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PRODUCTION OF *TRANS*-β-FARNESENE BY CALLUS OF *HUMULUS LUPULUS*

DEREK V. BANTHORPE, JACQUELINE T. BROWN and GEORGE S. MORRIS*

Department of Chemistry, University College London, 20 Gordon Street, London, WC1H OAJ, U.K.

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Key Word Index—*Humulus lupulus*; Cannabinaceae; hop; tissue culture; *trans*-β-farnesene; mevalonate incorporation.

Abstract—Callus lines of *Humulus lupulus* have been established that accumulated *trans*-β-farnesene as the main component (*ca* 14%) of the extractable oil. Incorporation studies with 3*R*-[2-^{14}C] mevalonate resulted in very significant (*ca* 2%) incorporation of the applied tracer into (2*E*,6*E*)-farnesol and *trans*-β-farnesene.

INTRODUCTION

trans-β-Farnesene (7,11-dimethyl-3-methylene-1,6(*E*),10-dodecatriene) is an aphid alarm pheromone [1, 2] that because of its presumed direct derivation from 2(*E*),6(*E*)-farnesyl pyrophosphate or its nerolidyl isomer is an attractive target compound for genetic manipulation [3]. The sesquiterpene rarely occurs in quantity in plants, but a good source is oil of *Humulus lupulus* (wild hop: Cannabinaceae) [4]. We here report the establishment of callus lines of hop capable of providing biomass which would allow detailed molecular and biochemical studies. Tissue cultures of hop cultivars have been widely studied in the micropropagation of virus-free stock [5] but there is no previous report of the accumulation of secondary metabolites. *trans*-β-Farnesene has also been recorded in cultures of *Matricaria* spp. [6, 7].

RESULTS AND DISCUSSION

Friable, pale, sometimes brown, calli were readily initiated from explants of germinating seeds of *H. lupulus* using the conditions detailed in the Experimental. Under appropriately chosen regimes sesquiterpenes accumulated and were identified by GC-MS analysis of extracts. An assay of an optimum callus line after 12 sub-cultures from initiation is shown in Table 1. The solvent-extractable oil contained *trans*-β-farnesene as the only characterized sesquiterpene (other sesquiterpenes comprised <1% of the total oil) and smaller amounts (*ca* 2% of total) of monoterpenes, probably ocimene and myrcene as indicated by GC-MS, together with high-boiling materials (resins, steroids etc. cf.[8]) were present. Extracts were not made by steam distillation as this is known [9] to cause extensive breakdown of the components of hop oils. Growth of the culture had effectively ceased by the day 24, although the cultures never reached a true stationary phase and the maximum accumulation of oil and of *trans*-β-farnesene occurred reproducibly after *ca* eight days incubation. The decrease in oil accumulation in the period days eight to ten may be due to degradation, release into the medium and/or reduction of metabolite synthesis during the period of maximum culture growth. At the time of maximum oil formation, the proportion of *trans*-β-farnesene in the extractable oil (*ca* 14%) was close to that (16–19%) in oil from field-grown plants [8], but the total yield of oil from callus was only 4% of that from the latter (wet/fresh weight). However, such a yield of oil from any culture is, in general experience, highly acceptable and sesquiterpenes have rarely been reported to accumulate under such conditions [10]. Because of the relatively high water-content of the callus, the comparison is much more favourable to the latter (*ca* 60%) if the weight of oil is calculated on a dry weight basis.

de novo Synthesis of *trans*-β-farnesene was never in doubt given that the number of passages before assay must have reduced the levels of any secondary metabolites carried over from the original explant to quite negligible proportions. However, such *de novo* synthesis was proven, and in addition activities of the enzymes *in callo* that synthesized *trans*-β-farnesene were demonstrated, by the incorporation experiments as recorded in Table 2. The total incorporation into the fraction extractable with ethyl acetate was very significant (*ca* 6%) with *ca* 33% of the recovered label present in the sesquiterpenes. The specific activity of the farnesol must have been extremely large as this compound must have been present in trace amounts, for it was not detected as a component (<0.1%) of the extractable oil. Feeding experiments involving the administration of tracer to callus cultures by injection or standard infiltration techniques have generally yielded low incorporations into terpenoids [cf. 11]—presumably owing to compartmentation, localization, and lack of transport of the added tracer-containing materials. Our high incorporations may result from the use of a novel feeding technique. The callus was supported on a cellulose wick into which the tracer was injected. On surrounding the wick with liquid culture medium, the tracer was carried up to bathe the callus by the 'transpiration' stream with little dilution.

Our results demonstrate the high activities of the synthases of 2(*E*),6(*E*)-farnesol and *trans* β-farnesene *in callo*, and this coupled with the high growth rate of the

* Present address: Advanced Technologies (Cambridge) Ltd., Cambridge, U.K.

Table 1. Analysis of hop callus

Days*	*trans*-β-Farnesene†	Oil‡	% *trans*-β-farnesene§	Wt callus (% increase)‖
0	0.9	41.2	2.2	0
4	1.0	17.5	5.7	12
8	18.0	124.8	14.5	52
11	0.7	46.8	1.5	124
21	1.8	28.8	6.3	214
24	1.4	21.2	6.6	459

*After subculture.
†μg *trans*-β-Farnesene per g callus.
‡μg Total oil per g callus.
§% Of *trans*-β-farnesene in oil.
‖% Increase in fresh weight of callus: inoculum *ca* 1 g.

Table 2. Incorporation of 3*R*-[2-^{14}C]-mevalonate into components of callus of *H. lupulus*

Compound	MVA*	Unidentified	*trans*-β-Farnesene	2(*E*),6(*E*)-Farnesol
% Recovery†	32	35	14	19

*MVA activity; largely absorbed on callus–Sorbarod interface (See Discussion and Experimental).
†Percentage of recovered activity in the various compounds/fractions). Feeding conditions in Experimental.

callus makes the system ideal as a source of biomass for the previously mentioned gene-transfer investigations that are underway.

EXPERIMENTAL

Culture methods. The callus lines were established from an explant derived from seed of *H. lupulus* L. (ex. Chiltern Seeds, Ulveston, Cumbria U.K.). The initiation and callus maintenance medium was Gambourg's [12] supplemented with casein (3 g/l), 2,4-D (1 mg/l), BAP (1 mg/l) and sucrose (30 g/l) adjusted to pH 5.5. The seeds were sterilised by rinsing with abs. EtOH (30 sec); treatment with 'Domestos' (2% aq. NaOCl; 1 hr) followed by washing with sterile dist. H_2O (× 5) and were germinated and formed callus at 25° in continuous illumination (Philips tubes 'Warm White'; λ_{max} 580 nm; 600 lux). Some 10% of the seeds had formed callus after 13 weeks and this was subcultured and maintained on a 14-day growth cycle. After 12 passages the assays were carried out.

Product analysis. Cultures (2 g) were frozen in liq. N_2 and ground up with acid-washed sand (1 g) and EtOAc (redistilled; 4 ml) containing longifolene (*ca* 1 μg) to act as an internal standard. The slurry was shaken (2 hr; 0°) and the supernatant decanted and evapd in a stream of N_2. The residue was redissolved in EtOAc (0.2 ml) and aliquots (1 μl, 1 μl and 10 μl respectively) were subjected to GC; GC-MS and TLC. GC-Analysis was carried out on 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 column coupled to a Shimatzu 80 mass spectrometer (at 70 eV), or a chromatographic system of SE-30(20% on 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 MS25 mass spectrometer (70 eV) coupled to a Kratos data system. TLC was carried out against standards on 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 phosphomolybdic acid in EtOH (5%), followed by heating at 100° for 2 min.

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 an aliquot of 3*R*-[2-^{14}C]-MVA (10^5 dpm; 56.7 mCi mmol; 500 μl H_2O). A callus (*ca* 1 cm^3) was placed on each rod and the covered dish was incubated at 25° for 2 hr under the previous illumination regime. Culture medium (minus agar; 30 ml) was then added to the base receptacle and the system was incubated as above for a further 22 hr. The callus was then extracted as before and analysed by TLC systems (a) to (c) and the tracer-containing bands corresponding to the sesquiterpenes and to unreacted MVA were located. Radiochemical analysis was by LSC-counting with Optiscint scintillation solution (LKB Ltd, Croydon, UK) and 4×10^4 dpm were accumulated so that 2σ was ± 1%.

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