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S-Carvone as a natural potato sprout inhibiting, fungistatic and bacteristatic compound

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Abstract

S-Carvone, a common monoterpene found in caraway (*Carum carvi* L.), inhibits the sprouting of potatoes very efficiently at continuous low head space concentrations.

The length growth of potato sprouts was inhibited within 2 days following exposure to S-carvone. Sprouts were able to convert S-carvone into more reduced compounds.

In addition, growth of the plant-pathogenic fungi *Fusarium solani* and *Fusarium sulphureum* was found to be inhibited by S-carvone at concentrations between 1 and 3 mM. At sub-inhibitory concentration, both *F. solani* and *F. sulphureum* converted S-carvone into more reduced compounds which were shown to be less toxic as compared to S-carvone.

Finally, the growth rate of *Streptococcus thermophilus*, *Lactococcus lactis* and *Escherichia coli* decreased at S-carvone concentrations above 1 mM. This decrease correlated with a dissipation of the proton motive force generated in these cells.

Keywords: Growth inhibition; Monoterpenes; Potato tubers; S-Carvone; *Solanum tuberosum* L.

1. Introduction

Monoterpenes form a class of natural compounds biogenically derived from two isoprene units, with acyclic, monocyclic and bicyclic carbon skeletons. These compounds represent the major components of essential oils and are produced in different parts of various plants. Although the function is not completely understood, there is considerable evidence that they play an ecological role (Fischer, 1986). The monoterpenes have an attractive or a deterrent effect on in-

sects, and many monoterpenes are known to be toxic for micro-organisms (Knobloch et al., 1986, 1987; Smid et al., 1994). In addition, the monoterpenes inhibit germination of seeds (Asplund, 1968; Reynolds, 1987).

Monoterpenes are used as flavouring compounds in foods or perfumes, of which menthone and menthol, isolated from *Mentha* sp., and S-carvone, isolated from caraway or dill seeds, are well known examples.

Some monoterpenes inhibit the sprouting of potato tubers (Meigh, 1969; Beveridge et al., 1983; Vaughn and Spencer, 1991; Oosterhaven et al., 1993; Vokou et al., 1993). From these monoter-

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penes S-carvone has a promising potential as a commercial sprout inhibitor (Hartmans et al., 1995).

Besides the reversible sprout-inhibiting effect, S-carvone has interesting side effects. It inhibits the growth of several potato storage pathogens (Hartmans et al., 1995).

The question arises how S-carvone influences potato sprout growth and growth of microorganisms. There is little biochemical and physiological knowledge on the action of monoterpenes on plants in general. The inhibition of germination of seeds by several alicyclic and heterocyclic compounds, however, is well described (Asplund, 1968; Fischer, 1986; Reynolds, 1987, 1989). Reynolds (1987) concluded that the lipophilic nature of the molecule is one of the main factors contributing to the inhibitory activity of alicyclic compounds. However, some highly lipophilic compounds like limonene and α -pinene showed much less inhibition of germination than would be expected on the basis of their lipophilicity. On the other hand, the inhibitory activity of other compounds was much greater than would be expected in comparison with compounds of related structures. For example, the α,β -unsaturated keto group is important in its germination-inhibiting effect (Asplund, 1968; Reynolds, 1989).

The biological activities of monoterpenes have been studied in somewhat more detail in mitochondria isolated from plant material. β -Pinene acts as an uncoupling agent in mitochondria at low concentrations (on a μM scale) (Douce et al., 1978; Pauly et al., 1981). Cineole inhibits mitochondrial respiration at 5 mM (Muller et al., 1969; Lorber and Muller, 1980b). Membrane disturbances were also detected in roots of *Allium cepa* following three days exposure to a high concentration of cineol (most probably 1,8-cineole) (Lorber and Muller, 1976, 1980a).

For micro-organisms the general view on the mode of action of monoterpenes is based on the lipophilic nature of the compounds, i.e. interference with membranes, disturbance of the integrity and barrier function which leads to dissipation of ion gradients (metabolic energy) and eventually results in cell death (Sikkema, 1993). The generally observed trend that good solubility of

monoterpenes in water results in a stronger antimicrobial activity is partly outruled by the nature of the functional groups of the monoterpene (Knobloch et al., 1987).

In conclusion, lipophilicity plays a role in the biological activity but the spatial orientation of the functional groups is also important. Furthermore, these data indicate that there is no general mechanism by which monoterpenes act on physiological processes and it remains to be determined for particular compounds such as S-carvone.

Oosterhaven et al. (1993) described specific effects of S-carvone on 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, E.C. 1.1.1.34), the key enzyme in the mevalonate pathway. HMGR activity of potato sprouts was almost completely inhibited within four days following exposure to low head space concentrations of S-carvone.

In this paper we describe the effects of S-carvone on potato sprouting and on bacterial and fungal growth.

2. Materials and methods

2.1. Potato sprout growth

Seed potatoes (*Solanum tuberosum* cv. Bintje), were stored at 4°C until further use and the experiments were carried out as described by Oosterhaven et al. (1993).

Briefly, 30 eye pieces were isolated from different tubers and placed on moist perlite in a 20-l container. The volatiles tested (R- and S-carvone and R- and S-limonene from Merck, Amsterdam, The Netherlands; carvenone, dihydrocarvone (isomeric mixture consisting of 80% trans and 20% cis-isomers), dihydrocarveol (isomeric mixture of the 4 isomers, with dihydrocarveol as the predominant compound, >80%), 2-methylcyclohexenon, 3-methylcyclohexenon, dihydrocarvylacetate, menthylacetate and cuminaldehyde from Roth, Karlsruhe, West Germany) were applied as a liquid (250 μ l in Petri-dishes) after which the containers were sealed with tape. Sprout length was measured at time zero and after various days of treatment. In order to determine the concentration of the added monoterpenes in the

sprouts, sprouts were extracted according to Oosterhaven et al. (1995). The extracted volatiles were analyzed by gas liquid chromatography (GLC) as described below.

2.2. Respiration measurements

Potato tubers were wounded as described by Oosterhaven et al. (1995). The wounded tubers were exposed to S-carvone for four days and at several time intervals the respiration was measured as O₂-consumption and CO₂-production as described by Peppelenbos et al. (1993).

Mitochondria were isolated from the perimedullar zone of intact potato tubers and mitochondrial respiration was measured according to Gude (1989) with a Clark-type O₂ electrode system from Yellow Springs Instruments Co. Ltd. (Ohio, USA). The reaction medium contained 0.7 M mannitol, 5 mM EDTA, 10 mM potassium phosphate buffer (pH = 6.8), 0.1% (w/v) bovine serum albumin and known quantities of mitochondrial protein (2–4 mg) in a volume of 600 μ l (Gude, 1989). The O₂ concentration in air-saturated medium was taken as 250 μ M. Mitochondrial integrity was estimated using Cyt *c*-dependent O₂ uptake (Douce et al., 1987).

State III respiration was measured with succinate as a substrate (10 mM final concentration) and 200 μ M ADP. The oxygen consumption of uncoupled mitochondria was measured in the presence 2 μ M CCCP (carbonyl cyanid *m*-chlorophenylhydrazone).

2.3. Fungal strains

Fusarium solani var. *coeruleum* and *F. soliphureum* were obtained from culture stocks (IPO-DLO, Netherlands) and maintained on PDA-plates: 22 g/l potato extract (Oxoid), 2 g/l glucose and 0.8% agar at 9°C. Liquid cultures were grown in 75 ml of chemically defined medium (CDM) in 300-ml erlenmeyers at 20°C, shaking with 150 rev/min. The CDM contained (g/l): K₂HPO₄·3H₂O, 1.1, adjusted to pH 6.2 with KH₂PO₄; glucose, 10; asparagine, 2; MgSO₄·7H₂O, 0.1; CaCl₂, 0.01; NaCl, 0.03; biotin, 0.001; thiamin, 0.001 and Vishniac solution, 1‰ (vol/vol).

S-Carvone and related compounds were dissolved in dimethylsulfoxide (DMSO) and additions were made till concentrations were reached as indicated in the text. The same amount of DMSO was added to controls. The concentration of DMSO was always lower than 0.1% and had no effect on the growth of the fungi. The concentration of monoterpenes in the medium was checked at different time intervals from time zero.

2.4. Glucose determination

Glucose consumption was taken as a measure for biomass of the fungi. The Anthron method was used for the determination of total sugars (Hodge and Hofreiter, 1962). A sample of the culture medium was diluted 50 times and 500 μ l was used in the Anthron assay.

2.5. Monoterpene determination in growth medium

Samples of 1 ml were taken from the culture medium and extracted with 1 ml pentane/diethylether (1:2, v/v). Before extraction a known amount of naphthalene was added to the sample as an internal standard. After 20 s vortexing, the samples were centrifuged (10 min 3000 g) and the organic solvent layer was analyzed by GLC. The GC conditions were: CP-sil 58 CB column (25 m, 0.25 mm inner diameter, 0.20 μ m film thickness; Chrompack, Middelburg, Netherlands); temperature program: 1 min 70°C followed by 10°C/min to 120°C, 7 min isothermic at 120°C. With these conditions a separation could be established between the stereo-isomers of dihydrocarveol.

2.6. Bacterial growth and Δ pH and $\Delta\psi$ measurements

E. coli was grown aerobically or anaerobically at 37°C (Poolman et al., 1989); *S. thermophilus* was grown anaerobically in Belliker medium with 10 mM lactose at 42°C (Poolman et al., 1989); *L. lactis* was grown semi-aerobically in MRS medium with 10 mM lactose at 30°C (De Man et al., 1960). The influence of S-carvone on the pH gradient (Δ pH) and membrane potential ($\Delta\psi$) in a liposome model system was analyzed as described previously (Sikkema et al., 1992).

3. Results and discussion

3.1. Potato sprout growth inhibition

Exposure of potato tubers to a continuous low concentration of S-carvone led to a complete inhibition of sprout growth on a laboratory as well as on a practical scale. The inhibition of sprout growth was reversible since removal of S-carvone led to immediate and normal growth of the sprouts on the tubers.

The sprout growth inhibiting properties of various volatiles were tested in a model system consisting of potato tuber eye pieces with one sprout each. S-Carvone, among others, turned out to be a very effective sprout growth-inhibiting compound (Table 1). After addition of limonene the sprouts became necrotic within one day. However, after removal of limonene, lateral sprouts from the eye (bud) developed, indicating that the eyes were not completely damaged by a limonene treatment (5 days). It should be stressed that it is difficult to compare the sprout growth-inhibiting properties of the volatiles. The head space concentration as well as the residual content of each compound were different in the studies (Table 1). Further-

more, most of the volatiles were converted into more reduced compounds.

Because S-carvone seemed to be the most promising compound for practical use for several reasons (Hartmans et al., 1995), further research was focussed on this particular compound. Exposure of potato sprouts to S-carvone at a head space concentration of about 5–10 $\mu\text{g/l}$ resulted (already after two days) in complete inhibition of sprout growth (results not shown; Oosterhaven et al., 1993). The growth inhibition was reversible, since removal of S-carvone led to immediate re-growth of the sprouts, either by continued top growth or by branching. The sprouts exposed to S-carvone appeared thick and pale/yellow compared to control sprouts and no necrotic spots occurred (results not shown).

The sprouts converted S-carvone mainly into neoisodihydrocarveol (Fig. 1). The conversion went via dihydrocarvone, an intermediate that could be detected in significant amounts in the sprouts. Isodihydrocarveol, carvylacetates and hydroxylated carvones were formed in trace amounts.

Since the volatility of dihydrocarveol and dihydrocarvone are different to S-carvone, the effect

Table 1
Potato sprout growth inhibiting effect of various volatiles

Compound	Sprout growth inhibition ^a	Residue in sprouts (mg kg ⁻¹ FW) ^b	Bioconversion ^c
S-Carvone	+++	60– 75	+
R-Carvone	++	40– 60	+
S-Limonene	necrotic within 24 h	300–400	–
R-Limonene	necrotic within 24 h	300–400	–
2-Methyl cyclohexenon	++	40– 50	+
3-Methyl cyclohexenon	+	400–500	–
(+) Menthol	+	10– 15	–
Dihydrocarvone	++	100–130	+
Dihydrocarveol	–	3– 5	–
Carvenone	+	50– 60	–
Menthylacetate	++	20– 25	+
Dihydrocarvylacetate	++	40– 50	+
Cuminaldehyde	+	8– 10	–

Sprouts were exposed to 250 μl of each compound, at 15°C in the dark as described in Section 2. Sprout growth inhibition and the residual content of the volatiles and the possible bioconversion were determined after five days of treatment.

^a +++ = very effective, + = effective, – = no sprout inhibition.

^b Content of added compound including conversion products in mg kg⁻¹ fresh weight.

^c + = bioconversion, – = no bioconversion.

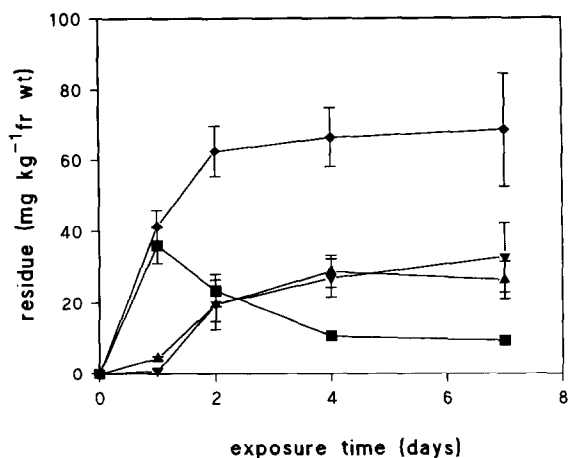


Fig. 1. In-vivo bioconversion of S-carvone by potato sprouts. Sprouts were exposed to S-carvone (head space concentration 5–10 $\mu\text{g/l}$), and at several time intervals the sprouts were extracted; ◆ = total, ■ = S-carvone, ▼ = neoisodihydrocarveol, ▲ = sum of isodihydrocarveol, dihydrocarveol and isodihydrocarveol. Error bars indicate S.D., $N = 3$.

of these compounds on the growth of sprouts in isolated potato eye pieces could not be compared. Potato tissue cell culture experiments showed that S-carvone was a much more effective growth inhibitor than dihydrocarveol and dihydrocarvone: the growth of the tissue cultures was inhibited at 1 mM S-carvone whereas >2 mM dihydrocarvone and >5 mM dihydrocarveol were needed to affect growth (results not shown). Again, it is difficult to compare the action of these compounds directly, since they are continuously converted in these tissue cultures. The end product of both S-carvone and dihydrocarvone was predominantly neoisodihydrocarveol.

Accumulation of S-carvone in potato tissue was dependent on the type of tissue used. The residual content of intact tubers was 5 mg kg^{-1} FW (after four weeks exposure). About 90% was associated with the peel fraction, indicating that the peel is a very efficient barrier to S-carvone. The peeled potato tuber fractions contained maximal 0.5 mg kg^{-1} FW and no bioconversion products could be traced inside the tuber. The concentration of S-carvone in wounded potato tubers (half potato tubers) never exceeded 20 mg kg^{-1} FW (at 15°C), even not till three weeks of exposure,

Table 2

Residual content of S-carvone in different tuber tissues exposed to S-carvone vapour (5–10 $\mu\text{g l}^{-1}$) for five days at 15°C in the dark

Tissue	S-Carvone concentration (mg kg^{-1} FW)	Conversion products (mg kg^{-1} FW)
Intact tuber	5	–
Peeled tuber	<1	–
Peel	10	–
Half tuber	20	30
Sprouts	20	30

but since S-carvone is converted into other more reduced compounds the total amount of monoterpenes is higher, reaching levels of 50–70 mg kg^{-1} FW (Table 2). Sprouts contain about the same concentration of S-carvone and its derivatives as wounded potato tubers (Fig. 1 and Table 2).

In order to test the influence of S-carvone on respiration, we measured mitochondrial oxygen consumption in the presence of S-carvone (Fig. 2). Only at concentrations of 1 mM or higher respira-

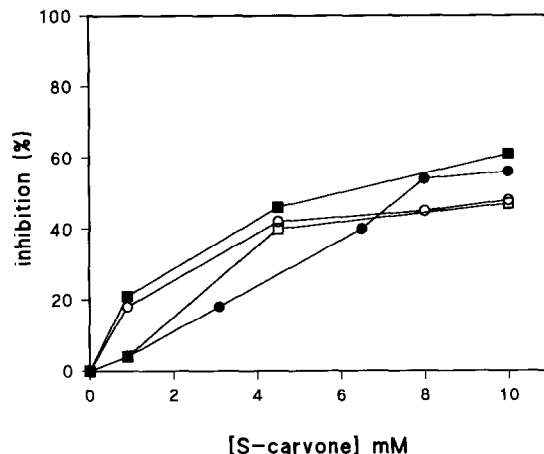


Fig. 2. Mitochondrial oxygen uptake in response to various concentrations of S-carvone and dihydrocarveol, expressed as % inhibition of control (oxygen uptake of control mitochondria). State III respiration of mitochondria (with succinate as the substrate in the presence of ADP (200 μM)) was measured in the presence of S-carvone (●) and dihydrocarveol (■); oxygen uptake of decoupled mitochondria treated with S-carvone (○) and dihydrocarveol (□) in the presence of 2 μM of the decoupler CCCP (carbonyl cyanid *m*-chlorophenylhydrazine).

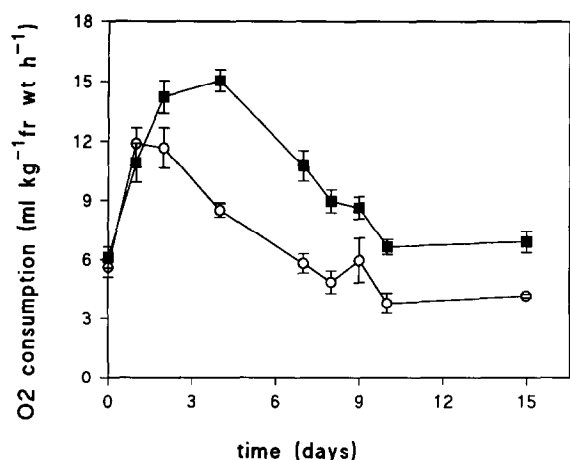


Fig. 3. Respiration, expressed as O₂-production, of wounded potato tubers: ○ = control, ■ = S-carvone, 20 mg kg⁻¹ FW. Error bars indicate S.D., *N* = 3.

tion was inhibited, concentrations which are never reached in whole potato tubers nor in sprouts. Wounded potato tubers contain higher amounts of S-carvone compared to whole tubers, and the effect of S-carvone on the respiration of wounded potato tubers was studied. Control wounded tubers showed the wound-induced respiration as described by Kahl (1974). Fig. 3 shows that wounded tubers treated with S-carvone (20 mg kg⁻¹ FW) exhibited a significantly higher O₂ consumption (as well as a higher CO₂ production; results not shown) as compared to control wounded tubers. This indicates that the rate of respiration is increased following exposure to S-carvone, suggesting that S-carvone has an uncoupling effect at low concentrations. However, uncoupling was not observed in in-vitro experiments with intact mitochondria (results not shown).

3.2. Effect of S-carvone on fungal growth

In a liquid culture medium, supplemented with 2.5 mM S-carvone, a reduced but significant growth of the plant pathogenic fungi *F. solani* and *F. sulphureum* was observed (Table 3). At higher concentrations growth of both fungi was found to be fully abolished. Comparable growth characteristics were observed in culture medium supplemented with dihydrocarvone (Table 3). In

Table 3

Comparison of the bioconversion and the inhibitory action of S-carvone, dihydrocarvone and dihydrocarveol on the growth of *F. solani* and *F. sulphureum* in liquid medium

		Conversion		Growth	
		<i>F. sol.</i>	<i>F. sulph.</i>	<i>F. sol.</i>	<i>F. sulph.</i>
S-Carvone	2.5 mM	+	+	+	+
	3.0 mM	+	+	-	-
Dihydrocarvone	2.2 mM	+	+	+	+
	3.0 mM	+	+	-	-
Dihydrocarveol	2.4 mM	-	-	+	+
	3.0 mM	-	-	+	+

+ = visible growth or apparent conversion detected, - = no growth detected.

addition, no growth inhibition was detected with dihydrocarveol at 3 mM.

Both fungal species showed metabolic conversion of S-carvone and dihydrocarvone. On the other hand, no conversion of dihydrocarveol could be detected in liquid cultures of *F. solani* and *F. sulphureum* (Table 3). To study the metabolic conversion of S-carvone in more detail, *F. sulphureum* was cultured in a medium containing a sub-inhibitory concentration of S-carvone (1.4 mM). After a lag phase of approximately 100 h, a rapid decline of the S-carvone concentration in the *F. sulphureum* culture was observed (Fig. 4a). The culture was found to be essentially depleted from S-carvone after 230 h of incubation (Fig. 4a). During the course of incubation, significant amounts of isodihydrocarvone, neoisodihydrocarveol and isodihydrocarveol were shown to accumulate. In addition, trace amounts of dihydrocarveol and neodihydrocarveol were detectable. The accumulated dihydrocarvone subsequently disappeared from the medium between 230 and 340 h incubation, indicating that this reduced derivative of S-carvone is further metabolized by *F. sulphureum*. The amounts of accumulated neoisodihydrocarveol and isodihydrocarveol remained essentially constant during prolonged incubation (Fig. 4a). Except for the accumulation of trace amounts of dihydrocarveol and neodihydrocarveol, comparable results were obtained with *F. solani* (data not shown).

No significant decrease of the S-carvone concentration was observed in fungi-free medium in-

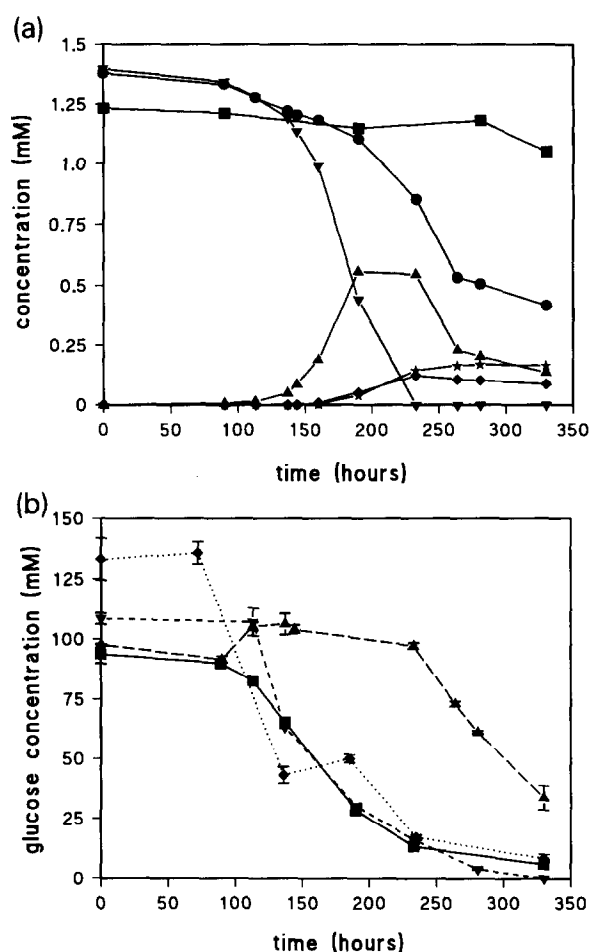


Fig. 4. (a) Time course of conversion of S-carvone by *F. sulphureum* and evolution of monoterpene breakdown products; ∇ = S-carvone, \blacktriangle = isodihydrocarveol, $*$ = neoisodihydrocarveol, \blacklozenge = isodihydrocarveol, \bullet = sum of S-carvone and its conversion products, \blacksquare = S-carvone containing medium without fungi. (b) Glucose consumption of *F. sulphureum* grown in liquid medium supplied with 1.8 mM dihydrocarveol (∇), dihydrocarvone (\blacklozenge), S-carvone (\blacktriangle). \blacksquare = control. Error bars indicate S.D., $N = 3$.

dicating that during the course of the experiment, evaporation of the monoterpene did not play a significant role. The sum of the concentrations of S-carvone and its more reduced derivatives declined significantly during the course of the experiment (Fig. 4a). This suggests that more volatile or undetectable compounds are produced in the culture.

These results show that *F. sulphureum* and *F. solani* possess enzymes which convert S-carvone into more reduced compounds.

To establish whether conversion of S-carvone into dihydrocarvone and a mixture of dihydrocarveols leads to detoxification of the medium, the effect of all different compounds on growth of *F. sulphureum* was studied in more detail. Since glucose consumption correlates well with biomass growth (Oosterhaven et al., in prep.), the glucose consumption of *F. sulphureum* was measured in the presence of S-carvone, and the more reduced metabolites. Fig. 4a shows that 1.85 mM S-carvone completely inhibited glucose consumption for over 200 h. The onset of glucose consumption after 220 h coincides with the moment that the conversion of S-carvone is complete. Furthermore, neither dihydrocarvone (1.85 mM) nor an isomeric mixture of dihydrocarveol (1.85 mM) inhibited glucose consumption, indicating that under these conditions, the tested compounds did not affect fungal growth. This experiment indicates that conversion of S-carvone into more reduced compounds acts as a detoxification process.

3.3. Bacterial growth inhibition

Bacterial growth was followed for aerobic, anaerobic and fermentatively growing *E. coli* and the lactic acid bacteria *S. thermophilus* and *L. lactis*. The growth rate decreased with increasing concentrations of S-carvone (Fig. 5). At concentrations higher than 1 mM, the growth rate of all the bacteria and conditions tested was influenced. Below 1 mM the growth of anaerobically and fermentatively growing *E. coli* and of the lactic acid bacteria was affected but not that of aerobically growing *E. coli*. Since anaerobic growth (fermentative and in the presence of an exogenous electron acceptor) yields less metabolic energy than aerobic growth, any condition that affects metabolic energy conservation by the cell will have greater impact on anaerobic than on aerobic growing organisms.

Because of its lipophilicity, S-carvone partitions preferentially into lipid membranes; the partition coefficient of S-carvone in a liposome-buffer system is 26 (Sikkema et al., 1992). Very lipophilic

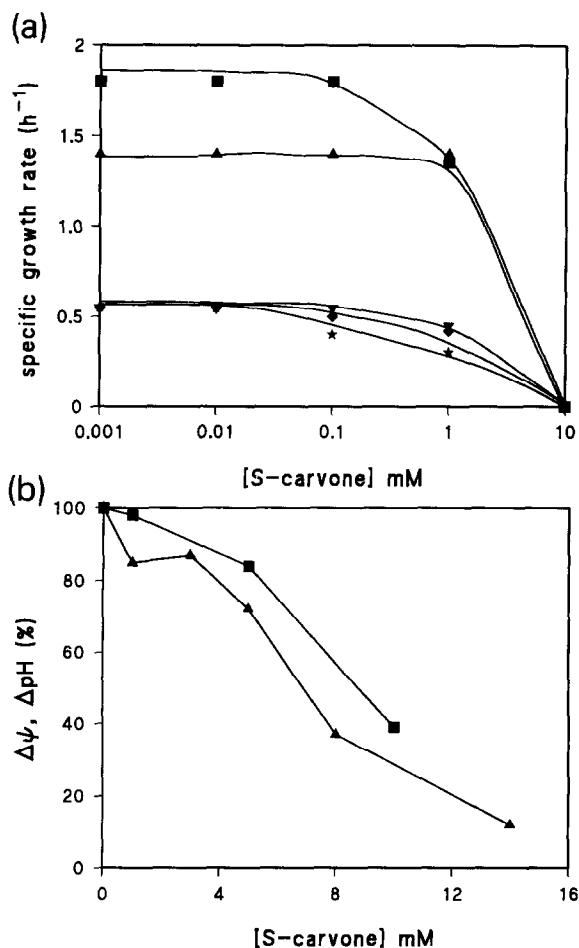


Fig. 5. (a) Growth rate of *Streptococcus thermophilus* (■), *Lactococcus lactis* (▼) and *Escherichia coli*, (▲ aerobic, ◆ anaerobic, in the presence of KNO₃ as exogenous electron acceptor, and fermentative (*) growing) in response to increasing concentrations of S-carvone. (b) Influence of S-carvone on ΔpH (▲) and Δψ (■) in a liposome model system. A ΔpH and Δψ were generated in the cytochrome *c* oxidase containing liposomes upon addition of 10 mM potassium-ascorbate, 200 μM TMPD (N,N'-tetramethyl-p-phenylenediamine) and 20 μM horse heart cytochrome *c* and in the presence of oxygen.

compounds, like tetralin, have a partition coefficient over 1000. This leads to excessive accumulation in the membranes and impairment of different membrane functions, including metabolic energy conservation (Sikkema, 1993).

In order to test the hypothesis that growth of the bacteria was influenced by S-carvone because of loss of membrane integrity and consequently

metabolic energy conservation, the effect of S-carvone on the generation of a proton motive force was tested in a liposome model system. S-carvone led to dissipation of ΔpH and Δψ but only at concentrations above 1 mM (Fig. 5). By comparison, the ΔpH was 50% reduced by 200 μM tetralin (Sikkema et al., 1992) but only at 6 mM S-carvone (Fig. 5).

The decreasing growth rate of the bacteria with increasing S-carvone concentrations correlated with a decrease of ΔpH and of Δψ, suggesting that S-carvone acts by disturbing the metabolic energy status of the bacterial cells.

4. Conclusions

S-Carvone is a naturally occurring volatile with interesting properties that can be commercially exploited. The use of S-carvone is based on a wide range of biological effects occurring in different unrelated organisms. Potato sprout growth is affected by low concentrations of S-carvone in the head space as well as in potato tubers. The observed growth inhibition of potato sprouts and of sprouting of potato tubers could not be explained by inhibition of respiratory processes. Probably more specific effects of S-carvone play a role in the sprout growth of potato tubers, such as inhibition of the key enzyme in the mevalonate pathway, HMGR (Oosterhaven et al., 1993). Bacterial growth is most likely affected by partitioning of S-carvone in lipid membranes which disturbs the membrane barrier function and consequently, metabolic energy conservation. Fungi are also inhibited by S-carvone, but various organisms are able to convert it into less toxic compounds.

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