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Inhibition of potato sprout growth by carvone enantiomers and their bioconversion in sprouts

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Summary

The monoterpenes (R)-(-)-carvone and (S)-(+)-carvone inhibited sprout growth in a model system consisting of sprouts growing from potato eye pieces. The sprout tissue was not necrotic after carvone treatment and the inhibition was reversible, since after treatment the sprouts showed regrowth either by continued top growth or by branching. However, the effect of both isomers on sprout growth differed, and (S)-(+)-carvone inhibited the elongation of the sprouts sooner than did (R)-(-)-carvone. This might be explained by a faster uptake of the former, since the concentration of (S)-(+)-carvone and its derivatives was twice as high during the first 4 days compared with (R)-(-)-carvone-treated sprouts.
The sprouts were able to reduce (R)-(-)-carvone mainly into neodihydrocarveol, and (S)-(+)-carvone into neoisodihydrocarveol; in addition hydroxylated compounds were also detected.

Introduction

Potatoes are usually stored cold (5-7 °C), in combination with the application of synthetic sprout inhibitors. It has been known since 1969 that naturally occurring volatile substances like pulegone and carvone can also inhibit sprouting (Meigh, 1969; Beveridge et al., 1983; Vaughn & Spencer 1991; Oosterhaven et al., 1993; Vokou et al., 1993). Furthermore, various biological effects of monoterpenes have been observed: many of them inhibit seed germination (Asplund, 1968; Fischer, 1986; Reynolds, 1987), and Lorber & Muller (1980) observed a reduced mitotic activity and a hyperconcentration and breakage of chromosomes in *Allium cepa* L. following exposure to 1,8-cineole. Root tip cells of *Cucumis sativus* L. showed a disruption of membranes and accumulation of lipid globules in the cytoplasm, a reduction in the number of a variety of organelles, including mitochondria, and a disruption of membranes surrounding nuclei and mitochondria after exposure to the volatiles (predominantly cineole and camphor) emanating from *Salvia leucophylla* L. (Lorber & Muller, 1976). In these studies a very high concentration of monoterpenes was

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used. Pauly et al. (1981) described the uncoupling effect of low concentrations of β-pinene on photosynthetic electron transport. Attempts to produce monoterpenes in more than trace amounts using plant cell cultures have hitherto failed, probably because of the toxicity of the products (Brown et al., 1987).

In the papers cited above (Asplund, 1968; Lorber & Muller 1976, 1980; Reynolds, 1987) no attention was given to the effective concentration of the monoterpenes used or the possible bioconversion of these compounds, although monoterpenes can be converted by intact plants and by plant cell cultures (Hirata et al., 1982).

We are interested in the mechanism by which (S)-(+)-carvone acts as a sprout inhibitor (Oosterhaven et al., 1993). This paper describes a model system by means of which the effects and the bioconversion of (R)-(-)-carvone and (S)-(+)-carvone (hereafter referred to as R carvone and S carvone respectivily) could be studied on potato sprouts.

Materials and methods

Chemicals. R carvone and S carvone were obtained from Merck. Samples of dihydrocarvone (80% *trans*-isomer and 20% *cis*-isomer), dihydrocarveol (mixture of the four isomers) and dihydrocarvyl acetate (mixture of the isomers) were purchased from Roth, and ^{13}C-labelled methyltriphenylphosphonium iodide ($Ph_3P^{13}CH_3I$) was from Janssen Chimica. All reagents were of analytical grade.

Potato sprout cultures. In order to study the effect of carvone on a growing system related to potato, we used potato eye pieces placed on moist perlite (Agraperlite, Pull B.V., Rhenen, The Netherlands). Seed potatoes (*Solanum tuberosum* L.) of cv. Bintje were stored at 4 °C until use. Prior to the experiment, the potatoes were placed at 18 °C to sprout. Eye pieces of about 25 mm diameter and 20 g weight, cut from corresponding regions of different potatoes, were placed on a 4 cm thick layer of moist perlite in a 20 l tray. The trays, with 30 eye pieces each, were stored at 15 °C in the dark until the average sprout length was about 30 mm. Two Petri dishes, each with 25 µl, 75 µl or 125 µl of the (liquid) carvone enantiomers to be tested, were placed on top of the perlite, to ensure a uniform concentration of the monoterpene vapour in the atmosphere. The trays were covered with a plastic lid and sealed with tape. Because light inhibits potato sprout growth, the trays were placed in the dark at 15 °C. The sprouts were exposed to the carvone vapour over several days, whereupon they were harvested for further examination. Some carvone-treated sprouts were placed on fresh perlite in order to observe their regrowth. Growth was determined by measuring sprout length as well as the sprout and root weights.

Flow cytometric analysis. The nuclear DNA content of control and S-carvone-treated potato cell suspensions was determined according to Van der Valk et al. (1988). S-carvone was added in various concentrations up to 1.5 mM, the suspensions were incubated for 4 days at 20 °C on a gyratory shaker in the dark, and protoplasts were isolated at intervals from small samples of the suspension (Van der Valk et al., 1988).

Extraction of carvone and its conversion products from potato sprouts. Whole potato sprouts, grown as described above, were removed from the eye pieces. The lipid extraction method of Bligh & Dyer (1959) was used for extraction of the monoterpenes. A chloroform ($CHCl_3$) phase was obtained and dried over anhydrous Na_2SO_4 and concentrated to 5 ml using a rotary evaporator. The extract was subsequently analysed by gas-liquid chromatography, using naphthalene as an internal standard. Concentrations were expressed as mg kg^{-1} fresh weight (ppm).

Gas chromatography. The $CHCl_3$ extracts, containing carvone and its conversion products, were analysed by GC using an apolar capillary phase column (CP-Sil 5cb column WCOT, 25 m x 0.32 mm i.d., Chrompack, Middelburg, The Netherlands). The compounds were identified by a comparison of retention times, using standards, and by GC-Mass Spectrometry (GC-MS). Spectra were taken as electron impact spectra (70 eV).

GC conditions were as follows: oven temperature 80-300 °C, at 10 °C min^{-1}; injector temperature 260 °C; detector temperature 300 °C; carrier gas H_2 at a flow rate of 2.5 ml min^{-1}; split ratio 1:20. Carvone concentrations in the atmosphere were determined by taking a 10 ml head space sample (with a syringe), from the tray with the potato sprouts. The sample was passed over Tenax adsorbens (Chrompack) in order to trap fatty acids. The monoterpenes were desorbed by thermodesorption at 300 °C for 20 min. Using a cold trap (-70 °C) the volume was reduced, and the sample was analysed by GC using a CP-Sil 19cb column (WCOT, 25 m x 0.32 mm i.d., Chrompack).

Synthesis of [^{13}C]S-(+)-carvone. This was synthesized in a two-step reaction. In the first step, C-9 was exchanged for oxygen. S carvone (9 g) was dissolved in 120 ml methanol-dichloromethane (MeOH-CH_2Cl_2, 1:5, v/v) and cooled at -78 °C. After ozonolysis, 2.5 g thioureum were added and the mixture was stirred for 2 h at 22 °C. The solution was concentrated using a rotary evaporator, water was added and it was extracted three times with 75 ml diethylether (Et_2O). The combined Et_2O layers were washed twice with water, once with NaCl-satured water, and dried over $MgSO_4$. The mixture was submitted to column chromatography over silica-gel and eluted with ethylacetate-petroleum ether (30:70, v/v). The second step involved the exchange of the introduced oxygen for ^{13}C. 2.5 g $Ph_3P^{13}CH_3I$ were dissolved in 25 ml dry tetrahydrofuran (THF), then 4 ml 1.6 M *n*-butyl-lithium (in hexane) were added, and the mixture was stirred for 30 min, and cooled to -78 °C; 0.97 g of the product from the first reaction step was dissolved in 3 ml dry THF and then added. After 60 min the reaction was completed. [^{13}C]S carvone was purified by column chromatography with ethylacetate-petroleum ether (10:90). The overall yield was 47%.

NMR analysis. [^{13}C]S carvone was added to the trays with the potato sprouts, which were extracted as described above. The aqueous phases were lyophilized and dissolved in deuterated water (D_2O). After a second lyophilization the samples were dissolved in 0.5 ml D_2O and analysed. ^{13}C-NMR experiments were conducted at room temperature (50.323 MHz, using a spectral width of 15 kHz, 90° pulses and

repetition times of 1 s, with broadband proton decoupling). Chemical shifts were measured relative to the signal of $CDCl_3$ (deuterated chloroform: 77.1 ppm).

Results and discussion

Growth inhibition. R- and S carvone showed a concentration-dependent effect on sprout growth (Table 1). Addition 250 µl S carvone gave a strong inhibition of growth, whereas 150 µl and 50 µl had much smaller effects. This was reflected by the sprout length after 7 days of treatment as well as by the fresh weight. The dry matter content of the carvone-treated sprouts was higher than the controls. At all additions, the effect of R carvone was less pronounced than that of S carvone, although for both isomers the concentration in the atmosphere was about 5-10 µg l^{-1} after the addition of 250 µl of either (liquid) compound.

After a few days of exposure to carvone, the growth of the sprouts was inhibited (Fig. 1). Exposing them to S carvone resulted in quicker inhibition than exposure to R carvone (Fig. 1B vs 1C). The inhibition was reversible, because removing the carvone vapour led to regrowth. It seemed that the effect of 2 days of S carvone treatment was critical with respect to regrowth, since the variance was large, as some sprouts grew whereas others did not. The same was true for R carvone-treated sprouts, but then only after 4 days treatment. Seven days exposure to either R- or S carvone completely inhibited sprout growth (Fig. 1D).

Carvone treatment at the concentrations applied did not result in necrotic tissues. The carvone-treated sprouts were thicker, just below the top, than the control sprouts, and they did not show the purple colour exhibited by the tips of the control sprouts. Such swelling has also been described by Lorber & Muller (1980). They reported root tip swelling of *Allium cepa*, when exposed to the vapour of macerated leaves of *Salvia leucophylla* (mainly consisting of camphor, 1,8-cineole, α-pinene, camphene and β-pinene), and reduced mitotic activity in the root tips. However,

Table 1. Effect of R carvone and S carvone on length, fresh weight, and dry matter content of potato sprouts after 7 days treatment.

Treatment	Length (ratio of control at t = 0)	Fresh wt (g/sprout)	Dry matter (%)
Control	7.09	5.35	6.9
250 µl R carvone	1.93	1.11	7.4
250 µl S carvone	1.24	0.59	7.6
150 µl R carvone	2.69	1.34	ND[1]
150 µl S carvone	2.01	1.19	ND
50 µl R carvone	5.81	3.48	ND
50 µl S carvone	5.51	2.44	ND

[1] ND = not determined.

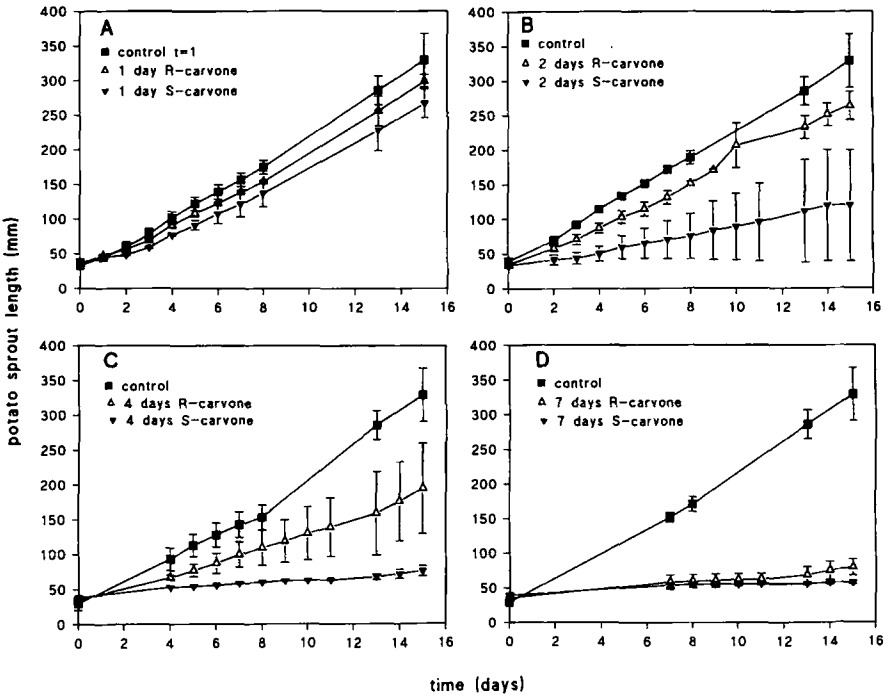

Fig. 1. Effect of R carvone and S carvone on potato sprout length. Potato sprouts were exposed to carvone vapours for 1, 2, 4 and 7 days (Figs A-D, respectively).

microscopic analysis of the sprout tips did not reveal abnormal cells; all stages of cell division were detected in treated as well as in control sprouts. In addition, studies with potato cell cultures were performed in order to determine the nature of the growth inhibition. At S carvone concentrations higher than 1 mM, cell growth was reduced. However, the cells did not show an arrest in cell cycle (Fig. 2). Flow cytometric analysis of relative DNA contents from the control and S carvone-treated cell suspensions showed similar DNA contents in the G1 and G2 phases during 4 days treatment.

The sprouts were able to recover after carvone treatment, showing either continued top growth or branching, i.e. development of lateral sprouts on the main sprout. Which of the two phenomena occurred was dependent on the duration of the treatment and on the enantiomer used. The percentage of branched sprouts was much higher for S carvone-treated sprouts. Sprouts which had been exposed to S carvone for 4 days had no further growth, and showed lateral sprouts in all cases, whereas only 32% of the R carvone-treated sprouts showed branching; the remaining

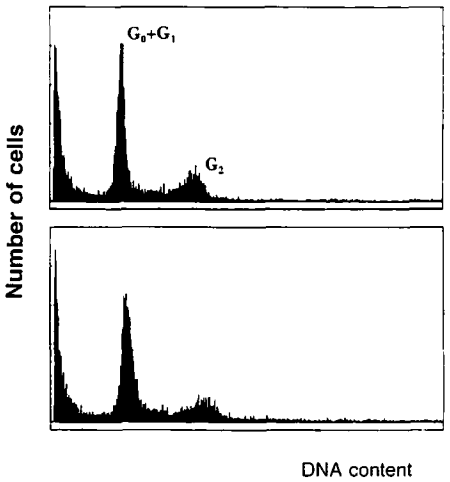

Fig. 2. Flow cytometric histograms showing the relative DNA content of nuclei per cell (arbitrary units), isolated from control (upper graph) and S-carvone (lower graph) treated potato cell suspensions, after 28 h. G_0, G_1 and G_2 refer to the nuclear stages of cell division: G_0 and G_1, cells with one set of chromosomes; G_2, cells which double their DNA content due to mitosis. The horizontal axis expresses the relative DNA content (determined by the amount of fluorescent dye bound by DNA). Identical results were obtained for 2, 3 and 4 days of treatment.

68% had continued top growth. Control sprouts did not branch (Table 2). This indicates a loss of apical dominance due to carvone treatment.

Besides the effect on sprout length, there was a pronounced effect of both carvone enantiomers on root growth. Only a small root system developed as a result of the carvone exposure (Table 3), but after stopping the treatment normal growth of the root system was observed. However, the effect of carvone on rooting depended on the physiological age of the tubers. Sprouts of 6 months-old tubers showed almost no rooting, whereas sprouts from young tubers did show some rooting following carvone treatment.

Table 2. Effect of R carvone and S carvone on the development of lateral sprouts.

Exposure time	Sprouts with lateral sprouts (%)		
	Control	R carvone	S carvone
1 day	0	15	18
2 days	0	19	79
4 days	0	32	100
7 days	0	69	100

Table 3. Effect of R carvone and S carvone on the growth of potato roots and sprouts.

Exposure time (days)	Control		R carvone		S carvone	
	Fresh wt per eye piece (g ±SE)					
	Root	Sprout	Root	Sprout	Root	Sprout
0	0	0.39 (0.02)	0	0.39 (0.02)	0	0.39 (0.02)
1	0.08 (0.01)	0.51 (0.02)	0	0.53 (0.05)	0.02	0.67 (0.15)
2	0.20 (0.01)	0.75 (0.10)	0	0.53 (0.05)	0.03 (0.01)	0.51 (0.03)
4	0.32 (0.05)	1.04 (0.08)	0.05 (0.01)	0.85 (0.01)	0.04 (0.01)	0.56 (0.06)
7	0.45 (0.06)	1.62 (0.13)	0.07 (0.01)	0.70 (0.08)	0.07 (0.01)	0.57 (0.14)

The observation that 4 days of R carvone treatment caused a similar growth inhibition to 2 days of S carvone, as mentioned above, might be explained by a difference in the concentration of both enantiomers in the potato sprouts (Figs 3A and 3B). During the first 4 days the total monoterpene concentration in the S carvone-treated sprouts was almost twice as high as that in the R carvone-treated

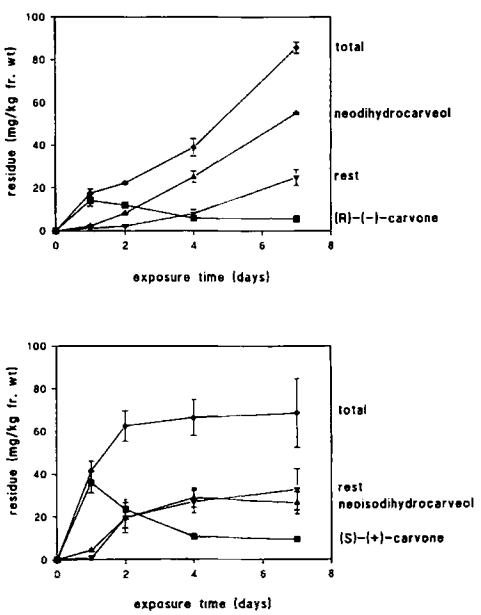

Fig. 3. Bioconversion of R carvone (A) and S carvone (B) by potato sprouts.
■ = R carvone (A), ■ = S carvone (B), ▲ = neodihydrocarveol, ▼ = rest (volatiles consisted mainly of dihydrocarvone isomers, dihydrocarveol isomers, and traces of dihydrocarvyl acetates and hydroxylated compounds), ◆ = sum of the conversion products.

ones. Also, bioconversions occurred in the sprouts; this will be discussed in the next section.

Stereospecific effects of R and S carvone were also described by Reynolds (1987), the latter being more effective. A 50% decrease in apple seed germination was obtained by 0.38 mM R carvone and by 0.058 mM S carvone (Reynolds, 1987). Apart from the optical rotation, their physical properties are similar, and R carvone is no less volatile than S carvone. It seems that the uptake of R carvone is slower than that of S carvone. Recent findings indicate a significant discrimination between R and S carvone by phospholipid monolayers (Pathirana et al., 1992). R carvone interacted more strongly than its enantiomer because the monolayers were more expanded. Although these results have been obtained using synthetic phospholipid monolayers, this may explain the differences observed after treatment with carvone.

Occurrence of carvone and its conversion products in potato sprouts. The total concentration of carvone and its derivatives in the sprouts of the model system, determined by GC, reached 85 mg kg^{-1} fresh wt after 7 days (Fig. 3). R and S carvone were converted into more reduced compounds (Figs 3A and 3B). The conversion was stereoselective: R carvone was mainly converted into neodihydrocarveol and S carvone into neoisodihydrocarveol. Some other compounds were also detected in the extracts.

Using [^{13}C]S carvone, labelled at the C-9 position in the isopropenyl group, we were able to prove that all the volatile products found in the sprouts were indeed conversion products of carvone. Table 4 shows the mass spectral characteristics of the carvone derivatives isolated after 4 days of exposure to [^{13}C]S carvone. The structure of three compounds could not be elucidated. One of these had a molecular weight of 168. After silylation of the $CHCl_3$ extracts of the sprouts containing the volatile

Table 4. Volatile compounds, and their Mass Spectrometry (MS) characteristics, isolated from potato sprouts after 4 days exposure to [^{13}C]S carvone.

Compound	M_r	Characteristic m/z fragments (relative intensity in parentheses)[1]
Dihydrocarvone (*cis*-isomer)	152	41(40), *68*(100), *96*(66), *110*(30), *153*(15)
Dihydrocarvone (*trans*-isomer)	152	41(40), *68*(100), *96*(77), *110*(14), *153*(24)
Neodihydrocarveol	154	43(68), *80*(75), 93(81), *108*(100), *122*(68), *137*(64), *155*(2)
Isodihydrocarveol	154	43(62), *80*(79), 93(100), *108*(92), *122*(48), *137*(50), *155*(8)
Neoisodihydrocarveol	154	41(70), *80*(82), 93(90), *108*(100), *122*(65), *137*(32), *155*(2)
Carvone	150	54(45), 82(100), *94*(28), *109*(29), *136*(5), *151*(8)
Unknown	?	59(32), *80*(100), *81*(82), *109*(71), *136*(18), *151*(50), *161*(5)
Unknown	168	42(50), 56(48), *70*(100), *85*(55), *98*(55), *141*(15), *154*(9), *169*(18)
Unknown	?	43(100), *71*(60), 82(45), 95(40), *139*(32), *153*(8)

[1] Values in italics represent m/z fragments containing [^{13}C], as determined by comparison of the GC-MS spectra of the compounds in the chloroform extracts from carvone and [^{13}C]carvone-treated sprouts.

compounds, this particular compound showed a shift in retention time comparable to the shifts of the compounds known to have a hydroxyl group, thus indicating that it possessed a hydroxyl group. The other two unknown compounds were also most probably hydroxylated compounds, because they showed the same behaviour after silylation.

Acetylation did also occur, because dihydrocarvyl acetate was detected in the extracts, although in very small amounts. Its identity was confirmed by comparing its retention time with that of an authentic compound on two GC columns of different polarity as well as by comparison of their mass spectral data.

The $CHCl_3$ extracts and the aqueous phases obtained during the extraction were subjected to ^{13}C-NMR spectroscopic analysis. The spectra of the $CHCl_3$-soluble compounds from the S carvone and [^{13}C]S carvone-treated potato sprouts are shown in Figs 4A and 4B, respectively. The spectra differ qualitatively in the region of 100-120 ppm. Six peaks could be designated to carvone and its derivatives. The peak at 110.1 ppm is due to carvone and the one at 108.1 ppm could be explained by a shift caused by a hydroxyl group at C-4. The other signals in the spectrum could not be assigned. Analysis of the aqueous phase also showed clear ^{13}C-signals in the region 100-120 ppm (Figs 5A and 5B) which pointed to the presence of water-soluble [^{13}C]carvone-derived compounds. Carvone could not be present in the aqueous phase since it was extracted three times with $CHCl_3$, and the third extract did not contain any carvone, dihydrocarvone or dihydrocarveol. So the compounds in question must be conjugated to a water-soluble compound, for example glucose, or they must be highly hydroxylated.

Monoterpene glycosides have been found in all kinds of monoterpene-producing plants. However, they have not been described in relation to monoterpene

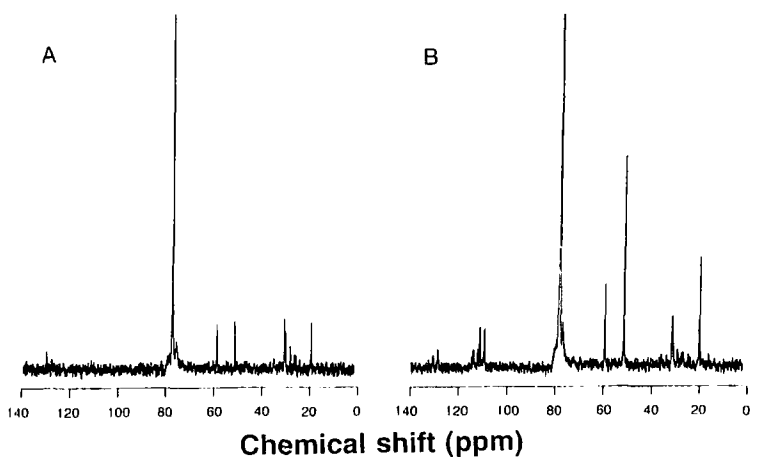

Fig. 4. ^{13}C-NMR spectrum of chloroform extracts from sprouts treated with S carvone (A) and [^{13}C]S carvone (B).

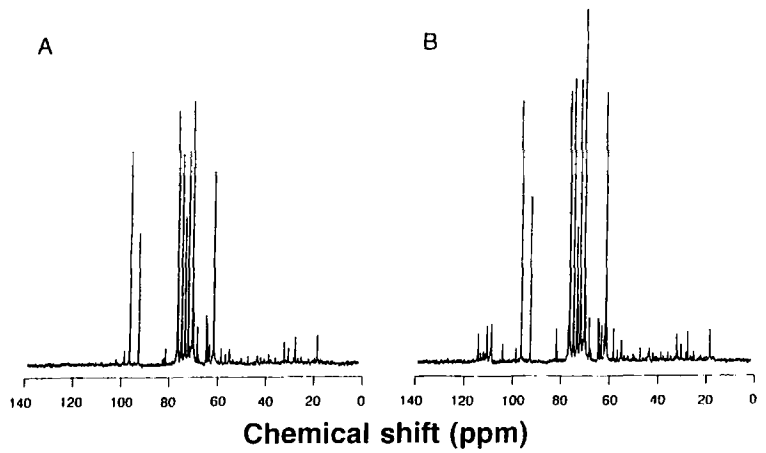

Fig. 5. ^{13}C-NMR spectrum of aqueous phases of extracts from sprouts treated with S carvone (A) and [^{13}C]S carvone (B).

bioconversions by plant structures that do not contain endogeneous monoterpenes. Further studies will deal with the nature of these water-soluble compounds.

Hirata et al. (1982) described the bioconversion of R carvone and S carvone by cell suspension cultures of *Nicotiana tabacum* L.: R carvone was predominantly converted into neodihydrocarveol and S carvone into neoisodihydrocarveol. Thus our findings are in agreement with their results. However, their *N. tabacum* cell suspensions were not able to hydroxylate or acetylate R and S carvone, as did the potato sprouts in our experiments.

The quantitatively different effects of R and S carvone on apple seed germination described by Reynolds (1987) were explained by stereospecific effects at a molecular level. However, an alternative explanation might be that the different effects of both enantiomers, as also found in our experiments, are due to a different uptake of the compounds in the seeds. This means that enantioselective effects may already play a role in the uptake of carvone.

Effect of carvone conversion products on sprout growth. Because we found a number of conversion products of carvone in potato sprouts, e.g. *trans*-dihydrocarvone, dihydrocarveol and dihydrocarvyl acetates, we also studied their possible effect on sprout growth in our model system. Dihydrocarvone inhibited sprout growth in the same way as S carvone, whereas dihydrocarveol did not (Table 5). However, the concentration of dihydrocarveol in the sprouts as well as in the atmosphere was very low (Table 5), so no definite conclusion with regard to the effect of dihydrocarveol on sprout growth can be drawn.

Dihydrocarvone was mainly converted into neodihydrocarveol. The stereochemistry of the conversion could not be determined in detail, as the sample applied consisted of a mixture of *cis*- and *trans*-dihydrocarvone. Besides the

Table 5. Effect of dihydrocarvone and dihydrocarveol on potato sprout growth.

Exposure time (days)	Control	Dihydrocarvone[1]			Dihydrocarveol[2]		
	Length (ratio of control at t=0)	[Residue][3] (mg kg^{-1})	[Air][4] (µg l^{-1})	Length (ratio of control at t=0)	[Residue][3] (mg kg^{-1})	[Air][4] (µg l^{-1})	Length (ratio of control at t=0)
0	1.00	-	-	1.00	-	-	1.00
1	1.05	54 (10)	ND[5]	1.05	6.3 (0.8)	ND	1.07
3	1.39	105 (23)	30.4 (8.2)	1.10	3.9 (0.8)	2.3 (0.3)	1.43
5	2.17	100 (21)	15.3 (6.0)	1.24	4.1 (1.0)	1.8 (0.4)	2.33

[1] Mixture of 20% *cis*- and 80% *trans*-isomer.
[2] Mixture of all four dihydrocarveols.
[3] Residual content of the compound tested, including the conversion products (SE in parentheses).
[4] Concentration of the compound tested, in the headspace of the container (SE in parentheses).
[5] ND = not determined.

dihydrocarveol isomers, at least six hydroxylated compounds were detected in the extracts analysed. Although the bioconversion of dihydrocarveol could not be fully studied because the concentration in the sprouts remained low, some hydroxylated compounds were detected.

Dihydrocarvyl acetates also inhibited the growth of potato sprouts. They could hardly be detected in the sprouts, but the corresponding alcohols were found at a level of 40 mg kg^{-1} fresh wt. Therefore the deacetylation of the dihydrocarvyl acetates must have been very efficient. This was confirmed by the finding that cell-free extracts of sprouts were also able to deacetylate the esters, producing dihydrocarveols. This was an enzymatic process, since boiled cell-free extracts did not deacetylate the esters. All four isomers of dihydrocarveol and at least eight hydroxylated compounds were detected.

Conclusions

Other authors (Asplund, 1968; Lorber & Muller, 1976, 1980; Reynolds, 1987) have reported on the mode of action of monoterpenes but not their effective concentration in tissue or their possible bioconversion. Our study shows that R carvone and S carvone are readily reduced and that the concentration of these compounds in the tissue depends on their structure. Although the concentration of the carvones in the atmosphere around the sprouts was similar, S carvone was found sooner in the sprouts than was R carvone. Thus the differences in growth or germination may also be due to a derivative (or even more than one) of the applied compound, and its concentration in the atmosphere may not be related to that in the tissue.

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