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ABILITY OF PLANT CALLUS CULTURES TO SYNTHESIZE AND ACCUMULATE LOWER TERPENOIDS

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Key Word Index—*Anethum graveolens*; Umbelliferae; *Jasminum officinale*; Oleaceae; *Lavandula angustifolia*; *Ocimum basilicum*; *Rosmarinum officinalis*; labiatae; *Pinus radiata*; Pinaceae; *Tanacetum vulgare*; Compositae; tissue culture; biosynthesis; cell-free extracts; monoterpenes; sesquiterpenes.

Abstract—Callus cultures derived from seven oil-producing plants have been maintained under different regimes of media, temperature and illumination, and have been assayed for ability both to synthesize and to accumulate mono- and sesqui-terpenes. Callus of *Pinus radiata* (the sole gymnosperm) accumulated α- and β-pinenes at levels comparable with those in the parent stem and needles; and that of *Jasminum officinale* accumulated traces of several monoterpenes (< 0.1% the amount in petals), but cultures of *Rosmarinus officinalis*, *Lavandula angustifolia*, *Anethum graveolens*, *Ocimum basilicum* and *Tanacetum vulgare* did not detectably accumulate the lower terpenoids or secrete them into the medium. However, all seven culture lines yielded cell-free extracts containing prenyltransferase and an isomerizing system (as assayed by conversion of IPP into GPP, NPP and FPP) with activities some 3–400-fold greater than those extracted from the parent mature plants, or up to 90-fold the levels extractable from young seedlings of the various species. Callus of five of the species (*A. graveolens* and *O. basilicum* were not assayed) also contained MVA-kinase, MVAP-kinase, MVAPP-decarboxylase and IPP-DMAPP isomerase at levels comparable with those in the parent tissue. Hence the angiosperms yielded cultures that presumably contained the crucial enzymic machinery necessary for the synthesis of the lower terpenoids, although accumulation of those compounds did not occur. Reasons for this unexpected situation are discussed. These results imply that callus cultures may be a convenient source of biomass for studies on the enzymes of terpenoid biosynthesis.

INTRODUCTION

Accumulation of monoterpenes resulting from *de novo* synthesis has been demonstrated in tissue cultures of several oil-bearing plants: e.g. in *Mentha* [1,2], *Pelargonium* [1], *Thuja* [3], *Tanacetum* [4], *Perilla* [5] and *Gardenia* species [6; and see a recent review, 7], in addition to several examples to be cited below. *de novo* Synthesis and identification of products has been inconclusive in many other reports [cf. 8–10], and the minute quantities of monoterpenes (and other secondary metabolites) claimed to accumulate after a few subcultures may be the consequence of carry over from the explant. However, in many of the valid examples the yields, types and proportions of monoterpenes differed widely from those in the parent tissue. Spectacular syntheses of certain sesquiterpenes have also been demonstrated in several culture lines [cf. 3, 11, 12], and production of this class in culture has been induced by fungal infection and by cell-wall degrading enzymes [13; Brooks, C. J. W. and Watson, D. G., unpublished results].

There is an obvious but apparently not distinguished difference between the ability of a tissue to synthesize monoterpenes or other secondary metabolites and its capacity to accumulate these products. For example, nascent terpenoids may be degraded by routes that have been demonstrated to exist in plants and in tissue cultures [14, 15] or they may be functionalized *in situ* or be secreted into the medium there to undergo unsuspected secondary reactions. One way to evaluate the intrinsic ability of a culture to synthesize monoterpenes is to prepare and assay suitable cell-free extracts. Such extracts that are exceptionally active in promoting sesquiterpene biosynthesis have been prepared for *Andrographis* cultures [16]; and recently we have found that cultures of *Rosa damascena* that did not accumulate detectable quantities of monoterpenes nevertheless yielded extracts that contained all the enzymes necessary for synthesis of geraniol and nerol (the parents of monoterpenes) from MVA at levels of activity up to 300-fold that obtainable from the parent tissue [17].

We now record some systematic studies on these two capabilities for callus derived from seven oil-producing plants: *Tanacetum vulgare* L., *Pinus radiata* D. Don, *Jasminum officinale* L., *Rosmarinus officinalis* L., *Lavandula angustifolia* L. Mill., *Ocimum basilicum* L. and *Anethum graveolens* Benth. Other aspects of terpenoid

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Abbreviations: MVA, mevalonate; MVAP, 5-phosphomevalonate; MVAPP, 5-pyrophosphomevalonate; IPP, isopentenyl pyrophosphate; DMAPP, 3,3-dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; NPP, neryl pyrophosphate; FPP, farnesyl pyrophosphate; Pi, phosphate (inorganic).

metabolism by cultures of the first five species have previously been reported from these laboratories [15, 18–20]. Cultures of *O. basilicum* have been established [21] and claimed to accumulate thymol [22] or linalool [23], although *de novo* formation and unambiguous characterizations were not achieved. *Anethum graveolens* has been cultured but no accumulation of essential oils was reported [24–26]. Except for *P. radiata* (cf. ref. [19]; to be discussed later) no comparable studies using cell-free extracts have been made for these, or any other monoterpene-producing species save for the *Rosa* species previously mentioned [17].

Our present work used callus tissue. There are good reasons for concluding that synthesis and accumulation of the lower terpenoids (unlike alkaloids and steroids) may usually require a degree of cytodifferentiation in cultures with the formation of specific glandular structures or resin ducts in both angiosperms [27, 28] and gymnosperms [29]. This is not achieved in cell suspensions where there is anyway appreciable selection pressure to favour fast-growing cell lines that may be deficient in secondary metabolism [30–32]. Our experience with suspension cultures (see later) does indicate a much inferior synthetic capacity, if any, for secondary metabolism as compared with the situation for callus tissue.

RESULTS AND DISCUSSION

Nature of cultures

All seven species easily formed callus after a 4- to 10-week induction period with success from 20 to 50% of explants when a modified Murashige and Skoog or Nash and Davies medium was used (see Experimental). All derived cultures were stable as regards morphology, ploidy (where examined) and the assayed biosynthetic properties for at least 2 years (when experiments were discontinued) and the cultures from *R. officinalis* and *L. angustifolia* have been thus maintained for six and five years respectively. Subculturing was usually performed after 2–6 weeks depending on the species. Use of recently recommended media [33] or addition of 4-chlorophenylacetate which is claimed to induce growth in Compositae [34] were found to be ineffective.

Callus of all species were friable with little apparent differentiation. They mainly comprised vacuolated parenchyma-type cells with some formation of tracheids and centres of meristematic activity [35, 36]. Root formation persisted in all conditions for cultures of *O. basilicum*, but was minimized by addition of kinetin and NAA (0.5 mg/l) to the media. *Anethum graveolens* was unique in that there was no tendency for polyphenol formation (i.e. 'browning') even in cultures that were extensively illuminated.

Many studies of plant tissue culture have used isothermal conditions with constant illumination—although these are hardly physiological conditions for plants! Similarly, many studies have involved use of conditions chosen to maximize growth: but these may not be good choices to favour secondary metabolite production. The latter may be stimulated when cell division is slow or has ceased, and metabolites and cofactors become available for alternative processes to primary metabolism [37]. Consequently, we have monitored synthesis and accumulation of mono- and sesquiterpenes in cultures maintained under a variety of conditions of light and temperature, and also with various additions to the medium.

The external conditions used, which will be referred to in the following sections, were: (A) natural illumination (south-facing room; *ca* 400 lx max.), 28°; (B) constant illumination (Thorn 'White' tube, λ_{max} 580 nm, *ca* 600 lx), 28°; (C) periodic cycle: 'white' light *ca* 1500 lx for 16 hr, 30°, dark for 8 hr, 25°; (D) constant illumination (Thorn 'Growlux' tube, λ_{max} 660 nm, *ca* 600 lx), 28°; (E) natural illumination (*ca* 400 lx max.), 37°; (F) natural (summer) room temperature and illumination (1500–2500 lx max.), *ca* 10–20°; (G) total darkness, 28°. Variations in media are listed in Table 2, and in the later text.

Accumulation of terpenes by callus cultures

The lower terpenoids produced by the parent plants of all species have been recorded [38], and recent analyses have been reported for *R. officinalis* [39] and *A. graveolens* [40]. Our analyses of leaf material were in broad agreement with these. Our general procedure was to maintain stock callus with regular subculturing under conditions (A), and to introduce regimes (B)–(G) for 1–6 cycles of subculture before assay for accumulation of terpenes or for capacity for such synthesis (see next section and Table 1 for growth rates under the different conditions). Both callus and medium were analysed for free and conjugated (i.e. α- and β-glucosides; phosphate esters) terpenes. The only callus to accumulate such products (free terpenes) was that from *P. radiata*, as has previously been briefly reported [19]. The anatomy of these *Pinus* cultures appeared the same as those of the other six types, with no obvious differentiation to form secretory or storage structures [cf. 19, 30]: but for conditions (A)–(F), α- and β-pinenes were detected in yields in the range $0.13–2.3 \times 10^{-2}$% wt/wet wt. These levels are at least 10^6-fold greater than any possible 'carry-over' and at best represent 20–40% of the levels accumulated in needles and stem of the parent plant [19]. No conjugated terpenes were found in the callus; and no terpenes (free or conjugated) were detected in the media under any conditions.

Callus of *Jasminum officinale* (conditions A and C) accumulated nerol, linalool, citronellal, citronellol and citrals (all free) in total *ca* 1×10^{-5}% wt/wet wt. Although small, this value is real and is some 10^3-fold greater than the minimum level detectable by the GC-assay used. With regard to age and cultural history of the culture line, it could not represent any carry-over: indeed, the original explant from stem lacked detectable quantities of monoterpenes. The observed accumulation was, however, less than 0.1% of the level of oil in petals of the parent plant (the latter contained mainly linalool, benzyl acetate and benzyl benzoate). Callus of the other five species under conditions (A)–(G) did not produce detectable quantities of lower terpenoids: less than 10^{-2} μg/g (if any) was present: this corresponds to less than 10^{-4}% (if any) of the levels in the storage organs in the parent plants.

After this work was completed, it was reported that *Thuja* cultures secreted monoterpenes into the medium and these were largely (but not completely) lost by volatilization unless trapped in a layer of a commercially available triglyceride [3, 41]. Previously we had attempted similar trapping experiments using toluene, liquid paraffin or alkanes, but these led to cessation of growth or death of the callus, presumably owing to coating and inhibition of gas and nutrient exchange. The failure to

detect even traces of terpenoids or their known products of metabolism, even when precautions were taken to prevent volatility loss, leads us to conclude that negligible secretion could have occurred with our examples. In addition, blanks showed that the level of secretion reported for *Thuja* would have been detected by our methods.

Callus of *R. officinalis* and *T. vulgare* were also grown with the medium supplemented with casein hydrolysate and with an extract of necrotic cells of *L. angustifolia* (this species was chosen as its callus is biosynthetically active for non-terpenoid secondary metabolites [42]); and also on N and C deficient media (levels 10–70% those in the standard medium). No terpenoid biosynthesis was detected, although growth was significantly slower (down to 20% of controls) in the latter experiments.

Attempts to detect terpene synthesis in several (*P. radiata*; *T. vulgare*; *J. officinale*) callus lines by injection with intermediates of the pathway also failed. Thus, ^{14}C-labelled MVA and IPP were incorporated less than 1×10^{-4}% if any. This type of approach is however limited by possible restriction or prevention of access of the exogeneous material to any existing biosynthetic sites.

Synthetic capabilities of cell-free extracts of callus

There are many advantages in assaying the capability of cell-free extracts from cultures for carrying out terpene biosynthesis rather than relying on accumulation of the products as an index of synthetic ability. Thus (a) conversion of specific intermediates into a few particular terpenoids can be assayed, rather than assay of conversion into possibly unsuspected and unknown conjugates; (b) complicated assays of media are eliminated; (c) assays are rapid and reproducible and can be easily carried out on extracts of callus subjected to various regimes; (d) secondary reactions of the product under study frequently do not occur in the extracts and in any case can be usually eliminated; (e) the presence (or absence!) of specific steps in a biosynthetic chain of reactions can be demonstrated; and (f) the predictability and paucity of likely products simplifies chemical and radiochemical assays.

Table 1 records the growth rates for callus of *R. officinalis* and *L. angustifolia* under a variety of environmental conditions. Typically, stock cultures that had been maintained under conditions (A) were transferred to conditions (B)–(G) as described in the previous section. The rapid growth in complete darkness (condition G) is noteworthy: a similar effect has been reported for *Pinus* cultures [43]. Conditions (C), (D) and (E) induced greening in each species. Maximum levels of chlorophyll in *R. officinalis* were 0.06 mg/g ($a:b = 18:1$) comparable with *ca* 0.6 mg/g ($a:b = 22:1$) in leaves. No carotenes were detected. *Lavandula angustifolia* produced less chlorophyll (0.03 mg/g at optimum) but this was now localized into intensely pigmented areas.

Also recorded are the synthetic capabilities of cell-free extracts prepared by methods known to be effective when applied to plant tissue generally [19, 44]. Products of incubations with [^{14}C]-IPP were routinely separated and monitored by GC-TLC procedures and similar results were obtained (within the experimental error) when rigorous purification via derivatization to constant specific radioactivity was carried through. Such simple GC-TLC purifications certainly would not have been adequate for products biosynthesized by the whole cultures.

IPP was incorporated by all extracts tested into geraniol, nerol and 2-*E*- and 2-*Z*-farnesols, although the former farnesol isomer was always predominant ($E:Z$, *ca* 20:1). Thus, prenyltransferase (EC 2.5.1.1) which catalyses the formation of geraniol and 2*E*-farnesol from IPP (and the derived DMAPP), IPP-DMAPP isomerase (EC 5.3.3.2) and an $E \rightarrow Z$ isomerase system (perhaps redox) which converted the two primary products into nerol and 2*Z*-farnesol (or their pyrophosphates) were present in the extracts. It is noteworthy that the four recorded products are not important components of the leaf oil of either species, but are usually considered to be the parents of acyclic and cyclic mono- and sesquiterpenes that do there occur. However, the cell-free extracts also produced higher terpenoids. Thus for *R. officinalis*, with little variation over conditions (A)–(F) incorporations into TLC-fractions provisionally assigned as sesquiterpene hydrocarbons (mainly caryophyllene and longifolene, *ca* 0.9%); diterpenes (mainly phytol 4%) and higher terpenoids (mainly squalene, 0.2%) as well as into the

Table 1. Prenyltransferase and related activities in cell-free extracts from callus maintained under different temperature–illumination regimes; IPP as substrate

	R. officinalis					*L. angustifolia*				
Conds*	Growth†	Pigment‡	G	N	F§	Growth	Pigment	G	N	F
A	0.32	—	0.3	0.1	28	0.40	—	0.5	0.1	17.1
B	0.16	g	—	—	—	0.11	—	—	—	—
C	0.28	g″	0.8	0.1	26	0.31	g	0.3	0.1	15.8
D	0.23	g	0.3	0.1	27	0.26	g″	0.4	0.1	14.9
E	0.21	—	—	—	—	0.37	—	—	—	—
F	0.04	g	0.2	0.1	23	0.08	g	0.1	0.03	9.5
G	0.36	—	0.8	0.7	9	0.38	—	—	—	—

*Cultures maintained under conditions (A)–(G) (see Discussion) for 2–3 subculture cycles.

†Index of growth. $G = (m_t - m_0)/m_t \cdot t$, where m_0 and m_t are masses of explants at initiation of last subculture and its termination at t days (s.e. $ca \pm 10$% actual values).

‡Pigmentation: g indicates greening; g″ most intense colouration, —indicates creamy-white callus.

§% Incorporation of tracer into products. G = geraniol; N = nerol; F = farnesol ($2E + 2Z$ isomers); s.e. $ca \pm 10$%.

monoterpenes α- and β-pinene (0.05 %). The above assignments are by R_f values in TLC only, but imply the existence of mono- and sesquiterpene cyclases and C_{20} and C_{30} synthetases in the extracts. These subjects will be amplified in a future communication. It is noteworthy that 'greened' cultures that might be expected to possess enhanced biosynthetic abilities over those of their colourless counterparts, yielded extracts with no particular potency.

The recorded products (Table 1) refer to callus maintained under the specified conditions for 2 or 3 subcultures. Assays carried out after longer periods under the regimes generally gave lower but constant values. Thus for *R. officinalis* under conditions (A) the % incorporation of tracer into combined lower terpenoids by extracts prepared after 2, 5 and 17 subcultures was 28.4, 18.2 and 18.1 %. In a series of ancillary experiments, it was shown that (a) MVA was incorporated into lower terpenes by the extract, but at levels 10–20-fold less than IPP; (b) iodoacetamide and SKF 625 (known inhibitors of IPP-DMAPP isomerase) blocked all production of lower terpenoids; (c) incorporations from IPP as precursor were closely paralleled when equivalent quantities of IPP and DMAPP were incubated together with iodoacetamide; and (d)—what is implied in (a)—that MVA was converted by typical extracts into MVAP, MVAPP, IPP (in the presence of iodacetamide to block further progression) in yields, typically, 0.1, 0.3 and 9 %—conversions with a similar profile to that obtained from extracts from the parent plants.

A detailed analysis of the above results would require a monitoring of the individual enzyme activities involved in the building up from MVA (C_6) to terpenoids (C_{10} and C_{15}); but the general conclusion is that although the callus maintained under the various conditions did not accumulate the lower terpenoids, all the relevant enzymes for the production of these compounds from MVA could be demonstrated to be present in all the conditions that were investigated.

Table 2 records a limited study of the effect on the extractable prenyltransferase and related systems from callus maintained under conditions (A) when the culture medium was varied. Incorporations were observed that were outside experimental errors of measurement, but no spectacular effects were found. The rationale for the variation of sucrose concentration was a report that an increase in this level often causes an increase in secondary metabolism in cultures [45]. Not shown in the table is a very significant increase in incorporation of IPP into the sesquiterpene hydrocarbon fraction (see before) when either $NADP^+$ or NADPH was added (1.3 mM) to the assay system. For *R. officinalis*, the percentage incorporations into this fraction were increased by *ca* 10- and 3-fold respectively when callus that had been subject to 10 and 25 subcultures was assayed. The effect was reproducible and was mirrored by a corresponding decrease in incorporation into farnesol. A very similar effect was also found for callus of *L. angustifolia*, and presumably indicates a stimulation of sesquiterpene cyclase activity.

Metabolism of IPP by extracts of callus of other species that had been maintained on temperature–illumination regimes are recorded in Table 3. Again, prenyltransferase and its associates were detected wherever sought; and for *T. vulgare* and *P. radiata* the extracts probably sustained the synthesis of bicyclic monoterpenes. The latter, however, are provisional identifications by TLC that have not been confirmed rigorously by purification *via* derivatization. The formation of sabinene by callus of *T. vulgare* recalls the accumulation of this bicyclic monoterpene in a previous callus line of the species [18].

Table 4 records the overall prenyltransferase activities (total incorporation into geraniol, nerol and derived monoterpenes and the farnesols) from cell-free extracts of callus (conditions A) and compares these with the activities in extracts from seedlings, mature plants and in shoots regenerated from callus, all extracted and assayed under the same conditions. It was previously shown that shoots regenerated from callus of *R. officinalis* [20] and *L. angustifolia* [20, 46] were capable of accumulating monoterpenes characteristic of the parent plants, although the callus was devoid of this ability. The extracts from callus of all seven species were uniformly higher in enzymic activity by 3–400-fold than the levels from mature plants, and up to 90-fold than those from seedlings.

Biosynthetic capabilities of suspension cultures

Suspension cultures of *P. radiata* (callus of which showed the greatest ability for both accumulation and synthesis of lower terpenoids) produced negligible, if any, amounts of stored lower terpenes (< 0.001 % that of the mature plant), and no excretion into the medium could be detected. Less detailed studies with cell cultures of *R. officinalis*, *T. vulgare* and *J. officinale* led to the same

Table 2. Prenyltransferase and related activities in cell-free extracts from callus maintained on modified media; IPP as substrate

Conditions*	% Incorp. (*R. officinalis*) G	N	F†	% Incorp. (*L. angustifolia*) G	N	F
Standard (A)	0.3	0.1	28	0.5	0.1	17.1
Sucrose (0.5-fold)		39		0.3	0.3	27.1
Sucrose (3-fold)		27		0.3	0.1	21.8
Coconut milk (10 % v/v)		26		—	—	—
2,4-D (1 mg/l)	1.0	0.3	37	0.8	0.2	25.6
Extract of callus‡		24			35	

*Modification of standard medium.

†Percentage incorporation of tracer: products as Table 1.

‡Extract (100 g/l) of necrotic tissue of *L. angustifolia* (see text).

Table 3. Prenyltransferase and related activities in cell-free extracts from callus of other species: IPP as substrate; culture conditions (A)

Species	Conditions	% Incorporation G	N	F	Others
A. graveolens	A	0.9	0.9	1.2	—
	C*	0.8	0.8	1.0	—
O. basilicum	A	0.1	0.1	5.7	—
J. officinale	A	0.1	0.1	—	0.2†
	C	0.0	0.1	—	0.3†
T. vulgare	C*	0.0	4.1	—	4.0‡
P. radiata	A	3.9	42.1	—	0.4§
	C*	1.8	40.1	—	0.2§

*Green cultures (chlorophyll 0.01–0.1 mg/g).
†Linalool.
‡Sabinene.
§α- and β-Pinene.

conclusion. Similarly, negligible levels of prenyltransferase could be detected in extracts of these cultures. However, much more detailed work must be carried out before it can be assumed that these suspensions lack the ability to synthesize these terpenoids. More so than for callus, the predominant cell type in fast-growing suspensions may be selected for growth at the expense of differentiation and (presumed) concomitant ability for secondary metabolism, and the predominant type may crucially reflect the nature of the explant and the culture conditions.

General

Only callus from *P. radiata*, the sole gymnosperm investigated, showed a capacity to accumulate the lower terpenoids that rivalled that of the parent plant. No terpene accumulation was previous recorded in callus of this species [47], but then a different medium (including 2,4-D and BAP) was used. We have consistently avoided use of 2,4-D as it often inhibits secondary metabolism in culture [48] and our attempts to use the new medium led to poorly growing cultures with negligible synthetic ability. *Jasminum officinale* showed very low levels of accumulation of monoterpenes and the other five angiosperms did not accumulate detectable levels of these metabolites, if any.

It is especially disconcerting that callus of *T. vulgare* did not accumulate terpenoids, as a line established in 1966 and maintained for three years showed considerable differentiation and accumulated monoterpenes at levels *ca* 50% that in the parents [18]. This line could not be re-established despite considerable efforts, and use of the previous culture medium resulted in material that ceased growth after two or three subcultures. Presumably, the establishment of the successful line necessitated fortuitous choice of material for the initial explant, and this may be a general phenomenon. Detailed studies of the properties of cultures from explants of different ages and history from different clones are necessary. Detailed studies are also under way to ascertain whether accumulation such as occurs in *P. radiata* occurs in cultures of other gymnosperms.

The monoterpene-accumulating line of *P. radiata* [cf. 19], a recent reported culture line of *Thuja occidentalis* that secreted lower terpenoids [41] and the previously successful line of *T. vulgare* [18] all were slow growing, with *G*-values (see legend to Table 1) of *ca* 0.15, 0.03 and 0.01 respectively compared with values of 0.2–0.4 for most of our other lines. Slow growth may reflect an unadapted culture prone to necrosis that leads to stressed conditions suitable for the onset of secondary metabolism [37, 49]. It is interesting that the above three cultures produced monoterpene hydrocarbons, and those compounds are predominant for callus that accumulates monoterpenes [3, 5, 7, 20]. Oxidative elaboration of these primitive types may require further differentiation.

Despite the general lack of accumulation of lower terpenoids, all seven callus lines maintained under a variety of conditions did possess the crucial enzymic machinery for the construction of the parents of the lower terpenoids (Tables 3 and 4), and in addition the MVA-activating pathway: MVA-kinase (EC 2.7.1.36); MVAP-kinase (EC 2.7.4.2); MVAPP-decarboxylase (EC 4.1.1.33); and IPP-DMAPP isomerase (EC 5.3.3.2) was present and

Table 4. Prenyltransferase activities in cell-free extracts from callus, regenerated callus, seedlings and mature plants

	% Incorporation of IPP → C-10 + C-15 compounds				
	Callus	Seedlings*	Mature plant	Reg. callus†	Ratio‡
R. officinalis	33.6	0.6	0.1	12.1	336
L. angustifolia	21.9	12.5	1.1	15.6	20
A. graveolens	3.0	0.05	0.01	—	300
O. basilicum	5.9	0.8	0.1	—	59
J. officinale	0.4	0.1	<0.001	—	>400
T. vulgare	8.0	9.2	2.3	—	3
P. radiata	46.4	0.5	0.3	—	150

*Five weeks after germination.
†Shoots regenerated from callus.
‡Ratio of prenyltransferase and related activities in extracts from callus (maintained under conditions A) and mature plant.

could be extracted. Others have reported the isolation of the MVA-activating sequence (but not prenyltransferase) from callus of *Nepeta cataria* that did not accumulate monoterpenes [50]. In our work, the levels of prenyltransferase and related enzymes of terpene biosynthesis did not change greatly with the variation of a few (though possibly crucial) components of the medium or on the temperature-illumination regime. It could be argued that the prenyltransferase activity demonstrated is not involved in direct mono- and sesqui-terpene formation *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 not supported by our observations that prenyltransferase activity as demonstrated here can only be detected in plants that accumulate the lower terpenoids (i.e. as essential oils). Thus, callus of *Acer pseudoplatanus* (sycamore), *Phaseolus vulgaris* (broad bean) and *Zea mays* (maize) that certainly must have had the capacity to biosynthesize steroids yielded cell-free extracts devoid of prenyltransferase activity using our assay methods. Also, any artefactual isolation of the enzymic pathway to steroids in callus should surely be mirrored by similar artefacts from suspension cultures. But in the event, no prenyltransferase activity could be detected in extracts from suspension cultures of several of the species investigated in this paper. In addition, formation of C_{15} and C_{20} compounds (not on the pathway to steroids) were also detected in our extracts.

The lack of accumulation of lower terpenoids in most cultures despite the apparent presence of active enzymes mediating the steps from MVA may be due to the secondary reactions mentioned earlier. It may also be due to the virtual absence or low activity of an enzyme for a step before MVA, e.g. HMG-coenzyme A reductase. Such enzymes must be present as inferred from the steroid synthesis, but they may be compartmented from the enzymes of the terpenoid pathway.

The demonstration of prenyltransferase activity from *Rosa* cultures [17] which is now reproduced here in every example (Table 4) may represent a general property of callus cultures of oil-bearing plants. But the high levels of the activity relative to the levels from seedlings or mature plants may not be intrinsic but may be a consequence of the ease of the disruption of the cell walls and internal membranes and the lack of phenolics and other inhibitors in the culture compared with the situation in the plant. The enhanced values could also mean that some or all of the enzymes of the terpenoid pathway are derepressed in the cultures. Whatever the explanation, the present results suggest that callus cultures might provide readily available biomass for enzymic studies on terpenoids and other secondary metabolites.

EXPERIMENTAL

Culture conditions. Seeds and mature plants were obtained from the Royal Botanic Gardens, Kew and the U.C.L. Botanic Gardens. Explants from seedlings (30 days after germination) or shoots of young plants were initiated on a variety of media containing Oxoid agar (0.8–1.2%; adjusted for different batches so that the tissue 'bedded down') under conditions (A). The optimal media found were: *T. vulgare* (stem and leaf), Murashige and Skoog (MS) or Nash and Davies media [51, 52]; *J. officinale* (stem and leaf) and *A. graveolens* (stem), M and S; *O. basilicum* (stem), M and S with kinetin (0.5 mg/l) and NAA (1 mg/l) to suppress root formation; *R. officinalis* (stem) and *L. angustifolia* (stem), M and S medium modified [20]; and *P. radiata* (stem), M and S medium modified [53]. Coconut milk (10% v/v) was added to all, but weaned at the first or second subculture. Callus formed (4–10 weeks) with a success rate of 10–50%. Subsequently, subculture was performed at 2–6 week passages depending on the species and cultural conditions. All callus was maintained, apparently unaltered in its characteristics, for at least 1 year, and that of *L. angustifolia* and *R. officinalis* much longer (see text). Suspension cultures were maintained on M and S liquid medium at 100 to 120 rpm with passage times of 18–25 days after cell densities of *ca* 10^5 cm^{-3} were achieved. Shoots were regenerated ex callus of *R. officinalis* and *L. angustifolia* as described [20]. Extracts of necrotic callus of *L. angustifolia* (100 g; after 4 days at −20°) in H_2O (1 l.) were microfiltered and added to the appropriate media.

Assay for accumulation of terpenoids. Typically, plant material (callus or suspension; 30 g) was ground up in liquid N_2 and extracted (Soxhlet; 3 hr) with Et_2O (200 cm^3). The extract was dried (Na_2SO_4) and concd (to 1–2 cm^3) at 0° in a stream of N_2 for analysis. The plant debris was added to acetate buffer (pH 5: 25 cm^3; 0.1 M) and incubated with β-D-glucosidase (10 mg; ex Sigma Chem. Co.) for 3 hr at 40°, extracted with *n*-C_6H_{14} (3 × 20 cm^3), and the aq. layer adjusted to pH 6 and incubated with α-D-glucosidase (10 mg; ex Sigma Chem. Co.) and re-extracted. These extracts contained any terpene residues that had been bonded as glucosides. Another fraction of the debris was incubated in turn at pH 6 and pH 9 with acid phosphatase (ex wheatgerm; Sigma) and apyrase (ex Sigma) and similarly extracted to yield any terpenes conjugated as phosphate esters. For these, especially for the extraction and concn procedures, internal standards were added to allow compensation for volatility losses (generally not more than 20%). Media were liquefied by addition of H_2O (*ca* 4 vols), warmed in a sealed flask at 80° for 10 min, cooled to 2° and extracted with petroleum spirit, bp 60–80° and the aq. residue treated to cleave glycosides or phosphate esters as above. Identification of lower terpenoids was made by GC (10% FFAP or 20% Carbowax 20 M on Chromosorb W, 60–80 mesh; 3.5 m × 4 mm; 90–135° programmed; FID, N_2 3.6 l/hr); by capillary GC (SE 30, Carbowax 20 M; FFAP, as before; He 2–6 l/hr; and by GC-MS, Kratos MS-2S mass spectrometer with data accumulation on a Kratos 65-505 system; source 200°; interface 230°; ionization potential 70 eV).

Assay procedure: cell-free extracts. Plant material (for comparison purposes) was 5 week-old seedlings grown in summer under natural conditions or young leaves from potted mature specimens. Callus or this tissue (10 g) was washed with aq. EDTA (1%) and then well rinsed and pulverized in liquid N_2. PVP (insoluble Polyclar AT, ex GAF Ltd Manchester) was purified [54] and suspended in Pi buffer (25 cm^3; 0.05 M, pH 7.0) containing sucrose (0.25 M), sodium metabisulphite (5 mM) and ascorbate (5 M), and either dithioerythritol, 2-mercaptoethanol or thiourea [cf. 55] (1 mM) at 2°, and the mixture was stirred in over 10 min; and the whole then allowed to warm up to *ca* 4° [56]. For *P. radiata* cultures or plant tissues, the endogenous monoterpenes were removed at this stage by stirring in Amberlite XAD7-resin (10 g) [cf. 57] for 10 min. The extract was then filtered through glass wool and centrifuged (2.7 × 10^4 *g*; 20 min), to yield a supernatant for assay: this had a protein concn *ca* 0.3–0.6 mg/cm^3 by Potty's [58] or the bromophenol blue method [59]. This soln (1.5 cm^3) in Pi buffer (1 cm^3; 0.05 M; pH 7.0) containing $MgCl_2$ (15 mM) and dithioerythritol (0.05 mM) and [1-^{14}C]-IPP (1 μCi; 17 mCi/mole, ex Amersham International) was incubated in sealed tubes at 30° for 3–5 hr to reach the plateau region. Four replicate assays and a boiled enzyme control were carried out for each extract. After incubation, the soln was cooled (to 0°) and extracted with Et_2O (2 × 2 cm^3). The aq. layer

was treated with apyrase and acid phosphatase as above, and re-extracted. The combined extracts were reduced (to 1 cm^3) in a stream of N_2 at 0°. Losses in the last step were < 10%.

Minor modifications were made to the above procedure for extracts from *P. radiata* and *R. officinalis*. Now, $MnCl_2$ (0.5 mM) was added to the incubation buffer. For extracts from *J. officinale*, MES buffer rather than Pi was used and the supernatant after centrifugation was passed through a Biogel P2 column (27 cm × 2 cm), eluted with MES (0.01 M; pH 7.0) containing 2-mercaptoethanol (1 mM) and the forerun collected.

Characterization of products from cell-free extracts. Routine assays for terpenes were carried out, after addition of carrier (10 μl of appropriate standard) either by GC (20% FFAP or Carbowax 20 M on G-cel 80–100 mesh; 100° N_2 at 3.6 1hr) when the products were collected in *n*-C_6H_{14} at 0° or more usually by a sequential TLC procedure. The latter involved chromatography on silicic acid at 0–4° with (a) toluene–EtOAc (1:1 and (b) *n*-C_6H_{14}–EtOAc C 99:1) to separate the C_5, C_{10} and C_{15} compounds, which were then further separated on silica gel H–$AgNO_3$ (9:1) at 0° with toluene–EtOAc followed by hexane–Et_2O (both 99:1). Chromatograms were developed with 10% phosphomolybdic acid followed by heating at 120° for 2 min. The collected products (GC or TLC) were > 99% chemically pure (capillary GC) and > 98% radiochemically pure (2π-scan). These simple assays gave results identical within the experimental error (±5%) with those obtained when the products were purified to constant specific activity as their acetates, or were oxidized to aldehydes and purified to constant specific radioactivity as their 2,4-dinitrophenylhydrazones.

Miscellaneous. (a) *De novo* synthesis in callus was probed via injection with sterile (via Millepore filter 0.22 μ pore size) solns (1-^{14}C]-IPP (0.43 μCi/cm^3) containing (variously) ATP (0.1 mM) or glucose (0.2 mM). After 3–10 days the material was extracted and assayed as above. (b) Chlorophyll in callus was extracted and assayed by measurement at 645 and 663 nm [60]. (c) Radiochemical assays involved LSC with Butyl PBD in toluene as scintillant. 4×10^4 Dpm were accumulated so that 2σ was ±1%.

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