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Short Communication

Site of Monoterpene Biosynthesis in *Majorana hortensis* Leaves[1, 2]

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ABSTRACT

Excised epidermis of *Majorana hortensis* Moench (sweet marjoram) leaves incorporates label from [U-^{14}C]sucrose into monoterpenes as efficiently as do leaf discs, while mesophyll tissue has only a very limited capacity to synthesize monoterpenes from exogenous sucrose. These results strongly suggest that epidermal cells, presumably the epidermal oil glands, are the primary site of monoterpene biosynthesis in marjoram. Using a leaf disc assay, it was demonstrated that label from [U-^{14}C]sucrose is incorporated into monoterpenes most efficiently in very young leaves.

Plants that synthesize large quantities of monoterpenes and sesquiterpenes generally, if not always, contain specialized oil gland structures (5, 6). In many species, including *Majorana hortensis* (sweet marjoram), these oil glands are modified epidermal hairs. The oil glands are probably the major site of storage of monoterpenes, and are generally assumed to be the primary site of monoterpene biosynthesis (5, 6). That such structures are the actual site has been difficult to demonstrate, however, since technical problems are involved in isolating sufficient quantities of glands for biosynthetic experiments.

Fortunately, the epidermis of marjoram leaves is sufficiently sturdy to allow its excision from the mesophyll layer, thus providing a tissue greatly enriched in oil glands. When followed by a recently developed microsteam distillation technique (3), this procedure can be used to determine whether the epidermis is, in fact, capable of monoterpene biosynthesis. Results described in this communication clearly demonstrate that marjoram epidermis is capable of incorporating label from [U-^{14}C]sucrose into monoterpenes, while internal leaf tissue, under the same conditions, does not incorporate label into monoterpenes. Epidermal strips resemble leaf discs in their ability to synthesize monoterpenes. Using a leaf disc assay system, it is shown that young rapidly expanding leaves of marjoram synthesize monoterpenes most rapidly, and that the ability to synthesize monoterpenes from exogenous sucrose decreases as the leaves expand.

MATERIALS AND METHODS

Month-old sweet marjoram (*Majorana hortensis* Moench) plants, which were grown from seed (Northrup King) in a peat moss-perlite-sand mixture (1:1:1, v/v) under summer greenhouse conditions, were used for all biosynthetic experiments. Plants were selected so the leaf pairs of each plant were of comparable ages. For studies on the effect of leaf age on biosynthetic capacity, 20 discs (5 mm diameter) from each leaf type were cut with a cork borer and placed in one chamber of a 10-cm quadrant Petri dish with 0.1 ml of an aqueous solution containing 5 μCi of [U-^{14}C]sucrose (New England Nuclear, 4.9 Ci/mol) and 20 μg of Tween 20. The discs were incubated in the radioactive solution for 4 hr at 30 C in a water bath shaker. For studies with epidermis, epidermal strips (approximately equal proportions of the abaxial and adaxial surface) were excised from young leaves (7 × 12 mm) in cold 0.1 M sodium phosphate buffer (pH 6.5) containing 1 mM ascorbic acid and 0.1 mM dithioerythritol. The epidermal strips and pieces of mesophyll were then rinsed in distilled H_2O and patted dry. Leaf discs (5 mm) were cut from similar leaves in the same buffer, and rinsed and dried as before. Each of these tissues was then incubated with 5 μCi of [U-^{14}C]sucrose exactly as described above.

At the end of the incubation period, the tissue was extracted with 15 ml of diethyl ether (3- × 5-ml portions) by grinding in a small amount of Na_2SO_4 with a mortar and pestle. Internal standards (25 μl of distilled marjoram oil supplemented with additional monoterpenes) were added to the extract, which was then concentrated to 1 ml in the steam distillation apparatus and distilled. The ether phase was removed from the distillate and the aqueous phase was extracted with two additional 1-ml portions of diethyl ether. This combined ether extract was dried over Na_2SO_4, concentrated to small volume at 0 C under a stream of N_2, and an aliquot removed for determination of radioactivity by liquid scintillation spectrometry (72% efficiency for ^{14}C). The remaining extract was further concentrated and subjected to radio GLC. Procedures for steam distillation and radio GLC, and other analytical techniques, have been described elsewhere (3).

RESULTS AND DISCUSSION

Steam-distilled oil from young marjoram tissue (shoot tip and first two leaf pairs) was analyzed by combined gas chromatography-mass spectrometry (3), and shown to contain (by weight) sabinene (14%), α-terpinene (5%), limonene (3%), γ-terpinene (8.5%), *trans*-sabinene hydrate (3%), *cis*-sabinene hydrate (21%), linalool (6%), terpinen-4-ol (23%), and α-terpineol (5%), as well as a number of minor components. By contrast, oil from mature fully expanded leaves of the same plants contained lower levels of sabinene (8%) and terpinen-4-ol (17%), and a higher level of *cis*-sabinene hydrate (35%). Variation in oil composition between young and mature tissue of peppermint has been reported (1), an observation we have also noted in all species we have examined. Recent studies on the composition of

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the volatile oil from marjoram (4, 7) have indicated that two chemical variants exist: one form predominant in *cis*-sabinene hydrate, and the other predominant in terpinen-4-ol. Based on the oil composition of mature leaves, the marjoram plants employed in this study appeared to resemble more closely the variant predominant in *cis*-sabinene hydrate.

Table I. *Incorporation of [U-^{14}C]Sucrose into Monoterpenes of Marjoram Leaf Tissue*

In experiment 1, 20 discs (5 mm diam) were cut from leaves of the size indicated and incubated at 30 C for 4 hr with 0.1 ml of solution containing 5 μc of [U-^{14}C]sucrose (4.9 c/mol). In experiment 2, approximately 8 cm^2 of excised epidermis, 4 cm^2 of excised mesophyll tissue, and 4 cm^2 of leaf discs (20 × 5 mm diam) were prepared from young leaves (7 × 12 mm) and incubated as described in experiment 1. The incorporation into volatile monoterpenes in experiments 1 and 2 was measured as described in the text.

Experiment 1--Leaf Size	Incorporation of [U-^{14}C]Sucrose
mm	*dpm* × 10^{-4}
6 × 10	8.11
9 × 14	3.01
12 × 20	2.11
15 × 28	1.65
20 × 35	0.87

Experiment 2--Tissue	Incorporation of [U-^{14}C]Sucrose
	dpm × 10^{-4}
Epidermis	6.69
Mesophyll	0.40
Leaf discs	6.51

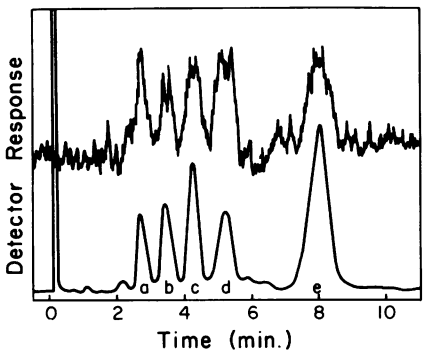

FIG. 1. Radio gas-liquid chromatogram of the steam-distilled lipids isolated from marjoram leaf epidermis that had been incubated with [U-^{14}C]sucrose. The smooth bottom tracing is the flame ionization detector response obtained from coinjected authentic monoterpene standards. The major components are sabinene (a), α-terpinene (b), γ-terpinene plus *trans*-sabinene hydrate (c), *cis*-sabinene hydrate plus linalool (d), and terpinen-4-ol plus α-terpineol (e). The top tracing shows radioactivity recorded by a model 7357 Nuclear-Chicago radioactivity monitor attached to the gas chromatograph. Gas-liquid chromatography was performed on a stainless steel column (2.4 m × 3 mm o.d.) packed with 5% OV-1 on 60/80 mesh Gas-chrome Q, and programed from 70 C at 2.5 C/min with an argon flow rate of 150 cm^3/min.

To determine which age of leaves was most suitable for the biosynthetic experiments described here, discs prepared from leaves of various stages of development were incubated with [U-^{14}C]sucrose, and incorporation of label into steam-volatile lipids determined. Sucrose is a relatively efficient precursor of monoterpenes in cuttings of peppermint (2), and a very suitable substrate for measuring monoterpene biosynthesis in tissue slices of marjoram. As seen in Table I (experiment 1), the youngest (smallest) leaves possessed the greatest biosynthetic capacity, and the ability to synthesize labeled volatile lipids from exogenous [U-^{14}C]sucrose decreased rapidly as the leaves expanded. In subsequent biosynthetic experiments, epidermal strips and mesophyll tissue were excised from the smallest leaves that were practical to handle. Table I (experiment 2) shows the amount of radioactivity incorporated into the steam-volatile lipids derived from [U-^{14}C]sucrose in equivalent amounts of epidermis, mesophyll tissue, and leaf discs of marjoram. Radio GLC of steam-volatile lipids obtained from the epidermal strips verified that radioactivity derived from [U-^{14}C]sucrose was associated with typical monoterpene components of marjoram oil (Fig. 1). Radio GLC of labeled monoterpenes obtained from leaf discs gave, essentially, an identical pattern, but the incorporation of label into monoterpenes of mesophyll tissue was too low to permit adequate radio chromatographic analysis.

Our results constitute the first direct experimental proof that leaf epidermis is capable of monoterpene biosynthesis, and that this tissue is as efficient as leaf discs are in incorporating the label from [U-^{14}C]sucrose into monoterpenes. Mesophyll tissue, on the other hand, is almost devoid of such biosynthetic activity, and the low level of incorporation observed may have been due to small fragments of epidermis adhering to the mesophyll. In conclusion, these findings provide strong evidence that the primary site of monoterpene biosynthesis is the epidermis, if not specifically the epidermal oil glands.

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