Phytochemistry, Vol. 22, No. 11, pp. 2459–2463, 1983.
Printed in Great Britain.

0031–9422/83 $3.00+0.00

BIOSYNTHESIS OF GERANIOL AND RELATED MONOTERPENES IN *PELARGONIUM GRAVEOLENS*

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(*Received* 29 *November* 1982)

Key Word Index—*Pelargonium graveolens*; Geraniaceae; biosynthesis; geraniol; metabolism of monoterpenes; enzymes.

Abstract—Tracer studies on *Pelargonium graveolens* have shown that the extractable activity of geranyl pyrophosphate synthetase passed through a sharp maximum in July, being then some 390-fold greater than the levels during winter. Similar profiles, although less extreme and slightly seasonally displaced, were found for extracted isopentenyl pyrophosphate isomerase and mevalonic acid kinase. Feeding experiments (isopentenyl pyrophosphate and mevalonic acid as precursors) on whole plants showed a similar profile for geraniol synthesis to the one given by geranyl pyrophosphate synthetase. Under conditions of optimum incorporation, the moieties of geraniol derived from both isopentenyl and 3,3-dimethylallyl pyrophosphates were equivalently labelled with tracer. No evidence could be obtained to support the view that valine or leucine were precursors of the 3,3-dimethylallyl-derived portion. Cell-free extracts were shown to contain a monoterpenol-oxidase (FMN-dependent), a monoterpenol dehydrogenase and a monoterpenol reductase (both NADPH-dependent). All three enzyme systems accepted geraniol and nerol, and some of their properties are described.

INTRODUCTION

Pelargonium graveolens Ait. (rose geranium) produces an essential oil (*ca* 0.2% wt/wet wt) comprising virtually only geraniol and nerol (3,7-dimethylocta-*trans*-2,6-dien-1-ol and its *cis* isomer) and citronellol (3,7-dimethyl-6-en-1-ol) in proportions *ca* 80:17:3. Thus, it is excellent material for the study of the biosynthesis of geraniol, the pyrophosphate of which is the generally-accepted parent of monoterpenes. In a search for a convenient source material for the purification of geranyl pyrophosphate synthetase (GPP synthetase; a sub-type of prenyltransferase; EC 2.5.1.1), we investigated the seasonal variation in the extractable activity of the enzyme from the plant. As a consequence of the spectacular activity profile obtained, resulting in cell-free extracts at optimum conditions of unprecedented activity, we further examined the seasonal dependence of two other enzymes involved in the biosynthesis of geraniol, viz. isopentenyl pyrophosphate isomerase (IPP isomerase; EC 5.3.3.2) and mevalonic acid kinase (MVA kinase; EC 2.7.1.36). In the course of these studies, we obtained cell-free extracts containing a monoterpenol oxidase, a similar dehydrogenase and a reductase involved in the formation of nerol and citronellol from geraniol. We briefly report some properties of these systems and record some additional data pertaining to the biosynthesis of the three monoterpenols.

RESULTS AND DISCUSSION

Seasonal variation of enzymes

Specimens of *P. graveolens* were kept at 50 ± 5° under natural illumination in the period October–April and otherwise outdoors, and cell-free extracts were prepared at monthly intervals. GPP synthetase was assayed using a 1:1 mixture of [1-^{14}C]IPP and 3,3-dimethylallyl pyrophosphate (DMAPP) as substrates in the presence of iodoacetamide (15 mM) to inhibit any IPP isomerase. Controls showed that the additive effectively (96–8%) blocked the conversion of IPP into DMAPP without, however, effecting the activity of the synthetase: similar selective inhibition has been reported for other plant species [1, 2]. Cleavage of the substrates by endogenous phosphatases was small (< 5%) under the conditions of the assay: this was fortunate as addition of F^- to inhibit these (cf. [3]) also inhibited (up to 50%) the synthetase. Use of other additives and variations in techniques (which did not lead to significant improvements to the standard cell-free extract) are briefly recorded in the Experimental.

The seasonal variation in synthetase activity is shown in Table 1. The sharp maximum in extractable enzyme activity in July (39% incorporation of tracer under standard conditions) resulted from one of the simplest, yet most efficient, cell-free systems for monoterpene biosynthesis that has been reported. The maximum levels were some 390-fold greater than those in January–December. In the latter months, the plant was not dormant (in a heated greenhouse) but was growing actively and forming new shoots, and so terpene-synthetase activity might have been expected if this was a characteristic of young tissue. The maximum occurred well after the onset of flowering (mid-May onwards) and did not correlate with visual estimates of the period of maximum leaf expansion (early May); immature leaves of *Salvia officinalis* were reported to synthesize and accumulate camphor most readily in the latter stage of development [4]. Despite the enormous variations in activity of extractable enzyme,

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Table 1. Seasonal variation of enzyme activities from *P. graveolens**

Month	GPP synthetase: Cell-free extract†	GPP synthetase: Whole plant‡	IPP isomerase§	MVA kinase‖
January	<0.1	<0.1, <0.1	6.1	2
February	1.2	—	7.0	2
March	5.0	0.2, 0.1	5.1	5
April	16	—	10	14
May	23	1.2, 0.7	12	22
June	23	1.5, 0.7	21	19
July	39	2.3, 1.6	31	17
August	30	2.0, 1.5	31	12
September	13	1.1, 0.8	18	8
October	5	—	15	5
November	0.5	0.2, 0.2	10	5
December	<0.1	<0.1, <0.1	8	5

*The table records % incorporations of tracer into products, under conditions defined in the Experimental. Standard error *ca* ±0.5% actual value.

†IPP + DMAPP as substrate.

‡IPP or MVA as substrate.

§Cell-free extract; IPP as substrate.

‖Cell-free extract; MVA as substrate.

accumulation of geraniol was practically constant (C_{10}/g wet wt leaf, 0.21 ± 0.03%) over the period March–October.

It could be argued that the profile obtained represented ease of extraction of the synthetase, rather than its intrinsic activity, or even that part of the pathway to phytosterols and carotenoids rather than that to monoterpenes was being isolated. The former suggestion seems unlikely in that younger plants with less robust cell walls and low levels of phenolics yielded relatively low levels of extractable enzyme. The latter situation would almost certainly involve the formation of farnesol (derived from farnesyl pyrophosphate, FPP) in the products (cf. [5]); in the event no such chain-lengthening of geranyl pyrophosphate could be detected (< 0.1% incorporation; if any). GPP synthetase activity in higher plants is usually associated with that of FPP synthetase, but *P. graveolens* is unusual (as is *Tanacetum vulgare*, cf. [1]) in that virtually no (< 1%) sesquiterpenes occur in the extractable oil. The results of feeding IPP, IPP + DMAPP, and MVA to whole plants for metabolism periods monitored to be optimum for incorporation into geraniol (Table 1), and the consequent time–incorporation profiles that were similar (although incorporation was not as efficient for obvious reasons; in particular it is not known whether IPP, etc. can cross the cell membrane) suggest a similar profile of monoterpene synthetase activity in this material (i.e. *in vivo*). Incorporations were still at a maximum in July, and were some of the highest recorded for monoterpene biosynthesis from *in vivo* feedings (cf. [6]). The implications of these observations for studies on the biosynthesis of secondary metabolites, and for enzyme purification, are obvious.

Time–incorporation profiles for IPP isomerase and for MVA kinase are also recorded in Table 1. Similar, but less spectacular variations, in the latter case displaced towards spring, were obtained. It has been reported that cell-free extracts from a variety of species incorporated the pyrophosphate of 2-methylbut-3-en-2-ol (dimethylvinylcarbinyl pyrophosphate, DMVC-PP) into various monoterpenes more efficiently than IPP or DMAPP [1, 7]. In the course of the assays of IPP isomerase, we particularly looked for the presence of DMVC or DMVC-PP (by TLC) or its likely breakdown product isoprene (by MS) but no evidence for the intervention of this possible precursor could be found. Consequently, the observed efficient incorporations may have no fundamental biosynthetic significance.

Further feeding experiments on whole plants

Feeding excised stems and leaves of a variety of plant species with [2-^{14}]MVA usually resulted in the IPP-derived moiety of monoterpenes being preferentially (and position-specifically) labelled: this was attributed to the existence of a metabolic pool of the biogenetic equivalent of DMAPP [G]. A rare exception was for *P. graveolens* where both IPP and DMAPP-derived portions were equally labelled [8]. It has been suggested that the asymmetric labelling may reflect the utilization of leucine or valine as precursors of the DMAPP-derived portion, and tracer experiments on the formation of various monoterpenes (including geraniol and citronellol) in several species (including *Pelargonium roseum*) have been held to support this [9, 10]. However, incorporations were low and the amino acids may have been degraded to acetate and the latter incorporated into the DMAPP pool.

Feeding [4-^{14}C]IPP to *P. graveolens* under conditions of maximum incorporation (July) yielded geraniol which was degraded to oxalic acid, levulinic acid and acetone in which the tracer was distributed *ca* 0:51:49. This confirms equal (within the experimental error ± 1%) labelling of the C_5 portions and implies that here DMAPP (or its equivalent) used in monoterpene biosynthesis was essen-

tially all derived from IPP, and no significant pool of the former either pre-existed, or was built up during the period of metabolism. Co-feeding of [4-^{14}C]IPP together with [U-^{3}H] valine or [U-^{3}H] leucine at mole ratios of 1:1 or 1:10 yielded geraniol in which the ^{14}C:^{3}H was > 99:1. Similar results were obtained for feeding experiments carried out in April when the % incorporations were 10-fold less. No evidence was thus obtained for the utilization of amino acids as monoterpene precursors.

Formation of nerol and citronellol

Our 'standard' cell-free extract biosynthesized geraniol together with nerol (1%) and traces of citronellol (< 0.1%) from [1-^{14}C]IPP. No farnesol could be detected. It is likely that the minor products were derived from geraniol, and attempts were made to characterize the enzyme system(s) involved.

Reconstitution of an acetone powder prepared from a cell-free extract in July led to the detection of a geraniol oxidase of such low reactive activity (< 5%) that it would not have significantly interfered with the assay of geraniol synthetase, and the seasonal variation of which closely paralleled that of the latter. The activity was assigned as an oxidase on the basis of concurrent assays of H_2O_2 and citral as previously described for a monoterpenol oxidase from *T. vulgare* [11], the only such enzyme previously reported. Some characteristics of the enyzme are in Table 2: the activity was not significantly affected by reagents that react with thiol groups or which chelate metals. The activity appeared slightly dependent on FMN but not on FAD or oxidized or reduced pyridine nucleotides either before or after extended dialysis at pH 6.3 or 8.5; and the activity was not restored by addition of any of these cofactors to the apoenzyme obtained after heat or guanidine treatment. The enzyme may be a flavoprotein: it has been frequently observed that the holoenzyme of such types are not easily reconstituted [12]. Under optimum conditions the reactivity of substrates tested was: geraniol 100; nerol 23; citronellol 2.

A similar cell-free extract showed no activity in the reduction of citral in the pH range 4–8 but addition of NADPH stimulated effective reduction to geraniol and nerol. Some properties of this system are recorded (Table 2). The reverse (geraniol dehydrogenase) activity was dependent on $NADP^+$ at pH 9.0. Finally, addition to the extract of NADPH uniquely of the cofactors investigated stimulated geraniol reductase activity to form citronellol. Nerol was less effectively converted (at optimum conditions: geraniol 100, nerol 73). Properties of the reductase are in Table 2: it was effectively inhibited by 'thiol' reagents. The enzyme system resembled that detected in extracts of *Rosa* petals [13].

These experiments demonstrate the entire machinery for the formation of nerol and citronellol from geraniol, and it can surely be assumed that phosphatases that convert the initially formed GPP into geraniol are present in *P. graveolens*. It is noteworthy that geraniol is probably oxidized *in vivo* by an oxidase to give geranial which spontaneously equilibrates to neral which is then reduced by a dehydrogenase to form nerol, rather than the sequence being effected by a simple redox system involving a dehydrogenase working in the two directions at different pH values. A similar situation probably exists in the redox

Table 2. Properties of enzymes of monoterpenoid metabolism in *P. graveolens*

	Geraniol dehydrogenase	Geraniol oxidase	Geraniol reductase
Substrate	Citral*	Geraniol	Geraniol
Product (%)†	Geraniol/nerol (5, 3)	Citral (21)	Citronellol (28)
pH optimum	7.0	6.3	8.2
pH range‡	6.5–7.3	5.2–8.7	7.1–9.9
Control activity§	0	100	0
Additives‖			
NADPH	100	100	100
$NADP^+$	0	102	0
NADH	2	98	1
NAD^+	0	101	0
FMN	0	123	0
FAD	0	101	0
SH inhibitors¶	—	95–102	0–15
Metal chelators etc**	—	93–100	89–100

*Equilibrium mixture of geranial and neral (*ca* 7:3).

†% Conversion under standard assay conditions (after additions of cofactors if necessary), see Experimental.

‡pH values for half-maximum activity.

§Activity of standard cell-free extract: no cofactors added.

‖Cofactors, inhibitors, etc. added at various 0.5–2 μM (cofactors); up to 0.5 mM (potential inhibitors). The values for the three enzymes refer to the activities, relative within a vertical column. Zero conversion signifies < 0.1%, if any, reaction (cf. 100% at maximum activity).

¶*p*-Hydroxymercuribenzoate; iodoacetamide; iodoacetate; *N*-ethylmaleimide.

**CN^-; EDTA; α,α-dipyridyl; 1:10-phenanthroline; 8-hydroxyquinoline; F^-, N_3^-.

interconversion of geraniol and nerol in extracts of *Rosa* petals [14]: the forward reaction involved loss of the pro-(1*S*) hydrogen, whereas the reverse led to loss of the pro-(1*R*) atom. This is difficult to understand if a dehydrogenase system was involved.

EXPERIMENTAL

Materials. Specimens of *P. graveolens* were a clone propagated vegetatively. The potted plants were kept outdoors April–September and in a greenhouse at *ca* 50 ± 5° during the rest of the year. [1-^{14}C]IPP was purchased from Amersham International. [4-^{14}C]IPP, [2-^{14}C] geraniol and [2-^{14}C] nerol (all 4–9 mC/mmol) were prepared as previously described, as was DMAPP [1, 11, 14]. [2-^{14}C] Citral was prepared (MnO_2 oxidation) from geraniol.

Preparation of cell-free extracts. Leaves (10 g) were destalked, washed (1% aq. EDTA) and ground in liquid N_2. The powder was allowed to warm up (5 min) and then stirred into 0.1 M MES (pH 7.0; 20 ml) containing sucrose (0.3 mM), ascorbate (0.1 mM) and redistilled 2-thioethanol (1 mM) and allowed to warm to *ca* 4° (90 min). The mixture was filtered through glass wool and centrifuged (10 000*g*; 30 min) and the supernatant passed down a column of Sephadex G-25 (50 × 0.5 cm) and eluted with 0.05 M MES (pH 7.0) to yield the protein in the exclusion vol., whereas phenolics, etc. were retarded. Protein concns in the eluant were *ca* 0.1 mg/ml as assayed colorimetrically [15]. This 'standard extract' or the Me_2CO powder derived from it was used for all the subsequent assays.

Preparation of the cell-free extract by a 'freeze thaw' method [16] of 6 cycles followed by vacuum-infiltration of the incubation soln gave very poor results: maximum extractable activities were 10–25% of those obtained by the conventional procedure. Me_2CO powders prepared from extracts obtained by the latter procedure lost 50% synthetase activity in 4 months at −20°.

Enzyme assays. (a) *GPP-synthetase.* The extract (1 ml) was added to MES (0.1 M; pH 7.0; 2.5 ml) containing ATP (10 mM), $MgCl_2$ (40 mM) and 2-thioethanol (10 mM) and incubated with substrates (28°; 3 hr). Preliminary expts had shown that the synthetase was optimally active at pH 7.0 (falling to half-maximum activity at pH 5.8 and 8.9) in the presence of the above additives. Addition of aprotinin (ex Sigma Chem. Co.) or trypsin inhibitor from egg white or soya bean did not alter the synthetase activity. The substrates were [1-^{14}C]IPP and DMAPP (1:1; 0.2 μCi; 1 mg) in the presence of iodoacetamide (14 mM). The SKF drugs 525A and 3301A (1 mM) were equally effective in inhibiting IPP isomerase (cf. [17]). After incubation, the soln was extracted (Et_2O; 3 × 3 ml) and the aq. layer boiled (3 min) and reincubated (30°/2 hr) with apyrase (5 mg). The pH was then adjusted to 10.5 (Na_2CO_3 buffer), alkaline phosphatase (5 mg) was added and a further incubation carried out, and any freshly-liberated terpenols extracted with Et_2O as before. Controls showed that these procedures cleaved > 93% of any terpenyl mono- or pyrophosphates. Most (> 85%) of the geraniol was in the first Et_2O extract; the subsequent boiling of the aq. layer converted some (~10%) of the residual GPP into the corresponding neryl and linalyl compounds. The assay was calibrated to allow for this. Geraniol, nerol and citronellol (5 μl) were then added to the combined Et_2O extracts, which were concd and purified by a 2-step TLC procedure: i.e. (a) silica gel H with toluene as eluant (R_f for geraniol, nerol, citronellol 0.85, 0.72 and 0.61, respectively); and (b) silica gel H with 3 consecutive elutions with EtOAc–toluene (1:4) which gave R_f values: 0.70, 0.68, 0.61. All TLC separations were carried out at 4° and spots were visualized by spraying with phosphomolybdic acid (10% ag.) and heating (100°; 5 min). Geraniol and nerol were also separated by GLC on Carbowax 20 M (10% on Chromosorb W 80–100; 2 m × 0.6 cm; 150°; N_2 flow rate 1.8 l/hr) when the relative retention times were 1.00 and 0.89, respectively. Products from the above separation procedures were shown to be chemically and radiochemically pure (> 99%) by GLC on capillary columns (50 m × 0.02 mm) of Carbowax 20 M and SE30 at 110°, and by monitoring with a 2π radiochromatogram scanner under various conditions of solvent and supporting phase. (b) *IPP isomerase.* This was assayed using procedures well established for the enzyme from hog liver with [1-^{14}C]IPP as substrate in Na maleate buffer, pH 6.0 [17; method 1] (c) *MVA kinase* was assayed using [2-^{14}C]MVA as substrate in Tris–HCl, pH 7.9, as described for *Pinus* species [18]. (d) *Geraniol oxidase* was assayed using [2-^{14}C] geraniol solubilized with Tween 80 in MES buffer, pH 6.3 [11]. (e) *Geraniol dehydrogenase* accepted [2-^{14}C] citral similarly solubilized in MES buffer, pH 7.0 [14]. (f) *Geraniol reductase* was assayed with [2-^{14}C] geraniol as substrate (again solubilized) in Pi buffer, pH 8.2 [13]. In assays (a) to (f), typically 0.1–1 μCi (0.1–1 mg) of substrate per aliquot was employed. Coenzymes were added at 0.1–0.5 mg per aliquot. All assays were performed in duplicate and those monitoring the seasonal dependence were made within the last 2 weeks of each month.

Experiments on intact plants. Methods of feeding [4-^{14}C]IPP and [2-^{14}C]MVA were as described for other species [8]. Material was harvested for extraction and chemical assay 15 and 24 hr after the administration of the tracer. Methods for purification of geraniol and nerol, their radiochemical assay and their cleavage to locate tracer within the IPP or DMAPP-derived portions have been fully described [8]. Metabolism periods of 15–40 hr were allowed when [4-^{14}C]IPP was co-fed with either [U-^{3}H]Leu or [U-^{3}H]Val.

Radiochemical methods. Typically, aliquots containing 500–1000 dpm were assayed. Reproducibility between duplicates was ±5% at most and usually better than ±2%. 4×10^4 disintegrations were accumulated in each assay so that 2σ was ±1%.

Acknowledgements—We thank the SERC for studentships to D.R.S.L. and C.R.P., and Messrs. A. Jocelyn, P. Hallwood and Miss U. Frame for experimental assistance.

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