Terpene Biosynthesis. Part VI.[1] Monoterpenes and Carotenoids from Tissue Cultures of *Tanacetum vulgare* L.

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Culture lines of *Tanacetum vulgare* L. which synthesise monoterpenes and carotenoids have been maintained *in vitro* for up to two years. The monoterpene pattern differed from that in the whole plant in that sabinene rather than isothujone was the main component. Xanthophylls were the only pigments formed. Specialised culture lines produced additionally β,β-carotene and chlorophyll.

Tracer studies revealed the formation of mevalonyl 5-phosphate, mevalonyl 5-pyrophosphate, and isopentenyl and 3,3'-dimethylallyl pyrophosphates in these cultures, and mevalonic acid was rapidly and significantly (*ca.* 0·01%) incorporated into monoterpenes.

In theory, the culture of explants of tissue of higher plants in sterile media provides a method for studying biosynthetic pathways and their control mechanisms without either the complications of translocation and compartmentation found in intact plants or the loss of organisation inherent in cell-free systems. In practice, such aims are limited by the great difficulty of maintaining cell lines and their low rate of growth, and by the

[1] Part V, D. V. Banthorpe, H. J. Doonan, and A. Wirz-Justice, preceding paper.

frequent absence of secondary metabolites.[2-6] Nevertheless, alkaloids [7,8] and sesqui- and higher terpenoids [9-15] *inter alia* have been demonstrated in cultures from various species and *de novo* synthesis has been proved.[11-17] Culture conditions have been developed for plants producing essential oils [18,19] but no studies have been carried out on monoterpene synthesis. We here report the establishment of various culture lines of *Tanacetum vulgare* L. (tansy) and the investigation of monoterpene and carotenoid synthesis in these. Several interesting differences from the situation in whole plants were revealed.

RESULTS

(*a*) *Growth and Morphology.*—Most cultures were made on agar supplemented with medium A (see Experimental section), but several other media were used and over 500 cultures were established. Suspension cultures were invariably unsuccessful. Tansy explants always grew slowly and sub-culturing was performed at 6—8 week intervals. The callus tissue grown on medium A became colourless after the first few sub-cultures even with extended periods of illumination [20] or with starch as carbon source,[21] but addition of kinetin [22] to the media caused chloroplast development. Cultures from leaf and root tissue did not survive 3 sub-culturings but those from stem were maintained for up to 2 years. Media for *Thuja plicata* and *Juniperus sabina* were also developed (see Experimental section).

No specific morphological pattern was discernible in 8 or 18 month-old cultures. The bulk of the tissue was undifferentiated parenchyma cells interspersed with xylem possessing extensive spiral thickening of the walls. Occasional cells contained small plastid-like bodies and older tissue had lignified outer walls.

Root tips of 2 week-old seedlings (intact plant) had a chromosome number $2n = 18$. The same value was obtained from callus tissue at 4—14 days after sub-culture of lines that had been maintained for 10 or 18 months.

(*b*) *Monoterpenes in Callus Cultures.*—The monoterpenes in 6 or 18 month-old callus tissue comprised *ca.* 0·10% of the wet weight.

Twenty-six monoterpenes were characterised in intact plants [23] and all but four were present in cultures in essentially the same (±2% actual value) percentage of the wet weight of the tissue. The differences were that sabinene [thuj-4(10)-ene], *p*-cymene, isothujone (*trans*-thujan-3-one), and carvone (*p*-mentha-6,8-dien-2-one) were present to the extent of 3·5, 1·3, 79·6, and 2·3% in plants and 79·3, 7·3, 1·6, and 0·0% in cultures, respectively.

(*c*) *Pigments in Callus Cultures.*—The percentages of pigments in cultures and various intact plant material are shown in the Table. The unidentified compound fluoresced

Pigments from leaf and culture tissue

	Tissue [a]				
Compound [b]	A	B	C [c]	D	E
Unidentified	+	+[d]	+	+[d]	+
β,β-Carotene	180	26	42	0	0·3
Lutein	213	35	123	5	6
Violaxanthin	17	4	12	1	1
Neoxanthin	20	5	20	15	16
Chlorophyll	2223	423	253	0	14

[a] A, Plant : leaf (3 month plant); B, plant : stem (3 month plant); C, plant : necrotic leaf at point of abscission (8 month plant); D, tissue culture : colourless type (8 month culture); and E, tissue culture : green type (8 month culture). [b] In μg pigment per g wet weight tissue. [c] Over 50% of carotenoid fraction was unidentified xanthophylls. [d] *Ca.* 4 and 1 μg g^{-1} tissue, respectively. Determined by weighing (Cahn microbalance).

and had $\lambda_{max.}$ (pentane) at 245, 253, and 263 nm. Phytoene (15-*cis*-7,8,11,12,7′,8′,11′,12′-octahydro-ψψ-carotene) has the shortest wavelength absorption of carotenoids [$\lambda_{max.}$ (light petroleum) 275, 285, and 296 nm] and so if the unknown is a carotenoid it might be lycopersene (7,8,11,12,15,7′,8′,11′,-12′,15′-decahydro-ψ,ψ-carotene) (for which spectral data are unavailable). The spectrum obtained suggests a diene; steroids should have been eliminated in the work-up.

(*d*) *Tracer Studies.*—Radioactive mevalonyl 5-phosphate and 5-pyrophosphate (MVAP and MVAPP) were detected within 8 min of injecting [^{14}C]mevalonic acid into cultures. The activity of both increased during 1—3 h as indicated by semi-quantitative radioscanning of chromatograms and then stayed appreciably constant for 24 h before decreasing. Labelled isopentenyl pyrophosphate (IPP) and 3,3-dimethylallyl pyrophosphate (DMAPP) were detected after 1 h and showed similar time profiles. Chlorophyll was labelled (*ca.* 0·01% of added tracer) after 2 h and by 24 h monoterpenoids and carotenoids were also significantly labelled (*ca.* 0·01% each, respectively).

The maximum incorporations into MVAP, MVAPP, IPP, DMAPP, and total monoterpenes were all 0·001—0·01%. The later intermediates of monoterpene biosynthesis could not be unambiguously characterised.

DISCUSSION

Conditions have been found for maintaining stable green and colourless culture lines of *Tanacetum vulgare* L., *Thuja plicata* L., and *Juniperus sabina* L., suitable for biosynthetic studies. Chromosomal analysis showed

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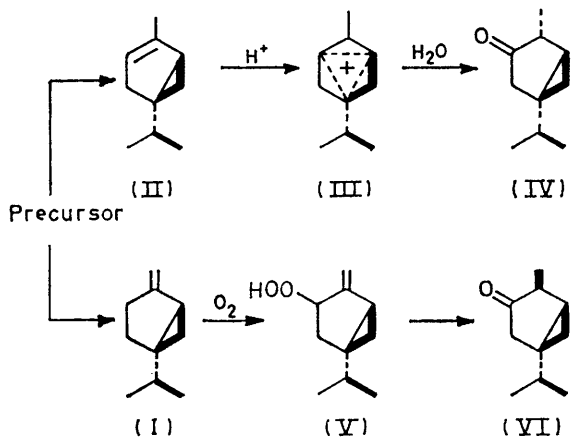
that growth of callus tissue was by cell division rather than by a gibberellin-like response.

Colourless cultured tissue contained about half the monoterpene content of the parent stem after 8—20 sub-cultures and almost all of this must have been synthesised *de novo*. Although no new monoterpenes were produced and most components were unchanged in their relative proportions, the formation of oxygenated derivatives was almost suppressed. In particular, the main monoterpene of cultures was sabinene (I) rather than isothujone (VI), the main component of leaf tissue, whereas the level of thujone (IV) was very similar (0·3 or 0·5%) in cultures or plants. The formation of some isothujone in the cultures suggested that the low proportions of this compound might not be due to the absence of the appropriate enzymes but to the shortage of oxygen in the culture conditions. If this is so, the accumulation of sabinene is consistent with the Scheme, which has been proposed tentatively on the basis of time–incorporation studies [23] and which implicates sabinene



SCHEME

rather than thuj-3-ene (II) as the precursor of isothujone. On this interpretation, thujone (IV) may be derived from anti-Markownikoff addition of water to its precursor, the route being facilitated by the formation of a non-classical species (III); whereas isothujone would be derived from radical-type oxidation at the 3-position. There are ample *in vitro* analogies for both the ready formation of non-classical species akin to (III) and for the oxidation step.

Tracer studies showed that label had passed significantly (*ca.* 0·01%) into monoterpenes and in particular into isothujone and sabinene within two days of injecting [2-^{14}C]mevalonic acid into callus tissue: this confirmed *de novo* synthesis.

Chloroplasts did not develop in the colourless callus tissue, and the carotenoids (which comprised one third of the quantity in the intact plant) were probably associated with the primitive primordia. The relative proportions of the carotenoids in intact plants were typically those found in other species [24] but β,β-carotene was absent from the cultures and neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol) was the main xanthophyll.

Oxygenated carotenoids predominated in both green and colourless tissue to a greater extent than in normal stem tissue. This is unexpected if the pattern of monoterpenes is due to oxygen deficiency. The possibilities are (*a*) our previous suggestion is incorrect, (*b*) the carotenoids capture the available oxygen more efficiently (and the basic dehydrogenation sequence to the carotenoids requires molecular oxygen), or (*c*) the oxygen of xanthophylls is derived from water rather than from air. Experimental evidence on the last point is contradictory.[25] Neoxanthin was also the predominant xanthophyll in (well-aerated) shake cultures from Paul's Scarlet Rose,[13] but neither lutein nor β,β-carotene was formed in this case.

The xanthophyll pattern in the cultures neither paralleled that occuring during fruit maturation [24] (where neoxanthin is rarely formed) nor that in senescent or necrotic leaf tissue of *T. vulgare* (see Table) or of other species.[26] These latter physiological processes involve the degradation of chloroplasts to chromoplasts, and the results from cultures indicate a situation where such modified organelles are absent. The pigments of leaf and stem tissue of mature *T. vulgare* plants were qualitatively similar and may reflect the relative concentrations of chloroplasts in the two tissues.

The green cultures contained chlorophyll but only *ca.* 3% of that in the parent stem tissue. Similar low levels have been reported for greening cultures from oats.[27] Addition of chloramphenicol is reported to inhibit chloroplast development in intact leaves if added early in the life cycle,[28] and actidione and streptomycin are claimed to behave similarly in certain species.[29] Chloramphenicol did not prevent greening of our cultures, but the last two did inhibit (reversibly) chlorophyll formation, if maintained in the medium (at 1 mg l^{-1}) through several sub-cultures. However these two cultures produced xanthophylls and monoterpenes in essentially the same yields and proportions as in the untreated cultures. This confirms our general experience that monoterpenoid and xanthophyll synthesis can occur independently of the existence of chloroplasts, whereas the synthesis of chlorophyll and the accumulation of ββ-carotene is linked to the formation of these organelles.

Use of double [^{14}C,^{32}P]-labelling enabled the characterisation of MVAP, MVAPP, IPP, and DMAPP from the cultures. The first two intermediates have recently been identified in cultures of *Kalanchoe crenata*.[30]

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EXPERIMENTAL

(*a*) *Culture Media.*—A previously described [13] medium containing basic medium (BM), coconut milk preparation (CM; 10% v/v), 2,4-dichlorophenoxyacetic acid (2,4-D; 6 mg l^{-1}), and mesoinositol (10 mg l^{-1}) (to prevent tannin formation) was used for most of the phytochemical studies (medium A). After the second sub-culture, the level of 2,4-D was reduced to 2 mg l^{-1}.

Later screening suggested that this medium was not the best and medium B [BM, CM(10%), copper(I) chloride (50 mg l^{-1}), and sodium molybdate (50 mg l^{-1})], with the phosphate concentration in the first component reduced to 50 mg l^{-1}, gave faster growth.

Other media were: medium C, BM and α-naphthylacetic acid (NAA, 0·2 mg l^{-1}) (this gave compact and faster growing tansy cultures than either medium A or B); medium D, medium C and kinetin (0·1 mg l^{-1}) (this gave green callus tissue and was used for experiments on green cultures); medium E, medium D and indol-3-ylacetic acid (IAA, 0·2 mg l^{-1}) (this gave similar growth to that in medium C); and medium F, BM, soya peptone (Oxoid, 1·0 g l^{-1}), and NAA (0·2 mg l^{-1}) (this gave good growth of tansy petioles).

Mesoinositol was added to media A—F. Catechol [31] or cytokinins [32] had no growth-promoting effects in our screening tests. Media A—F were the most successful of an extensive screening programme that we have carried out.

Purest available commercial chemicals were always used and the pH of the media was adjusted to 5·5 ± 0·2 after treatment in a autoclave or sterile filtration. Unsuccessful attempts were made to set up liquid suspension cultures (inoculated with fragments from agar-cultures) on medium A supplemented with sodium nitrate and sucrose,[33] and maintained in rotating vessels.

Medium A was unsuitable for cultures of *Thuja plicata* and *Juniperus sabina*, but fast growth of explants of these species were obtained on BM and CM (10%) with either IAA (6 mg l^{-1}) or NAA (0·2 mg l^{-1}) respectively. These cultures grew relatively rapidly and could be sub-cultured every 2—3 weeks.

Antibiotics were added to the green cultures at levels of 1 mg l^{-1}.

(*b*) *Methods.*—Standard methods [34] were used to set up agar cultures of the various media in small tubes (6 × 1 in). The consistency of the gel was very important. The agar concentration was correct if the gel would not set if the pH was lowered to 4·0. Batches were monitored with this criterion.

Explants (0·5—1 cm) of meristematic stem tissue of greenhouse specimens of *T. vulgare* were half-embedded in the medium in a lateral position and the cultures were kept at 25° in either natural light or on 16-h days in culture cabinets. The day-length did not appear to be critical for growth or terpenoid formation once the former had started.

Most cultures initiated callus growth in 8—15 days to form a mass of whitish translucent tissue burgeoning from the top of the cut stem. Sub-cultures of small fragments (3—6 mm) were carried out every 3—8 weeks when the callus tissue became brownish. Some cultures, however, took 3—4 months to initiate callus growth.

Chromosome analyses and histological examinations were made by standard methods.[35]

(*c*) *Phytochemical Investigations.*—Monoterpenes were isolated from individual cultures (6—18 month) and analysed by g.l.c. as described.[23] Major components were obtained from extracts of large numbers of cultures by g.l.c. or column chromatography and were characterised by i.r. spectroscopy. Lesser components were identified by comparison of the relative retention times on columns packed with Carbowax 20M or FFAP with those of standards and with the known [23] components of the oil from whole leaves.

Pigments were extracted with ether from the macerated cultures and were separated by standard methods [13,36] with precautions to prevent oxidation.[37] In addition, good separations were achieved on columns (7 × 1 in o.d.) of zinc chloride–G-cell 60—80 mesh (5 : 2 w/w); hexane. Fractions were monitored by t.l.c.[1] Identification was by (*a*) u.v. spectra; [36] (*b*) co-chromatography with authentic standards on several [1] t.l.c. systems; and (*c*) observation of the characteristic [38] shifts of $\lambda_{max.}$ of xanthophylls after addition of acid. Violaxanthin (5,6:5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol) was best identified and assayed by paper chromatography.[39]

Neoxanthin, lutein, and phytoene for use as standards were prepared from barley,[13] grass,[13] and tomatoes [40] respectively.

(*d*) *Tracer Studies.*—[2-^{14}C]Mevalonic acid (2 μCi), and ATP (0·1 mg) in water (10 μl) were injected into callus cultures (8 month; 4 weeks following sub-culture) and, after various time intervals, the tissue was macerated, the extract was deproteinated by boiling and then chromatographed.[30,41] Autoradiograms of the developed t.l.c. plates or papers were compared with standards and with traces obtained after feeding cultures inactive acid plus sodium [^{32}P]phosphate (10 μCi) and ADP (0·1 mg). The separated components were eluted, incubated with either 7N-hydrochloric acid for 7 min or with semen phosphatase at pH 5·4 in acetate buffer, and the location of tracer (^{14}C or ^{32}P) in products was compared chromatographically with standards.

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