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LIGHT-DEPENDENT MONOTERPENE SYNTHESIS IN *PINUS RADIATA* CULTURES

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Key Word Index—*Pinus radiata*: Pinaceae; biosynthesis; tissue cultures; monoterpenes; α- and β-pinene; toluene.

Abstract—Callus cultures of *Pinus radiata* that synthesized monoterpenes *de novo* and which were stable for at least 1 year have been established. The products differed from those of parent plants in that α-pinene (87–100%) rather than β-pinene was the main component. The best lines accumulated monoterpenes (*ca* 2×10^{-3}% wt/wet wt) in yields 40–20% of that in the parent stem and needles. The composition of the extractable oil depended on the light regime. After culture in total darkness toluene and acetone accumulated. These compounds also occurred (at low levels) in dark-grown seedlings and in seeds of *P. radiata* and a route for their formation from α-pinene is suggested. Cell-free extracts of the culture lines converted [^{14}C] IPP into geraniol, nerol and α- and β-pinenes in up to 46% total yield. These are the most active crude extracts for monoterpene biosynthesis that have been reported from either tissue cultures or higher plants.

INTRODUCTION

Secondary metabolism in plant tissue cultures has been extensively studied in recent years [1] and some spectacular examples of sesquiterpene biosynthesis have been achieved [2, 3]. However, few authenticated cases of *de novo* synthesis of monoterpenes are available [4–9], and in these the proportions and products often differed from those of the parent material. Cultures of *Pinus* species have often been established; but although, in some, the occurrence of secondary metabolites (e.g. phytosterols) has been reported, there are no records of the synthesis of lower terpenoids [10–14]. We now report culture lines of *Pinus radiata* D. Don that displayed unprecedented levels of monoterpene accumulation and which furthermore were light-dependent.

RESULTS AND DISCUSSION

Cultures that produce monoterpenes

All our work used callus cultures. The literature suggests that these are more effective than suspension cultures with respect to monoterpene synthesis. This may be a result of greater differentiation, perhaps consequent on the possibility of establishing gradients (e.g. of stages of the cell cycle; of metabolites; of inducers etc.) within the former tissue. Such cultures from stem tissue were readily established and were routinely sub-cultured every 3–4 weeks after *ca* 4-fold increase in volume. Young cultures (up to four sub-cultures) were often green; the % pigmentation, as estimated by absorbance at 680 nm was *ca* 0.5% of that in needles. Growth rates and analyses of the oils produced are recorded in Table 1. After 4–6 sub-cultures, the callus lines maintained their recorded properties (±20%) up to at least 15 sub-cultures when the experiment was terminated. The optimum line (B) produced extractable oil some 20–40% of that from the needles and stem respectively of the parent plants; but whereas that from the cultures (except A_3) comprised essentially α-pinene(pin-2-ene), that from the parent tissue contained 60–90% β-pinene(pin-2(10)-ene), the residue being almost entirely the α-isomer. It is conventional to quote percentages as wt/wet wt (i.e. superficially-dried tissue) and these are the values given above and in Table 1. However, particularly for *Pinus* species, the cultures and the parent material differ considerably in water content and values expressed in terms of dry wt (shredded material oven dried 110°/72 hr) are of interest. In these terms the culture line B that produced extractable oil (2×10^{-3}%) at levels 20 and 40% of that in needles and stem (all in terms of wet wt) produced oil (7×10^{-3}%) at levels of *ca* 58 and 117% respectively. The other values in Table 1 may be calculated *pro rata*. The trace components in the cultures (Table 1) corresponded to those occurring in the parent plants [cf. 15]. The variation in the α-pinene: β-pinene ratio in the cultures and parent material is consistent with the view [16] that two separate pathways led to these isomers from the acyclic or monocyclic biosynthetic precursors. The yields of monoterpenes from the cultures were unaffected by the continued presence of coconut milk (10% v/v) in the medium throughout the sub-cultures or by variation of sucrose in the medium from 10–200 g per l. Both growth rates and yields fell by 30–60% on culture at 15° rather than 27°. Such a reduction in temperature had resulted in a marked increase in alkaloid production in appropriate cultures [17].

The change in ratio of α- and β-pinenes between A_2 and A_3 and again between the latter and A_4 was reproducible and may reflect some physiological characteristic or result from inbalance in the components of medium, perhaps as

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Abbreviations: IPP, Isopentenyl pyrophosphate; MVA, mevalonate; DMAPP, 3,3-dimethylallyl pyrophosphate.

Table 1. Monoterpene accumulation in callus of *P. radiata*

Line*	Temp. (°)	Illumination† (lux)	G‡	10^2.(%)§	Components‖ α-Pinene	β-Pinene	Others
A_2	23	Natural (200–450)	0.11	0.13	87	13	0
A_3	23	Natural (200–450)	0.10	0.27	24	76	trace
A_4	23	Natural (200–250)	0.13	0.29	90	10	trace
B_2	27	Constant (150)	0.16	2.1	100	trace	0
B_4	27	Constant (150)	0.18	2.1	100	trace	0
C_4	27	12 hr (250)	0.21	0.67	100	trace	0
C_6	27	12 hr (250)	0.23	0.53	100	trace	0

*Cultures originated from stem tissue. Letter refers to regime; subscript indicates number of sub-cultures before assay.

†Regime: natural illumination refers to diurnal variation in a shaded aspect [at > 500 lux, extensive phenol formation ('browning') occurred in all culture lines].

‡Growth rate. $G = (m_t - m_0)/m_t \,.\, t$, where m_t and m_0 are masses of explants at initiation of last sub-culture and at its termination at time t (days).

§% (wt/wet wt) of extractable oil from culture. Mean of three independent determinations.

‖GC/MS analysis: 'others' refers to camphene, β-phellandrene and limonene. 'Trace' refers to < 0.5%; 0 to < 0.1%, if any. Means of three independent determinations.

a consequence of differences in permeability and hence in uptake of nutrients, peculiar to this stage of the growth cycle [cf. 18]. Assay of the medium from a selection of culture lines gave no indication of excretion of monoterpenes from the callus. Solvent extraction (18–24 hr) of the B_4 line resulted in a yellow oil with IR and mass spectra almost identical with those of a mixture characterized as consisting of polyisoprenol acetates from needles of *P. sylvestris* [19] or from a similar mixture prepared from needles of our specimens of *P. radiata*. The yield was 1.5% (w/w) from callus and 0.65% from fresh needles.

Controls indicated that 'carry-over' could have accounted for only *ca* 6% of the observed oil content at the end of the second sub-culture but for less than 1% by the fourth. Synthesis *de novo* was directly demonstrated by injection of the callus with [2-^{14}C] MVA and [1-^{14}C] IPP alone or in a mixture with ATP and glucose (see Experimental). A_4 cultures converted the precursors into pigments (chlorophyll a and b, β-carotene; *ca* 6:1 present at *ca* 0.3% that in needles, see previously) in conversions 10–30%, α-pinene (0.01–0.3%) and β-pinene (0.08–0.2%) after 3 days metabolism following injection. All the products were purified to constant specific radioactivity and the variations may reflect differences in access to the biosynthetic sites. [2-^{14}C]MVA added to the medium was taken up into callus (*ca* 13% in 3 days) and the percentage conversions of this into pigments and monoterpenes were within the same ranges.

In summary, our cultures produced all the monoterpenes of the parent tissue and one line (B) accumulated these even after 15 sub-cultures. Seedlings grown aseptically on a solid medium similar to that for the culture medium (except that no auxin, coconut milk or *m*-inositol were present) under illumination as used for the cultures gave yields and proportions of monoterpenes similar (±5%) to that of seedlings grown under conventional conditions, rather than those of the cultures.

Secondary metabolites in 'dark' cultures

Most studies of secondary metabolism in tissue cultures have involved isothermal conditions at constant illumination. The data in Table 1 suggest an influence of light on the yield and composition of extractable oil from the callus, and cultures of *Tanacetum vulgare* that actively synthesised monoterpenes in high yield had in fact been maintained under near physiological ranges of light and temperature [20]. Brief studies also indicate the influence of light on monoterpene formation in cultures of *Mentha* species [21] and on the formation of alkanes in callus of *Ruta graveolens* [22, 23]. Diurnal variation (periods 6–14 hr daily) of temperature (16–27°) and light intensity (0–450 lux) gave results with callus of *P. radiata* little different from those presented in Table 1. However, maintenance of the culture lines A–C (pretreated as

Table 2. Production of secondary metabolites in 'dark' cultures of *P. radiata*

Origin*	Period†	10^2.(%)‡	Components§ α-Pinene	β-Pinene	Toluene
A	42	0.11	29.3	0	70.7
B	42	0.23	60.2	0	39.8
C	42	0.17	36.7	0	63.3
A	63	0.12	76.2	0	23.8
B	63	0.15	32.7	0	67.3
C	63	0.08	82.4	0	17.6

*Letters refer to the culture lines maintained for 4–6 subcultures under conditions as in Table 1, before being maintained in total darkness at 27°.

†Period of darkness (days) before assay. Subculture was every 19–22 days.

‡% (wt/wet wt) of extractable oil from the culture: means of three independent determinations.

§GC/MS analysis. Acetone (volatile and difficult to extract from the aqueous solutions) was always detected in amounts ~ 0.05%. All figures are means of two independent determinations.

described in Table 1) in total darkness for periods of 6 or 9 weeks (with sub-culture at 3 week intervals) led to analyses as in Table 2. Growth rates were rapid; *G ca* 0.32 (cultures of *R. graveolens* also flourished better in the dark [22]) but the yields of oil were low and varied little for different culture lines.

The outstanding feature was the formation by all the culture lines of toluene often as the predominant product together with acetone. It was rigorously checked that the latter was a metabolite and not an artefact of, e.g. the solvents used in the work-up process. The proportions of toluene and α-pinene varied depending on the period of darkness and also on the pre-history of the cultures.

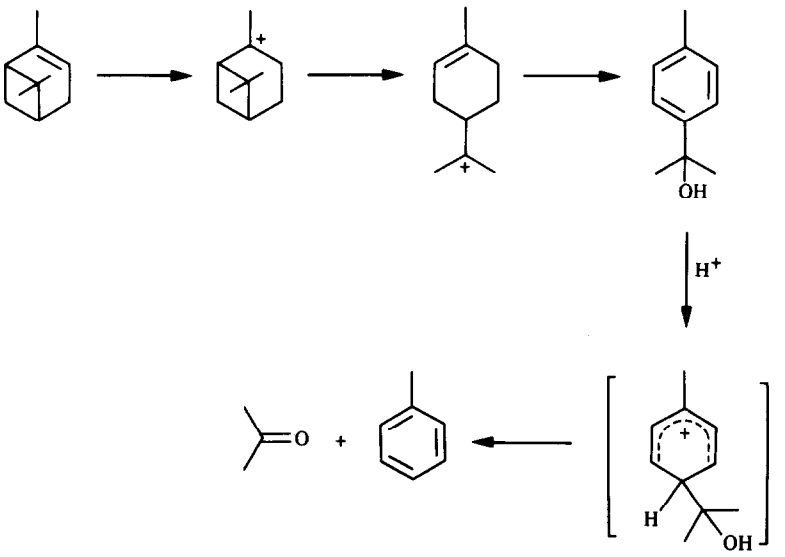
It is premature to try to interpret these reproducible fluctuations. More significant was the finding that all the lines lost their ability to produce toluene within 4 weeks of being returned to their original regime. After this time the metabolism had reverted to that reported in Table 1, and was reversible in that another period of darkness (6 or 9 weeks) reintroduced toluene formation. These observations are novel, the one related study being the observation that monoterpene synthesis in *P. pinaster* ceased in the dark [24]. We suggest that in total darkness the accumulation of α-pinene is reduced by degradation as outlined in Scheme 1.

Occurrence of toluene in P. radiata

Although not usually considered a natural product, toluene occurs in tolu balsam, perhaps being derived from breakdown of monoterpenes during distillation [25]. It is formed by microbial degradation of terpenoids [26] and is present in avocado fruit [27]. On extraction of seeds of *P. radiata* we found 4.2% (wt/wt) toluene (together with β-pinene; 44.0%, α-pinene 30.9%; limonene 7.9%; camphene 7.5% and myrcene 5.5%). Seeds of *P. sylvestris* also contained toluene (3.2%). α-Pinene has previously been reported in seeds of a *Thujopsis* species [28]. Seedlings of *P. radiata* (3 or 12 months after germination) contained α- and β-pinene in the ratios *ca* 39:61 and 35:65, respectively, but no toluene ($< 1 \times 10^{-3}$%, if any) could be detected. However, on maintaining the seedlings in the dark for 4 weeks before harvesting, toluene accumulated i.e. the β-pinene:α-pinene:toluene ratios were 35.5:60.3:4.2 for the 3 month seedlings and 24.1:75.8:0.1 for the 12 month seedlings. Thus the unexpected metabolism discovered in the cultures could be replicated in whole plants.

Cell-free extracts of tissue cultures

A soluble extract was prepared from callus (line B) using methods developed for other plant species [29, 30] and this was tested for efficiency in synthesis of monoterpenes with [1-^{14}C]IPP as substrate (Table 3: where optimum concentrations of Mg^{2+} and Mn^{2+} are recorded). Incorporation using [2-^{14}C]MVA as substrate were *ca* 10-fold less. The main products were geraniol and nerol (not components of the extractable oil) rather than α- and β-pinene. Addition of NaF, a phosphatase inhibitor, increased (at 15 mM) the yield of geraniol and nerol and (at 80 mM) that of pinenes. This may result from different phosphatases being responsible for cleaving IPP and DMAPP (the precursors of geraniol and nerol) and the pyrophosphates of the latter C_{10} alcohols (which are the presumed precursors of the bicyclic monoterpene hydrocarbons). There is evidence that endogenous phosphatases in other species have different specificities towards terpenyl pyrophosphates [31]. The optimum system (46.4% incorporation under the standard conditions) is by far the most active crude extract for the preparation of monoterpenes that has been prepared from either tissue cultures or higher plants, and is some 150-fold more active than the best corresponding extract (assayed under identical conditions) from needles or seedlings of *P. radiata*. This factor may, however, represent not merely the endogenous levels of enzymic activity, but both the ease of disruption of the walls of the callus and the lack of deactivating phenolics compared with the parent plant. Cell-free extracts from 'dark' cultures (see previous sec-



Scheme 1. Suggested route for the production of toluene and acetone in cultures of *P. radiata*.

Table 3. Incorporation of IPP-[1-^{14}C] into monoterpenes by extracts of callus of *P. radiata*

System*†	Conc (mM)	% Pinenes ($\alpha+\beta$)	% Nerol‡	% Geraniol‡	Total %
Standard	—	0.14	14.6	1.0	15.7
+ Mn^{2+}	0.5§	0.43	42.1	3.9	46.4
+ Zn^{2+}, Cu^{2+}, Hg^{2+}	15	‖	‖	‖	0 to 0.2
+ –SH inhibitors¶	1	‖	‖	‖	0 to 0.3
+ NaF	15	0.56	18.2	1.8	20.6
+ NaF	80	2.0	9.9	1.3	13.2
+ NaF	120	1.4	5.6	0.5	7.5

*Standard cell free extract as described in Experimental. Metal salts added as chlorides unless otherwise specified.

†Polyclar AT, Amberlite XD-4, $NADP^+$, NADPH, ATP had little (±30%) effect on the standard system.

‡Normalized % incorporations [to allow for small (±3%) variations] in values from different extracts. α- and β-Pinenes were formed in the ratio *ca* 3:1.

§Precipitation occurred at greater concentrations of Mn^{2+}.

‖% Incorporation into total monoterpenes, only, was assayed.

¶*p*-Hydroxymercuribenzoate, iodoacetamide.

tions) showed levels of activity for monoterpene synthesis some 60% of those from 'light' cultures. A freeze-thaw technique followed by vacuum infiltration of substrate that has been claimed effectively to measure the *in vivo* activity of certain plant enzymes [32] was 2-fold more effective for incorporation of IPP than was cell-free extracts from pine needles. However, the method was *ca* 10-fold less efficient than the conventional cell-free extract from callus. Iodoacetamide (2 mM; a known inhibitor of IPP–DMAPP isomerase) inhibited the formation of monoterpenes when IPP was substrate (Table 3). However, when IPP and DMAPP (1:1) were employed, the yield of acyclic and bicyclic monoterpenes was unaffected. Hence no significant pool of DMAPP or its biogenetic equivalent such as has been demonstrated in many plant species [33] could have been present in the callus.

The culture lines described in this paper should provide material for several fundamental investigations: e.g. (a) the presence and nature of light receptors (phytochromes?) in the callus; (b) the mechanism of the formation of toluene and its metabolic fate; (c) ^{13}C NMR studies on the biosynthesis of monoterpenes; (d) the achievement of biomass for the characterization of the monoterpene cyclases involved in the formation of the pinenes.

EXPERIMENTAL

Materials and methods. [1-^{14}C]IPP (17 mCi/mmol) was available from a previous study [34]. Stem of *P. radiata* (terminal branches, tree 1 m) were sterilized, washed, cut longitudinally (15 mm × 1 mm) and explanted with the cut surface in contact with Murashige and Skoog medium as modified for *Pinus* species [35, 36] made up in agar (0.8% w/w) at pH 5.7. We found that asparagine in the medium (rather than glycine which inhibits pine cultures [36]) was not essential, and could be replaced by aspartic acid, but callus formation and growth were more rapid in its presence. Agar at 1.0% (w/w) was too 'stiff' and did not allow the cultures to bed down. After 4 weeks, callus was induced in *ca* 30% explants and the first sub-culture (6 weeks) omitted coconut milk from the medium. Subsequently, sub-culture was at 3 week intervals (when vol. increase was *ca* 4-fold). Attempts to induce callus formation from needles was unsuccessful. Cultures were maintained under fluorescent strip lighting (150 lux; Thorn 30 W 'Daylight'), or Gro-lux strips (425 lux; Thorn 40 W) or in an incubator with natural lighting from a south facing aspect (150–400 lux). Callus (10 g) were extracted (Soxhlet; Et_2O 150 ml; 12 hr), the extract concd (to 20 ml; N_2 stream; 0°) and after removal of pigments etc. by passage through Al_2O_3 (15 × 0.5 cm) it was analysed by GC (Carbowax 20 M or FFAP on diatomite 60–80; 6 m × 4 mm; 120°; N_2 50 ml/mm) and by GC/MS (Carbowax 20 M; 3 m × 2 mm, He 40 ml/min; sources 200°; interface 230°; ionization potential 70 eV) and the data accumulated on a Kratos 65–505 computor system. Exhaustive (18–24 hr) extraction of callus resulted in a yellow oil (IR ν_{max}^{KBr} cm^{-1}: 1025, 1250, 1730, 1945, 2100) which was characterized (see Discussion) as a mixture of polyisoprenyl acetates.

Cell free extracts. Plant or callus (10 g) was washed with aq. EDTA (1%) and H_2O, ground in liq. N_2, and stirred into a suspension of insoluble PVP (25 g; Polyclar AT ex G.A.F. Ltd., Manchester, purified as in [37]) in phosphate buffer (25 ml 0.05 M; pH 7.0) at 0° containing sucrose (0.25 M), sodium metabisulphite (5 mM), ascorbate (5 mM) and dithioerythritol (1 mM) over 10 min. The supernatant was then stirred with Amberlite XAD-4 resin (10 g) for 10 min at 0°, and filtered through glass wool. The filtrate was centrifuged ($2.7 \times 10^4 \times g$; 20 min) and the supernatant was used for assay. This (1.5 ml) and phosphate buffer (1 ml; 0.05 M; pH 7.0) containing $MgCl_2$ (15 mM) and diethioerythritol (0.05 mM) comprised the 'standard' incubation system (Table 3) to which additives were made. The protein concn [cf. 38] was *ca* 0.3 mg per ml and incubations were made for 3–5 hr (to reach the plateau region) at 30° in sealed tubes with [^{14}C]IPP (1 μCi). After incubation, the reaction was cooled at 0°, extracted (Et_2O, 2 × 2 ml) and the aq. layer incubated for 3 hr at 27° with apyrase and alkaline phosphatase (1 μg) in order to cleave any phosphate esters to alcohols. The incubate was re-extracted, the Et_2O extracts were combined and reduced (to *ca* 1 ml) in a stream of N_2 at 0°. Geraniol, nerol and α- and β-pinenes were characterized by GC/MS and purified to constant specific radioactivity (after addition of carrier; 10 μl of each component) by GC (Carbowax 20 M; FFAP; conditions as before) and (for geraniol and nerol) by TLC on silicic acid with, in sequence as eluant (a) toluene–EtOAc; (b) hexane–EtOAc; and (c) $CHCl_3$–ethyl carbonate (all 85:15). The alcohols were also converted into their acetates and purified thus by GC. The

pinenes were purified by TLC on silica gel G, silicic acid and $AgNO_3$–silica gel G (10% w/w) eluted with (a) toluene–EtOAc and (b) hexane–Et_2O (both 99:1); and in addition reduced to *cis*- and *trans*-pinane and purified thus by GC. All products were chemically purified (> 99% by GC Carbowax 20 M and SE-30 capillary columns, 50 m × 0.02 mm) and radiochemically pure (2π scanning of a variety of the above TLC separations. Cell free extracts of *P. radiata* plants, for comparison purposes, were made by the above methods using growing tips of seedlings (30 days) and of young trees (1 m; ca 5 year old)

Miscellaneous. Callus (that had been subcultured three times) was injected with sterile (via millipore filter 0.22 μ pore size) solns (1 ml) of [12-^{14}C]MVA (0.5 μCi) or [1-^{14}C]IPP (0.43 μCi) containing (variously) ATP (0.1 mM) or glucose (0.2 mM). After 3 days the material was extracted and assayed as above. Freeze thaw-vacuum infiltration was carried out for two or six cycles as described [32]. Radioactive measurements were made using Butyl-PBD (0.8% v/v) in toluene as scintillant. Typically aliquots containing 10^3 to 10^4 dpm were assayed, and 4×10^4 disintegrations were accumulated so that the 2σ error was $\pm 1\%$.

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REFERENCES

1. Alfermann, A. and Reinhard, E. (eds) (1978) *Production of Natural Compounds by Cell Culture Methods*, Gessell. Strahlen und Umweltforschung MbH, München.
2. Overton, K. H. and Picken, D. J., (1977) *Prog. Org. Chem. Nat. Prod.* **34**, 249.
3. Anastrasis, P., Freer, I., Gilmour, C., Mackie, H. and Overton, K. H. (1982) *J. Chem. Soc. Chem. Commun.* 268.
4. Charlwood, B. V. and Banthorpe, D. V. (1979) *Prog. Phytochem.* **5**, 67.
5. Lang, E. and Horster, H. (1977) *Planta Med.* **31**, 112.
6. Cashgap, M. M., Kueh, J. S., Mackenzie, I. A. and Pattenden, G. (1978) *Phytochemistry* **17**, 544.
7. Bricout, J., Garcia-Rodriquez, M. J., Paupardin, C. and Saussay, R. (1978) *C. R. Hebd. Seances Acad. Sci. Ser. D.* **286**, 1585.
8. Nabeta, K., Ohnishi, Y., Hirose, T. and Sugisawa, H. (1983) *Phytochemistry* **22**, 421.
9. Ueda, S., Kobayashi, K., Muramatsu, T. and Inouye, H. (1981) *Planta Med.* **41**, 186.
10. Brown, C. L. and Sommer, H. (1975) *Atlas of Gymnosperms in Vitro*, 1924–74, Georgia Forestry Res. Council, Atlanta, Georgia.
11. Hasegawa, M., Higuchi, T. and Ishikawa, H. (1960) *Plant Cell. Physiol.* **1**, 173.
12. Laseter, J. L., Evans, R., Walkinshaw, C. H. and Weete, J. D. (1973) *Phytochemistry* **12**, 2255.
13. David, M. A. and Plastira, P. V. (1976) *C. R. Hebd. Seances Acad. Sci. Ser. D.* **282**, 1159.
14. Burnman, D. H. and Jansson, E. (1980) *Z. Pflanzenphysiol.* **96**, 1.
15. Valenzuela, P., Cori, O. and Yudelevich, A. (1966) *Phytochemistry* **5**, 1005.
16. Banthorpe, D. V. and Le Patourel, G. N. J. (1972) *Biochem. J.* **130**, 1055.
17. Courtois, J. and Guern, J. (1980) *Plant Sci. Letters* **17**, 473.
18. Yeoman, M. M., Aitchison, P. A. and Macleod, A. J. (1977) in *Regulation of Enzyme Synthesis and Activation in Higher Plants* (Smith, H., ed.) p. 63. Academic Press, London.
19. Hannus, K. and Pensar, G. (1974) *Phytochemistry* **13**, 2563.
20. Banthorpe, D. V. and Wirz-Justice, A. M. (1972) *J. Chem. Soc. Perkin Trans.* 1, 1769.
21. Paupordin, C. (1979) *Prod. Subst. Nat. Cult. in Vitro Tissus Cell Veg., Journ. Etud.*, 119; *Chem. Abs.* 1981, **95**, 49188.
22. Courduan, G. and Reinhard, E. (1972) *Phytochemistry* **11**, 917.
23. Nagel, N. and Reinhard, E. (1975) *Planta Med.* **27**, 264.
24. Gleizes, M., Pauly, G., Bernard-Dagan, C. and Jacques, R. (1980) *Physiol. Plant.* **50**, 16.
25. Hildring, O. V., Bergstrom, K. N. and Trobeck, K. C. (1936) *U.S.P.* 2052917; *Chem. Abs.* 1936, **30**, 7130.
26. Hunt, J. M., Miller, R. J. and Whelan, J. K. (1980) *Nature* **288**, 577.
27. Jansen, E. F. and Wallace, J. M. (1965) *J. Biol. Chem.* **240**, 1042.
28. Hasegawa, J. and Hirose, Y. (1981) *Phytochemistry* **20**, 508.
29. Banthorpe, D. V., Bucknall, G. A., Doonan, H. J., Doonan, S. and Rowan, M. G. (1976) *Phytochemistry* **15**, 91.
30. Croteau, R. and Felton, M. (1981) *Arch. Biochem. Biophys.* **207**, 460.
31. Croteau, R. and Karp, F. (1979) *Arch. Biochem. Biophys.* **198**, 523.
32. Rhodes, D. and Stewart, G. R. (1974) *Planta* **118**, 133.
33. Allen, K. G., Banthorpe, D. V., Charlwood, B. V., Ekundayo, O. and Mann, J. (1976) *Phytochemistry* **15**, 101.
34. Banthorpe, D. V., Doonan, S. and Gutowski, J. A. (1977) *Phytochemistry* **16**, 85.
35. Murashiga, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.
36. Brown, C. L. and Lawrence, R. H. (1968) *Forest Sci.* **14**, 62.
37. McFarlane, W. D. and Vader, M. J. (1962) *J. Inst. Brew. London* **68**, 254.
38. Potty, V. H. (1969) *Analyt. Biochem.* **29**, 535.