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MONOTERPENE BIOSYNTHESIS IN EXTRACTS FROM CULTURES OF *ROSA DAMASCENA*

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Key Word Index—*Rosa damascena*; Rosaceae; tissue cultures; enzymes; biosynthesis; monoterpenes; geraniol; nerol.

Abstract—Callus from the stem of *Rosa damascena* did not accumulate monoterpenes. However, cell-free extracts of the cultures converted isopentenyl pyrophosphate (IPP) into geraniol and nerol some 300-fold more efficiently than did the optimum extracts from the parent plants. The former extracts also effectively converted mevalonate (MVA) into IPP. Thus the crucial enzymes for monoterpene synthesis were present in the cultures.

INTRODUCTION

In few cases have either monoterpenes or sesquiterpenes been shown to be synthesized *de novo* by plant cell cultures [1–5] and even here the products often differed in type and pattern from those in the parent material. For *Rosa* species, cultures of the cv Paul's Scarlet did not produce these lower terpenoids [6], and although callus of *R. damascena* was claimed to synthesize geraniol and linalool, characterization (via GC) and proof of *de novo* synthesis were inadequate [7]. However, for these and other cultures it seems important to distinguish between accumulation and the capacity to synthesize a particular metabolite. Detection of the former may depend not only on the correct choice of assay for a derivative (e.g. glycoside, ester) but also on the ability of the culture to degrade any nascent metabolite. Thus, cultures of *R. dilecta* could metabolize exogenous geraniol [8].

RESULTS AND DISCUSSION

Callus and suspension cultures of *R. damascena* cv Trigintpetalla were established from petal, leaf, stem and root on a variety of media. None accumulated the lower terpenes (geraniol, nerol, linalool, citronellol, farnesol) characteristic of the species (assayed as free compounds; glycosides and esters; $< 10^{-4}$ % w/w, if any, above carry-over). Most efficient induction and growth were for stem tissue on a medium developed for the *Rosa* cv Paul's Scarlet [9]. When callus cultures thus maintained were exposed to natural illumination, harvested after 6 weeks in subculture (see Experimental) and disintegrated, extracts could be obtained that showed remarkable levels of activity of key enzymes for monoterpene synthesis. The results of assays using extracts from callus harvested 6 weeks after subculture are compared in Table 1 with interconversions found using a freeze–thaw and vacuum infiltration technique [10] on intact callus and also with direct injection of precursor into callus. Geraniol and nerol (*ca* 8:1) comprise *ca* 95% of the steam-volatile fraction from *R. damascena*: the residue, farnesol, linalool and citronellol could not be detected ($< 10^{-4}$ % interconversion if any, by any of the three techniques). The extractable enzymic activity depended on the age of the subculture: 12-week-old material gave interconversions of IPP* into DMAPP in extracts (*ca* 1.5%) and also by the freeze–thaw method (*ca* 1%) but none by injection, and no method indicated synthesis of geraniol or nerol. *De novo* synthesis of geraniol in the younger cultures was proved by recrystallization of its 1-naphthylurethane to constant specific radioactivity. No suitable solid derivative of nerol has been reported or could be found and therefore the radioactive product was characterized by three GC and three TLC systems.

It was also demonstrated that MVA was converted into its phosphate and pyrophosphate and also into IPP (3, 8 and 10% at optimum when further reaction of IPP was inhibited by addition of iodoacetamide) in the extracts. The significance of these novel results is that although the cultures did not accumulate detectable quantities of monoterpenes, the crucial enzymic machinery for formation of these compounds was present and could be extracted. Preliminary studies on *Pinus*, *Tanacetum* and *Rosmarinus* species indicate that this situation may be general. For *R. damascena*, it proved difficult to prepare efficient cell-free extracts from petal, stem or leaf material. Best results were from the first, but here the levels of geraniol/nerol synthetase were, at best, some 300-fold lower than those obtained from the cultures. This may reflect the ease of breakage of the cell walls, and also the lower levels of phenolics, in the latter material. It could also mean that some or all enzymes of the monoterpenoid pathway are derepressed in the cultures such that the overall level is 300-fold higher than in the differentiated plant.

EXPERIMENTAL

Explants of stem (0.5 × 0.1 cm) were cultured on 0.7% agar containing Nash and Davies medium [9]. Initially 10% coconut milk was present, but this was weaned off at the second subculture. At 27° under natural illumination (south-facing window) *ca* 50% of explants formed cultures within 6 months; subculture was then performed at 6 or 12 week intervals when the vol. was *ca* 1–2 ml. Optimum results were achieved with white

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

Table 1. Metabolism of [4-^{14}C]IPP by callus of *R. damascena*

Method*	% Recovery†	Incorporation (%) DMAA‡	Geraniol	Nerol
Cell-free extract	93 ± 2	22.9 ± 0.9	3.6 ± 0.2	0.4 ± 0.1
Freeze–thaw technique	94 ± 1	17.7 ± 1.0	1.9 ± 0.2	0.3 ± 0.03
Direct injection	92 ± 2	1.9 ± 0.1	0.0	0.0

*See Experimental. [4-^{14}C]IPP as substrate in each case.

† Percentage recovery of tracer: the balance over the three products was isopentenol. Estimated standard errors are recorded.

‡ 3,3-Dimethylallyl alcohol.

friable callus that had been subcultured 6 times at 6 week intervals. Tissue (10 g) was washed (H_2O, 1% EDTA), dried and pulverized in liquid N_2. The resultant powder was mixed with MES buffer (0.1 M, pH 7.0, 20 ml) containing $NaHSO_3$ (10 mM), 2-mercaptoethanol (1 mM) and sucrose (0.3 M), the slurry thawed (4°, 30 min), was filtered through glass wool and centrifuged (10^4 *g*, 20 min). The supernatant was desalted (Biogel R2 or Sephadex G-25 column, 10 × 30 cm) by elution with MES (0.01 M, pH 7.0, + 2-mercaptoethanol, 1 mM). The forerun (16 ml; 0.1–0.25 mg protein per ml) was collected. For assay of monoterpene formation, this extract (2 ml) and MES (0.1 M, pH 7.0, 1 ml) containing 2-mercaptoethanol (1 mM), ATP (2 mM) and $MgSO_4$ (40 mM) was incubated (25°, 3 hr) with [4-^{14}C]IPP (10 μg; 0.5 μCi). The resultant soln was extracted with Et_2O (2 × 3 ml), the pH adjusted to 9.0 (Na_2CO_3 buffer), reincubated (30°, 3 hr) with alkaline phosphatase and apyrase (1 mg of each), and re-extracted. After addition of carrier, [^{14}C]-labelled products were isolated (GC; Carbowax 20 M; 150°) and converted into 1-naphthylurethanes (isopentenol derivative, mp 67°; 3,3-dimethylallyl alcohol mp 97°; geraniol, mp 48°) which were recrystallized (EtOH, Me_2CO) to constant sp. act. and characterized by ^{1}H NMR and by C, H and N analysis. A suitable derivative of nerol could not be prepared and this product was purified by GC on Carbowax 20 M, FFAP and SE-30, and by TLC on silica gel H (EtOAc, $CHCl_3$, C_6H_6, variously) The freeze–thaw method was as described [10]. Direct injection of precursor into callus (2–4 g) was via a millepore membrane (0.22 μm); the tissue was then reincubated for 24–72 hr before work-up and assay (as for cell-free extract). MVA- and MVAP-kinases and formation of IPP from MVA in the extracts were assayed by standard methods [11, 12].

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