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MEVALONATE-ACTIVATING ENZYMES IN CALLUS CULTURE CELLS FROM *NEPETA CATARIA**

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Key Word Index—*Nepeta cataria*; Labiatae; mevalonate activating enzymes; monoterpenes; nepetalactone; terpenoid biosynthesis; tissue culture.

Abstract—The 30000 *g* supernatants from cell-free extracts of *Nepeta cataria* leaf tissue and leaf callus tissue have mevalonic acid kinase, mevalonic acid phosphate kinase and mevalonic acid pyrophosphate decarboxylase activities. The callus tissue cell-free extract produced mevalonic acid pyrophosphate and isopentenyl pyrophosphate; however, very little mevalonic acid phosphate was observed. The leaf cell-free extracts incubated with [^{14}C]-mevalonic acid produced higher amounts of mevalonic acid phosphate. When both the leaf cell-free extract and the callus cell-free extract were incubated with [^{14}C]-mevalonic acid in the presence of iodoacetamide, the ion exchange column elution profile was cleaner, which was confirmed by PC. Apparently the callus tissue 30000 *g* supernatant contains mevalonic acid phosphorylating enzymes even though there is no production of the methyl cyclopentane monoterpenes.

INTRODUCTION

Tissue cultures from volatile-producing plants have been successfully obtained [1, 2]; however, the presence of volatile oils in the cultures has not been reported [3]. Vasil and Hildebrandt [4] observed that endive tissue plantlets produced a mild aromatic flavor similar to that produced by the normal intact plant. It has been reported that mint grown in culture [5] smells like "freshly cut watermelon". Thomas and Stobart [6] have investigated the products in assays of mevalonate activating enzymes in acetone powders prepared from callus cultures of *Kalanchoe crenata*.

The incorporation of radioactivity from [$^{14}CO_2$]- or [^{14}C]-mevalonic acid into the essential oil nepetalactone has been shown to occur [7, 8] in *Nepeta cataria* plants. We have demonstrated the presence of mevalonic acid kinase, mevalonic acid phosphate kinase and mevalonic acid pyrophosphate decarboxylase in cell-free homogenates of *N. cataria* leaves [9]. The present paper describes our investigation of mevalonic activating enzymes in callus cultures of *N. cataria*.

RESULTS AND DISCUSSION

The growth of leaf and stem callus was tested on three different media. The SH media in the light gave the best growth. The callus tissue grew very poorly in the dark. Even though the callus tissue initially grew very well on the SH media, the STABA media proved to be good for growth. The STABA media produced a more friable callus than the SH media. For the development of suspension cultures, the STABA media was used rather than the SH media. The fact that the callus tissue no longer produce secondary metabolites (nepetalactone) is not unusual. The leaf callus tissue appeared to grow faster than the stem and after the fourth week both leaf and stem callus cultures began to turn brown. Subculturing at the initial browning period on fresh media produced new callus growth. The callus tissue had no odor of nepetalactone or other essential oils from *N. cataria* but smelled like 'watermelon'.

The relative growth rate of the *N. cataria* callus cells on SH medium was obtained by weighing the callus at the appropriate time periods. The greatest amount of growth occurred within the first 4

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Table 1. Radioactivity distribution in phosphorylated intermediates

	Incorporation of radioactivity into phosphorylated intermediates (dpm)	% Distribution of radioactivity				
		MVA	MVA Lactone	MVA 5 P	MVA 5 PP	IPP
Pellet						
Leaf	47000	63·66	31·33	5·01	0·00	0·00
Callus	0	60·04	39·96	0·00	0·00	0·00
Super						
Leaf	836400	17·18	2·76	45·05	10·76	24·25
Callus	597200	22·90	3·42	1·62	1·94	70·12
Super (+ iodoacetamide)						
Leaf	876800	10·21	1·42	37·94	23·33	27·10
Callus	905400	4·83	0·71	1·07	6·32	87·07

MVA = mevalonic acid; IPP = isopentenyl pyrophosphate.

weeks. We subsequently performed all subcultures after 4 weeks.

The callus tissue was analyzed for nepetalactone [10] by grinding and extracting with hexane. TLC of the crude oil showed a band with an R_f similar to that of standard nepetalactone; however, GLC did not confirm the presence of nepetalactone. Thus, the callus tissue does not produce nepetalactone; on the other hand, there are some hexane-soluble non-volatile compounds produced which we have not identified. Our cells grown under conditions of darkness or in the light did not develop chloroplasts, unlike the situation in *Kalanchoe* [6].

When [^{14}C]-mevalonic acid was incubated with the 30000 *g* pellet from a fresh leaf extract and from leaf callus tissue extract, only mevalonic acid, mevalonolactone and mevalonic acid phosphate were identified. Table 1 shows the results of the distribution in phosphorylated intermediates as determined by ion exchange chromatography and confirmed by PC. Besides mevalonic acid phosphate, there were no phosphorylated intermediates eluted in the pellet extract.

Mevalonolactone, mevalonic acid, mevalonic acid phosphate, mevalonic acid pyrophosphate and isopentenylpyrophosphate are present in both the leaf and callus incubations. However, callus tissue extracts produced much less mevalonic acid phosphate than did those from leaf tissue and there was a greater percentage of the radioactivity found in isopentenyl pyrophosphate isolated from the callus cells.

Iodoacetamide which is an inhibitor [11] of isopentenyl pyrophosphate isomerase was added to a 30000 *g* supernatant incubation medium. Since this would prevent the formation of prenyl phosphates, which are inhibitory to mevalonic kinase, one would expect to see a better utilization of mevalonic acid toward phosphorylated intermediates. The elution profile from the ion exchange column showed more symmetrical peaks and the purity was verified by PC indicating that in the absence of iodoacetamide higher prenylphosphates are synthesized by the crude enzyme preparation. Again the callus assays showed less mevalonic acid phosphate than the leaf extract. It was suggested by Threlfall *et al.* [12] that there were within the cell of green leaf two distinct sites of terpenoid biosynthesis with distinct mevalonic kinase enzymes, one inside and one outside the chloroplast, and that the enzyme inside the chloroplast is involved in terpenoid biosynthesis in the greening plastids and the extrachloroplastidic enzyme is involved in the synthesis of terpenoids. Gray and Kekwick [13] have suggested that there are two distinct systems and that the two mevalonic kinase enzymes are indistinguishable by pH optima and other kinetic parameters. The pH profile for mevalonic acid kinase was nearly constant between 6·5 and 9·0 for the enzyme preparations from leaf tissue, callus tissue and a chloroplastidic enzyme preparation. We could detect no maximum of mevalonic acid kinase activity in our leaf preparations, callus tissue preparations or chloroplastidic enzyme preparations.

It appears that the enzymes of the leaf tissue and leaf callus tissue from *N. cataria* are indistinguishable under the conditions of our preparations and we can also conclude that under our homogeniza-

tion procedure there should be chloroplastidic and extrachloroplastidic enzymes. Our 30000 *g* preparation of leaf tissue is still green whereas the callus preparation has no color; however, we have not observed isoenzymes of mevalonic acid kinase.

EXPERIMENTAL

Plants of *Nepeta cataria* used for leaf and stem cultures were maintained in the green house. Mature leaves from 2–3-month-old plants were used. All chemicals were reagent grade.

Media preparation: Three types of media were initially used for this study. The three media are referred to as STABA [2], SH [15] and SM [16]; all were prepared with glass distilled H_2O, at 0·75% agar. For the STABA media we chose 2 ppm of 2,4-dichlorophenoxyacetic acid and used pyridoxine hydrochloride in place of pyridoxal. In the SH media, pyridoxine is 10× more conc than published. Leaves and stems were surface sterilized by shaking in 100 ml of 0·1% Tween-20 in 70% EtOH for 2 min, decanting the EtOH soln, shaking with 100 ml 2·75% aq. NaClO for 10 min, decanting and finally washing 6× with sterile glass dist. H_2O. Leaves were sliced into 6–8 mm strips and stems sliced longitudinally. The explant tissue was then placed on the appropriate medium. All operations and transfers were carried out in a Laminar flow hood. After 4 weeks the callus tissue (6 10 mm dia) from the initial explant was excised and placed in new media for subculture. *Ca* 4–6 weeks of growth is completed before second subcultures are prepared. Unless noted all cultures were grown at 27° under continuous light.

Callus cell-free preparation: A 48 g quantity of *N. cataria* leaf callus (fourth subculture) from SH media was ground with 5 g acid-washed Polyclar AT and sand in the presence of 40 ml grinding soln consisting of 10 mM KCl, 5 mM $MgCl_2$, 500 mM sucrose, 1 mM EDTA, 5 mM dithiothreitol and 0·1% Triton X-100 in 500 mM phosphate buffer pH 7·5. The homogenate was squeezed through 6 layers of cheese cloth and centrifuged at 120 *g* for 20 min. The crude extract was then centrifuged at 3000 *g* for 20 min and the pellet discarded. The supernatant was centrifuged at 30000 *g* for 20 min. This supernatant is referred to as the 30K super and the pellet called the 30K pellet.

Leaf cell-free preparation. A 33 g quantity of fresh leaves from mature plant (4 months old) was ground with 4 g Polyclar AT and sand in the presence of 30 ml grinding soln. The centrifugation procedure used for cell-free extract of callus tissue was followed.

Incubation and chromatography. The incubation medium contained 20 mM phosphate buffer pH 7·2 with 3·4 mM $MgCl_2$, 3·4 mM $MnCl_2$, 20 mM KF, 3·4 mM dithiothreitol, 20·7 mM ATP and 6·9 mM NADP; 5 ml enzyme extract, 1 μCi of mevalonic acid (2-^{14}C, 14·32 mc/mM) and appropriate phosphate buffer to make the final vol. 10 ml. Iodoacetamide at 5 mM was used in the appropriate incubations. After 3 hr the reaction was stopped by placing the tubes in a boiling H_2O bath for 5 min. The protein was removed by centrifugation and the pellet washed with 1·0 ml H_2O. The pooled rinse and supernatant were applied to 0·5 × 5 cm Dowex-1-formate column as described by Suzue *et al.* [17]. The ion exchange column was eluted successively with 25 ml H_2O, 25 ml 2 N formic acid, 50 ml 4 N formic acid, 50 ml 4 N formic acid + 0·4 M ammonium formate and finally, 50 ml 4 N formic acid + 0·8 M ammonium formate at 4°. Three ml fractions were collected and 50 μl removed for scintillation counting in toluene–EtOH.

Essential oil isolation. A 1·6 g quantity of *N. cataria* callus tissue (fourth subculture) from SH media was extracted in a blender with 5·0 ml *n*-hexane and centrifuged at 2000 *g* for 5 min. The pellet was extracted again with 5·0 ml hexane, the hexane fractions pooled and conc under N_2. 2·3 mg crude oil was obtained. This was dissolved in hexane and chromatographed [10] on silica gel thin layer plates (along with standard nepetalactone) and used for GLC [10].

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