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SYNTHESIS AND ACCUMULATION OF POLYGODIAL BY TISSUE CULTURES OF *POLYGONUM HYDROPIPER*

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Key Word Index—*Polygonum hydropiper*; Polygonaceae, water pepper; tissue culture, tracer studies; sesquiterpenes; polygodial; drimenol.

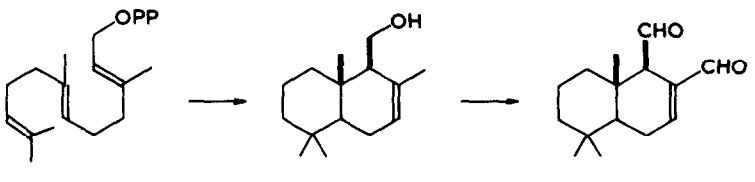
Abstract—Callus and suspension cultures of *Polygonum hydropiper* accumulated the insect anti-feedant compound polygodial (and to a lesser extent its parent drimenol) at levels of up to 0.25 and 29% of those in the flowering heads and foliage respectively. Feeding experiments with 3*R*-[2-^{14}C]-mevalonate showed that (weight for weight) the apparent synthetic ability for these sesquiterpenes was up to two-fold greater in the callus than in the flowerhead or leaf of the parent plant. Thus, cell cultures provide suitable biomass for studies on the purification of drimenol synthetase.

INTRODUCTION

Polygonum hydropiper L. Polygonaceae; water pepper, syn. = arsesmart) is a widely used medicinal and culinary herb which contains a variety of sesquiterpenes of the drimane type [1–3] The main constituent of the foliage and the seeds is (−)-polygodial (**3**; ≡(−)-tadeonal≡drim-7-en-11,12,-dial) which also occurs in liverworts [4], tree bark [5, 6] and nudibranchs [7] and is also reported (variously) to be cytotoxic; an inhibitor of plant growth and of complement; an allergen; an anti-microbial, molluscicidal and piscicidal agent [2, 8, 9] and in particular a potent insect anti-feedant [10]. This last property cannot be directly exploited in agriculture owing to the volatility and instability of the dialdehyde. However, as the latter is presumably formed in only a few steps from the ubiquitous (2*E*,6*E*)-farnesyl pyrophosphate (**1**, FPP) via (−)-drimenol (**2**), the possibility exists of gene-transfer from *P. hydropiper* to a commercially important crop or to horticultural plants [11]. Such transfer is the more attractive as specific secretory or storage structures are apparently not present in *P. hydropiper* and the anti-feedant is stored throughout the aerial parts. As part of a detailed study of the sequence (**1**) → (**3**) (Fig. 1), and as a first step for gene-transfer studies we have established, for use as an efficient source of biomass, cell cultures of *P. hydropiper* that accumulate polygodial. Production of this and related compounds has not hitherto been demonstrated *in vitro*; nor indeed have cell cultures of *P. hydropiper* been previously reported.

RESULTS AND DISCUSSION

Callus was readily initiated from explants of stem (success *ca* 90%; induction period *ca* 2 weeks) when Murashige and Skoog (MS) medium [12] was supplemented with 1-naphthaleneacetic acid (NAA), benzylaminopurine (BAP) and, in particular, gibberellin A_3 (GA_3). Two callus lines (A and B) were initiated at Glasgow and London, as light brown, friable and greenish hard calli respectively. Initially, line A did not produce drimane-type sesquiterpenes, but after transport to London, formation of such metabolites was detected after 12 passages. Line B, in contrast, produced the desired compounds from initiation. As the culture media were nominally similar for the two cell lines, temperature or other factors attending or subsequent to transfer may have triggered the particular biosynthetic capacity. Line A was subsequently used to initiate cell suspensions consisting largely of aggregates up to *ca* 2 mm diameter.



1 2 3

Fig. 1. Outline of biosynthetic route to polygodial.

Callus from line A (18–24) passages was extracted at different times in the growth cycle and the products of interest were characterized by comparison with authentic standards using TLC (3 systems), GC and GC–MS. The crude extracts predominantly were composed of waxes and high M_r compounds that were not identified, but they also contained phytosterols (stigmasterol and sitosterol) as well as polygodial, drimenol and 2(*E*),6(*E*)-farnesol. The time profiles of accumulation of the drimanes in a typical callus of line A are shown in Fig. 2 The maximum occurred at *ca* day eight of the growth cycle, and a similar pattern occurred for the accumulation of these products at the same day after sub-culture of line B, notwithstanding the different passage time for the latter (see Experimental). At *ca* day eight of the cycle, the drimanes comprised *ca* 20% (wt/wt) of the extractable oil together with *ca* 1% 2(*E*),6(*E*)-farnesol. The accumulation of sesquiterpenes by a typical cell. suspension culture derived from callus line A after 12 passages is recorded in Table 1. Again, the recoveries of products varied within the cell cycle, and here the yield of extractable oil also followed a similar profile, both being greatest at *ca* day four The reason for such fluctuations, which occurred with all callus and suspension lines analysed, is not clear. One factor may be the occurrence of extensive necrosis (the cell cultures never entered a true stationary phase). Another may be instability which may mask the true rates of synthesis Polygodial is known to be unstable at room temperature, and we have found that the cell suspensions were capable of rapidly metabolising exogenous drimenol (5 mg per 500 ml of culture per 24 hr) to form water solubles. We carried out several experiments using Miglyol as a sink in a two-phase system, but this did not significantly improve the overall recovery of the sesquiterpene

The accumulations of sesquiterpenes in suspension cultures and callus cultures (days 4 and 8 respectively) is compared in Table 2 with those in organs of the parent plant at the flowering stage. The extremely high level of polygodial (*ca* 10% wt/fr wt) in the flowerhead is noteworthy. At optimum, the levels of polygodial in callus and suspensions are *ca* 0.25 and 29% respectively of those in the flowerhead and foliage

Feeding experiments with 3*R*-[2-^{14}C]-mevalonate (MVA) designed to illustrate the synthetic as opposed to the accumulative abilities of both the plant and cultures are recorded in Table 3. Not only is *de novo* synthesis confirmed, but the high synthetic capability of the callus material—and by implication of cell suspensions—is clear. One novel feature of these experiments was that the tracer was introduced by a wick-feeding technique using cellulose rods, and the observation of the preferential labelling of sesquiterpenes rather than steroids suggests restriction of growth under these conditions. The results indicate that callus (and presumably cell suspensions) not only accumulate polygodial and its parent in useful amounts, but they also contain active enzyme systems for

Table 1 Production of sesquiterpenes by cell suspension cultures of *Polygonum hydropiper*

Day*	Farnesol†	Drimenol†	Polygodial†	Oil‡	%§
0	3(1)	2(0)	13(6)	102	0
4	4(1)	13(7)	237(53)	406	46
6	7(1)	4(3)	28(14)	223	22
8	0(0)	0(0)	1(0)	19	59
11	0(0)	0(0)	0 3(2)	6	596

*Day from subculture· passage period seven days.

†Accumulation of sesquiterpenes (μg/g fresh weight) in cells and (in parenthesis) in medium (μg/g fresh weight of producing cells). Formal s e *ca* ±6% actual value in the range 100–200 μg/g.

‡Total extractable oil (μg/g fresh weight)

§% Increase in fresh weight of culture

Table 2 Accumulation of sesquiterpenes by cultures and plant

Tissue	Farnesol*	Drimenol*	Polygodial*
Callus A	6	2	52
Callus B	1 3	3	62
Suspension	4 2	13	237
Root	1.6	2	81
Leaf	5 6	30	813
Stem	1 0	82	625
Flowerheads	46 4	$6\,3 \times 10^4$	9.6×10^4

*μg/g wet weight Formal s e *ca* ±3% actual value in the range 100–200.

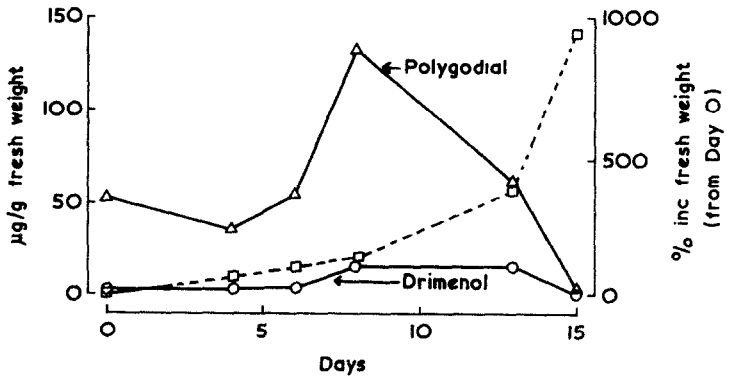


Fig 2. Production of drimenol and polygodial across the culture cycle △—Polygodial, ○—drimenol, □—% increase of culture

Table 3. Incorporation of 3*R*-[2-^{14}C]-MVA into lower terpenoids in plant and culture

Tissue	MVA*	Farnesol	Drimenol	Polygodial	Balance (to 100)
Callus A	35	36	10	17	2
Callus B	36	33	15	13	3
Shoot	68	8	8	10	6
Flowerhead	71	6	8	8	7

*Incorporation into MVA band and lower isoprenoids after incubation period (see Exptl). Callus at day five of cell cycle. Incorporations represent % of ^{14}C recovered in the fractions assayed.

cyclisation of FPP and the subsequent oxidations, the mechanisms of which are unknown. Such culture material is much more convenient to handle than the fibrous and highly-irritant slow growing plants; and in addition we have found that the relevant enzyme systems can be extracted and fractionated much more efficiently from callus cultures than from field-grown material.

EXPERIMENTAL

Materials P hydropiper was field grown (R. Parr, Mitterdale Drug Farm, Royston, Herts, U K.), harvested at flowering (September) and processed the same day by separation of tissues (root, leaf etc.), flash freezing (liq. N_2) and storage at $-20°$. Under these conditions the composition of the tissues did not appreciably vary over at least six months. Seeds (for initiation of cultures) and highly purified (−)-polygodial were kindly provided by Dr J. Pickett (Rothampstead Exptl. Station, Harpenden Herts, U K.) and polygodial was stored at $-20°$ in the dark. (−)-Drimenol was extracted from bark of *Drimys winterii* [5] and stored at 0°.

Culture methods. Callus line A was established from an explant of stem from a mature plant using MS supplemented with GA_3 (10 mg/l), BAP (0.5 mg/l), NAA (2 mg/l) and sucrose (30 g/l), solidified with agar (plant tissue culture grade, Flow Laboratories, Irvine, Argyll, U.K.) and taken to pH 5.5. After initiation (*ca* 2 weeks), passages were carried out every 14 days for 34 months before harvesting and assay. The cultures were maintained at 25° in continuous illumination (Philips tubes 'Warm White'; λ_{max} 580 nm; 600 lux). Line B was established from stem explants of freshly germinated seedlings and cultured similarly. Passages were made every 28 days for 12 months before assays were started. Cell suspensions were established from callus line A in the above medium minus agar and maintained under similar environmental conditions on a gyrorotatory shaker (120 r.p.m.). Subculture was every 7 days for 3 months before assay.

Product analysis. Plant or culture material (2 g) was frozen (liq. N_2) and ground up with acid-washed sand (1 g) together with redistilled EtOAc (4 cm^3) containing longifolene and phytol (*ca* 1 μg) as int. standards. The pulverized slurry was shaken (2 hr; 0°), and the supernatent decanted and evapd to dryness at 0° in a stream of N_2. The residue was redissolved in EtOAc (0.2 cm^3) and aliquots (1, 1 and 10 μl respectively) were subjected to GC, GC–MS and TLC. GC analysis was carried out using an OV-101 W.C.O.T. capillary column (26 m × 0.32 mm i.d., 60–250° programmed at 8° min; injector and detector at 250°; He 0.01 l/hr). GC-MS involved either the above GC system coupled to a Shimatzu 80 mass spectrometer (70 eV) or SE-30 (20%, Chromosorb W 100–200; 3 m × 8 mm i.d; 60° for 5 min, then 2°/min to 160° and 6°/min to 230°; N_2 3.6 l/hr) linked to a Kratos MS 25 mass spectrometer (70 eV) coupled to a Kratos 65–505 data system. TLC utilised silica gel G (0.25 mm) with (a) EtOAc–*n*-C_6H_{14} (3:7), (b) EtOH–$CHCl_3$ (3 100) and (c) C_6H_6–Me_2CO (3:10). Visualization was with 5% vanillin in conc. H_2SO_4 or 5% phosphomolybdic acid in EtOH (followed by 100°, 2 mins).

Radioactive feeding. Three sterile cellulose rods (Sorbarods; ex Baumgartner S.A., Lausanne, Switzerland; 0.5 × 2 cm) in the base of a petri dish were each inoculated (via a millepore filter; 0.22 μm) with 3R-[2-^{14}C]-MVA (10^5–10^6 dpm; 56 7 mCi/mmol; 500 μl water). A callus (*ca* 1 cm^3) was placed on each rod or a shoot (stem apex, 4 leaves and flowerhead; *ca* 5 cm) was inserted into each, and the dish (covered for the case of callus) was incubated at 25° for 2 hr under illumination as previously specified. Phosphate buffer (0.02 M; pH 7.1; 30 cm^3) containing sodium molybdate (1 mM), $MgCl_2$ (5 mM), $MnCl_2$ (2 mM) and DTT (5 mM) or liquid culture medium (30 cm^3; see before) was then added to the base receptacle and the system was incubated for a further 22 hr. The plant material was then extracted as before and separated by TLC with systems (a) to (c) and the ^{14}C-containing bands corresponding to the sesquiterpenes, and to unreacted MVA (most of which had probably not penetrated the tissue) were located and assayed by LSC with Optiscint (LKB Ltd, Croydon, U.K.) to give $2\sigma \pm 5\%$.

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REFERENCES

1. Asakawa, Y. and Takemoto, T. (1979) *Experientia* **35**, 1420.
2. Fukuyama, Y., Sato, T., Asakawa, Y. and Takemoto, T (1982) *Phytochemistry* **21**, 2895.
3. Fukuyama, Y., Sato, T., Miura, I. and Asakawa, Y. (1985) *Phytochemistry* **24**, 1521
4. Asakawa, Y., Toyota, M., and Takemoto, T. (1978) *Phytochemistry* **17**, 457
5. McCallion, R. F., Cole, A. J L., Walker, J. R. C., Blunt, J. W. and Munro, M H. G. (1982) *Planta Med.* **44**, 134.
6. Cortes, M. J., Urrejola, R. and Oyarzun, M. L. (1982) *Bol. Soc. Chil. Quim* **27**, 307.
7. Okunda, R. K., Scheur, P. J., Hochlorski, J. E., Walker, R. P. and Faulkner, D J. (1983) *J. Org. Chem.* **48**, 1866.
8 Stampf, J L., Benezra, C. and Asakawa, Y. (1982) *Arch Dermat Res.* **273**, 277.
9. Kubo, I. and Taniguchi, M. (1988) *J. Nat Prod* **51**, 22.
10. Van Beek, T. A. and de Groot, A. R. (1986) *Rec Trav Chim.* **105**, 513.
11. Pickett, J. A. (1985) *Phil. Trans. R. Soc. Lond* **310B**, 235.
12. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.