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The accumulation of essential oils by tissue cultures of *Pelargonium fragrans* (Willd.)

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Essential oils are not generally accumulated in morphologically undifferentiated cell suspension cultures. A correlation between morphological differentiation and accumulation is confirmed but it is demonstrated that this level of differentiation is not a prerequisite for product synthesis. In this paper we demonstrate that fine cell suspensions do retain their ability to synthesise essential oils and that accumulation can be induced by manipulation of the photoperiod. It is also shown that morphologically undifferentiated immobilised and aggregate cell systems are capable of essential oil synthesis.

Monoterpene *Differentiation* *Tissue culture* *Photoperiod* *Immobilization* *Aggregation*

1. INTRODUCTION

The genus *Pelargonium* contains a large number of scented variants which produce between them a wide range of essential oil components including mono- and sesquiterpenes. These are commercially important plant products used widely in the perfume, pharmaceutical and flavour industries. The possibility of obtaining these lower isoprenoids, along with many other useful secondary products, from plant cells in culture has been considered as an attractive alternative to whole plant cropping techniques [1]. Previous studies on the synthesis and accumulation of monoterpenes by plant cells in vitro have recently been reviewed [2], and although this area of research has received much attention, only limited success has been achieved.

Generally, monoterpenes and other lipophilic compounds are sequestered in specialised cells or tissues, e.g. glandular hairs, resin canals and schizogenous glands. It has often been noted that these facets of differentiation are usually absent in non-producing cell lines and it has therefore been implied that there is an actual requirement for this level of differentiation in the culture before product synthesis can take place [3].

We have recently investigated the control of callus formation and differentiation within this genius [4] and have confirmed a correlation between monoterpene production and morphological differentiation in vitro. However, further studies indicated that neither organogenesis nor the formation of specialised tissues is a prerequisite for monoterpene synthesis in plant cell cultures. This has recently been discussed [5].

Here, we confirm the correlation between monoterpene accumulation and morphogenesis and further report on the induction of monoterpenes in undifferentiated, suspension cultures of *P. fragrans*.

2. MATERIALS AND METHODS

Callus of *P. fragrans* was initiated as described [4] and maintained on Murashige and Skoog medium [6] (Flow Laboratories) supplemented with 5 $mg \cdot l^{-1}$ benzylaminopurine (BAP), 1 $mg \cdot l^{-1}$ naphthaleneacetic acid (NAA), 3% sucrose and 1% agar adjusted to pH 5.5. Incubation was carried out at 26°C in a light regime of 16 h light (photosynthetic photon flux density

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3 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 8 h dark and callus was subcultured at 4-weekly intervals.

Partial differentiation and complete plant regeneration were obtained by subculture of callus material onto MS medium supplemented with 0.5 $mg \cdot l^{-1}$ BAP-0.5 $mg \cdot l^{-1}$ NAA and 0.5 $mg \cdot l^{-1}$ BAP-0.05 $mg \cdot l^{-1}$ NAA, respectively.

Suspension cultures were maintained on MS medium (supplemented with 5 $mg \cdot l^{-1}$ BAP and 1 $mg \cdot l^{-1}$ NAA) incubated at 26°C in continuous light (3 $\mu E \cdot m^{-2} \cdot s^{-1}$; Philips 'Daylight' fluorescent source) with a shaking rate of 115 rpm. Suspension cultures were subcultured at 7 day intervals (10 ml cells into 60 ml of medium) for a period of 6 months prior to use in the following investigations. (i) Manipulation of photoperiod. Immediately after subculture, cell suspensions were transferred to 4 separate light regimes, viz. continuous dark, continuous light, 18 h light/6 h dark or 12 h light/12 h dark. (ii) Immobilisation of plant cells. Cell suspensions were subcultured into flasks containing 16 cubes (each 1 cm^3) of reticulate polyeurethane foam (8 pores $\cdot cm^{-1}$) and loaded cubes were transferred to new medium on a 7 day cycle. (iii) Formation of aggregates. Cell suspensions were subcultured into MS medium supplemented with 0.5 $mg \cdot l^{-1}$ BAP and 0.2 $mg \cdot l^{-1}$ NAA.

Monoterpenes were extracted from the culture medium with a 3-fold excess of hexane for 2 h; callus and plant material were ground with sand to a fine powder before extraction. Hexane extracts were reduced to 0.1 ml under an atmosphere of nitrogen before analysis.

Monoterpenes were analysed by gas liquid chromatography using a glass column (6 mm diameter, 2 m long) packed with Carbowax 20M (20%) on Gas Chrom Q operating in the temperature range 70–170°C with an initial temperature hold of 5 min and a column heating rate of 3°C $\cdot min^{-1}$. Monoterpene products were identified by retention and mass spectral data. GC-MS was carried out on a Perkin Elmer Sigma 3 GC interfaced to a Kratos MS25 mass spectrometer (acceleration voltage, 1300 V; beam current, 100 μA; ionising potential, 70 eV) fitted with an all-glass jet separator.

Extractions and analyses were carried out after 10 days incubation for photoperiod studies, after 3 weeks for aggregates systems and after 8 weeks for immobilised systems, undifferentiated callus and partially differentiated callus. Regenerated plants were cultivated for 3 months in greenhouse conditions prior to analysis. The analytical results are means calculated from an average of 10 replicate experiments.

3. RESULTS

The monoterpene components of the parent plant, regenerated plant, undifferentiated and partially differentiated callus are shown in fig.1. Whilst undifferentiated callus (trace B) accumulated negligible amounts of monoterpene, there was a gradual restoration of monoterpene accumulation as morphological differentiation was induced. The callus grown on 0.5 $mg \cdot l^{-1}$ BAP and

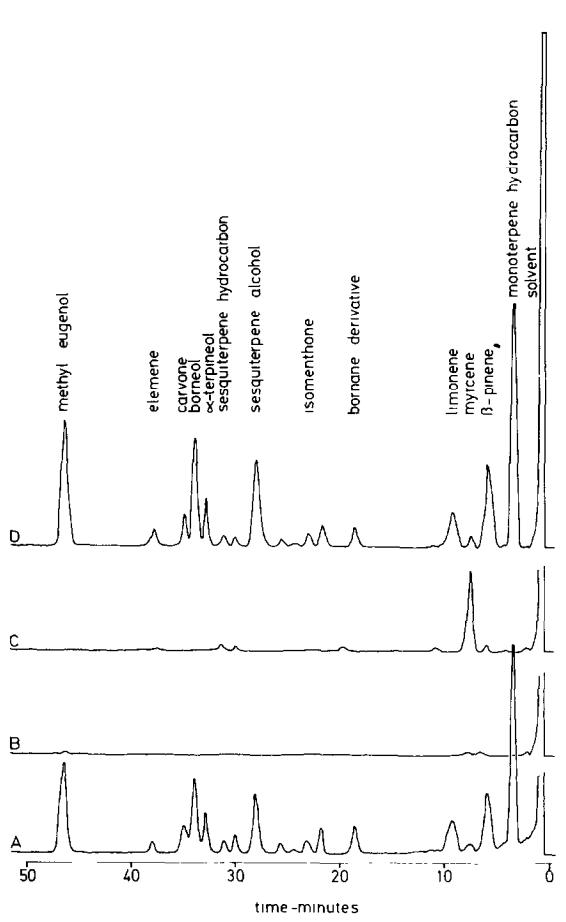

Fig.1. Essential oil profiles of (A) parent plant, (B) undifferentiated callus, (C) partially differentiated callus, (D) regenerated plant.

0.5 mg·l^{-1} (trace C) exhibited the development of glandular hairs but not true shoot formation. Such cultures accumulated myrcene as the major monoterpene component and trace amounts of other terpenoids characteristic of the parent plant, the total oil production being 0.3% that of the parent plant. The regenerated plants produced oil with an identical profile to that of the parent plant (traces D and A, respectively).

Fig.2 shows the induction of monoterpene accumulation in fine suspension cultures which have been subjected to a range of photoperiods. Cultures grown in both continuous dark (trace D) and in a regime of 18 h light/6 h dark (trace B) show monoterpene accumulation, with production levels being 1.0 and 3.0% that of the parent plant.

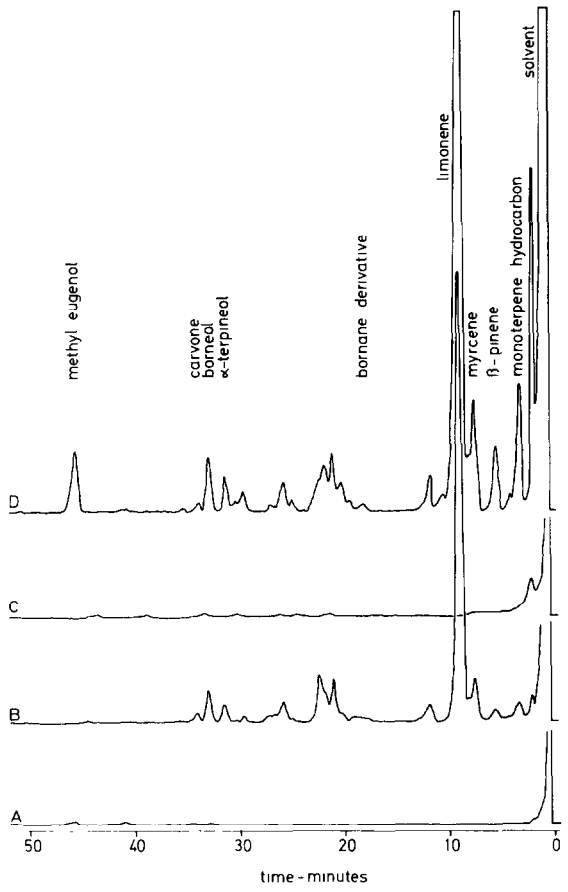

Fig.2. Essential oil profiles of photoperiod treated suspension cultures. (A) Continuous light, (B) 18 h light/6 h dark, (C) 12 h light/12 h dark, (D) continuous dark.

The monoterpenes accumulated within the cells are qualitatively and quantitatively similar to those excreted into the medium. Cultures grown in continuous dark exhibit an essential oil profile similar to that of the parent plant but with an enhanced limonene component (oil containing 5% limonene in parent plant compared to 50% in dark grown cultures). Similar enhancement of the monoterpene hydrocarbon fraction could be observed in the oil from the cells grown in a light regime of 18 h light/6 h dark, immobilised and aggregate culture systems (fig.3, traces B and C, respectively), with only negligible accumulation of methyl eugenol in these cases. The oil products compared with the parent plant in immobilised and aggregate systems were 20 and 1% respectively, with at least 90% being present in the medium. Only negligible amounts of monoterpenes were observed in suspension cells grown in continuous light or in a 12 h light/12 h dark regime.

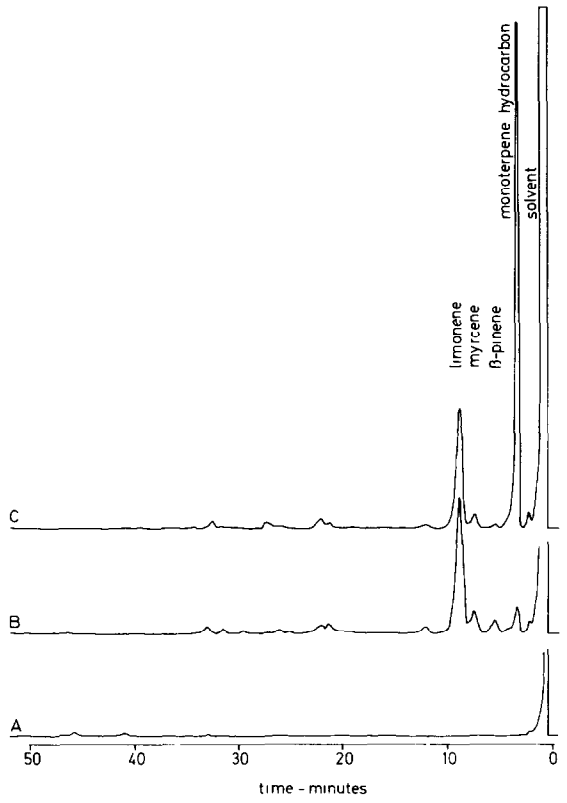

Fig.3. Essential oil profile of (A) fine suspension culture grown in continuous light, (B) immobilised culture system, (C) aggregate culture system.

4. DISCUSSION

Although it is well established that monoterpene and sesquiterpene production in the intact plant is affected by photoperiod, few investigations on this aspect have been carried out in vitro. Watts et al. [7] have studied the effect of photoperiod on flavour production in celery cultures and found that there was no stimulation with a continuous dark or a 12 h light/12 h dark regime. Banthorpe and Njar [8] reported a light-dependent synthesis of monoterpene hydrocarbons with degradation products appearing predominately in dark grown cultures of *Pinus radiata*. Further, a stimulation of monoterpene formation was observed when pericarp and endocarp cultures of *Citrus limonia* were grown in continuous dark [9]. It is considered that the regulatory enzyme in the early stages of the isoprenoid pathway is hydroxymethylglutaryl-CoA reductase. Since there is some indication that the regulation of this enzyme is mediated by light via phytochrome [10,11], it may very well be that the induction of lower terpenoid accumulation by light treatment is itself phytochrome mediated.

Neither the immobilised nor aggregate cultures exhibited morphological differentiation. It has been suggested that cell aggregation and immobilisation can induce secondary product formation by causing a change in the cellular environment and that this arrangement is closer to that of the cells in the intact plant. This theory has recently been discussed by Yeoman et al. [12].

It is demonstrated that morphological differentiation is not necessary for product synthesis. However, without such differentiation accumulation levels are low compared to the parent plant. This level of differentiation or its synthetic equivalent (two-phase system) may therefore be necessary to obtain accumulation levels comparable to that of the parent plant.

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