoid agar No. 2 (the concentration being varied batchwise to ensure bedding-down). Both media were

Table 3. Metabolic fate of ^{14}C-labelled substrates in culture (ex *R. damascena*)

			Time‡					
Culture	Substrate*	Oxid. prod.†	0.5	1	3	12	24	48
Callus	3*R*-MVA	$C_5 + C_{10}$§	0.5, 0.6	0.02, 0.02	0.0, 0.0	0.0, 0.0	0.0, 0.0	0.0, 0.0%
Callus	IPP	$C_5 + C_{10}$§	0.0	0.0	0.0	0.0	0.0	0.0
Callus	Geraniol	$C_5 + C_{10}$§	0.6, 0.6	0.1, 0.1	—	—	—	0.0, 0.0
Callus	Citronellol	$C_5 + C_{10}$§	0.0	0.0	—	—	0.0	0.0
Suspension	Geraniol	Citrals‖	0.8	0.7	0.1	0.3	0.2	0.1

*Typically 10 μCi, 0.22 mmole^{-1}.

†Epoxides of C_5 and C_{10} terpenols and polyols derived therefrom by hydration. The relative quantities of epoxides, diols, triols and polyols were *ca* 1:0.2:0.2:0.1 for C_5 compounds and 1:0.5:0.1:0.1 for the C_{10} compounds respectively.

‡Time of incubation (hr); % incorporation of tracer. Injected into callus (6 weeks in subculture) or added (0.2 mM) to suspension culture (4–6 weeks in culture after two, four or six passages). Geraniol was toxic to cultures at > 2 mM.

§Oxidation products of IPA, DMAA, geraniol and nerol; the balance was largely in water solubles and in metabolic products not assayed or identified.

‖Citrals a and b (geranial and neral).

—Indicates no assay was made at this time period.

supplemented by 10% (v/v) coconut milk. Callus formed (5-fold increase in vol.) in *ca* 50% cases within 3–9 months under standard conditions at 27° with natural illumination from a south-facing aspect at max. 400 lux. Greater light intensities caused extensive browning of cultures. The passage time after *ca* 5-fold increase in vol. (for conditions A Table 1: conditions B–F gave similar growth rates) was 6–8 weeks and the coconut milk was weaned after the first sub-culture. Greening was induced by incorporation of raffinose (2%) in place of sucrose as the carbon source and use of a 2,4-D to kinetin ratio of 0.02:1 (mg l^{-1}). Potential inhibitors were added to the medium at ranges of concns that did not alter the growth rate: viz. ethidium bromide (5–30 mg l^{-1}), chloroamphenicol (200–500 mg l^{-1}), KCN (0.1–7 mg l^{-1}), ferulic acid (1–3 mg l^{-1}) and others (1–20 mg l^{-1}). Experiments with C, N and P deficient media involved 5–20% of the normal levels of these elements. Heat stress was obtained by high incubation temps up to 37° for varying periods or by application of a hot platinum wire loop to areas of the callus. Suspension cultures were routinely maintained in Murashige and Skoog's liquid medium at 27°/600 lux at 120–150 rpm and lines were developed under conditions A–F (Table 1). The passage time was *ca* 28 days, and typically the suspension comprised clusters of up to 20 cells of characteristically elongated shape. Chromosome numbers were determined in the latter half of the exponential phase: mitotic arrests were achieved with the use of colchicine and 8-hydroxyquinoline.

Assay of callus and suspensions for terpenoids. Cultures (10–20 g) were pulverized in liq. N_2, and extracted (Soxhlet) with Me_2CO (40 cm^3; 3 hr) and Et_2O (40 cm^3; 3 hr). The aq. residue was combined with the insoluble plant debris, treated with 10 M HCl (6 cm^3; 60°/2 hr), extracted (Et_2O; 3 × 20 cm^3) and combined with the previous extract: thus free and bonded terpenoids were collected. This extract was concd to 5 cm^3; flash distillation) and assayed by TLC on SiGel H_{60} (PhMe–EtOAc, 17:3 v/v) or GC (FFAP; 15% on Chromosorb W, 80–100 mesh; 0.5 cm × 10 m; 3.5 l hr^{-1}; 100–200° at 1° min^{-1}). For the few cases where positive identifications were made (by comparison with authentic standards), the products were confirmed by GC-MS on the above columns using a Kratos MS-25 mass spectrometer linked to a Kratos 65-505 data system and computer.

Cell-free extracts. [1-^{14}C]-IPP for use as substrate was purchased (Amersham International), [^{14}C]-DMAPP was prepared [32] and labelled geraniol and nerol were obtained by feeding $^{14}CO_2$ to *Pelargonium graveolens* [33]. The epoxides of IPA, DMAA, geraniol and nerol, and their hydration products (diols and triols) were all prepared as standards, and their spectral and chromatographic properties have been recorded [29].

Soluble extracts of callus and suspensions were prepared as previously described for terpenoid formation [6] and for oxidative systems [27]. Additionally, for the studies involving oxidative degradation, the substrates (when largely water-soluble) were incubated in a rotary shaker (100 rpm), and the extraction procedure (for the very water-soluble products) involved use of solns satd with NaCl, cf. [25]. The efficiencies of extraction by the latter method were: C_5-epoxides (94%); -diols (80); triols (40); C_{10}-epoxides (90); C_{10}-diepoxides (70); C_{10}-triols and diols (40–60%); as determined by controls using authentic substances.

Products were routinely separated and purified for radioactive assay by TLC and GC as described in the citations, as controls confirmed that very similar results (s.e. ± 10%) were obtained after rigorous, time-consuming, purification via recrystallization of solid derivatives to constant specific radioactivity. The rapid (GC, TLC) assay was valid as few contaminants occurred in the cell-free preparations: much more complex procedures involving extensive purification would have been necessary for studies of metabolism in intact callus or suspensions.

Degradation of terpenes in callus and suspension cultures. ^{14}C-labelled substrates (0.5 μCi; 0.2 mCi $mole^{-1}$) were emulsified with Lubrol 12 AG (ex ICI Dyestuffs Division, Manchester) in MES (pH 7.0; 0.1 M; 0.5 cm^3) and injected into callus via a millepore membrane (pore size 0.22 μm). Additives to cell suspensions were injected (10% v/v in DMSO; 5–10 cm^3) into the medium (100 cm^3). After the appropriate metabolism times the cultures were extracted and assayed as above.

Radiochemical assays. These were standard using LSC with butyl-PBD in toluene (0.8% w/v) as scintillant. 4×10^4 disintegrations were accumulated so that 2σ was ± 1%.

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