Different aspects of S-carvone, a natural potato sprout growth inhibitor

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aan mijn ouders
aan Regina, Jente en Ger

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Abbreviations

AMPPD	3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)phenyl-1,2-dioxetane
ABA	abscisic acid
ADP	adenosine diphosphate
AP	ascorbate peroxidase (E.C. 1.11.1.11)
BCIP	5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt
BSA	bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CDNB	1-chloro-2,4-dinitrobenzene
CH_2Cl_2	dichloromethane
$CHCl_3$	chloroform
CIPC	isopropyl N-(3-chlorophenyl)carbamate (chlorpropham)
CK	cytokinin
DMN	dimethylnaphthalene
DTT	dithiothreitol
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetraacetate
Et_2O	diethyl ether
fr. wt	fresh weight
GA	gibberellic acid
GC-MS	gas chromatography-mass spectrometry
GR	glutathione reductase (E.C. 1.6.4.2)
GRAS	generally recognized as safe
GSH	reduced glutathione
GSSG	glutathione disulphide (oxidized glutathione)
GST	glutathione S-transferase (E.C. 2.5.1.18)
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase (E.C. 1.1.1.34)
I	deoxyinosine
IPC	isopropyl N-phenylcarbamate (propham)
MeOH	methanol
MH	maleic hydrazide
MVA	mevalonic acid
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced)

Stellingen

1. Door de combinatie van spruitremmende en schimmelgroeiremmende eigenschappen en door het reversibele karakter van de spruitgroeiremming heeft S-carvon goede mogelijkheden om als commercieel spruitremmingsmiddel voor (poot)aardappelen dienst te doen.
   - Dit proefschrift

2. Vergelijkende studies naar de effecten van monoterpenen op plantaardige weefsels worden bemoeilijkt door de omzetting van deze verbindingen in afgeleide produkten.
   - Dit proefschrift

3. Het verschil in gevoeligheid voor S-carvon van *Fusarium solani* en *F. sulphureum* var. *coeruleum* bij toepassing op de aardappel kan niet worden verklaard door een verschil in omzettingssnelheid.
   - Dit proefschrift

4. Door het hanteren van bemiddeling en contractteelt in de groenten- en fruitafzet wordt de veilingklok ondermijnd.

5. De kosten die berekend worden voor meerwerk aan nieuwbouwwoningen staan veelal niet in verhouding tot de hoeveelheid meer te leveren werk.

6. Het is cynisch dat berichten over treinongevallen met dodelijke afloop in de krant worden afgedaan met de mededeling dat de trein vertraging opliep.

7. De toenemende individuele (auto)mobiliteitsvrijheid leidt tot een steeds beperktere bewegingsvrijheid.

8. Aardappelen met het predikaat "MBT" of "Milieukeur" dienen te worden behandeld met een S-carvon-bevattend spruitremmingsmiddel of met een gelijkwaardig natuurvriendelijk alternatief indien ze langere tijd moeten worden bewaard.

9. Het gebruik van spruitremmingsmiddelen voor aardappelen op basis van monoterpenen bij de oude Inca-culturen betekent dat de Inca's daarmee hun tijd ver vooruit waren, of dat wij ver achterlopen bij de toepassing van natuurstoffen voor deze doeleinden.

10. De overwinning van de milieubeweging met betrekking het voorgenomen afzinken van het boorplatform Brent Spar in de oceaan hoeft nog geen winst voor het milieu te betekenen.

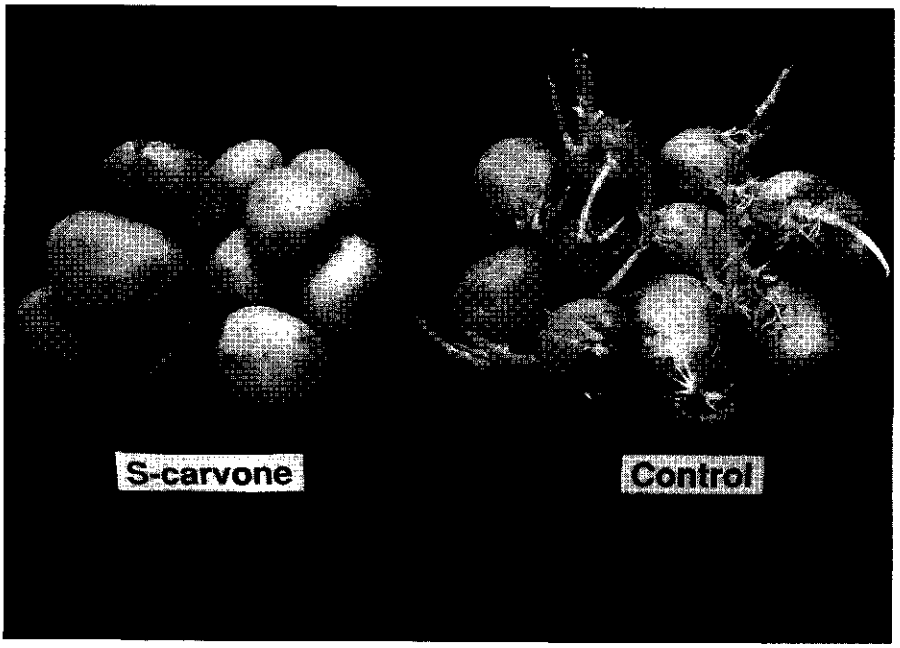
11. Invoering van "zuivere speeltijd" in de voetbalsport zal niet alleen de eerlijkheid, maar bovenal het kijkplezier, vergroten.

12. De toenemende populariteit van vluchtige oliën om hun vermeende effect in een aromatherapie is eerder te verklaren door een goede marketing dan door feitelijk vastgestelde fysiologische activiteit.

Stellingen behorend bij het proefschrift, getiteld 'Different aspects of S-carvone, a natural potato sprout growth inhibitor' door Jacobus Oosterhaven.

Wageningen, 11 oktober 1995

NBT	*p*-nitro blue tetrazolium chloride
NMR	nuclear magnetic resonance
PAL	phenylalanine ammonia lyase (E.C. 4.3.1.5)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PMSF	phenylmethylsulphonyl fluoride
PVPP	polyvinylpolypyrrolidone
THF	tetrahydrofuran



S-carvone inhibits the sprouting of potato tubers. Tubers of the cultivars Bintje (light skin) and Bildtstar (dark skin) were stored in 20 l containers with 250 µl S-carvone for 4 weeks whereafter the sprout growth was established and this photograph was taken.

CHAPTER 1

General introduction

1.1 Potato production and storage

Potato production

The potato (*Solanum tuberosum* L.) forms a major crop in The Netherlands. In 1993, over 2 million tons of potatoes were produced. The major part is processed into crisps and French fries, whereas the remaining potatoes are used for direct consumption. Seed potatoes were produced in an amount of about 550,000 tons of which the major part is exported all over the world.

After harvest, potato tubers are dried and cured for about 2 weeks at a temperature of about 15°C, the optimal curing temperature (Burton *et al.* 1992). Curing is the process by which the wounds are healed that have occurred during the mechanical harvest. A proper wound healing is essential because wounds are ideal entries of (storage) pathogens and lead to loss of water and solutes (Burton *et al.* 1992).

After curing, the temperature is lowered to a cultivar-dependent temperature. If this temperature is too low, cold-sweetening will develop during storage, i.e. an accumulation of reducing sugars in the tuber. During processing, these reducing sugars react with free amino acids (the so called Maillard reaction, reviewed by Whitfield, 1992) leading to bitter, brownish compounds. If the storage temperature is too high, the dormant period is shortened and sprouts will develop, leading to weight loss and quality reduction.

Seed potatoes have to be stored in such a way that the sprouting capacity and growth vigour are optimal at the planting date. Low temperatures (2-4°C) are suitable for this purpose, but in common practice, mechanical cooling is only used for about 25% of the seed potatoes. The use of outside air ventilation cannot every year guarantee a sufficiently low temperature throughout the storage period. Therefore, in some occasions the use of a reversible sprout inhibitor would be useful in order to control sprouting of the tubers temporarily.

Brief history of potato storage

The potato cultivation started at least 7000 years ago (Hawkes 1994). It is believed that it has been domesticated in the south of Peru and the north of Bolivia. From there it has been spread to other parts of South America as well as to other continents. The first record of the cultivation of potato in Europe is from 1573 (Hawkes and Francis-Ortega 1992), but export of potatoes from the Canary Isles to Antwerp was documented already in 1567.

The ancient people of the Inca cultures, living in South America, cultivated potatoes once a year. Although the potatoes had a long natural bud rest, they already had problems with the quality of potato tubers during storage. In order to cope with too early sprouting and insect attack of the tubers, they stored the harvested potatoes in a storage bin as drawn in Fig. 1.1. In a corner of a shed or outside, an area of a suitable size was boxed in with planks. Immediately before the potatoes were transferred to the bin, the floor and sides were lined with muña twigs. When the box was full, the potatoes were completely covered with the twigs (Stoll 1986). The use of twigs of muña, belonging to the genus *Minthostachys*, prevented sprouting as well as insect damage of the crop. Of the genus *Minthostachys*, 12 species are known from Venezuela to Argentina and these are found at altitudes of 2000-3800 m all over the Andes (Stoll 1986). This storage method is still in use in some parts of South America (Aliaga and Feldheim 1985; Stoll 1986).

In 1985 it became evident that volatile compounds, emanating from the muña leaves, controlled sprouting as well as insect attack (Aliaga and Feldheim 1985). Analysis of these volatiles showed that the active compounds, responsible for the sprout inhibition, were pulegone, menthone, menthofuran, isomenthone and *trans*-caryophyllene, making up more than 95% of the essential oil (Aliaga and Feldheim 1985; Baerheim Svendsen *et al.* 1987). The following section will deal in more detail with the prevention of sprout growth of potatoes in modern potato storage practice.

Dormancy, sprouting and sprout control

Potato tubers possess a natural period of bud rest, which is usually referred to as dormancy. According to the definition of a working group of the European Association for Potato Research, dormancy is defined as the physiological state of the tuber in which autonomous sprout growth will not occur, even when it is placed under ideal conditions for sprouting (darkness,

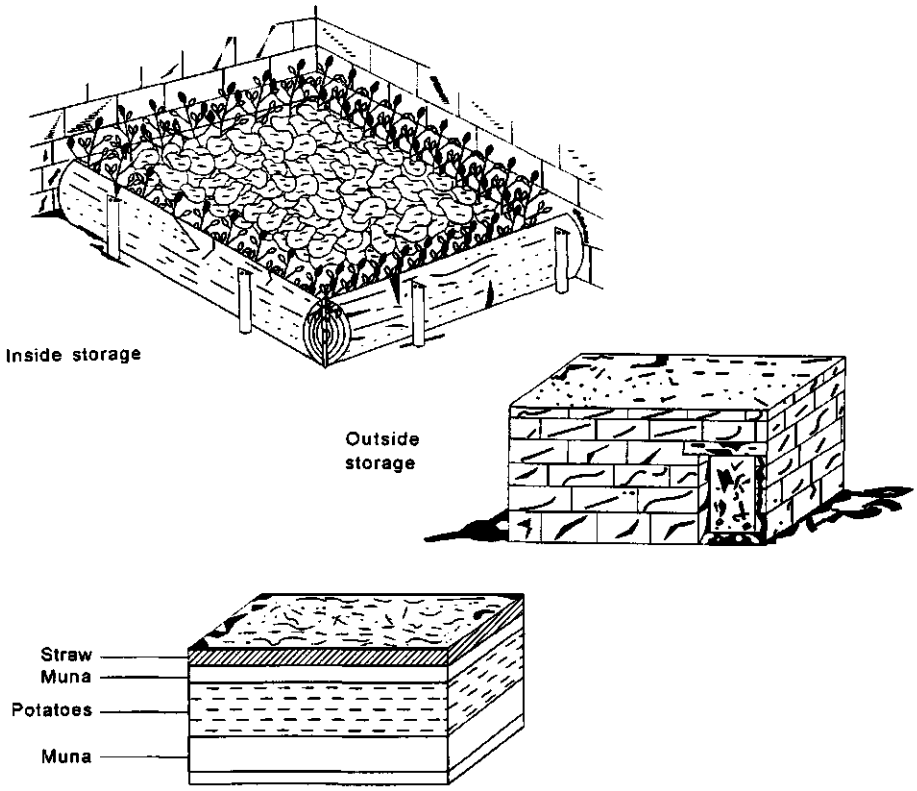


Fig. 1.1 Storage of potato tubers with the use of twigs of *Minthostachys* species, in order to prevent sprouting and insect damage, in South America (redrawn with permission from Stoll 1986).

temperature 15-20°C, relative humidity 90%) (Reust 1986). The length of the dormancy period depends on cultivar, maturity of the tuber, and soil and weather conditions during growth (Burton *et al.* 1992). Furthermore, storage conditions, such as temperature, humidity and light, are important factors determining the length of the dormancy period.

A dormant state is not characterized by a total lack of metabolic and synthetic activities. In fact, autoradiographic studies have demonstrated the continuous synthesis of proteins, RNA and DNA in potato tuber buds from the time of tuber harvest throughout dormancy (MacDonald and Osborne 1988). Breaking of dormancy and the appearance of the white tips of the emerging sprouts is associated with a rise in protein, RNA and DNA synthesis (MacDonald and Osborne 1988). Hormones, such as abscisic acid (ABA), gibberellic acid (GA) and cytokinins (CK), play a role in the duration and the

breaking of dormancy, but it is still uncertain whether changes in hormonal balances are the cause or the consequence of (changes in) the dormant state (Van der Plas 1987).

Sprouting has to be promoted in potatoes which are to be planted, but it has to be prevented in potatoes which are used for consumption and processing. Breaking of dormancy can be established with the use of various chemicals and treatments, such as cytokinins, gibberellins, rindite (a three component mixture containing ethylene chlorohydrin, ethylene dichloride and carbon tetrachloride), thiourea, carbon disulphide, mercaptoethanol, bromoethane, partial anaerobiosis and carbon dioxide (reviewed by Coleman 1987).

Sprout control

Control of sprouting is important when potato tubers have to be stored for a long period. A cold temperature regime (2-4°C) can control sprouting. However, low-temperature sweetening will develop leading to high concentrations of reducing sugars in the tuber. Therefore, storage temperatures are generally maintained at 6-8°C in combination with a sprout inhibitory treatment of the tubers (Burton *et al.* 1992). Several treatments are suitable in order to prevent sprouting, although only a few are used in practice.

The most widely used sprout suppressants are isopropyl N-phenyl-carbamate (propham or IPC) and isopropyl N-(3-chlorophenyl)carbamate (chlorpropham or CIPC). IPC and CIPC act as specific inhibitors of mitosis (Nurit *et al.* 1989), thereby preventing sprout growth. Because health authorities try to reduce residue levels of synthetic pesticides, maximum residue levels are determined. These values differ widely in the western European countries and vary between 0 mg kg^{-1} and 50 mg kg^{-1} for unpeeled potatoes (Burton *et al.* 1992).

Maleic hydrazide (MH) is a sprout inhibitor which must be applied as a foliar spray 3-6 weeks before destruction of the foliage (Weiss *et al.* 1980). It is, amongst others, used in the USA and leads to a satisfactory inhibition of sprout growth during storage. MH cannot be used for seed potatoes since the sprouting is irreversibly inhibited.

Tecnazene has been used in the UK for many years. Dalziel and Duncan (1980) described the antifungal as well as the sprout inhibitory properties of tecnazene. Although the sprout inhibitory properties are sufficient,

there are some drawbacks of its use because the efficacy decreases if dormancy has already been broken, the ventilation rate is too high or the storage temperature exceeds 10°C (Dalziel and Duncan 1980).

Alternative sprout inhibitors

Dimethylnaphthalenes (DMNs) appeared to inhibit sprouting at a laboratory (Meigh *et al.* 1973; Filmer and Thompson 1983) as well as on a practical scale (Duncan and Van Es 1988). DMNs are produced by potato tubers and especially 1,4-dimethylnaphthalene and 1,6-dimethylnaphthalene were potent inhibitors, comparable to IPC and CIPC (Meigh *et al.* 1973). However, a commercial use is possibly restricted by the fact that toxic effects were noticed. When several disubstituted methylnaphthalenes were applied to newly fertilized sea urchins and fish eggs, toxic effects occurred: abnormal and dead embryos were observed in the concentration range of 0.3-3.0 ppm (Sæthre *et al.* 1984). The authors suggested that the toxicity could be related to reactive metabolites of the various DMN derivatives. Especially the ability to form epoxides by means of an enzymatic bioconversion catalysed by cytochrome P-450 mono-oxygenase, could attribute to the toxicity since these epoxides react readily with nucleophiles and may be responsible for the binding to biopolymers within the cell (Sæthre *et al.* 1984).

An alkaloid from *Camptotheca acuminata* Decne, camptothecin, inhibited sprout growth completely for 4 weeks at 15°C (Wang *et al.* 1980). Camptothecin was found to inhibit sprouting by inducing structural changes in vascular tissues and interfering with cell division in the meristematic tissue of the sprouts (Wang *et al.* 1980). In mice bearing leukemia, camptothecin showed noteworthy life-prolongation activity by inhibition of topoisomerase I (Wall and Wani 1993). Clinical trials in China have reportedly shown responses in the treatment of patients with liver, gastric, head and neck, and bladder cancer, but clinical trials in the USA were terminated due to severe toxicity. However, several more soluble semi-synthetic derivatives of camptothecin with superior activity and reduced toxicity have recently entered new clinical trials (Cragg *et al.* 1993).

Several volatile compounds were also found to inhibit sprouting of potato tubers (Meigh 1969; Meigh *et al.* 1973; Beveridge *et al.* 1981). An interesting group of sprout growth inhibitory volatiles are (the components of) essential oils, in particular the monoterpenes. In 1969, Meigh reported the

effect of carvone, pulegone and piperitone. Furthermore, Beveridge *et al.* (1981, 1983), Aliaga and Feldheim (1985), Hartmans and Van Es (1988), Vaughn and Spencer (1991, 1993) and Vokou *et al.* (1993) described sprout inhibitory effects of several monoterpenes.

Although many of these monoterpenes and other volatile compounds possess a good sprout growth inhibitory capacity, only recently (in 1994) a commercial formulation, on the basis of the monoterpene S-carvone[1], was registered and brought on the market in The Netherlands. In a four years study using various potato cultivars, a regular application of S-carvone throughout the storage period led to excellent sprout control. Even when S-carvone was applied on a practical scale (250 tons), sprouting could be controlled for at least 10 months, resulting in good or even better storage results compared with the use of (C)IPC (Hartmans *et al.* 1995). Furthermore, because of the antimicrobial activity of S-carvone (see also section 1.3) the growth and development of some storage pathogens were markedly inhibited in the trials where S-carvone was applied (Gorris *et al.* 1993; Gorris *et al.* 1994; Hartmans *et al.* 1995). The mechanism by which monoterpenes control sprout growth of potato tubers is not known.

1.2 Essential oils

Volatile oils are the odoriferous principles found in various plant parts. Because they evaporate when exposed to air at ordinary temperatures, they are called volatile oils, ethereal oils or essential oils. Depending on the plant family, the volatiles may occur in specialized secretory structures such as glandular hairs, modified parenchymal cells or oil-tubes (called vittae; for example the vittae in the pericarp of caraway seeds) (Tyler *et al.* 1988). The oils consist of a mixture of secondary metabolites, usually monoterpenes, sesquiterpenes and phenylpropanoids.

Essential oils are known since antiquity and are frequently used because of their (supposed) pharmaceutical and antimicrobial properties, and because of the excellent flavour and fragrance characteristics of many of

[1] In nature two enantiomers of carvone are found: spearmint contains (R)-(-)-carvone, and dill and caraway contain (S)-(+)-carvone. In this thesis (S)-(+)-carvone will be referred to as S-carvone.

them. In European folklore it was believed that caraway, and its essential oil, would prevent the theft of anything that contained it. It was also included in love potions, given to a lover to prevent him from being "stolen" (Metcalfe 1992).

Essential oils are also used in aromatherapy where these oils are believed to promote and maintain health and vitality (Metcalfe 1992).

1.3 Monoterpenes

Monoterpenes are formed by the combination of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These two compounds are usually referred to as active isoprene units and form the building blocks of many natural compounds. Isopentenyl pyrophosphates are synthesized within the mevalonate route and lead to the synthesis of e.g. monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units), squalene (6 units) and natural rubber (> 100 units) (Paterson-Jones *et al.* 1990). Till now, more than 1,000 naturally occurring monoterpenoids are known, most of which have been isolated from higher plants (Charlwood and Charlwood 1991).

The central pathway of the mevalonate route is schematically drawn in Fig. 1.2. The pathway is present in animals, plants and most micro-organisms. However, it is virtually only in plants that side branches of the mevalonate pathway occur which lead to the accumulation of terpenoids, although not all of the different side branches are always displayed in any one plant species (Banthorpe 1991).

Monoterpenes are known to be cytotoxic to plant tissues as well as to micro-organisms and insects. The literature covering these aspects is briefly summarized below and will be described in more detail in the following chapters.

Plant growth regulation by monoterpenes

Inhibition of seed germination by several volatiles, including monoterpenes, is known since long (Evenari 1949). Furthermore, ecological studies revealed that the inhibition of growth and development of annual grass species in the vicinity of *Salvia leucophylla* was due to the production of vola-

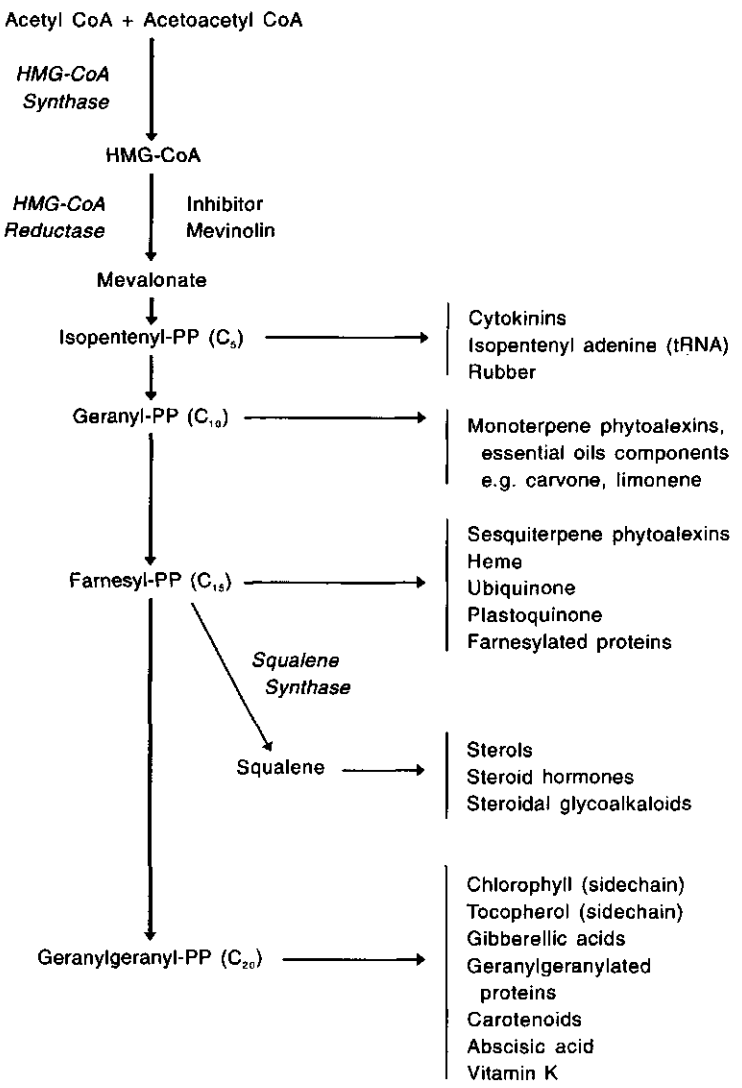


Fig. 1.2 A simplified schematic representation of the mevalonate pathway leading to a large number of secondary metabolites (adapted from Weissenborn *et al.* 1995).

tile monoterpenes, especially cineole (most probably 1,8-cineole is meant) and camphor (Muller and Muller 1964). Fischer (1986) reviewed the function of monoterpenes as plant germination and growth regulators, and stated that although the function of monoterpenes is not well understood, there is considerable evidence that they play an ecological role in the interaction of

plants with other organisms, thereby supporting the findings of Evenari (1949) and Muller and Muller (1964).

Various monoterpenes exert different effects, ranging from inhibition or stimulation of respiration to interactions with specific enzymes (Einheilig 1986). However, no clear case is known indicating that such effects are the primary causes of growth reduction. In view of the structural diversity of monoterpenes and their diverse effects, it is unlikely that all monoterpenes have an identical mode of action (Einheilig 1986). As a consequence, research into the mode of action of S-carvone on the sprout growth of potatoes and on fungal growth seems to be interesting.

Antimicrobial activity

Numerous reports have been published on the antimicrobial activity of essential oils. However, there are only a few reports describing the effects of (purified) monoterpenes on microbial growth. Deans and Ritchie (1987) screened 50 plant essential oils for their antibacterial properties against 25 genera of bacteria using the agar diffusion technique. The five most inhibitory oils were those of thyme, cinnamon, bay, clove and almond. Since synergistic, additive, and antagonistic effects have been described (Kurita and Koike 1982; Janssen *et al.* 1987), it is not possible to establish a relationship between oil composition and the biological activity (Piccaglia *et al.* 1993).

There are some papers on structure activity relationships and the possible mechanism of the antimicrobial activity of essential oils and some individual monoterpenes (Kurita *et al.* 1981; Knobloch *et al.* 1986, 1987 and 1989; Sikkema 1993). Knobloch *et al.* (1989) suggested that essential oils cause membrane damage due to their lipophilic properties. However, specific functional groups were additionally effective: phenolic and non-phenolic alcohols revealed the strongest inhibitory effects, followed by aldehydes and ketones, whereas the hydrocarbons were less active at similar mM concentrations.

The presence of one or more double bonds conjugated to a carbonyl group resulted in a relatively high antifungal activity (Kurita *et al.* 1981). Kurita *et al.* (1981) suggested that the antifungal activities of aldehydes like, for example, cinnamaldehyde are, at least in part, due to its ability to form charge transfer complexes with electron donors of a fungal cell.

Monoterpenes are assumed to be poorly soluble in water (Eastcott *et al.* 1988). However, great differences exist between the hydrocarbons (low solubility, <35 ppm) and oxygenated monoterpenes (solubilities from 155-6990 ppm) (Weidenhamer *et al.* 1993). Monoterpenes are supposed to dissolve in membranes, thereby dissipating the proton motive force and causing a loss of membrane integrity (Knobloch *et al.* 1986, 1989). Evidence for this hypothesis comes from experiments in which the membrane leakage as function of the monoterpene concentration was determined in liposome model systems (Sikkema 1993). In cytochrome *c*-oxidase containing proteoliposomes both components of the proton motive force, namely the pH gradient and the electrical potential, were dissipated with increasing concentrations of cyclic monoterpene hydrocarbons (Sikkema 1993). This was primarily due to an increased permeability of the membranes for protons.

Uribe *et al.* (1985, 1990) found that ß-pinene and limonene exerted their action at the membranes and the membrane-embedded enzymes. ß-Pinene and limonene inhibited respiration, and the inhibition depended on the ratio of the terpene to the amount of yeast cells. The effect on respiration could be localized in the cytochrome *b* region of the electron transport chain (Uribe *et al.* 1985).

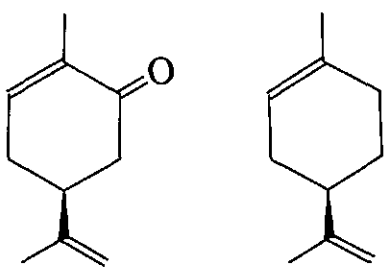


Fig. 1.3 S-(+)-carvone (left) and R-(+)-limonene.

Caraway seed oil, consisting mainly of S-(+)-carvone and R-(+)-limonene (Fig. 1.3), showed a growth inhibitory activity against several Gram-positive and Gram-negative bacteria (Kellner and Kober 1954; Farag *et al.* 1989; Rasheed and Chaudhry 1974; Elkhouly *et al.* 1978; Janssen 1989) and fungi (Farag *et al.* 1989; Janssen 1989; Gorris *et al.* 1994; Smid *et al.* 1994). Yousef and Tawil (1980) reported that the growth-inhibiting properties of caraway seed oil were more effective against fungi than against bacteria. Both S-carvone and R-carvone were antifungal against the potato storage pathogens *Fusarium sulphureum*, *Phoma exigua*, and *Helminthosporium sulphureum* in *in vitro* as well as *in situ* test systems (Smid *et al.* 1994; Gorris *et al.* 1994). Morris *et al.* (1979) tested R-carvone and S-

carvone in agar overlay and dilution assays. The enantiomers showed a different activity against *Staphylococcus aureus*. This was also found by Jay and Rivers (1984). However, Janssen (1989) did not find enantiomer effects towards *Bacillus subtilis* and *S. aureus*.

Insecticidal activity

Monoterpenes may have insect repellent as well as attractant activities. There are indications that coevolutionary processes have resulted in plant-insect interactions such that a monoterpene in a plant may be repellent to many insects but be attractant to certain host specific insect species (Rice and Coats 1994). Some monoterpenes are acute toxic. For example, S-limonene is toxic to rice weevils and cockroaches (Rice and Coats 1994, and references therein). Monoterpenoid activity against insects and the toxicity of various monoterpenes, and derivatives thereof, against the housefly (*Musca domestica*) were reviewed and compared by Rice and Coats (1994). Ketones were more active than the corresponding alcohols. Furthermore, the results of the study provided indications that minor structural variations in monoterpenes can elicit major differences in their toxicity.

Extracts of dill (*Anethum graveolens*) seeds, mainly consisting of S-carvone and R-limonene, had long lasting repellency against the confused flour beetle (Su 1985, 1987). *Sitophilus granarius* was found to be killed by the essential oils of dill, caraway and coriander (Zuelsdorff and Burkholder 1978).

Pharmaceutical properties of monoterpenes

Pharmacological and clinical tests, and the experience obtained by traditional medicine have shown that essential oils possess various properties, such as antiinflammatory, antiseptic, carminative, sedative and circulation-stimulating activities (reviewed by Schilcher 1985).

Amongst other essential oil components, S-carvone induced an increased activity of the detoxifying enzyme system gluthathione S-transferase in A/J mice (Lam and Zheng 1991). The α,β-unsaturated ketone system in S-carvone appeared to be critical for the high enzyme-inducing activity (Zheng *et al.* 1992). On the basis of these results, the authors hypothesized that these class of compounds could form a potential class of tumour-protective agents.

S-limonene containing diets significantly reduced the development of mammary cancer in rats, induced by DMBA (7,12-dimethylbenz[a]anthracene),

a tumour-inducing compound (Elegbede *et al.* 1984; Maltzman *et al.* 1991; Crowell *et al.* 1992). Furthermore, caraway oil has an inhibitory effect on chemically induced tumours of mouse skin. The inhibition was manifested by the disappearance of carcinomas, a reduced incidence and number of papillomas, and a regression of already established papillomas (Shwaireb 1993). The mode of action of caraway oil is not known. However, especially towards the role of limonene in cell metabolism, some clues are now beginning to emerge.

R- and S-limonene have been reported to inhibit cell growth, possibly by inhibition of the farnesylation of a special class of cellular proteins of 21-26 kDa in human mammary epithelial cells (Crowell *et al.* 1991). Farnesylation involves a post-translational modification of proteins by a cholesterol precursor, farnesyl pyrophosphate (reviewed by Glomset *et al.* 1990; Clark 1992; Tamanoi 1993). One of the substrates of farnesyltransferases are the so-called Ras-proteins which are involved in cell cycling. Schulz *et al.* (1994) have presented data that strongly suggest that mevalonate-derived products required for lymphocyte proliferation may include one or more isoprenylated proteins, and that the isoprenylation of these proteins is required for the progress of the cell cycle. So, limonene can block cell cycling and proliferation of tumour cells possibly by the inhibition of specific cell-cycling proteins. Furthermore, Elson and Yu (1994) hypothesized that the mevalonate pathway of tumour tissues is uniquely sensitive to the inhibitory action of dietary isoprenoids such as limonene, thereby exhibiting the chemo-prevention of cancer.

GRAS status of S-carvone

S-carvone was given the generally-recognized-as-safe (GRAS) status by the Flavoring Extract Manufacturers' Association in 1965, and was approved by the Food and Drug Administration for use in food in 1961 (Chan 1990 and references therein). The acceptable daily intake was established at 1.25 mg kg^{-1} body weight.

A two years trial covering several toxicity tests with S-carvone has been performed by the National Institute of Health in the USA (Chan 1990). Administering S-carvone to male and female mice, and addition of S-carvone to Chinese hamster cells and to cells of *Salmonella typhimurium* (for genetic

studies) did not give any evidence of carcinogenic activity. Furthermore, neither compound-related lesions nor histopathologic changes were observed.

1.4 Caraway and the Dutch Caraway Research Programme

The cultivation of caraway (*Carum carvi* L.) has a long tradition in The Netherlands; since the beginning of the 18th century, it has been grown as a field crop, ranging from 10 ha to 10,000 ha annually (Zijlstra 1916; Gildemeister and Hoffmann 1931). The plant originates from Asia and southern parts of Europe, but it can be cultivated in many countries of which Poland, Russia and Hungary are the major producers of the seeds (Van der Meer 1960). In The Netherlands, caraway is predominantly cultivated on heavy clay soils in the provinces of Groningen and Zeeland.

Yields of caraway seed and essential oil vary widely within and between years (Toxopeus and Bouwmeester 1993). The caraway fruits, usually referred to as seeds, contain 3-7% essential oil in oil ducts located in the pericarp. The oil consists mainly of S-carvone (50-60%) and R-limonene (35-45%) (Van der Meer 1960; Toxopeus and Bouwmeester 1993).

Caraway is a biennial (winter-caraway) as well as an annual (spring-caraway) crop. Till now, in The Netherlands mainly the biennial cultivars are cultivated. The plant is sown in early spring together with a cover crop. In the first year it reaches the rosette stage; during the second year stems grow and it starts flowering in spring. After seed set, fruits develop and the mature fruits are harvested during the beginning of July whereafter they are dried to about 11% moisture content (Heger 1956; Toxopeus and Bouwmeester 1993). The fruits can be stored for years without hardly any quality loss.

Most of the seeds were exported to Austria and the USA, where the distilled oil was used as bakery constituent and as a flavouring agent. However, due to an increased production in Poland, Hungary and Russia, the price of the seed fell sharply and the enthusiasm to grow caraway in The Netherlands vanished.

In order to promote caraway as a (profitable) alternative in crop rotation, besides traditional crops like potato, sugar beet and wheat, a Dutch national research programme was started. The aim of the programme was to look for new applications of caraway oil, and in particular of S-carvone, to develop

improved caraway cultivars by means of breeding and selection, and to get a better understanding of the factors that determine the essential oil yield of the crop, such as cultivation and diseases (for a review of the results of this programme, see: Meijer and Oosterhaven 1994, and a special issue of Industrial Crops and Products 1995).

In 1989, an industrial programme was started in order to develop the practical application of S-carvone as a potato sprout inhibitor on a large scale. The use of S-carvone was already mentioned by several authors, but no one ever attempted to apply S-carvone on a practical scale. The results of this programme were very promising and resulted in a market introduction of a formulation of S-carvone with the trade name "Talent" in 1994 (Hartmans *et al.* 1995).

1.5 Outline of the thesis

The thesis deals with several aspects of the use of S-carvone as a natural potato sprout growth inhibitor. In Chapter 2, the potato sprout inhibitory properties of several monoterpenes, including R-carvone and S-carvone, tested in a potato model system, are described. Because of the very promising results obtained with S-carvone and because of the lack of side effects (no effect of S-carvone on the taste of cooked potato tubers, and no side effects on the processing quality of the S-carvone treated tubers) (Hartmans *et al.* 1995) research was focussed on S-carvone.

Several fungi are affected in their growth by S-carvone. However, two related species infecting potato, namely *Fusarium solani* var. *coeruleum* and *F. sulphureum*, reacted differently when they were exposed to S-carvone. The hypothesis that different bioconversion mechanisms would account for the difference in susceptibility was tested and results from these studies are described in Chapter 2.

The fate of R-carvone and S-carvone in potato tissues is described in Chapter 3. Various bioconversion reactions took place, and S-carvone was mainly converted into neoisodihydrocarveol.

The basic hypothesis for the growth inhibitory mechanism of S-carvone came from animal studies in which cyclic oxygenated monoterpenes have been found to reduce the activity of the key enzyme of the mevalonate

pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). In order to test the hypothesis that S-carvone reduces the activity of HMGR and thereby influences the potato sprout growth, biochemical and molecular biological experiments (Chapter 4) as well as Western analyses (Chapter 6) were carried out.

Results of these studies made it essential to investigate the effect of S-carvone on wound healing processes. Wounding of potatoes occurs during their mechanical harvest, and application of S-carvone on a practical scale might imply the use of S-carvone immediately after the harvest, i.e. before the curing period. In the Chapters 5 and 7 the delayed wound healing due to the effect of S-carvone is described.

Stress related enzyme systems are induced in tissues exposed to S-carvone. The possible relation with the bioconversion and conjugation of S-carvone, and the biochemical changes coinciding with the bioconversion reactions are discussed in Chapter 8.

The general discussion, Chapter 9, deals with different aspects of the use of S-carvone and the metabolic and biochemical changes detected in potato tissue after an S-carvone treatment.

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CHAPTER 2[1]

S-carvone as a natural potato sprout inhibitory and fungistatic compound

Summary S-carvone, a monoterpene also found in caraway (*Carum carvi* L.), inhibits the sprouting of potatoes very efficiently when applied at constantly low headspace concentrations. The elongation 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* var. *coeruleum* and *F. sulphureum* was found to be inhibited by S-carvone at a concentration of 3 mM. At sub-inhibitory concentrations, both *F. solani* and *F. sulphureum* converted S-carvone into more reduced compounds which were shown to be less toxic than S-carvone.

2.1 Introduction

Monoterpenes form a class of natural compounds with acyclic, monocyclic and bicyclic carbon skeletons, which are derived from two isopentenyl pyrophosphates. The monoterpenes, that usually represent the major components of essential oils, are produced in different parts of various plants. Although their function is not completely understood, there is considerable evidence that they play an ecological role (Fischer 1986). The monoterpenes may have an attractant or a deterrent effect on insects, and many monoterpenes are known to be toxic for micro-organisms (Knobloch *et al.* 1986, 1987; Smid *et al.* 1994). In addition, a number of monoterpenes inhibit germination of seeds (Asplund 1968; Reynolds 1987).

[1] This chapter is based on a combination of two papers:
Oosterhaven, K., B. Poolman and E.J. Smid (1995). S-carvone as a natural potato sprout inhibiting, fungistatic and bacteriostatic compound. Ind. Crops Prod. 4: 23-31.
Oosterhaven, K., A. Chambel Leitao, L.G.M. Gorris and E.J. Smid (1995). Comparative study on the action of S-(+)-carvone, *in situ*, on the potato storage fungi *Fusarium solani* var. *coeruleum* and *F. sulphureum* (submitted).

Monoterpenes are used as flavouring compounds in foods or parfumes; menthone and menthol, isolated from *Mentha* species, 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; Vokou *et al.* 1993). From these monoterpenes, S-carvone has a promising potential as a commercial sprout inhibitor (Hartmans *et al.* 1995).

Besides the reversible sprout inhibitory effect, S-carvone has interesting additional effects because it inhibits the growth of several potato storage pathogens (Kurita *et al.* 1981; Smid *et al.* 1994; Hartmans *et al.* 1995).

The question arises how S-carvone influences the potato sprout growth and the growth of micro-organisms. There is little biochemical and physiological knowledge on the action of monoterpenes on plants in general. The inhibition of the germination of seeds by several alicyclic and heterocyclic compounds, among which seevral monoterpenes, 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. In contrast, the inhibitory activity of other compounds was much greater than would be expected in comparison with compounds with related structures. For example, the α,ß-unsaturated keto group of carvone is important for the germination-inhibiting effect (Asplund 1968; Reynolds 1989).

The biological activities of monoterpenes have been studied in somewhat more detail in mitochondria and chloroplasts isolated from plant material. ß-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 the mitochondrial respiration at 5 mM (Muller *et al.* 1969; Lorber and Muller 1980b). Membrane disturbances were detected in roots of *Allium cepa* following a 3 days exposure to a high concentration of cineole (most probably 1,8-cineole is meant) (Lorber and Muller 1976, 1980a).

With regard to micro-organisms the general idea of the mode of action of monoterpenes is based on the lipophilic nature of the compounds, i.e. an action due to interference with membranes, which leads to disturbance of their integrity and barrier function which then leads to dissipation of ion gradients

and eventually results in cell death (Sikkema 1993). The generally observed trend that, within a group of lipophilic compounds such as monoterpenes, a better solubility 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 nature and spatial orientation of the functional groups is also important. Furthermore, the data found in the literature indicate that there is no general mechanism by which monoterpenes interfere with physiological processes, thereby inhibiting growth, and the mode of action of S-carvone on sprout growth remains to be studied.

Growth inhibition of the potato storage fungus *F. sulphureum* by S-carvone was found to be reversible, whereas exposure to cinnamaldehyde resulted in an irreversible inhibition. Interestingly, *F. solani* var. *coeruleum* is, in contrast to *F. sulphureum*, quite resistant to S-carvone (Gorris *et al.* 1994). Since monoterpenes can be converted by micro-organisms (Noma and Tatsumi 1977; Vokou and Margaris 1988), we tested the hypothesis that the difference in susceptibility of both fungi towards S-carvone might be explained by different bioconversion mechanisms.

2.2 Materials and methods

Potato sprout growth Seed potatoes (*Solanum tuberosum* L. cv. Bintje) were stored at 4°C until further use. Prior to the start of the experiment, the potatoes were placed at 18°C to allow sprouting. Eye pieces of about 25 mm in diameter and 20 g, isolated from corresponding regions of different potatoes, were placed on a 4 cm thick layer of moist perlite (Agraperlite, Pull, Rhenen, The Netherlands) in a 20 l container. The containers, 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 125 µl of the volatile compound to be tested, were then placed on top of the perlite layer, to assure a uniform headspace concentration. The containers were covered with a plastic lid, sealed with tape and kept at 15°C. Because light inhibits potato sprout growth, the containers were placed in the dark.

The volatiles tested were: R- and S-carvone, and R- and S-limonene (Merck, Amsterdam, The Netherlands); carvenone, dihydrocarvone (mixture consisting of 80% *trans*-isomer and 20% *cis*-isomer), dihydrocarveol (mixture of the four isomers: dihydrocarveol, neodihydrocarveol, isodihydrocarveol and neoisodihydrocarveol, with dihydrocarveol as the predominant compound, >80%), (+)-menthol, 2-methylcyclohex-2-enone, 3-methylcyclohex-2-enone, dihydrocarvyl acetate (mixture of the isomers), menthyl acetate and cuminaldehyde (all from Roth, Karlsruhe, Germany).

Sprout length was measured at the moment of addition of the volatiles (= time zero) and after various days of treatment. In order to determine the concentration of the added monoterpenes in the sprouts, the sprouts were extracted as described in Chapter 3. The extracted volatiles were analysed by gas-liquid chromatography (GC) as described below.

Respiration measurements Potato tubers were wounded as described in Chapter 7. The wounded tubers were exposed to S-carvone for 4 days, and after several time intervals the respiration was measured as O_2 consumption and CO_2 production, as described by Peppelenbos *et al.* (1993).

Mitochondria were isolated from the perimedullar zone of intact potato tubers, and the mitochondrial respiration was measured according to Gude (1989) with a Clark-type O_2 electrode system (from Yellow Springs Instruments Co., 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 µl (Gude 1989). The O_2 concentration in air-saturated medium was considered to be 250 µM. Mitochondrial integrity was estimated by determining the cytochrome *c*-oxydase activity of the isolated mitochondria and that of solubilized mitochondria, using Tween (Douce *et al.* 1987).

State III respiration was measured with succinate as a substrate (10 mM final concentration) and 200 µM ADP. The O_2 consumption of uncoupled mitochondria was measured in the presence of 2 µM CCCP (carbonyl cyanide 3-chlorophenylhydrazone).

Fungal strains *Fusarium solani* var. *coeruleum* and *F. sulphureum* were obtained from culture stocks (IPO-DLO, The Netherlands) and maintained on PDA plates: 22 g l^{-1} potato extract (Oxoid), 2 g l^{-1} glucose and 0.8% agar.

Liquid cultures were grown in 75 ml of chemically defined medium (CDM) in 300 ml Erlenmeyer flasks at 20°C, shaking with 150 revs min^{-1}. The CDM contained (g l^{-1}): $K_2HPO_4.3H_2O$, 1.1, adjusted to pH 6.2 with KH_2PO_4; glucose, 10; asparagine, 2; $MgSO_4.7H_20$, 0.1; $CaCl_2$, 0.01; NaCl, 0.03; biotin, 0.001; thiamine, 0.001; and Vishniac solution, 1‰.

Conidia production Barley grains (50 g) with 2 g perlite (Agraperlite, Pull) were soaked in 250 ml water in a 500 ml Erlenmeyer flask. After 24 h, the water was decanted and the flasks sterilized. The mixture was kept at 25°C, and after another 24 h, the flask was closed with cotton and aluminium foil, and sterilized again. Because too much moisture may inhibit conidia production, the surplus of moist in the flasks was allowed to evaporate inside a laminar flow cabinet. After cooling to room temperature, the medium was inoculated with fungal hyphae grown on agar plates. The Erlenmeyer flasks were incubated at 18°C, and shaken vigorously 4-8 times daily. After hyphal growth was no longer visible (usually after 10-14 days), some grains were analysed microscopically for the presence of conidia.

Monoterpene treatment of fungi S-carvone, dihydrocarvone and dihydrocarveol, the samples described above, were dissolved in DMSO; 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 below 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 after their addition (= time zero). Monoterpene treatment of the fungi grown on solid medium (PDA medium as indicated above) in Petri dishes was performed as described by Smid *et al.* (1994). S-carvone (10 µl) was added when the mycelial mat reached a diameter of 1 cm.

Glucose determination Glucose consumption was taken as a measure of the biomass of the fungi. The Anthron method was used for the determination of total sugars (Hodge and Hofreiter 1962). A sample (ca. 0.5 ml) of the culture medium was diluted 50 times, and 500 µl were used in the Anthron assay.

Monoterpene determination Samples (1 ml) from the culture medium were extracted with 1 ml *n*-pentane-diethyl ether (1:2, v/v). Before extraction, a

known amount of naphthalene was added to the sample as an internal standard. After stirring (Vortex mixer) for 20 s, the samples were centrifuged at 3,000 *g* for 10 min, and the extraction liquid was analysed by GC. The GC conditions were as follows; column: CP-Sil 58cb (WCOT, 25 m x 0.25 mm i.d., film thickness 0.20 µm; Chrompack, Middelburg, The Netherlands); oven temperature programmed: 70°C for 1 min, subsequently up to 120°C at 10°C min^{-1}, and then held isothermal for 7 min. Using these conditions a separation of the four stereoisomers of dihydrocarveol was achieved.

2.3 Results

Potato sprout growth inhibition

Exposure of potato tubers to a constantly low headspace concentration of S-carvone led to a complete inhibition of sprout growth on a laboratory as well as on a practical scale (Hartmans *et al.* 1995). The inhibition of the sprout growth was reversible since removal of S-carvone led to an immediate and normal growth of the sprouts on the tubers.

The sprout growth inhibitory 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 inhibitory compound (Table 2.1). After addition of limonene the sprouts became necrotic within 1 day. However, after removal of limonene, lateral sprouts from the eye (bud) developed, indicating that the eyes were not completely damaged by the limonene treatment (5 days). It should be stressed that it is difficult to compare the sprout growth inhibitory properties of the volatiles.

The headspace concentration in the containers as well as the residual content of each compound in the sprouts were different in the studies (Table 2.1). Furthermore, a number 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.

Table 2.1. Potato sprout growth inhibitory effect of various volatiles. Sprouts were exposed to 250 µl of each compound, at 15°C in the dark as described under Materials and methods. The growth inhibition, the residual content of the volatiles, and their possible bioconversion were determined after 5 days of treatment.

Compound	Sprout growth inhibition [1]	Residue in sprouts (mg kg^{-1} fr. wt) [2]	Bioconversion [3]
R-Carvone	++	40-60	+
S-Carvone	+++	60-75	+
R-Limonene	necrotic within 24 h	300-400	-
S-Limonene	necrotic within 24 h	300-400	-
2-Methylcyclohex-2-enone	++	40-50	+
3-Methylcyclohex-2-enone	+	400-500	-
(+)-Menthol	+	10-15	-
Dihydrocarvone	++	100-130	+
Dihydrocarveol	-	3-5	-
Carvenone	+	50-60	-
Menthyl acetate	++	20-25	+
Dihydrocarvyl acetate	++	40-50	+
Cuminaldehyde	+	8-10	-

[1] +++ = very effective, ++ = effective, + = minor growth inhibition, - = no inhibition
[2] content of added compound including conversion products in mg kg^{-1} fr. wt
[3] + = bioconversion products detected, - = no bioconversion products detected

Exposure of potato sprouts to S-carvone at a headspace concentration of about 5-10 µg l^{-1} resulted (already after 2 days) in complete inhibition of the sprout growth (see Chapters 3 and 4). The growth inhibition was reversible, since removal of S-carvone led to an immediate regrowth of the sprouts, either by continued elongation or by branching. The sprouts exposed to S-carvone were thick and pale/yellow compared with control sprouts, and necrotic spots did not occur.

The sprouts converted S-carvone mainly into neoisodihydrocarveol (Chapter 3). The conversion went via dihydrocarvone, an intermediate that could be detected in significant amounts in the sprouts. Isodihydrocarveol, carvyl acetates and hydroxylated carvones were formed in trace amounts.

Table 2.2. Residual content of S-carvone in different potato tuber tissues after exposure to S-carvone (headspace concentration, 5-10 µg l^{-1}) for 5 days at 15°C in the dark.

Tissue	S-Carvone concentration (mg kg^{-1} fr. wt)	Conversion products (mg kg^{-1} fr. wt)
Intact tuber [1]	5	not detected
Peeled tuber [1]	< 1	not detected
Peel [1]	10	not detected
Half tuber [2]	20	30
Sprouts [3]	20	30

[1] exposure of intact tubers to S-carvone, whereafter the three samples were analysed separately
[2] exposure of wounded (half) tubers to S-carvone
[3] exposure of sprouts, in the one-eye model system, to S-carvone

Since the volatility of dihydrocarveol and dihydrocarvone is different from that of S-carvone, the effect of these compounds on the growth of sprouts in isolated potato eye pieces could not be compared. Experiments using potato cell suspension cultures showed that S-carvone was a much more effective growth inhibitor than dihydrocarveol and dihydrocarvone: the growth of the suspension cultures was inhibited at 1 mM S-carvone, whereas concentrations higher than 2 mM dihydrocarvone and above 5 mM dihydrocarveol were needed to affect the growth. Again, it is difficult to compare the action of these compounds directly, since they were continuously converted in the suspension cultures - like in the sprouts - into mainly dihydrocarvone and neoisodihydrocarveol.

The accumulation of S-carvone in potato tissue was dependent on the type of tissue used. The residual content of intact tubers was about 5 mg kg^{-1} fr. wt. 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 maximally 0.5 mg kg^{-1} fr. wt, and bioconversion products were not detected inside the tuber. The concentration of S-carvone in wounded potato tubers (half tubers) did not exceed 20 mg kg^{-1} fr. wt (at 15°C), not even after 3 weeks of exposure, but since S-carvone was converted into other more reduced compounds, the total amount of monoterpenes was higher, reaching levels of 50-70 mg kg^{-1} fr. wt (Table 2.2).

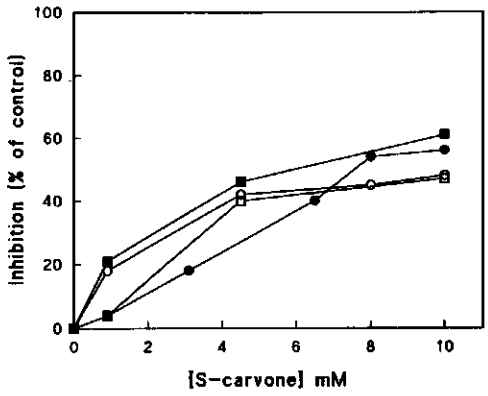

Fig. 2.1
Mitochondrial oxygen consumption in response to various concentrations of S-carvone and dihydrocarveol, expressed as percentage of inhibition compared with a control (O_2 uptake of control mitochondria). State III respiration of mitochondria, with succinate as the substrate in the presence of ADP (200 µM), was measured in the presence of S-carvone (●) and dihydrocarveol (■); Uncoupled respiration, with succinate as the substrate in the presence of CCCP (2 µM), was also measured in the presence of S-carvone (○) and dihydrocarveol (□).

Sprouts contained about the same concentration of S-carvone and its derivatives as the wounded potato tubers (Table 2.2).

In order to test the influence of S-carvone on respiration, we measured the mitochondrial O_2 consumption in the presence of S-carvone (Fig. 2.1). Only at concentrations of 1 mM or higher, the respiration was inhibited, i.e. at concentrations which are never reached in whole potato tubers nor in sprouts. Uncoupling effects of S-carvone on mitochondria were never observed. Since wounded potato tubers contained a higher concentration of S-carvone than whole tubers, subsequently the effect of S-carvone on the respiration of wounded potato tubers was studied.

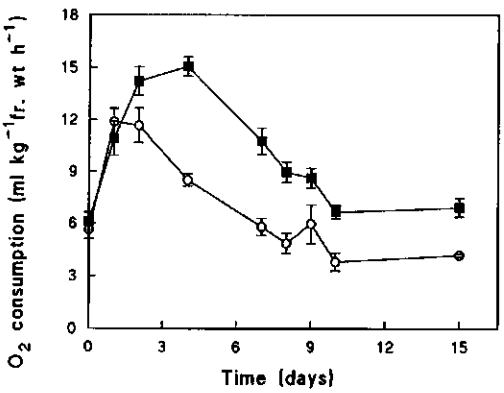

Fig. 2.2
Respiration, expressed as oxygen consumption of wounded potato tubers.
○ = control; ■ = S-carvone (tissue concentration was approximately 20 mg kg^{-1} fr. wt during the days 1-4; at day 4, S-carvone was removed from the containers).
Error bars indicate SD, N = 3.

Table 2.3. Inhibitory effect of S-carvone, dihydrocarvone and dihydrocarveol on the growth of *Fusarium solani* var. *coeruleum* and *F. sulphureum* in liquid medium, and comparison of the bioconversion of these monoterpenes.

		Growth [1]		Conversion [2]	
		F. sol.	*F. sulph.*	*F. sol.*	*F. sulph.*
S-Carvone	2.5 mM	±	±	+	+
	3.0 mM	-	-	+	+
Dihydrocarvone [3]	2.2 mM	±	±	+	+
	3.0 mM	-	-	+	+
Dihydrocarveol [4]	2.4 mM	+	+	-	-
	3.0 mM	+	+	-	-

[1] - = no growth, ± = reduced growth, + = normal growth
[2] + = conversion products detected in the medium, - = no conversion products detected
[3] 80% *trans*-isomer and 20% *cis*-isomer
[4] mixture of isomers containing >80% of dihydrocarveol

Wounded tubers used as a control showed the wound induced respiration as described by Kahl (1974). Fig. 2.2 shows that wounded tubers treated with S-carvone (20 mg kg^{-1} fr. wt) exhibited a significantly higher O_2 consumption (as well as a higher CO_2 production; results not shown) than the wounded tubers that were not exposed to S-carvone. This indicates that the rate of respiration is increased following exposure to S-carvone.

Fungal growth inhibition

In liquid culture, S-carvone and the sample of dihydrocarvone isomers applied, inhibited the growth of both *F. solani* var. *coeruleum* and *F. sulphureum* completely at 3.0 mM (Table 2.3); at lower concentrations (from 1 mM to 2.5 mM) the growth was reduced. In contrast, the sample of dihydrocarveol isomers was not inhibitory at 3.0 mM nor at 2.4 mM (Table 2.3). The germinating conidia and slowly growing hyphae from cultures of both *F. solani* var. *coeruleum* and *F. sulphureum* treated with S-carvone and dihydrocarvone were found to convert these compounds, whereas dihydrocarveol was not converted.

Samples from the fungal cultures incubated for at least 200 hours in the presence of 3.5 mM S-carvone, dihydrocarvone and dihydrocarveol, were transferred to solid media without each of the monoterpenes. Within 3 days,

regrowth was visible in all cases (data not shown). Apparently, none of the compounds was fungicidal.

Bioconversion of S-carvone

In order to start each experiment with the same amount of biomass, the media were inoculated with conidia. The germination of conidia of both *F. sulphureum* and *F. solani* var. *coeruleum* was not inhibited by the presence of 1.8 mM S-carvone as microscopic analysis of the culture media revealed. The slowly growing hyphae of both fungi converted S-carvone to such an extent that after 240 hours all S-carvone added had been converted. *F. solani* var. *coeruleum* was found to convert S-carvone into isodihydrocarvone, isodihydrocarveol and neoisodihydrocarveol. *F. sulphureum* converted S-carvone into the same compounds as main products, but additionally trace amounts of dihydrocarveol and neodihydrocarveol were detected. Thus, there was some difference in the bioconversion pattern between both fungi, since *F. sulphureum* produced all stereoisomers of dihydrocarveol, whereas in the case

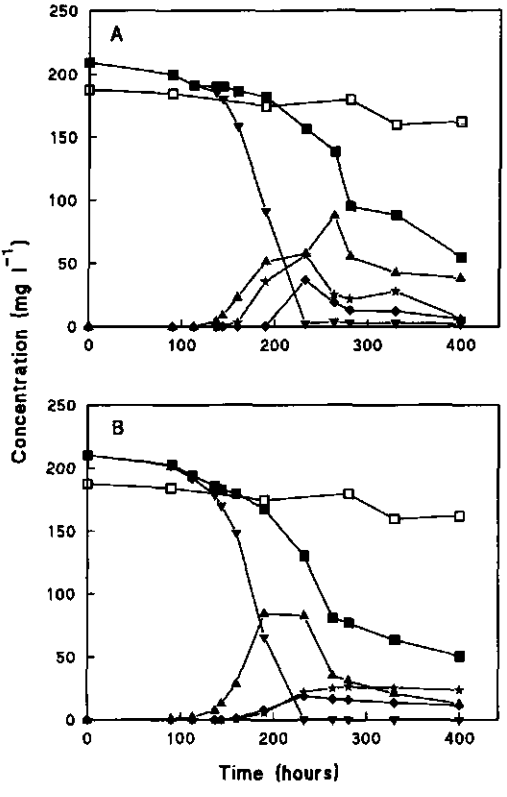

Fig. 2.3
Time course of conversion of S-carvone by *Fusarium solani* var. *coeruleum* (A) and *F. sulphureum* (B).
□ = control (without fungi); ■ = sum of S-carvone and its bioconversion products; ▼ = S-carvone; ▲ = isodihydrocarvone; ★ = neoisodihydrocarveol; ◆ = isodihydrocarveol.

of *F. solani* var. *coeruleum* only isodihydrocarveol and neoisodihydrocarveol were formed. With regard to the sum of the concentrations of all of these monoterpenes present in the growth medium, a decline was noticed (Fig. 2.3).This could not be explained by evaporation of the various monoterpenes, because in the control experiment - uninoculated culture medium to which S-carvone was added - the total concentration was found to change only slightly during 400 hours of incubation.

The same result was obtained for the samples of dihydrocarvone and dihydrocarveol added to the growth medium. The loss of monoterpenes from the growth medium could also not be explained by an accumulation of the compounds in the mycelium, because neither extraction of the lyophilized mycelium nor extraction of the medium including the mycelium yielded the lost amount of compounds.

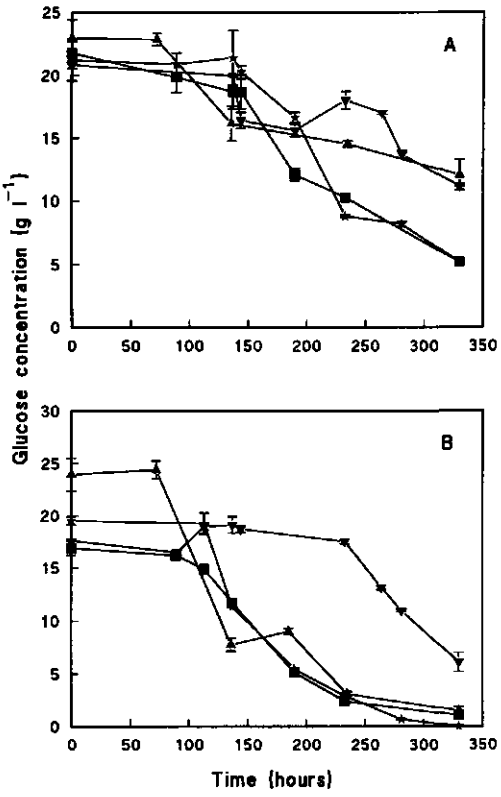

Fig. 2.4
Glucose consumption of *Fusarium solani* var. *coeruleum* (A) and *F. sulphureum* (B) in control, i.e. without monoterpenes (■), and in the presence of S-carvone (▼), dihydrocarvone (▲) and dihydrocarveol (★). The initial concentration of the monoterpenes was 1.85 mM.

Glucose consumption

Glucose consumption of *F. sulphureum* in the presence of 1.85 mM of the samples of dihydrocarvone or dihydrocarveol (the mixture of stereoisomers) was equal to that of a control in which no monoterpene was added (Fig. 2.4B). However, 1.85 mM S-carvone inhibited the glucose consumption completely. From the moment of complete S-carvone conversion, after 240 hours (Fig. 2.3), the glucose consumption started to proceed with the same rate as in the control.

In the case of *F. solani* var. *coeruleum* the situation was less clear (Fig. 2.4A). Addition of dihydrocarveol to the growth medium did not influence glucose consumption as compared to the control, but similarly to S-carvone also dihydrocarvone inhibited the glucose consumption to some extent.

In vitro growth

Because a growth assay in liquid medium does not simulate completely the *in situ* situation, the fungi were also grown on solid PDA medium in Petri dishes closed with a plastic ring. When the mycelium reached a diameter of about 1 cm, the fungi were exposed to S-carvone vapour. Growth of both fungi was inhibited following the S-carvone exposure (Fig. 2.5). At 280 hours of incubation, i.e. 160 hours after the exposure to S-carvone, a slowly inclining regrowth was observed which at 380 hours was at a similar rate as in the controls for both fungi. Conceivably the fungi had converted S-carvone to such an extent that its growth inhibitory activity was overcome.

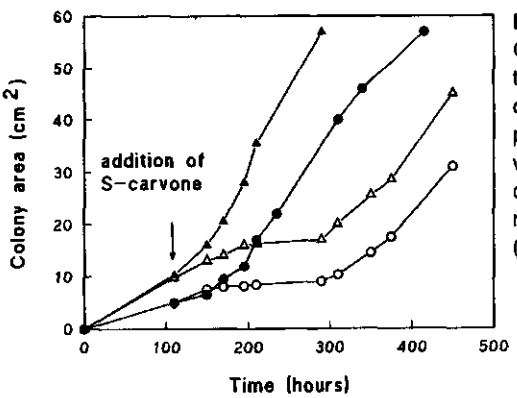

Fig. 2.5
Growth of *Fusarium solani* var. *coeruleum* in the presence (○) and absence (●) of S-carvone, and growth of *F. sulphureum* in the presence (△) and absence (▲) of S-carvone, when grown on solid medium in Petri dishes. When the diameter of the mycelial mat reached 1 cm, S-carvone was added (as indicated in the figure).

2.4 Discussion

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 various unrelated organisms. Potato sprout growth is affected by low concentrations of S-carvone in the headspace. The observed inhibition of the sprouting of potato tubers and of the growth of the potato sprouts could not be explained by inhibition of respiratory processes. Probably more specific effects of S-carvone play a role in the sprout growth inhibition of potato tubers (see following Chapters).

In a previous study (Gorris *et al.* 1994), a significant difference in the susceptibility of *F. solani* var. *coeruleum* as compared to *F. sulphureum* towards S-carvone was noticed. The observed *in situ* tolerance of *F. solani* var. *coeruleum* and the susceptibility of *F. sulphureum* for S-carvone cannot be explained by differences in the bioconversion mechanisms studied here. Both fungi were able to convert S-carvone, and at comparable rates, into mainly isodihydrocarvone, isodihydrocarveol and neoisodihydrocarveol. Comparing the degree of growth inhibition exerted by S-carvone and its various conversion products, we found that the conversion products had a smaller inhibitory effect than S-carvone (Fig. 2.4). However, dihydrocarvone was more inhibitory to *F. solani* var. *coeruleum* than to *F. sulphureum* (Fig. 2.4).

Microscopic studies revealed that S-carvone did not inhibit the germination of conidia. Using liquid growth medium supplemented with S-carvone, small amounts of hyphae of both fungi were able to convert S-carvone.

The mass balance of S-carvone and its bioconversion products was not complete; in all cases about 30-40% of the S-carvone added was found missing at the end of each experiment. This could not be explained by evaporation of S-carvone or its metabolites nor by accumulation in the mycelium. It is conceivable that S-carvone or its conversion products are bound by polar compounds that are not extracted by the method employed here. Also a further conversion or complete breakdown may occur. Future research must give an answer to these hypotheses.

Experiments using solid medium yielded similar results as those with the liquid media with regard to the anti-fungal effects of S-carvone. A solid

medium system is more related to the *in situ* situation in which S-carvone is applied as a volatile to control the growth of these fungi on a product surface. Apparently, other factors are responsible for the observed difference in the susceptibility of the two fungi towards S-carvone.

A possible explanation for the observed difference in susceptibility of both fungi towards S-carvone on potato, could be a (specific) interaction of *F. solani* var. *coeruleum* with bacteria. Duarte and Clark (1993) showed that symbiosis of *F. solani* with an *Erwinia* species can occur. Many bacteria have a relatively high tolerance level towards monoterpenes and some of them are able to convert S-carvone (Noma and Tatsumi 1977). Such a symbiosis could give *F. solani* var. *coeruleum* an advantage above *F. sulphureum* in the resistance towards S-carvone. Also the way of hyphal growth in and on the potato tuber could be a factor. *F. sulphureum* shows an aerial growth, whereas *F. solani* var. *coeruleum* shows a more inwardly directed growth (results not shown).

Another hypothesis relates to different interactions between the potato tuber and the fungi. For instance, infection of *F. sulphureum* may induce a stronger resistance response in the potato tuber than *F. solani* var. *coeruleum*, and as a result *F. sulphureum* may become more susceptible to S-carvone.

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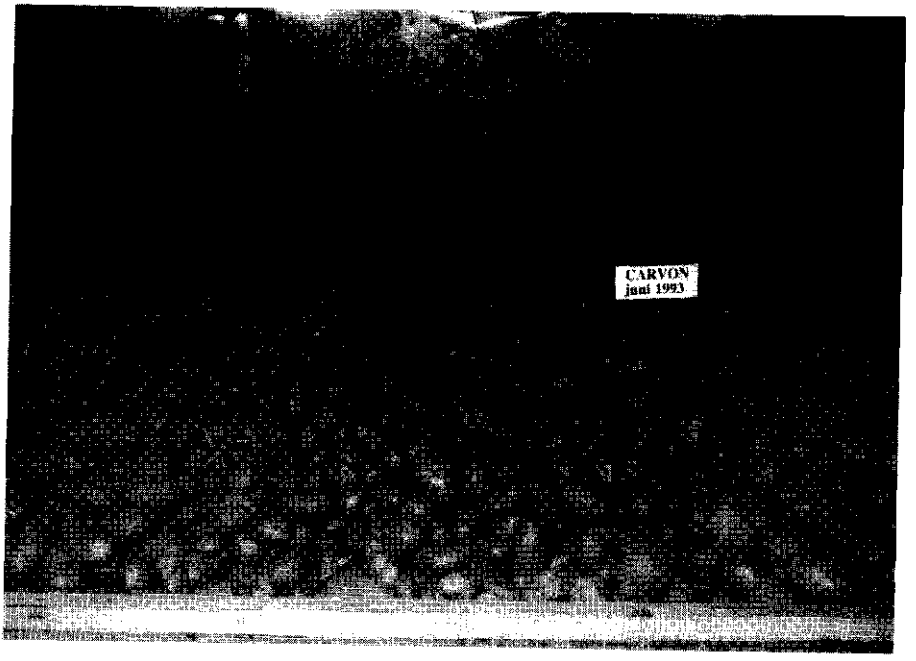

Addition of Talent®, the sprout inhibitor based on S-carvone, on a semi-practical scale (15 tons). Potato tubers of the cultivar Bintje were harvested in October, cured and dried for 14 days, and then stored at 6-7°C using outside air ventilation. At the end of the storage period (end of May), the tubers were reconditioned and the store was unloaded. The sprouting of the Talent®-treated tubers showed less infection with silver scurf (*Helminthosporium solani*) compared with (C)IPC-treated tubers.

CHAPTER 3[1]

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

Summary The monoterpenes R-carvone and S-carvone inhibited sprout growth in a model system consisting of sprouts growing on potato eye pieces. The sprout tissue was not necrotic after the carvone treatment, and the inhibition was reversible. After the carvone treatment, the sprouts showed regrowth either by continued elongation or by branching. However, the effects of both isomers on the sprout growth differed to some extent. S-carvone inhibited the elongation of the sprouts sooner than R-carvone. This might be explained by a faster uptake of S-carvone in the sprouts, 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 detected.

3.1 Introduction

Potatoes are usually stored under a cold regime (6-8°C), in combination with the application of synthetic sprout inhibitors. It is been known since 1969 that naturally occurring volatiles like pulegone and carvone can also inhibit sprouting (Meigh 1969; Beveridge *et al.* 1983; Vaughn and Spencer 1991; 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 and Muller (1980) observed a reduced mitotic activity and a hyperconcentration and breakage of chromosomes in *Allium cepa* L. following exposure to cineole (most probably 1,8-cineole is meant). *Cucumis sativus* L. root tip cells showed a disruption of membranes and accumulation of lipid globules in the cytoplasm, a reduction in

[1] This chapter corresponds with a paper, by Oosterhaven, K., K.J. Hartmans and J.J.C. Scheffer, published in Potato Res. 38: 211-222 (1995).

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 and Muller 1976). In these studies a very high concentration of monoterpenes was used. Pauly *et al.* (1981) described the uncoupling effect of low concentrations of ß-pinene on the photosynthetic electron transport. Attempts to produce monoterpenes in more than trace amounts using plant cell cultures failed so far, probably because of the toxicity of the products (Brown *et al.* 1987).

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

In our research on the application of S-carvone as a sprout inhibitor for potatoes we are interested in the mechanism by which carvone acts as inhibitor (see Chapter 4). This chapter deals with a potato sprout model system by means of which the (different) effects and the bioconversion of R-carvone and S-carvone are studied.

3.2 Materials and methods

Chemicals R-carvone, S-carvone and the samples of dihydrocarvone, dihydrocarveol and dihydrocarvyl acetate were as described in Chapter 2. ^{13}C-labelled methyltriphenylphosphonium iodide ($Ph_3P^{13}CH_3I$) was from Janssen Chimica. All reagents were of analytical grade.

Potato sprout growth In order to study the effect of carvone on a growing system related to potato, we used the model system of potato eye pieces as described in Chapter 2. Two Petri dishes, each with 25 µl, 75 µl or 125 µl of the carvone enantiomer to be tested were placed on top of the perlite layer in the containers. The sprouts, from seed potatoes (*Solanum tuberosum* L. cv. Bintje), were treated during several days, whereafter they were harvested for further examination. Some sprouts were placed on fresh perlite in order to

study a possible regrowth of the sprouts. The growth was determined by measuring the 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 suspension cultures was determined according to Van der Valk *et al.* (1988). S-carvone was added in various concentrations up to 1.5 mM, the cell suspension cultures were incubated for 4 days at 20°C on a gyratory shaker in the dark, and at several time intervals, protoplasts were isolated from small samples of the cell 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 and Dyer (1959) was used for extraction of the monoterpenes present. The potato material was cut in small pieces and 50.0 g were homogenized using a Warring blender with 100 ml methanol and 50 ml chloroform for 2 min, after which another 50 ml chloroform were added. After homogenization for 30 s, 50 ml water were added with a subsequent 30 s homogenization. The homogenate was filtered through glass-microfiber filters, and the chloroform phase was separated from the aqueous phase. The chloroform phase was then dried over anhydrous Na_2SO_4 and concentrated to 5 ml using a rotary evaporator. The extract was subsequentely analysed by gas-liquid chromatography, using naphthalene as an internal standard. Control sprouts did not contain any S-carvone nor its bioconversion products. Concentrations were expressed as mg kg^{-1} fr. wt (ppm).

GC analysis The chloroform extracts, containing carvone and its conversion products, were analysed by GC using a CP-Sil 5cb column (WCOT, 25 m x 0.32 mm i.d.; film thickness 0.20 µm; Chrompack). GC conditions were as follows; oven temperature programmed: 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. Identification of the compounds was performed by comparison of retention times, using standards, and by GC-MS. Spectra were taken as electron impact spectra (70 eV).

Carvone concentrations in the atmosphere were determined by taking a 10 ml headspace sample with a syringe, from the container with the potato

sprouts. The sample was passed over Tenax adsorbens (Chrompack). 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 then analysed by GC using a CP-Sil 19cb column (WCOT, 25 m x 0.32 mm i.d.; film thickness 0.20 µm; Chrompack).

Synthesis of [^{13}C]S-(+)-carvone The compound 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 MeOH-dichloromethane (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 diethyl ether (Et_2O). The combined Et_2O layers were washed twice with water, once with NaCl-satured water, and then dried over anhydrous $MgSO_4$. The mixture was submitted to column chromatography over silica gel and eluted with ethyl acetate-petroleum ether (30:70, v/v).

The second step involved the exchange of the introduced oxygen for ^{13}C. A sample of 2.5 g $Ph_3P^{13}CH_3I$ was dissolved in 25 ml dry tetrahydrofuran (THF); then 4 ml 1.6 M *n*-butyl-lithium (in *n*-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 ethyl acetate-petroleum ether (10:90, v/v). The overall yield was 47%.

NMR analysis [^{13}C]S-carvone was added to the containers with the potato sprouts which were extracted as described above. The aqueous phases were lyophilized and dissolved in 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 $CDCl_3$ (77.1 ppm).

Table 3.1 Effects of R-carvone and S-carvone on length, fresh weight, and dry matter content of potato sprouts after 7 days of treatment.

Treatment	Length [1]	Fresh weight (g sprout^{-1})	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[2]
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] expressed as ratio of the length at t = 0
[2] ND = not determined

3.3 Results and discussion

Potato sprout growth inhibition

R- and S-carvone showed a concentration dependent effect on the sprout growth in the potato sprout model system (Table 3.1). Addition of 250 µl S-carvone led to a strong inhibition of the sprout 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 that of the control. 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 carvone exposure, the elongation of the potato sprouts was inhibited (Fig. 3.1). Exposure of the sprouts to S-carvone resulted in an earlier growth inhibition than exposure to R-carvone (Figs 3.1B *vs* 3.1C). The inhibition was reversible, because removal of the carvone vapour led to regrowth. It seemed that the effect of 2 days of S-carvone treatment was critical with respect to the regrowth, since the variance in regrowth was large, as some sprouts did show top growth whereas others did not. The same was true for R-carvone treated sprouts, but then only after 4 days of treatment.

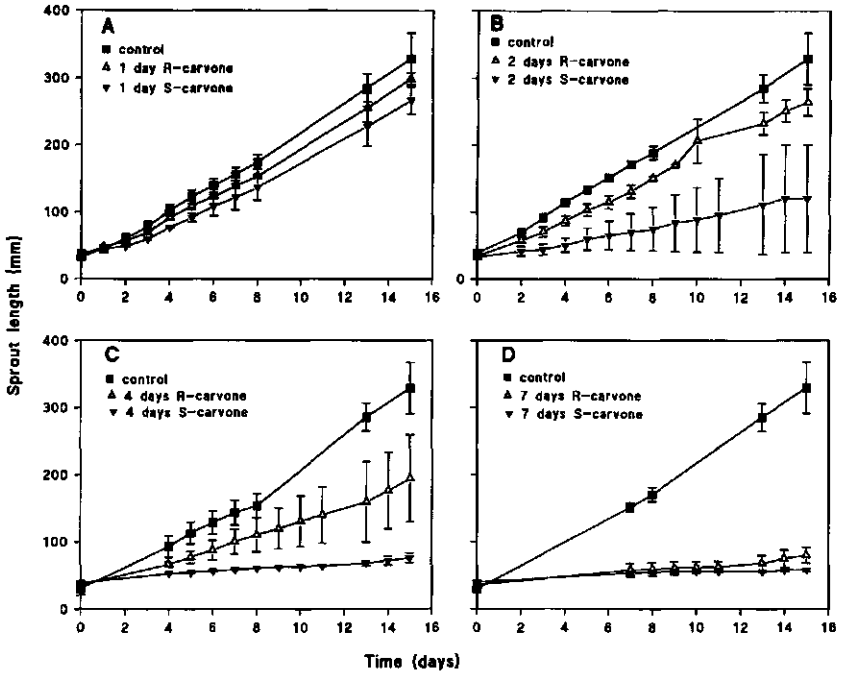

Fig. 3.1 Effects of R-carvone and S-carvone on potato sprout length. Potato sprouts were exposed to carvone vapours for 1, 2, 4 and 7 days (Figs A-D, respectively).

7 Days of exposure to either R- or S-carvone completely inhibited the sprout growth (Fig. 3.1D). In conclusion, R-carvone treated sprouts showed a later growth inhibition than sprouts exposed to S-carvone.

A carvone treatment at the concentrations applied did not result in necrotic tissues. The R- and S-carvone treated sprouts were thicker, just below the top, compared with the control sprouts, and they did not show the purple colour at the sprout tips of the controls. Such a swelling has also been described by Lorber and Muller (1980). They reported root tip swelling of *Allium cepa*, exposed to the vapour of macerated leaves of *Salvia leucophylla* (mainly consisting of camphor, 1,8-cineole, α-pinene, camphene and ß-pinene), and a reduced mitotic activity in the root tips. However, by microscopic analysis of the sprout tips we did not observe 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. 3.2). Flow cytometric analysis of relative DNA contents from the

control and the S-carvone treated cell suspensions showed similar DNA contents in the G_1 and G_2 phases during 4 days of treatment. This indicates that the cell cycle is not arrested after S-carvone treatment.

The sprouts were able to recover after the carvone treatment, showing either continued elongation 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 the S-carvone treated sprouts. Sprouts which had been exposed for 4 days to S-carvone were completely without further elongation and showed in all cases lateral sprouts, whereas only 32% of the R-carvone treated sprouts showed branching; the remaining 68% of the sprouts showed a continued elongation. Control sprouts did not branch at all (Table 3.2). This indicates a loss of apical dominance due to the carvone treatment.

Besides the effect on the sprout length, there was a pronounced effect of both carvone enantiomers on the root growth in the same experiment. Only a small root system developed as a result of the carvone exposure (Table 3.3), but after stopping the treatment a normal growth of the root system was observed.

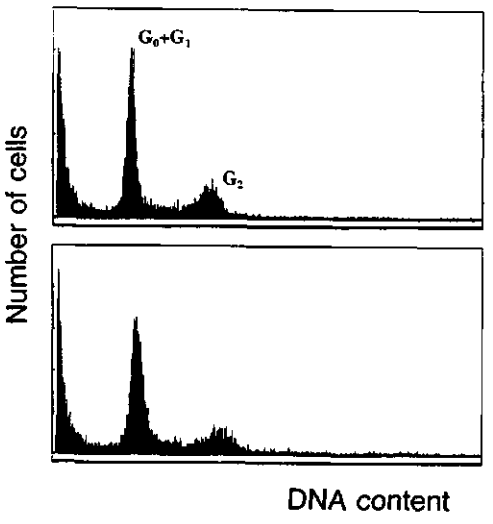

Fig. 3.2
Flow cytometric histograms showing the relative DNA content of nuclei per cell (arbitrary units), isolated from control (upper graph) and S-carvone (lower graph) treated potato cell suspensions, after 28 h. G_0, G_1 and G_2 refer to the nuclear stages of cell division: G_0 and G_1, cells with 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.

Table 3.2 Effects of R-carvone and S-carvone on the development of lateral sprouts, determined 7 days after various days of treatment.

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

However, the effect of carvone on rooting depended on the physiological age of the tubers used. Sprouts of six months-old tubers showed almost no rooting, whereas sprouts from young tubers did show some rooting following a carvone treatment.

The observation that a 4 days R-carvone treatment caused a similar growth inhibition as 2 days S-carvone, as mentioned above, might be explained by a difference in the concentration of both enantiomers in the potato sprouts (Figs 3.3A and B). 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 sprouts. 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), S-carvone being the more effective compound. 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, the physical properties of both compounds are similar, thus R-carvone is not less volatile than S-carvone. The quantitatively different effect of R-carvone and S-carvone on apple seed germination as described by Reynolds (1987) was explained by stereospecific effects on a molecular level. However, an alternative explanation may be that the different effects of both enantiomers, as also found in our experiments with potato sprouts, are due to a different uptake of the compounds in the seeds. This means that enantioselective effects may already play a role at the level of the uptake of carvone. It seems that the uptake of R-carvone is slower than that of S-carvone.

Table 3.3 Effects of R-carvone and S-carvone on the growth of potato roots and sprouts. Eye pieces with one sprout each were isolated from three months-old tubers.

Exposure time	Control		R-Carvone		S-Carvone	
	fresh weight per eye piece (g ± SE)					
(days)	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)

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 also form the basis of the explanation for the differences observed after the treatments with the carvone enantiomers.

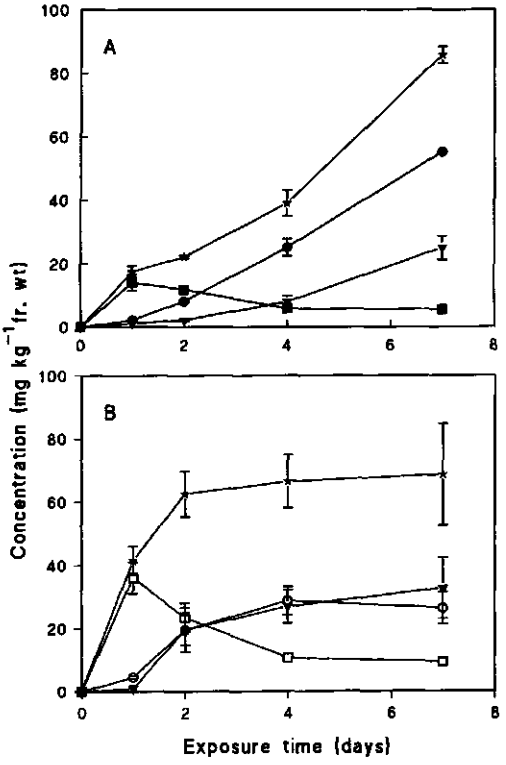

Fig. 3.3
Bioconversion of R-carvone (A) and S-carvone (B) by potato sprouts.
A: ■ = R-carvone; ● = neodihydrocarveol;
B: □ = S-carvone; ○ = neoisodihydrocarveol;
A and B: ▼ = rest (volatiles consisting mainly of dihydrocarvone isomers, dihydrocarveol isomers, and traces of dihydrocarvyl acetates and hydroxylated compounds); ★ = sum of the conversion products.

Table 3.4 Volatile compounds, and their mass spectrometric (MS) characteristics, isolated from potato sprouts after 4 days of exposure to [^{13}C]S-carvone.

Compound	M,	Characteristic m/z fragments (and their relative intensity)[1]
Dihydrocarvone (*trans*-isomer)	152	41(40), 68(100), 96(66), 110(30), 153(15)
Dihydrocarvone (*cis*-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] underlined values 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 treated and [^{13}C]carvone treated sprouts

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 levels up to 85 mg kg^{-1} fr. wt after 7 days (Fig. 3.3). R- and S-carvone were converted into more reduced compounds (Figs 3.3A and B). The conversion was stereoselective: R-carvone was mainly converted into neodihydrocarveol, and S-carvone into neoisodihydrocarveol. Besides these compounds, some other compounds were 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 added to the system. Table 3.4 shows the mass spectral characteristics of the carvone derivatives isolated after 4 days of [^{13}C]S-carvone exposure.

The structure of three compounds could not be elucidated. One of these compounds was characterized by a molecular weight of 168. After silylation of the chloroform extracts of the sprouts containing the volatile compounds, this particular compound showed a shift in retention time

comparable to the shifts of the compounds that were known to have a hydroxyl group. This clearly indicated 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 comparison of 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 chloroform extracts as well as the aqueous phases obtained during the extraction were also subjected to ^{13}C-NMR spectroscopic analysis. The spectra of the chloroform-soluble compounds from the S-carvone treated and [^{13}C]S-carvone treated potato sprouts are shown in Figs 3.4A and 3.4B, 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 3.4C and 3.4D) 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 chloroform and the third chloroform extract did not contain any carvone, dihydrocarvone nor 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 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, the *N. tabacum* cell suspensions were not able to hydroxylate or acetylate R- and S-carvone, whereas the potato sprouts in our experiments did.

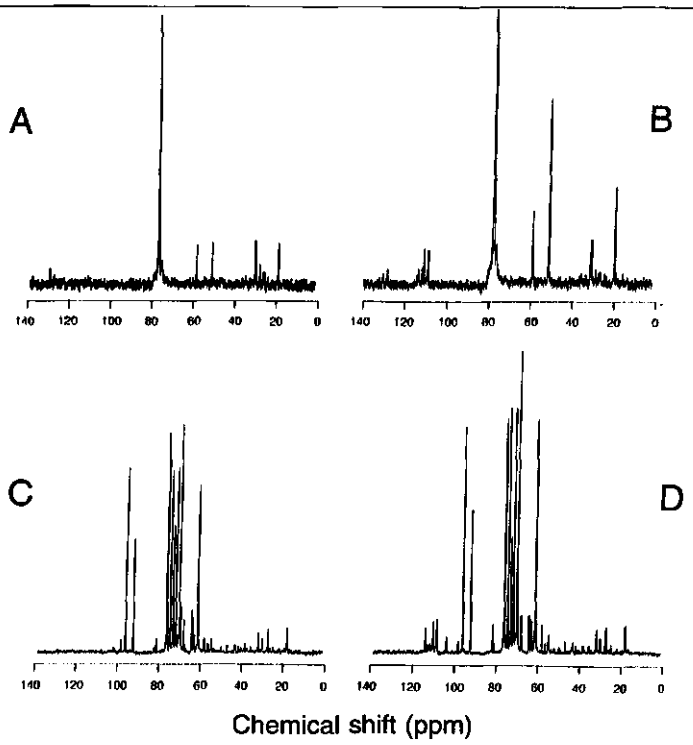

Fig. 3.4 ^{13}C-NMR spectra of chloroform extracts from potato sprouts treated with S-carvone (A) and [^{13}C]S-carvone (B), and ^{13}C-NMR spectra of aqueous phases of extracts from potato sprouts treated with S-carvone (C) and [^{13}C]S-carvone (D). Note the peaks in the range between 100 and 120 ppm (B) indicating the presence of water-soluble [^{13}C]S-carvone derived compounds.

Effect of carvone conversion products on potato sprout growth

As we found a number of conversion products of carvone in the potato sprouts, e.g. dihydrocarvone (*trans*-isomer), dihydrocarveol and dihydrocarvyl acetates, we also studied their possible effect on potato sprout growth in our model system. The sample of dihydrocarvone inhibited the sprout growth in the same way as S-carvone, whereas dihydrocarveol did not (Table 3.5). However, the concentration of dihydrocarveol in the sprouts as well as in the atmosphere was very low (Table 3.5). So a conclusion with regard to the effect of dihydrocarveol on sprout growth can not be drawn.

Dihydrocarvone was mainly converted into neodihydrocarveol. The stereochemistry of the conversion could not be determined in detail as the sample applied did also contain the *cis*-isomer (= isodihydrocarvone).

Table 3.5 Effects of dihydrocarvone and dihydrocarveol (250 µl per container) on potato sprout growth.

	Control	Dihydrocarvone[1]		Dihydrocarveol[2]		
Time days	Length[3]	[Residue][4] (mg kg^{-1})	[Air][5] (µg l^{-1})	Length[3]	[Residue][4] (mg kg^{-1})	[Air][5] (µg l^{-1})	Length[3]
0	1.00	-	-	1.00	-	-	1.00
1	1.05	54 (10)	ND[6]	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] 80% *trans*-isomer and 20% *cis*-isomer
[2] mixture of isomers containing >80% of dihydrocarveol
[3] expressed as ratio of the length at t = 0
[4] residual content of the compound tested, including the conversion products (SE indicated in parentheses)
[5] concentration of the compound tested, in the headspace of the container (SE indicated in parentheses)
[6] ND = not determined

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

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

Conclusions

Some authors (Asplund 1968; Lorber and Muller 1976, 1980; Reynolds 1987) have reported on the mode of action of monoterpenes. In none of these reports attention has been paid to the actual concentration in the tissue and

the possible bioconversion of the compounds tested. The study described here shows that R-carvone and S-carvone are readily reduced, and that the concentration of these compounds in the tissue is not just related to diffusion but also depends on the structure of the compound. Although the concentration of the carvones in the atmosphere around the sprouts was similar, S-carvone was found earlier in the sprouts than R-carvone. Thus differences in growth inhibition or germination inhibitory effects may also be due to a derivative (or even more than one) of the applied compound, and the concentration of the compound added to the atmosphere may not be strictly related to the concentration in the tissue. In addition to the reduced compounds, water-soluble compounds were found. Future research will be focussed on the identification of these products.

Acknowledgements

We thank Mr H. Zwarts and Dr B. Jansen (Department of Organic Chemistry, Wageningen Agricultural University) for the synthesis of [^{13}C]S-carvone, Mr P. van der Meer (ATO-DLO) for performing the NMR analysis, and Dr H.A. Verhoeven (CPRO-DLO, Wageningen, The Netherlands) for performing the flow cytometric analysis.

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Caraway (*Carum carvi* L.) grown in the field .

CHAPTER 4[1]

Inhibition of potato sprout growth by S-carvone: reduction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity without effect on its mRNA level

Summary The effect of S-carvone on potato (*Solanum tuberosum* L. cv. Bintje) sprout growth was studied in a semi *in vivo* system using potato eye pieces. S-carvone inhibited the sprout growth within 2 days. An extensive loss of the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; E.C. 1.1.1.34), a key enzyme in the mevalonate pathway, occurred concomitantly with the inhibition of sprout growth. The enzyme activity decreased to less than 3% of the control after 4 days of treatment. The loss of HMGR activity could not be attributed to a direct effect of S-carvone on the enzyme nor to an effect on *in vitro* translation processes. Northern analysis of the mRNA population at various time intervals during the treatment, using a specific HMGR potato probe, revealed a considerable hybridization signal after 4 days of treatment. This indicated that the effect of S-carvone on the activity of HMGR in potato sprouts is not regulated by transcriptional events, but by as yet unknown mechanisms.

4.1 Introduction

Monoterpenes which are found in a wide range of higher plants are the main components of many essential oils. Although the function of monoterpenes in plants is not completely understood, there is considerable evidence that they play a role in the interaction with other plants (Fischer 1986).

In general, allelochemicals have been evaluated for their impact on seed germination or some other aspects of plant growth and development. It was shown that several compounds, including monoterpenes, can inhibit the

[1] This chapter corresponds with a paper, by Oosterhaven, K., K.J. Hartmans and H.J. Huizing, published in J. Plant Physiol. 141: 463-470 (1993).

germination of seeds (Reynolds 1987). Some monoterpenes, like S-carvone, have been suggested for application as volatile sprout suppressants for potatoes (Meigh 1969; Beveridge *et al.* 1981). The mechanism of the sprout suppression or the germination inhibition is not known. However, some specific effects of particular monoterpenes on plants and animals have been described.

Lorber and Muller (1976, 1980a, 1980b) reported anatomical and physiological changes in various herb seedlings, following exposure to the volatiles, predominantly camphor and cineole, emanating from the leaves of *Salvia leucophylla* L. They found amongst others a drastic reduction in the number of a variety of intact organelles, including mitochondria, and a disruption of membranes surrounding nuclei and mitochondria. Mitotic activity was reduced, and hyperconcentration and breakage of chromosomes were observed (Lorber and Muller 1980a). Cineole (most probably 1,8-cineole is meant), in a concentration of 5 mM, was found to reduce the mitochondrial activity of *Avena fatua* L. (Lorber and Muller 1980b). Pauly *et al.* (1981) described the uncoupling effect of ß-pinene on photosynthetic electron transport of isolated chloroplasts. Furthermore, attempts to produce monoterpenes in more than trace amounts using plant cell cultures failed so far, probably because of the toxicity of the products (Brown *et al.* 1987). *In vitro* studies have shown that most cell lines either do not accumulate monoterpenes or accumulate them at very low levels.

An indication of the possible mode of action of S-carvone on the molecular level comes from animal studies. In rats, cyclic monoterpenes like cineole (most probably 1,8-cineole) or menthol, reduced the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (mevalonate: NADP oxido-reductase, CoA acylating, E.C. 1.1.1.34) (Clegg *et al.* 1982). This enzyme catalyses the rate-limiting reaction in the mevalonate pathway (Goldstein and Brown 1990). Mevalonic acid, the product of the reaction, is the precursor of a large number of terpenoids and derivatives thereof, which are vital for diverse cellular functions ranging from cholesterol biosynthesis to growth control.

Mevalonate starvation of an animal cell line, impaired in the mevalonate synthesis, resulted in a loss of protein synthesis (Sinensky and Logel 1985) and an arrest in cell cycling (Siperstein 1984), indicating the vital role of HMGR in cell growth and development.

In plants, HMGR is a key enzyme controlling the synthesis of phytoalexins, plant hormones like abscisic acid, gibberellins and cytokinins as well as chlorophyll, tocopherol, phylloquinone, ubiquinone, plastoquinone and carotenoids. HMGR activity can be regulated by phytochrome, herbicides, phytohormones, feedback mechanisms, and endogenous protein factors (Bach 1987). Bach and Lichtenthaler (1983) showed that the activity of HMGR in plants is crucial for growth and development, as treatment of radish seedlings with mevinolin, a potent HMGR inhibitor, inhibited the root elongation completely.

With respect to the possible application of S-carvone as a potato sprout suppressant, this chapter deals with the effect of S-carvone on potato sprout growth and on the HMGR activity in potato sprouts.

4.2 Materials and methods

Chemicals L-[^{35}S]methionine (37 TBq mmol^{-1}), [^{35}S]dATP (37 TBq mmol^{-1}), 3-hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A (1.92 GBq mmol^{-1}) were obtained from Amersham, and R,S-HMG CoA from Sigma. Restriction enzymes, DNA-modifying enzymes and nucleic acids were purchased from commercial sources. All reagents were of analytical grade.

Bacterial strains and media SURE-strains (Stratagene Inc.) served as hosts for pGEM5 Zf(+)-plasmids (Promega) and were grown on 2 x TY medium (Maniatis *et al.* 1982).

Potato sprout growth Using the model system of potato eye piece as described in Chapter 2, sprouts of seed potatoes (*Solanum tuberosum* L. cv. Bintje) were treated with S-carvone by placing two Petri dishes each with 125 µl S-carvone on top of the perlite layer. The containers were placed in the dark at 15°C. For inhibition of the *de novo* protein synthesis, 30 potato eye pieces were placed on perlite soaked with 500 ml cycloheximide solution (0.1 mM).

Cell fractionation The cell fractionation was carried out according to Oba *et al.* (1985), at 4°C under dim green safety light using six sprouts (1.5-3 g fresh

weight) in total. The sprouts were harvested and homogenized in a precooled mortar on ice with 2.5 volumes of homogenization buffer: 0.2 M potassium phosphate buffer (pH 7.5), 0.35 M sorbitol, 5 mM $MgCl_2$, 10 mM EDTA. Prior to use, 2-mercaptoethanol and insoluble PVPP were added to final concentrations of 20 mM and 5% (w/v), respectively. The slurry was squeezed through two layers of Miracloth filter, and the homogenate centrifuged at 1,200 *g* for 10 min. The supernatant was centrifuged for 40 min at 16,000 *g* to collect the organelle fraction; the microsomal fraction was obtained by subsequent centrifugation of the supernatant at 105,000 *g* for 1 h. Both pellets were resuspended in 0.5 ml resuspension buffer: 33 mM potassium phosphate buffer (pH 7.5) and 42 mM DTT. The resuspended fractions were frozen in liquid N_2 and stored at -80°C until further use.

Protein was estimated by the Pierce Protein Assay Reagent Kit (Rockford, ILL., USA) using bovine serum albumin (BSA) as a standard.

HMGR assay The assay was carried out as described by Stermer and Bostock (1987), with some modifications. The assay tubes contained 0.03 mg protein, 28.6 mM potassium phosphate buffer (pH 7.5), 7.4 mM DTT, 3.6 mM EDTA, 140 µg BSA, 0.57 mM $NADP^+$, 2.9 mM glucose-6-phosphate, 0.1 U glucose-6-phosphate dehydrogenase, 15 µM HMG CoA and 4.8 µM 3-hydroxy-3-methyl[3-^{14}C]glutaryl coenzyme A in a final volume of 100 µl. The assay was started by the addition of HMG CoA, and continued at 30°C for 20 min. In this assay system the reaction was linear for 45 min, and to a maximum of 0.1 mg protein. The reaction was terminated by the addition of 15 µl 3N HCl; subsequently the solution was allowed to stand at room temperature for at least 30 min to ensure a complete lactonization of the [^{14}C]mevalonate formed. The solution was then centrifuged for 5 min at 12,000 *g* to remove protein, and 25 µl of the supernatant was applied to silica gel (G1500) thin-layer plates (Shapiro *et al.* 1974). The plates were developed in chloroform-acetone (2:1, v/v). The area from Rf 0.45 to 0.7 was scraped off directly into a scintillation vial and counted.

Control experiments consisted of assays supplemented with 15 µl 3N HCl at t = 0, and assays without the NADPH-generating system.

RNA isolation The distal parts of potato sprouts were quickly frozen with liquid N_2 and then used for the isolation of total RNA as described by De Vries

et al. (1982). Poly(A^+) RNA was purified with HyBond messenger affinity paper (Amersham) according to the manufacturer's protocol.

Probe construction A cDNA HMGR gene fragment was obtained from genomic DNA using the polymerase chain reaction (PCR). First strand cDNA synthesis was carried out using Superscript MuMLV reverse transcriptase (BRL) on 0.5 µg poly(A^+) RNA, isolated from 'Bintje' potato sprouts. The oligonucleotides 5'-GGIGAT/CGCIATGGGIATGAA-3' and 5'-CCITGIC/TAICCICCICCITG-3', representing the 5' and 3' primer sequences, respectively, were synthesized with a Pharmacia GeneAssembler Plus. The fragments resulting from 30 PCR cycles starting from 1 µg 'Bintje' DNA and 1 min denaturation at 94°C, 1.5 min annealing at 50°C and 2 min polymerization at 72°C, were separated on a 1.5% agarose gel. The expected PCR product was purified from gel with QIAEX (QIAGEN Inc.), and phosphorylated with T4 polynucleotide kinase. After the kinase treatment, a 20-fold molar excess of adaptor, consisting of the oligonucleotides 5'-TC?GCGGCCGCT-3' and 5'GCGGCCGCG?-3', was ligated with the PCR fragment using a 3'dA present on many PCR fragments. Part of the ligation mixture was used for a second PCR reaction, employing the surplus of adaptors as primers. The resulting fragments were isolated, and digested with *NotI*, and inserted into alkaline phosphatase treated pGEM5-Zf(+). The ligation products were transformed into *E. coli* strain SURE (Stratagene Inc.) by electroporation.

Northern analysis Total RNA (10 µg per lane) was glyoxylated, and separated by electrophoresis on a 1.5% agarose gel (Thomas 1983). The separated RNAs were blotted onto HyBond N-membranes in an overnight transfer using 10 x SSC as blotting solution (Maniatis *et al.* 1982). The filter was air-dried, UV-irradiated (1200 µJ 100 cm^{-2}; Stratagene Inc.), and finally deglyoxylated by boiling for 5 min in buffer consisting of 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

The RNA blot was hybridized with digoxigenin-UTP labelled RNA probes at 65°C; subsequently, chemiluminescent (AMPPD) detection of the RNA/RNA hybrids was carried out according to the manufacturer's protocol (Boehringer Mannheim). The optical density of the hybridization signals on Kodak X-ray film was determined with a scanning densitometer (LKB).

In vitro translation. About 0.5-1 µg poly(A$^+$) RNA was used for *in vitro* translation in a wheat germ lysate (Promega) supplemented with L-[^{35}S]-methionine, according to the supplier's protocol. S-carvone and cycloheximide were dissolved in DMSO and added with increasing concentrations (0.1 µM to 1 mM). In the control experiment only DMSO was used.

Proteins were separated by SDS-PAGE (Laemmli 1970) (12.5% running gel and 5% stacking gel) and visualized by autoradiography.

Sequencing The cloned fragments were sequenced by the dideoxy method (T7-Sequencing Kit, Pharmacia) according to Sanger *et al.* (1977) using double-stranded DNA templates and [^{35}S]dATP. The DNA sequences were analysed using the DNASIS program (Pharmacia) and an LKB digitizer.

4.3 Results

Potato sprout growth inhibition

The potato sprout growth was almost completely inhibited within 2 days after the start of the S-carvone treatment. The control sprouts (no exposure to S-carvone) grew to a length of 4 times the initial length in 5 days (Fig. 4.1). Renewed sprout growth occurred within 8 days after the S-carvone treated sprouts were placed in a container with fresh perlite, and in an atmosphere without S-carvone, indicating that the sprouts had not died following the treatment.

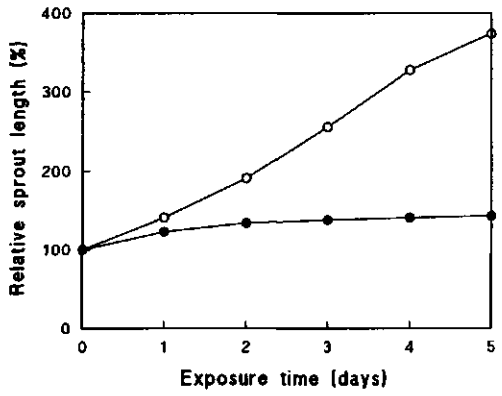

Fig. 4.1
Effect of S-carvone on potato sprout growth during incubation at 15°C in the dark.
○= control; ●= S-carvone.

A difference was observed with respect to the renewed sprout growth between physiologically young and physiologically old potatoes. The sprouts from eye pieces of the young potatoes (stored at 4°C for three months) developed new side sprouts just below the top of the sprout, whereas for the old potatoes (stored at 4°C for nine months) side sprouts developed, in general, at the basis of the sprout at the eye piece. Control sprouts grew linear without branching (see also Chapter 3, Table 3.2).

HMGR activity

The activity of HMGR in the potato sprouts was found in the 16,000 *g* and the 105,000 *g* pellets. The S-carvone treatment resulted in a dramatic decrease of the specific activity of the enzyme in both the 16,000 *g* (Fig. 4.2A) and the 105,000 *g* membrane fractions (Fig. 4.2B), whereas the specific activity of HMGR of the control remained almost constant over a 4 days period. This result was obtained in three repetitive experiments.

Cycloheximide treatment of the potato eye pieces gave an HMGR activity pattern similar to the S-carvone treatment, and led to a loss of enzyme activity within 4 days.

The reduction in specific activity, induced by S-carvone, could not be caused by a direct effect of S-carvone on the enzyme itself, because addition of S-carvone, at concentrations ranging from 1 µM to 0.01 M, in the HMGR assay system did not alter the enzyme activity.

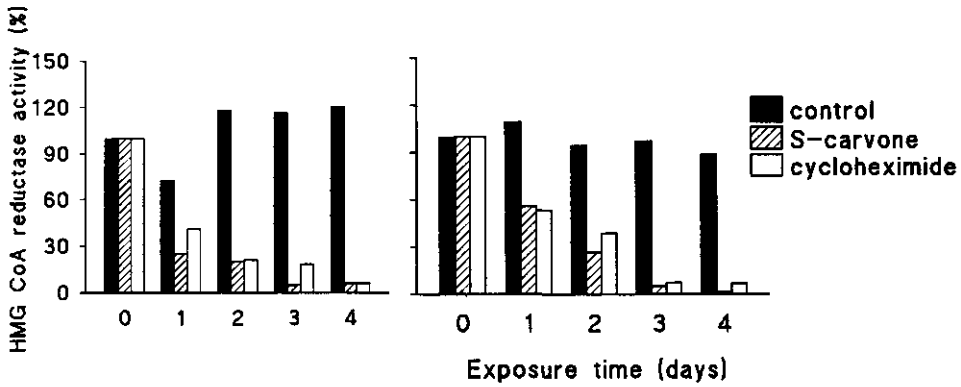

Fig. 4.2 The specific activity of HMGR in organelle (A) and microsomal (B) fractions following treatment of potato sprouts with S-carvone and cycloheximide. The activity is expressed as the percentage of enzyme activity of the control at day zero. A 100% activity corresponds with 30.0 nmol mevalonate h^{-1} mg^{-1} protein.

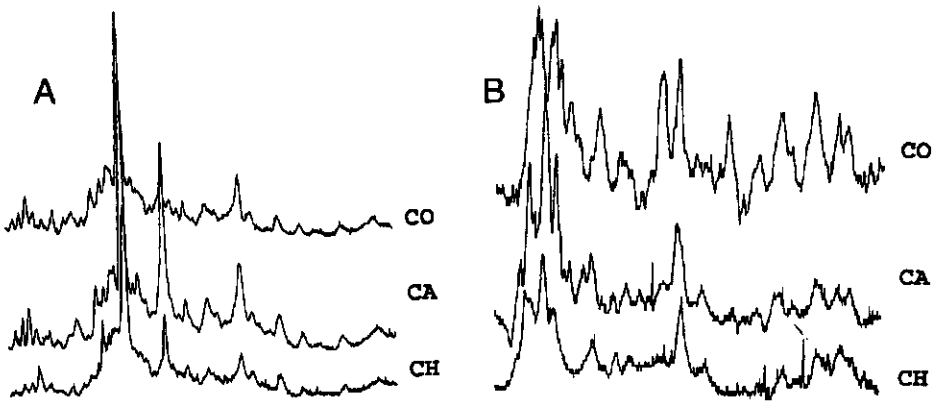

Fig. 4.3 Protein composition of the organelle (A) and microsomal (B) fraction of potato sprouts after 6 days of treatment with S-carvone and cycloheximide. Proteins were separated by SDS-PAGE, stained with Coomassie, and the lanes were scanned using a scanner.
CO = control at day 6; CA = S-carvone; CH = cycloheximide.

Membrane protein composition

The protein composition of the organelle fraction was not altered after the S-carvone treatment (Fig. 4.3A). The microsomal fraction, however, showed a loss of some proteins after 6 days exposure to S-carvone.

Cycloheximide treatment of the potato sprouts led to protein changes in the microsomal fraction which resembled those obtained by the S-carvone treatment (Fig. 4.3B). The protein composition in the organelle fraction did not significantly alter after the cycloheximide treatment.

***In vitro* translation**

Since S-carvone appeared to mimic the effects of the protein synthesis inhibitor cycloheximide, its effect on *in vitro* translation was investigated. Fig. 4.4B shows that S-carvone did not affect the translation in the way cycloheximide did (Fig. 4.4C). DMSO, the solvent of S-carvone and cycloheximide, did not influence the *in vitro* translation (Fig. 4.4A). Addition of cycloheximide to the *in vitro* translation system inhibited the translation at a concentration of 1.0 μM.

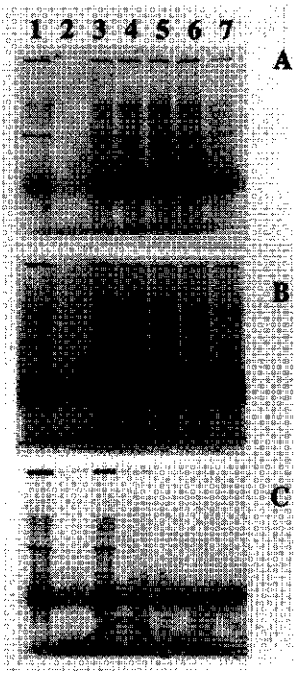

Fig. 4.4
Autoradiogram of labelled proteins after *in vitro* translation, using wheat germ lysate.
A: solvent (DMSO, lanes 3-7 of A, B and C contain the same amount of DMSO);
B: S-carvone (concentrations as indicated below);
C: cycloheximide (concentrations as indicated below).
Lane 1 = positive control (poly(A$^+$) RNA added);
lane 2 = negative control (no poly(A$^+$) RNA added);
lane 3 = 0.1 µM;
lane 4 = 1 µM;
lane 5 = 10 µM;
lane 6 = 0.1 mM;
lane 7 = 1 mM.

Transcription analysis

A 470 bp cDNA fragment representing the C-terminal part of the HMGR gene was obtained using the PCR technique with primers deduced from comparison of heterologous HMG CoA sequences from animal (Rajkovic *et al.* 1989) and plant (*Arabidopsis thaliana*) origin (Learned and Fink 1989). Comparison of the aligned sequences of the potato and *A. thaliana* genes showed 77% homology at the nucleotide level and 83% homology for the corresponding amino acid sequences (Fig. 4.5).

The cDNA clone cpotHMG was used as a probe in hybridization experiments with total RNA extracted from S-carvone treated and untreated potato sprouts. Slot blot hybridization revealed that the HMGR mRNAs were present in considerable amounts throughout the duration of the S-carvone treatment. In Fig. 4.6 the change in mRNA signal is compared with the decrease in specific activity of HMGR in the microsomal fraction of S-carvone treated sprouts. After 4 days of treatment, the specific activity had decreased to 3% of the control, but the mRNA content was almost twice the control value.

```
base sequence
pot       GCGGCCGCTGGGGACGCCATGGGGATGAACATGGTTTCCAAAGGTGTACAGAACGTTCTT
ara 1072            GGTGATGCTATGGGGATGAATATGGTTTCTAAAGGTGTGCAGAATGTTCTT

pot       GATTACCTTCAGAATGAGTACCCCGACATGGACATCATCGGCATATCTGGGAACTATTGC
ara 1123  GAGTATCTTACCGATGATTTCCCTGACATGGATGTGATTGGAATCTCTGGTAACTTCTGT

pot       TCGGACAAGAAACCAGCAGCAGTTAATTGGATTGAGGGGAGAGGAAGTCAAGTAGTTTGT
ara 1183  TCGGACAAGAAACCTGCTGCTGTGAACTGGATTGAGGGACGTGGTAAATCAGTTGTTTGC

pot       GAGGCAATCATCAAGGAAGAGCTGGTGAAGAAAGTTCTGAAAACTAGGGTTGCTACTCTA
ara 1243  GAGGCTGTAATCAGAGGAGAGATCGTGAACAAGGTCTTGAAAACGAGCGTGGCTGCTTTA

pot       GTTGAGCTGAACATGCTTAAAAACCTCACTGGCTCCGCCATGGCTGGTGCACTTGGTGGC
ara 1303  GTCGAGCTCAACATGCTCAAGAACCTAGCTGGCTCTGCTGTTGCAGGCTCTCTAGGTGGA

pot       TTCAACGCCCATGCCAGCAATATTGTCTCAGCTGTATATTTAGCCACTGGCCAGGACCCG
ara 1363  TTCAACGCTCATGCCAGTAACATAGTGTCTGCTGTATTCATAGCTACTGGCCAAGATCCA

pot       GCTCAAAATATTGAGAGCTCTCACTGCATCACTATGATGGAGGCTGTAAATGATGGCAAG
ara 1423  GCTCAAAACGTGGAGAGTTCTCAATGCATCACCATGATGGAAGCTATTAATGACGGCAAA

pot       GACCTCCATATTTCCGTCACCATGCCTTCAATCGAGGTGGGCACCGTCGGCGGCACA-
ara 1483  GATATCCATATCTCAGTCACTATGCCATCTATCGAGGTGGGGACAGTGGGAGGAGGA

          --notI--
pot       GCGGCCGC

amino acid sequence
pot       --notI---G  D  A  M  G  M  N  M  V  S  K  G  V  Q  N  V  L
ara  358            G  D  A  M  G  M  N  M  V  S  K  G  V  Q  N  V  L

pot       D  Y  L  Q  N  E  Y  P  D  M  D  I  I  G  I  S  G  N  Y  C
ara  375  E  Y  L  T  D  D  F  P  D  M  D  V  I  G  I  S  G  N  F  C

pot       S  D  N  P  P  A  A  V  N  W  I  E  G  R  G  K  S  V  V  C
ara  395  S  D  K  K  P  A  A  V  N  W  I  E  G  R  G  K  S  V  V  C

pot       E  A  I  I  K  E  E  L  V  K  K  V  L  K  T  E  V  A  T  L
ara  415  E  A  V  I  R  G  E  I  V  N  K  V  L  K  T  S  V  A  A  L

pot       V  E  L  N  M  L  K  N  L  T  G  S  A  M  A  G  A  L  G  G
ara  435  V  E  L  N  M  L  K  N  L  A  G  S  A  V  A  G  S  L  G  G

pot       F  N  A  H  A  S  N  I  V  S  A  V  Y  L  A  T  G  L  D  P
ara  455  F  N  A  H  A  S  N  I  V  S  A  V  F  I  A  T  G  Q  D  P

pot       A  Q  N  I  E  S  S  H  C  I  T  M  M  E  A  V  N  D  G  K
ara  475  A  Q  N  V  E  S  S  Q  C  I  T  M  M  E  A  I  N  D  G  K

pot       D  L  H  I  S  V  T  M  P  S  I  E  V  G  T  V  G  G  T
ara  495  D  I  H  I  S  V  T  M  P  S  I  E  V  G  T  V  G  G  G
```

Fig. 4.5 Nucleotide and deduced amino acid sequence of cpotHMG cDNA in comparison with the corresponding *Arabidopsis thaliana* HMGR gene fragment.

Northern blot analysis of total RNA showed that the transcript did not change in size after S-carvone treatment, indicating that no degradation products evolved (Fig. 4.7).

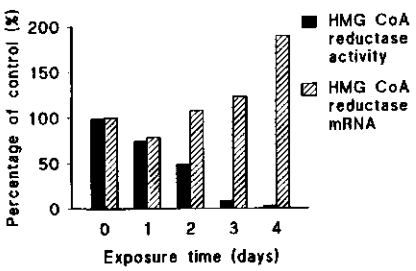

Fig. 4.6
Activity of enzyme and HMGR mRNA content in growing potato sprouts exposed to S-carvone. The data are presented as the percentage of the enzyme activity or mRNA concentration at day zero.

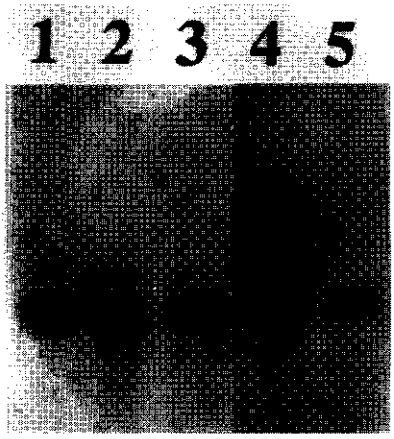

Fig. 4.7
Northern analysis of total RNA isolated from control and S-carvone treated potato sprouts.
Lane 1, 2 and 4 represent the HMGR mRNA of control potato sprouts after 0, 1 and 4 days respectively, lane 3 and 5 the mRNA content after 1 and 4 days exposure to S-carvone .

4.4 Discussion

Treatment of potato sprouts with S-carvone led to a growth inhibition within 2 days (Fig. 4.1) and was correlated with a decrease in HMGR activity in the organelle fraction as well as in the microsomal fraction (Fig. 4.2).

As HMGR is a key enzyme controlling the synthesis of many terpenoids, impairment of its activity could explain the observed phenomena. This statement is supported by studies from Bach and Lichtenthaler (1983) who showed that treatment of radish seedlings with mevinolin, a potent HMGR inhibitor, inhibited the root elongation and the development of lateral roots in etiolated seedlings. Mevinolin also reduced the elongation of hypocotyls in light-grown radish plants.

From animal systems, e.g. studies with Mev-1 cells, which have a block in the mevalonate-biosynthesizing enzyme HMG CoA synthase, it has become

evident that MVA starvation leads to a loss of DNA synthesis and of protein synthesis (Sinensky and Logel 1985), and to an arrest in cell cycling (Siperstein 1984).

Despite the indications that HMGR plays an important role in both animal and plant cells, it is still not clear how S-carvone regulates the HMGR activity in potato sprouts. There is no general toxicity of S-carvone towards the potato sprouts since the effect of the S-carvone exposure can be reversed by removal of S-carvone, which leads to renewed sprout growth.

In our experiments the loss of HMGR activity could not be attributed to a direct S-carvone inhibition of the enzyme, as adding S-carvone to an *in vitro* assay system showed no effect. Comparable data have been found in animal studies. Clegg *et al.* (1980) studied the effect of oral application of a variety of mono- and bicyclic monoterpenes in rats, and observed a specific hepatic HMGR activity reduction to 70%. These terpenes, or their major metabolites, did also not act as direct inhibitors of HMGR activity *in vitro* (Clegg *et al.* 1980). The inhibitory effect was still seen after solubilization of the enzyme from the microsomes. The reduction of the HMGR activity was not due to a general hepatoxicity, and they concluded that only oxygen-containing terpenes like menthol, borneol and 1,8-cineole, or terpenes such as limonene, which are rapidly converted into such compounds, are the inhibitory agents.

The cycloheximide treatment of the potato sprouts resulted in an altered protein composition of the microsomal membranes which resembled the protein pattern after the S-carvone treatment (Fig. 4.3B). The decreasing HMGR activity after the S-carvone or cycloheximide treatments showed a similar pattern (Fig. 4.2), indicating that the *de novo* synthesis of HMGR is blocked by the S-carvone treatment. However, S-carvone does not act like cycloheximide: *in vitro* translation processes are not inhibited by S-carvone (Fig. 4.4). Furthermore, the reduction in enzyme activity could not be related to a reduced HMGR mRNA level (Fig. 4.6).

Until now it is not clear whether the HMGR protein in S-carvone treated potato sprouts is present in an inactivated form or whether it is not translated from the mRNA. Apparently there are post-transcriptional control mechanisms that can modify the concentration of active HMGR.

The results from the cited animal studies concerning the influence of monoterpenes on HMGR are in agreement with the results presented in this chapter. Immunotitration of HMGR from terpene treated rats showed that the

activity loss was due to a smaller number of enzyme molecules, suggesting that the rate of enzyme synthesis or degradation had been altered. The precise action of monoterpenes on rat HMGR is not clear, but Clegg *et al.* (1982) supposed that the cyclic oxygen-containing monoterpenes acted as simple analogues of the inhibitory substituted cholesterol derivatives, and exerted their effect by an as yet unknown regulatory mechanism.

In animals, HMGR can be regulated at different levels. Phosphorylation and dephosphorylation is an important short term regulating mechanism controlling the inactivation and activation of HMGR respectively. Three different protein kinase systems have been described in this respect (Beg *et al.* 1987).

Long term control can be accomplished by changes in active-HMGR concentrations through transcriptional and post-transcriptional modifications as well as protein degradation.

Finally, the activity and degradation of HMGR can be changed by the microsomal membrane environment by altering the membrane fluidity (Davis and Poznansky 1987).

Only little is known about HMGR modulation in plants. Phosphorylation and subsequent inactivation of HMGR has been described for *Hevea brasiliensis* in *in vitro* experiments. Phosphorylation cannot be ruled out as an inactivating mechanism, but generally such a mechanism acts very quickly, within minutes or hours (Sipat 1982; Wititsuwannakul *et al.* 1990), whereas in our study the reduction in activity due to S-carvone treatment is in the order of days (Fig. 4.2).

Yang *et al.* (1991) described the effect of wounding and pathogen challenge on potato HMGR activity. The soft rot pathogen *Erwinia carotovora* as well as wounding induced increased HMGR mRNA levels followed by increased HMGR activity. But wounding induced another isogene of the HMGR family than the pathogen challenge. In our studies we used an HMGR probe from the highly conserved region of the gene, and therefore only general changes in HMGR transcripts will be detected. However, the almost complete loss of HMGR activity after the S-carvone and cycloheximide treatments, and presence of a clear HMGR mRNA signal on Northern blots after the S-carvone treatment indicate that the regulation is not at the level of isogenes.

Because of the lipophilic character of S-carvone, we suppose that it interacts with the membrane system of the plant cells, possibly by changing the membrane fluidity. The lipid micro-environment of HMGR might be altered after S-carvone treatment, resulting in an enhanced degradation and/or a disturbed insertion of the protein in the microsomal membrane.

Planned antibody-studies may answer the question whether the HMGR synthesis is inhibited during S-carvone treatment or whether the protein itself becomes inactivated.

Acknowledgements

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CHAPTER 5[1]

Inhibitory effect of S-carvone on wound healing of potato tuber tissue

Summary Wounded tuber tissue of potato (*Solanum tuberosum* L. cv. Gloria) exposed to S-carvone did show neither suberization nor cambium layer formation, whereas these processes started after 2-4 days in control tissue. Suberized tissue was clearly visible 24 days after the start of the S-carvone treatment, when the concentrations of S-carvone and its bioconversion products in the tissue were almost zero, but cambium layer formation had not yet started. The inhibition of wound healing coincided with a lack of induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; E.C. 1.1.1.34). The wounded potato tissue used as control, showed a transient induction of HMGR activity.

In S-carvone treated tissue, the activities of GR (glutathione reductase; E.C. 1.6.4.2) and AP (ascorbate peroxidase; E.C. 1.11.1.11) were induced, and the level of glutathione increased four- to five-fold.

5.1 Introduction

When plants are injured, they regenerate the injured surface by wound healing. Sliced tissue of potato tuber is a suitable model system to study this process as regards both the biochemical processes involved and the changes in chemical composition of the cell wall (Kolattukudy 1984). Elucidation of the processes involved in wound healing is of great economic importance since potatoes are sensitive to damage during mechanical harvesting. The wounded areas of potato tubers are ideal places for microbial attack, and they give rise to evaporation of water. Under optimal conditions, a period of two weeks suffices for completion of the wound healing (Burton *et al.* 1992). The result is a newly formed wound periderm, which consists of a specialized layer of cells arising from a cambium layer (Peterson *et al.* 1985). This is accompanied by

[1] This chapter corresponds with a paper, by Oosterhaven, K., K.J. Hartmans, J.J.C. Scheffer and L.H.W. van der Plas, published in Physiol. Plant. 93: 225-232 (1995).

suberization, which involves deposition of suberin within the cell wall and association of this polymeric material with waxes. The polymeric material appears to be primarily responsible for protection against pathogenic attacks, while the associated waxes provide an effective diffusion barrier (Kolattukudy 1984). Primary formation of suberin starts about 3 days after wounding, and is completed after 8 days. Secondary suberization, i.e. the suberization of the cells of the cambium layer, is completed within three weeks after wounding. However, the regulation of wound healing is causally poorly understood. Descriptively, potato tuber tissue reacts upon slicing with a dramatic increase in metabolic activity, including synthesis of proteins and RNA. For example, the level of polysomes increases immediately after slicing (Ishizuka and Imaseki 1989).

Abscisic acid (ABA) induces suberization by triggering a process that gives rise to the formation of a suberization-inducing factor (Cottle and Kolattukudy 1982; Kolattukudy 1984). An isoperoxidase associated with suberization was induced by ABA in potato cell cultures as well as in sliced potatoes. Another important change following wounding is the induction of HMGR (3-hydroxy-3-methylglutaryl coenzyme A reductase; E.C. 1.1.1.34) (Oba *et al.* 1985; Stermer and Bostock 1987; Yang *et al.* 1991; Choi *et al.* 1992). HMGR catalyses the conversion of 3-hydroxy-3-methylglutaryl CoA into mevalonic acid, a precursor of all terpenoid compounds, including among others phytoalexins, and plant hormones such as ABA and gibberellins. After slicing, the HMGR activity is maximal after 18 h and then it gradually decreases (Oba *et al.* 1985). The increased activity is due to a *de novo* synthesis of the enzyme. Elicitor treatment of the sliced tissue is followed by an increase of sesquiterpenoids, and HMGR appears to play a role in the complex regulation of the accumulation of sesquiterpenoid phytoalexins in potato (Oba *et al.* 1985; Stermer and Bostock 1987). Furthermore, HMGR isogenes are differentially activated by wounding or a pathogen attack (Yang *et al.* 1991; Choi *et al.* 1992).

S-carvone inhibits the growth of potato sprouts and reduces the activity of HMGR without affecting the HMGR mRNA-level (Chapter 4). S-carvone does not occur naturally in potato tubers. Because S-carvone can be used as a potato sprout inhibitor, it was found of interest to investigate the effect of S-carvone on wound healing in relation to its effect on HMGR.

In this chapter it is reported that S-carvone inhibits also wound healing. This may be caused by a lack of HMGR induction in potato wound tissue after exposure to S-carvone. Exposure of plants to environmental stress, caused for example by xenobiotics, usually leads to a marked change in glutathione metabolism (Alscher 1989). Since S-carvone can be regarded as a xenobiotic, it was also investigated whether glutathione contents did change in wound tissue of potato tuber exposed to S-carvone.

5.2 Materials and methods

Potato material Potato tubers (*Solanum tuberosum* L. cv. Gloria) were purchased from the local market, and stored at 4°C without sprout suppressants until further use. Tubers were cut in halves (transverse section from apex to base), and ten half tubers were placed on a stainless steel tray in a 20 l container. At time zero, two Petri dishes each containing 125 µl S-carvone (Merck) were placed in the containers with the material to be treated. Headspace concentrations were measured at each sampling date. In addition one Petri dish with about 25 g $Ca(OH)_2$ (technical grade) was placed in all containers to prevent a too high concentration of CO_2. The containers were closed, sealed with tape and then placed in the dark at 15°C. At each sampling date we analysed the half tubers from two containers with S-carvone, as well as the halves from two control containers (without S-carvone).

Sampling Tissue samples, about 3 mm thick, from the wound layer were taken in the dark with a potato peeler at different times. The samples (one sample from each container of both treated and untreated wounded potato tuber) were immediately frozen in liquid N_2, and then stored at -30°C until further biochemical analyses.

Staining procedures Suberization of the wound surface was assessed by microscopic analysis according to Vaughn and Lulai (1991), with some modifications. Freehand cross sections (minimal four per tuber) of the wound surfaces were cut with a razor blade from five tubers of each treatment (two containers with and two without S-carvone). The sections were stained: 60 min

in aqueous 0.1% (w/v) berberine-HCl (Sigma), 15 min in aqueous 0.5% (w/v) aniline blue, and a few min in 0.1% (w/v) $FeCl_3$ in 50% (v/v) aqueous glycerol. Between the staining treatments, the sections were washed with water. The sections were observed using a Zeiss microscope with excitation filter BP 450-490 (450-490 nm peak emission), chromatic beam splitter FT 510 (510 nm), and barrier filter LP 520 (520 nm).

Cell fractionation About 2.5 g frozen material was homogenized twice in an Ultra Turrax mixer for 30 s with 5 ml homogenization medium containing 0.2 M potassium phosphate buffer (pH 7.5), 0.35 M sorbitol, 5 mM $MgCl_2$ and 10 mM EDTA. Prior to use, 2-mercaptoethanol (20 mM, final concentration), insoluble PVPP (50 g l^{-1}) and PMSF (0.1 mM, final concentration) were added. The homogenate was squeezed through two layers of cheese cloth, and then centrifuged at 1,200 *g* for 8 min. The supernatant was centrifuged at 16,000 *g*, for 40 min, and the resulting pellet resuspended in 0.5 ml resuspension buffer containing 33 mM potassium phosphate buffer (pH 7.5) and 42 mM DTT. This resuspension is referred to as the organelle fraction. The 16,000 *g* supernatant was then centrifuged at 105,000 *g* for 90 min, and the resulting pellet resuspended in 0.4 ml resuspension buffer; this fraction is referred to as the microsomal fraction. Protein was estimated by the Pierce Protein Assay Reagent Kit (Rockford, ILL., USA) using BSA as a standard.

Enzyme assays HMGR was assayed as described in Chapter 4. GR was assayed by measuring the NADPH oxidation spectrophotometrically at 340 nm according to Foyer and Halliwell (1976). AP was measured according to Klapheck *et al.* (1990).

Samples were taken from two independent experiments, each with treated and untreated wound tissues of potato tuber. Extracts were made according to the descriptions cited, and enzyme determinations were performed in duplicate. Results are expressed as means ± SD.

Glutathione determination. Total glutathione, GSH and GSSG contents were essentially determined according to the specific enzyme method of Brehe and Burch (1976). Samples were taken from two independent experiments, each with treated and untreated wound tissue of potato tuber. Of each sample, about 2.5 g frozen material was homogenized for 1 min in an Ultra Turrax

mixer in 5 ml 2% (w/v) metaphosphoric acid, and then centrifuged at 6,000 *g*. The supernatant was diluted ten-fold in 0.28 M Na_2HPO_4 and this solution was used for further analysis. The sum of GSH and GSSG was estimated as follows: 0.8 ml of 125 mM potassium phosphate buffer (pH 7.5) containing 0.3 mM NADPH and 6.3 mM EDTA was mixed with 100 µl of a solution containing 6 mM 5,5'-dithiobis-(2-nitro)-benzoic acid, 125 mM potassium phosphate buffer (pH 7.5) and 6.3 mM EDTA, and with 100 µl of the ten-fold diluted potato extract. The reaction was started by the addition of 5 µl (0.5 U) GSH reductase (E.C. 1.6.4.2, type III; from baker's yeast, from Boehringer). The change in absorbance at 412 nm was recorded for 2 min. GSSG was determined after incubation of 1 ml of the ten-fold diluted potato extract with 40 µl 2-vinylpyridine for at least 1 h at 25°C. Then 100 µl of this mixture was assayed in the same way as described above. Total glutathione and GSSG contents were determined using a calibration curve with known amounts of GSH and GSSG. GSH was calculated from the difference in total glutathione and GSSG. Total glutathione, GSH and GSSG contents were calculated as nmol g^{-1} fr. wt, and expressed as means ± SD; the assays were performed in duplicate.

Gel electrophoresis SDS-PAGE (5% stacking gel and 12.5% running gel) was carried out using the discontinuous buffer system of Laemmli (1970). Proteins on the gels were stained by Coomassie.

Extraction of S-carvone and its conversion products The amount of S-carvone and its conversion products in the potato halves was determined using 50.0 g potato material in a chloroform/methanol extraction as described in Chapter 3. Samples were taken from two independent experiments with wound tissues of potato tuber treated with S-carvone. Control tubers did not contain any S-carvone nor its bioconversion products. The determinations were performed in duplicate. Concentrations were expressed as mg kg^{-1} fr. wt (ppm).

GC analysis The chloroform extracts, containing S-carvone and its conversion products, were analysed as described in Chapter 3. S-carvone concentrations in the containers were determined by taking a 10 ml headspace sample with a syringe, which was analysed as described before (Chapter 3).

5.3 Results

S-carvone inhibits wound healing

Potato tubers, wounded by cutting them in half, showed only little primary suberin formation after 2 days; however, suberization was clearly visible after 4 days and then went on until the end of the experiments (24 days) as was evident from the extension of the suberization to more cell layers, and the intensification of the fluorescence (Fig. 5.1A). Situated between the injured surface and parenchyma cells, and observed as rather small rectangular cells, the formation of a cambium layer was clearly visible after 7-8 days. Exposure of wounded potato tissue to S-carvone initially led to inhibition of both the primary formation of suberin and the formation of the cambium layer. After 12 days, some primary suberin formation occurred in about 40% of the tissue sections analysed; after 14 days, this had clearly occurred in about 70% and after 24 days in more than 95% of the sections analysed (Fig. 5.1B).

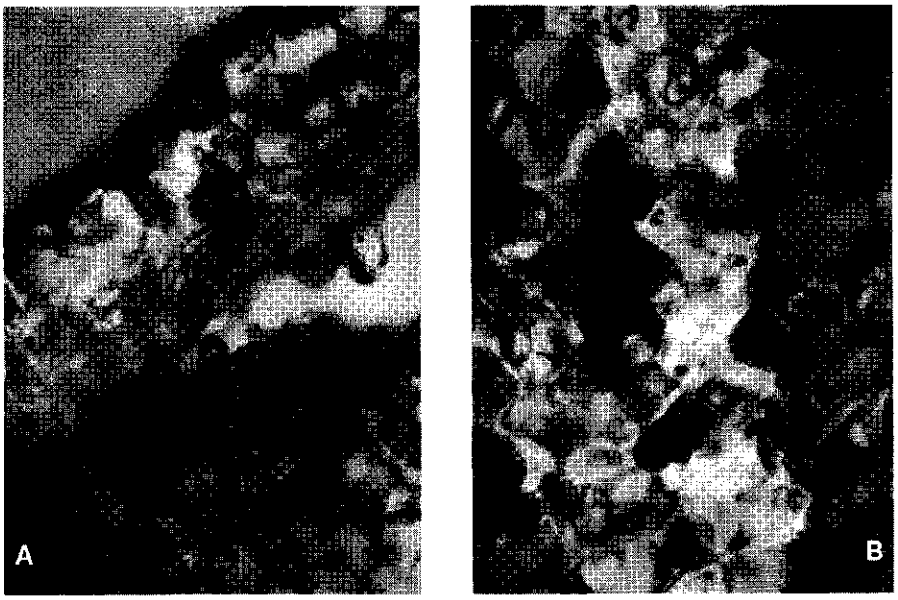

Fig. 5.1 Wound tissue of potato tuber after 24 days of wound healing. Control tissue (A) and tissue exposed to S-carvone (B). The fluorescent suberin cell layer was stained with berberine.

However, the cambium layer was still not present. Some cells looked like the brick wall structure that is characteristic for cambium cells, but an organized cambium layer such as seen in the control wound tissue (Fig. 5.1A) did not occur in the S-carvone treated tissue (Fig. 5.1B). In general, S-carvone delayed the suberin formation for about a week.

Bioconversion of S-carvone

After application as a liquid at time zero and volatilization, S-carvone was taken up by the wound tissue. In the tissue, S-carvone was converted into dihydrocarvone, isodihydrocarvone and neoisodihydrocarveol (Fig. 5.2A), and these compounds were partly released into the headspace (Fig. 5.2B). In containers with S-carvone but without wounded potato tubers, no conversion of S-carvone was found (results not shown). The S-carvone concentration in the headspace increased initially, and then decreased almost to zero after 14 days (Fig. 5.2B). Of the conversion products, mainly dihydrocarvone and isodihydrocarvone were released into the headspace, whereas neoisodihydrocarveol was the predominant compound in the tissue.

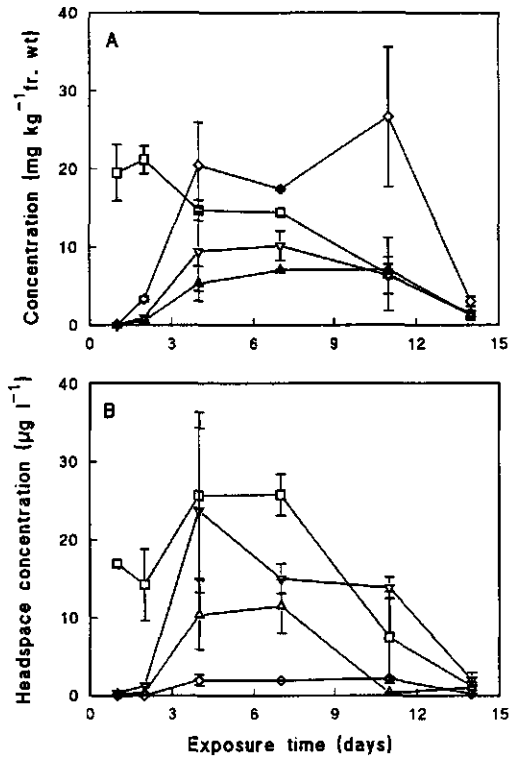

Fig. 5.2
Concentration of S-carvone and its bioconversion products in wound tissue of potato tuber (A) and in the headspace of containers with wounded potato tubers exposed to S-carvone (B).
□ = S-carvone; △ = dihydrocarvone; ▽ = isodihydrocarvone; ◊ = neoisodihydrocarveol.
Extracts were made from two independent experiments, and determinations were performed in duplicate per extract. Results are expressed as means ± SD.

After 14 days, the concentration of S-carvone and its derivatives was very low in the headspace as well as in the tissue. Coinciding with the decrease of the free monoterpenes in the tissue, the suberin formation became increasingly visible.

S-carvone prevents induction of HMGR activity

HMGR activity was strongly induced in the organelle as well as in the microsomal fraction in response to the wounding of the tuber tissue (Fig. 5.3, controls). The remaining supernatant fraction contained at most 5% of the HMGR activity. The induction was only transient, and after 2 days the activity rapidly decreased. The S-carvone exposure inhibited the induction of HMGR completely. As determined by the formation of [^{14}C]mevalonate from [^{14}C]HMG-CoA, the activity of HMGR in the S-carvone treated wound tissue was just above the detection limit of the assay.

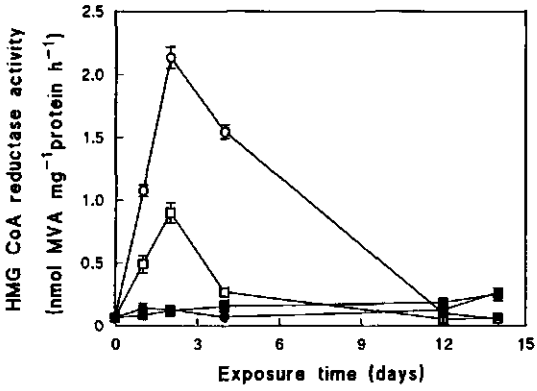

Fig. 5.3
3-Hydroxy-3-methylglutaryl coenzyme A reductase activity in wound tissue of potato tuber.
Organelle fraction: ○ = control; ● = S-carvone treated tissue. Microsomal fraction: □ = control; ■ = S-carvone treated tissue. HMGR activity is expressed as nmol mevalonate formed mg^{-1} protein h^{-1}. Extracts were made from two independent experiments, and enzyme determinations were performed in duplicate per extract. Results are expressed as means ± SD.

Protein composition

The protein pattern of the supernatant of a cell free extract after a 105,000 *g* centrifugation was altered after the S-carvone treatment (Fig. 5.4). After wounding, an induction of proteins took place in the region of 90 kDa (day zero and day 1 of control tissue). Some proteins of about 20 kDa were absent after 1 day. After 12 days of wound healing, an effect of S-carvone on the level of protein composition could be observed (Fig. 5.4 lanes 9 and 10, control (-) and S-carvone treated (+) tissue, respectively). The patatin protein, represented by the major protein band of 42 kDa, decreased in amount and the induced 84 kDa protein disappeared completely.

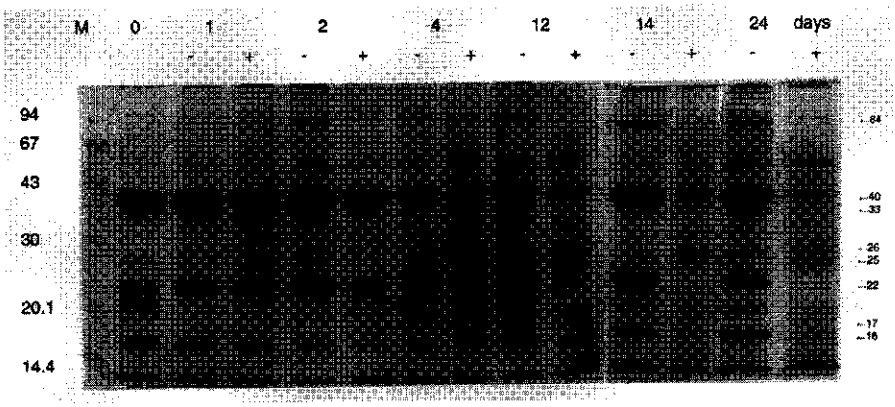

Fig. 5.4 SDS-PAGE analysis of tissue extracts from control (-) and S-carvone treated (+) wound tissue of potato tuber at different times during the treatment. Protein (15 µg) from 105,000 *g* supernatant extracts was applied to the gel. M: molecular mass markers. On the right hand side of the figure, marked differences in protein composition between controls and S-carvone treated wound tissues are indicated with arrows.

Three distinct proteins of about 26, 25, and 22 kDa, were induced after the S-carvone treatment. After 24 days, the patatin protein had almost completely disappeared, and the same was true for the proteins of about 84, 33, 22 and 16 kDa.

S-carvone influences glutathione levels

The glutathione metabolism in potato wound tissue changed rapidly after its exposure to S-carvone (Fig. 5.5). Initially the total glutathione content declined from 180 nmol g^{-1} fr. wt to 100 nmol after 1 day. In S-carvone treated tissue, the amount of total glutathione then increased from about 100 nmol g^{-1} fr. wt at day 1 to about 575 nmol g^{-1} fr. wt at days 12 and 14. Most of the increase could be attributed to GSH.

The GSH/GSSG ratio during the S-carvone treatment always remained higher than 3. Control tissue also reacted on wounding with an initial decrease in total glutathione, but the subsequent increase reached only 180 nmol g^{-1} fr. wt after 12 days, the major part of which came from GSH.

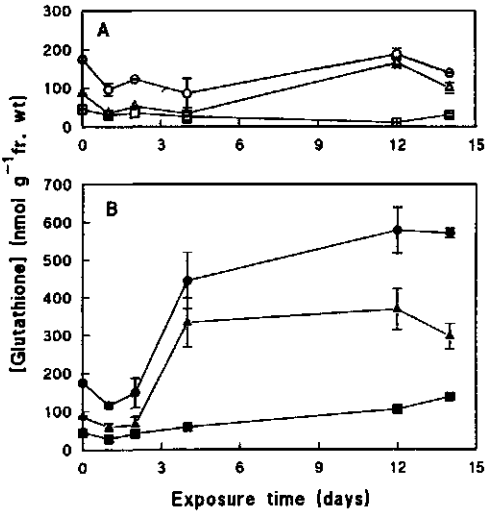

Fig. 5.5
Levels of total glutathione (○,●), GSH (△,▲) and GSSG (□, ■) in control (A) and in S-carvone treated (B) wound tissue of potato tuber.
Open symbols: controls; closed symbols: S-carvone treatment. Total glutathione is expressed as GSH equivalents, GSH and GSSG are expressed as nmol g^{-1} fr. wt. For statistics see legend to Fig. 5.3.

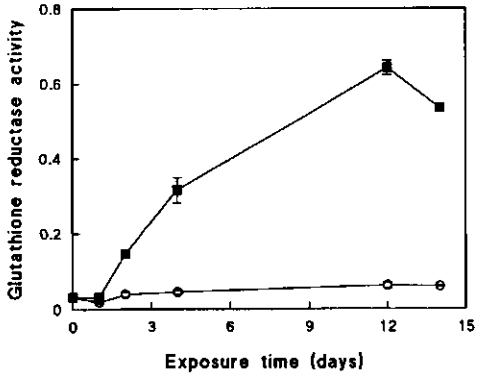

Fig. 5.6
Glutathione reductase activity in control (○) and in S-carvone treated (■) wound tissue of potato tuber. GR activity in U mg^{-1} protein. For statistics see legend to Fig. 5.3.

S-carvone influences stress related enzymes

The wound tissue appeared capable to keep glutathione in the reduced form (GSH), in control as well as in S-carvone treated tissue. Accompanying the altered GSH levels, the activity of GR increased over 20-fold in the S-carvone treated tissue (Fig. 5.6). Already after 2-3 days, the GSH level as well as the GR activity were significantly higher than in the control tissue.

Another enzyme in the glutathione-ascorbate pathway, ascorbate peroxidase (AP), also showed an increased activity in the S-carvone treated wound tissue (Fig. 5.7), whereas dehydroascorbate reductase remained more or less constant over a 14 days period. A significant difference between S-carvone treated and control tissue was not observed for this enzyme (results not shown).

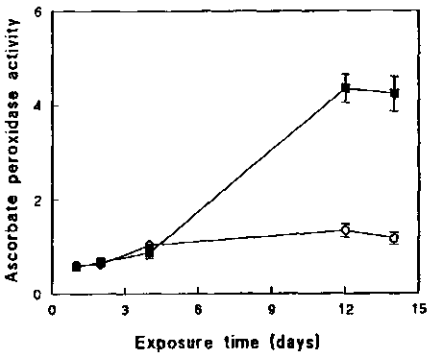

Fig. 5.7
Ascorbate peroxidase activity in control (O) and in S-carvone treated (■) wound tissue of potato tuber. AP activity in U mg^{-1} protein. For statistics see legend to Fig. 5.3.

5.4 Discussion

S-carvone inhibited wound healing in potato tuber tissue. Control tissue showed suberization already after 4 days, and the cambium layer was microscopically clearly visible after 7-8 days. After 24 days, when S-carvone and its bioconversion products were completely depleted from the tissue as well as from the headspace, a suberized layer was clearly visible (Fig. 5.1B), but the formation of the cambium layer had not yet started. Some wounded tubers showed the early beginning of cambium formation or the presence of some cambium-like cells, but none of these tubers showed an organized cambium layer such as observed in the control tubers. It seems that the disappearance of S-carvone and its bioconversion products from the wound tissue allowed suberin formation.

In wounded potato tubers the induction of HMGR, as observed in control tissue, did not take place after exposure to S-carvone. Therefore, S-carvone apparently prevents the induction of HMGR. In potato sprouts, S-carvone partly inhibited the activity of HMGR after 1 day and almost completely after 4 days (Chapter 4).

HMGR is widely accepted as playing a regulatory role in terpenoid synthesis (Bach 1987). Oba *et al.* (1985) showed that blasticidin, an inhibitor of protein synthesis, reduced both wound-induced and pathogen-induced HMGR activity as well as subsequent accumulation of phytoalexins. They suggest that increased HMGR activity is due to a *de novo* HMGR synthesis. This was confirmed by Stermer and Bostock 1987, Yang *et al.* 1991 and Choi *et al.* 1992, who showed that a defence related induction of HMGR activity is due to a *de novo* synthesis, resulting from an increase of HMGR mRNA levels. In animal tissue, the product of HMGR catalysed reactions, i.e. mevalonate, has at least two functions in the cell cycle: it acts as precursor in the cholesterol

synthesis (needed for membrane formation), and it has an essential function as initiator in DNA replication (Siperstein 1984). Furthermore, the essential role of HMGR in cell growth and cell division was illustrated by the effect of mevinolin on cell cultures of *Sylibum marianum.* The synthesis of plastidic prenyllipids decreased after mevinolin treatment, resulting in a general inhibition of cell growth and cell division (Bach 1987).

These data show the essential role of HMGR in cell division processes. So, the fact that S-carvone treated wound tissue of potato tuber did not form a cambium layer, a process that needs cell division, might be caused by the absence of a functional HMGR.

Suberization also needs the presence of ABA (Cottle and Kolattukudy 1982) since this compound appears to trigger a process that ultimately gives rise to a suberization-inducing factor (Cottle and Kolattukudy 1982; Kolattukudy 1984). The mevalonate route might partly provide the precursors for the synthesis of ABA. In S-carvone treated wound tissue the activity of HMGR is very low and, therefore, ABA will be synthesized only in limited amounts. This may explain the delay of suberization of about a week as compared with the control tissue.

The next question is whether the HMGR protein levels are affected by S-carvone. If so, this can be caused either by an increased degradation or by an inhibited synthesis. It is not plausible that S-carvone acts specifically on HMGR. The changes in the protein pattern during the S-carvone treatment show that not only HMGR but also other proteins are affected (Fig. 5.4). However, S-carvone does not inhibit protein synthesis in general, like cycloheximide does. *De novo* protein synthesis in potato sprouts was not inhibited by S-carvone, as determined with *in vitro* translation techniques (Chapter 4). Moreover, the S-carvone treated tuber wound tissue showed a rapid induction of GR (Fig. 5.6), AP (Fig. 5.7) and GST (Chapter 8) which indicates an altered instead of an inhibited protein synthesis.

A stress-induced change of glutathione metabolism is often mentioned in connection with protein synthesis (Alscher 1989). GSSG acts as an inhibitor of protein synthesis during drought stress of the moss *Tortula ruralis* (Dhindsa 1987). Although we found a significantly increased level of GSSG during the treatment with S-carvone, it is unlikely that HMGR was influenced in this way since the ratio of GSH/GSSG remained in favour of GSH. For the same reason, inactivation of HMGR by the formation of (mixed) disulphides due to a

low GSH/GSSG ratio, as occurs in animal tissue (Halliwell and Gutteridge 1989), is also not very likely. Bioconversion of S-carvone may lead to the formation of free radicals and H_2O_2, which inactivates HMGR in animal tissue (Omkumar and Ramasarma 1993). Potato HMGR is also sensitive to H_2O_2 since incubation of microsomes with 0.5 mM H_2O_2 for 5 min already resulted in a 75% loss of activity (unpublished results). However, S-carvone exerted its effect on HMGR already during the first and second day. At that period, little conversion products were found and, as a consequence, free radical formation should still be low. Moreover, the GSH/GSSG contents of control and wound tissues treated with S-carvone were not much different during these days. So, further studies will in the first place focus on the effect of S-carvone on the initiation of the wound healing process in relation to HMGR.

Glutathione is implicated in many processes occurring in plant cells: sulphur transport, detoxification of xenobiotics, and adaption of plants to environmental stresses such as air pollution, drought, and extreme temperatures (Alscher 1989). As far as we know, this chapter deals with the first report that describes the effect of a monoterpene, S-carvone, on the glutathione metabolism in plants. Since glutathione is involved in the scavenging of free radicals and H_2O_2, it is possible that the changed glutathione metabolism is a consequence of the bioconversion reactions that take place in the S-carvone treated wound tissue. Further studies must give answers to the question how the marked changes in enzyme activity and in glutathione contents in potato wound tissue are related to the exposure to S-carvone.

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CHAPTER 6

Preparation and use of 3-hydroxy-3-methylglutaryl coenzyme A reductase antibodies

Summary Following an S-carvone treatment the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) decreased in potato sprouts. Antibodies were raised against a synthetic peptide of the C-terminal region of potato HMGR. The decreasing HMGR activity in the sprouts correlated well with the disappearance of the HMGR signals on Western blots.

In wound healing potato tubers used as control, the induction of HMGR activity (Chapter 5) correlated with the appearance of an HMGR signal on Western blots. However, an S-carvone treatment prevented the induction of HMGR activity as well as the appearance of HMGR protein signals on the blots.

6.1 Introduction

Potato sprouts exposed to S-carvone showed a decreased HMGR activity while no change was observed in HMGR mRNA content (Chapter 4). The question was raised whether HMGR was inactivated or whether the *de novo* synthesis of HMGR was inhibited by some sort of post-translational or post-transcriptional process. The use of HMGR antibodies could be helpful in order to explain the action of S-carvone towards HMGR in potato sprouts. In this chapter the preparation and use of HMGR antibodies is described.

6.2 Materials and methods

Potato material Potato (*Solanum tuberosum* L. cv. Bintje) sprouts were exposed to S-carvone as described in Chapter 4. Wounding experiments, S-carvone treatment of wound tissue and tissue sampling were performed as described in Chapter 5.

Cell fractionation Potato sprouts were fractionated as described in Chapter 4. Wounded potato tissue was fractionated as described in Chapter 5.

Enzyme assay HMGR was assayed as described in Chapters 4 and 5.

Peptide synthesis On the basis of the DNA sequence of potato HMGR of the C-terminal region (see Chapter 4 for the details) the peptide (SGNYCSDNPPAAVNWIEGRGKSVVC) was synthesized using an Applied Biosystems 430 A peptide synthesizer, according to the FastMoc method on a 0.25 mmol scale (Fields *et al.* 1991). To circumvent extra, non-native charges, the peptide was synthesized with an N-terminal acetyl group and a C-terminal amide group (Talbot and Hodges 1981).

Preparation of antibodies The HMGR peptide was coupled to keyhole limpet haemocyanin (KLH) as a protein carrier by N-maleimidobenzoyl-N-hydroxysuccinimide through the cysteine of the peptide (Liu *et al.* 1979). Pre-bled rabbits were injected subcutaneously with 2 ml of an emulsion of peptide-KLH conjugate (1 mg ml^{-1}) in phosphate buffered saline (PBS; 0.8% (w/v) NaCL and 0.02% (w/v) KCl in 10 mM potassium phosphate buffer, pH 7.4) and Freund's complete adjuvant (1:1) (Sigma, St. Louis, USA). Rabbits were bled at 4-weeks intervals. A subcutaneous booster injection (2 ml) of peptide-KLH conjugate in PBS (0.5 mg ml^{-1}) was administered in Freund's incomplete adjuvant (1:1) (Sigma), 12 weeks after the first injection. Rabbits were bled 1 and 2 weeks after boosting. The serum of rabbit 375 collected 2 weeks after boosting was used in this study.

Coupling of the peptide to AminoLink Coupling gel The HMGR peptide was immobilized by coupling it to AminoLink gel according to the protocol of the manufacturer (Pierce, Rockford, ILL, USA). AminoLink gel slurry was washed with 0.1 M potassium phosphate buffer (pH 7.0; PBS). To 1 ml gel cake, 1.3 mg peptide dissolved in 1 ml phosphate buffer was added, and the gel cake was activated with 0.05 ml of 1.0 M sodium cyanoborohydride. The slurry was rotated gently end-over-end for 16 h. The unreacted sites were blocked with 1.0 M Tris-HCl (pH 7.4) after a second activation with sodium cyanoborohydride. The gel was washed with 1.0 M NaCl.

Purification of serum with the peptide-AminoLink column The column was washed with PBS. Rabbit 375 serum (0.5 ml) was allowed to completely enter the gel bed and incubated for 1 h. The column was washed again with PBS, and subsequently specific antibodies were eluted with 0.1 M glycine/HCl (pH 2.8). The glycine fraction was dialysed against PBS, concentrated with poly-ethylene glycol and again dialysed against PBS. The purified specific antibody fraction was used for the immunoblotting experiments.

Gel electrophoresis and Western blotting SDS-PAGE (12.5% gel) was run using the discontinuous buffer system of Laemmli (1970). Each slot of the gel was usually loaded with 15 µg protein and stained using Coomassie. For immunodetection, the proteins were transferred to poly-vinyl-difluoride (PVDF)-membranes using a semi-dry blot apparatus, and the sandwiches were constructed according to the manufacturer's protocol (Hoefer, San Francisco, USA). The transfer was routinely checked with a Coomassie stain of the blotted gel. The blots were blocked during 1 h with 2.5% BSA in PBS. The affinity purified Ig-G fraction from the peptide-AminoLink column was added to PBS containing 0.1% BSA, and incubated overnight with the membrane while gently shaking at room temperature. Immunodetection was carried out using alkaline phosphatase labelled goat anti-rabbit antibodies (Boehringer Mannheim). Incubation of the blot with *p*-nitro blue tetrazolium chloride (NBT, Sigma) and 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (BCIP, Sigma) resulted in a purple colour development after 15-30 min.

HMGR precipitation assay In order to precipitate HMGR and block its activity using HMGR antibodies, active HMGR fractions were incubated for 30 - 180 min at 30°C (gently shaking) with the purified antibodies (various amounts) whereafter the activity was assayed as described in Chapter 4. Protein A from Sigma (dissolved in PBS to 10 µg ml^{-1}; pH 7.0) or protein A sepharose (Sigma) were added to the fractions in various amounts to enhance precipitation. The fractions were centrifuged for 10 min in an Eppendorf centrifuge.

6.3 Results

HMGR in sprouts

Purified HMGR antibodies were raised against a synthetic peptide derived from the highly conserved C-terminal domain of potato HMGR. Using the antibodies, signals on blots corresponding with proteins of 95, 55 and 50 kDa were found in the organelle fraction of tissue extracts of the potato sprouts (Fig. 6.1A, lane 1).

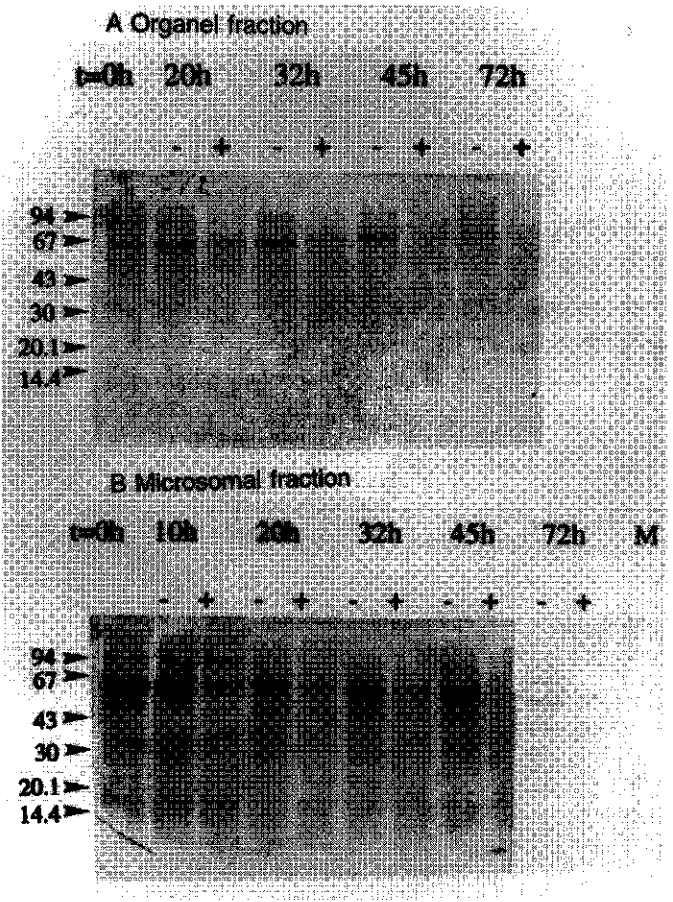

Fig 6.1 Western blot analysis of HMGR in organelle (A) and microsomal (B) fractions of potato sprouts after various hours (h) of treatment with S-carvone. Samples from the control sprout tissue are indicated by -, and those of S-carvone treated tissue by + (Molecular mass markers are indicated).

In the microsomal fraction, four major immunoreactive bands were detected corresponding with proteins with molecular weights of 95, 55, 50 and 46 kDa (Fig. 6.1B, lane 1). Minor bands in lane 1 and 2 (Fig. 6.1B) corresponded with 42 and about 35 kDa. S-carvone inhibits the activity of HMGR in potato sprouts (Fig. 4.2). The disappearance of HMGR signals on Western blots corresponded very well with the changes in HMGR activity (Figs. 6.1A and 6.1B).

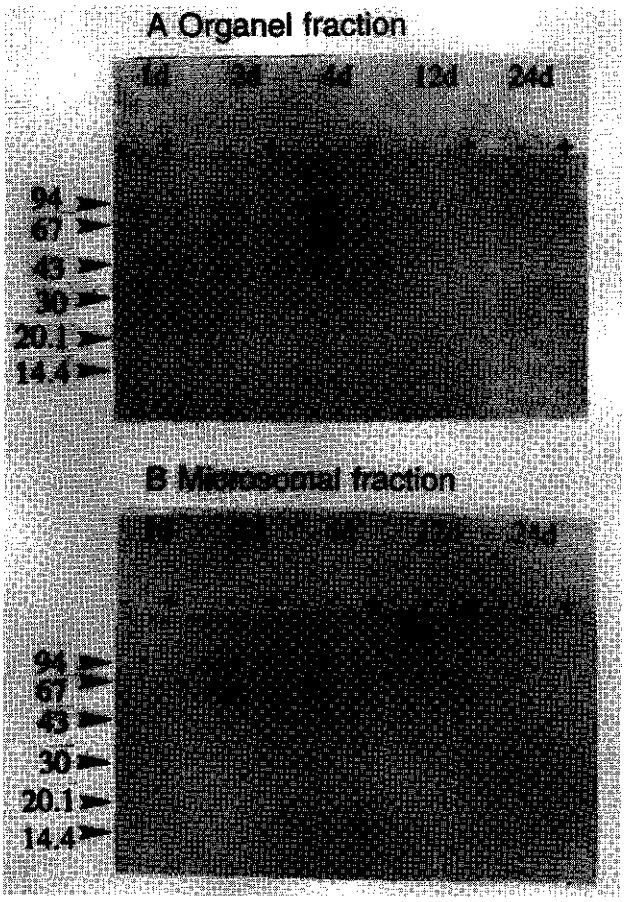

Fig 6.2 Western blot analysis of HMGR in organelle (A) and microsomal (B) fractions of wound healing potato tubers after various days (d) of treatment with S-carvone. Samples from the control wound tissue are indicated by -, and those of S-carvone treated tissue by + (Molecular mass markers are indicated).

HMGR in wound healing potato tuber tissue

Western blot analysis of tissue extracts of wounded potato tubers showed proteins corresponding with molecular weights of 95, 55, 50 and 35 kDa in the control wound tissue (Figs. 6.2A and 6.2B). The organelle and the microsomal fractions showed the same pattern.

In control wound tissue, the activity of HMGR (Fig. 5.3) correlated very well with the presence of the 95, 55, 50 and 35 kDa bands. This was observed for both the organelle and the microsomal fractions. S-carvone treated wound tissue generally did not show proteins of 95, 55 50 or 35 kDa (Fig. 6.2) in the Western blot. After 1, 2 and 4 days, the HMGR antiserum gave only a cross-reacting band with a protein of about 16 kDa which was not present in the control tissue of the same age, but only in the control at day 1.

The blots indicated that the lack of HMGR activity corresponded with the absence of the HMGR protein in the tissue. The blots also showed that after 12 days only the 55 kDa protein was present in the control tissue, although in very small amounts. In tissue treated with S-carvone for 12 days, a low but significant HMGR activity (Fig. 5.3) as well as a small but distinct protein band representing the 55 kDa protein in the microsomal fraction, could be distinguished (Fig. 6.2B). Furthermore, Figs 6.2A and 6.2B showed cross-reacting bands corresponding with proteins of 55 and 25 kDa at day 24.

HMGR specificity

Although the presence of HMGR signals on the blots correlated very well with HMGR activity, the specificity of the antibodies towards HMGR is thereby not proven. Attempts were made to block the *in vitro* activity of HMGR. However, using different amounts of purified HMGR antibodies, it was not possible to inhibit the HMGR activity *in vitro* significantly. Even treatment with protein A (either soluble protein A or insoluble protein A sepharose) could not precipitate HMGR and block its activity (Table 6.1).

Titration experiments with the antigen (the synthetic peptide of HMGR) showed that the HMGR signals from the blots disappeared, indicating that the antibody recognized the antigen, and this prevented binding of the antibody with HMGR antigens on the blot. However, this is only an indication and not a definite proof that the signals from the blot using the HMGR antibodies are specific for HMGR.

Table 6.1 HMGR precipitation using HMGR antibodies in combination with protein A and protein A sepharose. HMGR activity is expressed as dpm h^{-1}. All assays contained 30 µg protein.

Assay conditions	Control (HMGR active fraction)	HMGR antiserum (50 µl) added
Active HMGR fraction	2046 dpm[1]	1872 dpm
Active HMGR fraction + protein A (40 µl)	2086 dpm	1968 dpm
Active HMGR fraction + protein A sepharose	912 dpm	914 dpm

[1] The experiment was replicated three times (with different HMGR fractions from potato sprouts) and the data shown in the table are the results of a representative experiment.

6.4 Discussion

HMGR antibodies

HMGR has been purified from animal and plant sources and in a few cases antibodies were raised. HMGR was purified from yeast (Qureshi *et al.* 1976), chicken liver (Beg *et al.* 1977), rat liver (Nes *et al.* 1979; Edwards *et al.* 1980), human liver (Tanaka *et al.* 1982), radish (Bach *et al.* 1986), guayule (Reddy and Das 1986) and potato (Kondo and Oba 1986). Antibodies were also raised against a 21 amino-acids-synthetic peptide derived from *Hevea brasiliensis* (Chye *et al.* 1991). In *H. brasiliensis* two proteins showed reactivity with HMGR antiserum, at 59 and 50 kDa (Chye *et al.* 1991). The 9 kDa difference between these two proteins corresponded with the size of two membrane spanning domains which are presumably cleaved off when HMGR is solubilized during isolation (Chye *et al.* 1991). Ferrer *et al.* (1990) transformed *E. coli* with two radish HMGR cDNA fragments. The resulting proteins were identified using rat HMGR antibodies. Two immunoreactive bands of 45 and 63 kDa were detected, corresponding to the expected size. In addition, two smaller proteins (31.2 and 32.8 kDa) were found, possibly subfragments of HMGR.

Furthermore, antibodies have been raised against the soluble domain of radish HMGR2 (named HMG2) overexpressed in and purified from *E. coli* (Vollack *et al.* 1994). Western blot analysis of subcellular fractions isolated

from radish HMGR transformed yeast strains, showed immunoreactive bands in the range of 63 kDa.

Using affinity purified antibodies, raised against a synthetic peptide based on the amino acid sequence of the highly conserved C-terminal domain of potato HMGR, four major immunoreactive proteins of 95, 55, 50 and 35 kDa in wound tissue, and of95, 55 and 50 kDa in sprout tissue were detected. The 55 kDa protein is supposed to be the complete monomer of HMGR, whereas the 50 kDa protein might be a truncated form, missing the membrane region, or an isozyme. This corresponds with data reported for *H. brasiliensis.*

The 95 kDa protein, observed on the blots, might be a dimer of HMGR. In animal tissue, active dimers of HMGR have been found (Nes *et al.* 1985). In the microsomal fractions derived from tissues of day 1 (control) and of days 1, 2 and 4 (S-carvone treated tissue), the affinity purified HMGR antiserum reacted also with a protein of about 16 kDa (Fig. 6.2). This might be a break-down product of HMGR.

HMGR and S-carvone

Assuming that the affinity purified antibodies are able to detect potato HMGR, the following conclusions can be drawn. In sprouts, S-carvone inhibited the activity of HMGR already after 1 day and almost completely after 4 days (Chapter 4). This correlated very well with the disappearance of the HMGR signals on Western blots (Fig. 6.1). In wounded tubers the induction of HMGR, as observed in control tissue, did not occur following an S-carvone exposure. Neither HMGR activity nor the HMGR protein, as determined by Western blotting, could be detected (Fig. 6.2). Apparently, S-carvone prevents the induction of HMGR. In both sprout and tuber tissue, proteins with a similar molecular weight reacted with the affinity purified antibodies, indicating that most probably the same proteins were detected. However, the specificity of the antiserum against HMGR could not be proven by precipitation experiments.

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CHAPTER 7[1]

S-carvone inhibits phenylalanine ammonia lyase (PAL) activity and suberization during wound healing of potato tubers

Summary The periderm formation of wound tissue of potato tuber was inhibited temporarily after exposure to S-carvone. In wound tissue of tubers used as control a suberized cell layer was visible after 4 days, whereas in S-carvone treated tissue this was observed only after 14 days in most of the tubers. At day 21 all the S-carvone treated tubers had formed suberin, but only 10% of the tubers had developed a (begin of a) cambium layer. The appearance of suberin was related to the activity of phenylalanine ammonia lyase (PAL); in control and S-carvone treated tissues, maximum PAL activity preceded the appareance of the first suberized cell layers by 2 and 5 days respectively. The specific activity reached the same level in control and S-carvone treated wound tissues.

A short term treatment (2, 4 and 7 days) of wounded tuber tissue with S-carvone delayed the suberin formation. All the treated tubers showed suberization about 4 days after the exposure to S-carvone was finished, and this was closely related to the increase in PAL activity.

Due to the S-carvone treatment, the glutathione metabolism increased: after an initial decrease, as observed in the control, the total glutathione content increased four-fold after 9 days, and it remained high during the treatment. However, the length of the wound healing period before the start of a S-carvone treatment was of great influence on the increase of the glutathione metabolism and on the induction of glutathione reductase.

[1] This chapter corresponds with a paper, by Oosterhaven, K., K.J. Hartmans, J.J.C. Scheffer and L.H.W. van der Plas, published in J. Plant Physiol. 146: 288-294 (1995).

7.1 Introduction

It is known for about 25 years that volatile organic compounds are able to suppress sprouting of potato tubers (Meigh 1969; Beveridge *et al.* 1981; Vaughn and Spencer 1991; Vokou *et al.* 1993). Vaughn and Spencer (1991) described several volatile monoterpenes which inhibited potato tuber sprouting; among these, S-carvone is a potent sprout inhibitor. However, not only sprouting of the tubers was inhibited: also the process of wound healing of tuber tissue was inhibited following exposure to a single dosage of S-carvone (Chapter 5). Suberization as well as cambium layer development were blocked following the exposure to S-carvone. However, 14 days after the start of the exposure some suberized cells were observed, and after 24 days all the treated tubers had a clearly visible suberin layer but only a few showed the presence of a cambium layer. We suggested that the suberization in S-carvone treated wound tissue could appear because the concentration of S-carvone and its bioconversion products in the tissue was low again 14 days after the start of the treatment. Concomitant with the inhibition of the suberization a large increase in gluthatione content and in glutathione reductase activity were observed (Chapter 5). In accordance with the general view that glutathione serves as an antioxidant, protecting cells against damaging effects of stress (Alscher 1989), the suggestion was put forward that the observed change in glutathione metabolism following S-carvone treatment leads to a restored suberization.

Kolattukudy (1984) proposed a hypothesis for the structure of suberin. In this model, suberin consists of a complex polymer of phenolic compounds attached to the cell wall, associated with aliphatic components, thereby generating polyester domains. The formation of the phenolic compounds of suberin starts with the synthesis of *trans*-cinnamic acid from phenylalanine, which is catalysed by phenylalanine ammonia lyase (PAL; E.C. 4.3.1.5) (Stafford, 1974). PAL can be induced in plants by fungal elicitors (Wingate *et al.* 1988), reduced glutathione (GSH) (Wingate *et al.* 1988; Edwards *et al.* 1991) and wounding (Cottle and Kolatukkudy 1982).

We tested the hypothesis that S-carvone blocks the suberization by inhibition of the induction of PAL activity, and investigated the effects of a short term exposure of potato tuber wound tissue to S-carvone, i.e. for 2, 4 or

7 days. The possible link between the glutathione metabolism and the effect of S-carvone on suberization, was also studied.

7.2 Materials and methods

Potato material and sampling procedures Potato tubers (*Solanum tuberosum* L. cv. Bintje, size 45-50 mm) were harvested at the end of October 1993. After 3 days of storage at 15°C the wounding experiments were started; the physiological age of the material was thus very young, and the material had not formed a complete suberin layer at the skin yet. The tubers were cut in halves (from base to apex). One half served as control and the other half was treated with S-carvone. For each sampling date (t = 0, 0.5, 1, 2, 4, 7, 10, 14 and 21 days), four containers (20 l) were prepared, containing ten half tubers each. Two containers served as control and the other two containers were supplied with 250 µl S-carvone (Merck, 99% pure). To obtain a constant concentration of S-carvone in the headspace, new amounts of S-carvone were brought in the containers after 7 and 14 days. In addition one Petri dish with about 25 g $Ca(OH)_2$ (technical grade) was placed in all containers to prevent a too high concentration of CO_2. The containers were closed, sealed with tape and then placed in the dark at 15°C. At each sampling date the headspace concentration of S-carvone and its bioconversion products was determined as described in Chapter 5. The ten half tubers from each container were cut again from base to apex. One part was used for microscopic analysis and for monoterpene extraction, whereas from the other part the wound tissue was removed with a potato peeler. The slices, with a thickness of about 3 mm, were immediately frozen in liquid N_2 and stored at -30°C until further use. All handlings were performed in the dark under dim green safety light.

To determine the effect of a short term S-carvone treatment on tuber wound tissue, half tubers were also treated with 250 µl S-carvone for 2, 4 and 7 days. At several time intervals (0, 2, 7 and 14 days after ending the S-carvone treatment) samples were taken as described above.

In order to determine the effect of S-carvone on healing wound tissue, tuber tissue was treated with 250 µl S-carvone for one week after previous wound healing for 0, 2, 4 and 7 days. Thereafter samples were taken, and the tissue was analysed microscopically for the extent of suberization.

Cell fractionation Cell free extracts for the assay of glutathione reductase (GR; E.C. 1.6.4.2) were prepared as described in Chapter 5. For the extraction of PAL, about 1 g frozen material was homogenized twice in an Ultra Turrax mixer for 30 s with 4 ml extraction buffer containing 50 mM boric acid/sodium borate (pH 8.8), 5 mM 2-mercaptoethanol and PVPP (50 g l^{-1}), essentially according to Lisker *et al.* (1983). The homogenate was filtered through two layers of cheese cloth, and centrifuged at 20,000 *g* at 4°C for 20 min. The resulting supernatant was used for determination of PAL activity. Protein content was determined by the Pierce Protein Assay Reagent Kit (Rockford, ILL., USA) using BSA as a standard.

Enzyme assays GR was determined as described before (Chapter 5). PAL was measured essentially according to Lisker *et al.* (1983). Up to 250 µg protein was added to a reaction mixture containing 50 mM boric acid/sodium borate buffer (pH 8.8) and 10 mM phenylalanine in a total volume of 3 ml. After incubation at 37°C for 1 h the reaction was stopped by adding 100 µl 5N HCl, and then the absorbance at 290 nm was read against the same reaction mixture without L-phenylalanine (replaced by water). Enzyme activity was linear with respect to protein concentration up to at least 0.25 mg per assay and for up to 90 min of incubation time.

Samples were taken from two independent experiments, each with treated and untreated wound tissues of potato tuber. Extracts were made, and enzyme determinations were performed in duplicate. Results are expressed as means ± SD.

Glutathione determination Total glutathione, GSH and GSSG contents were determined as described in Chapter 5. Samples were taken from two independent experiments, each with treated and untreated wound tissue of potato tuber. Extracts were made, and the determinations were performed in duplicate. Total glutathione, GSH and GSSG contents were calculated as nmol g^{-1} fr. wt and expressed as means ± SD.

Staining procedures Suberization of the wound surface was assessed by microscopic analysis based on the method of Vaughn and Lulai (1991) as described in Chapter 5. Freehand cross sections (minimal four per tuber) of

the wound surfaces were cut with a razor blade from five tubers of each treatment (two containers with and two without S-carvone).

7.3 Results

Application of three times (t = 0, 7 and 14 days after wounding) 250 µl S-carvone to the containers with potato halves led to a more or less stable concentration of S-carvone in the headspace as well as in the potato tuber

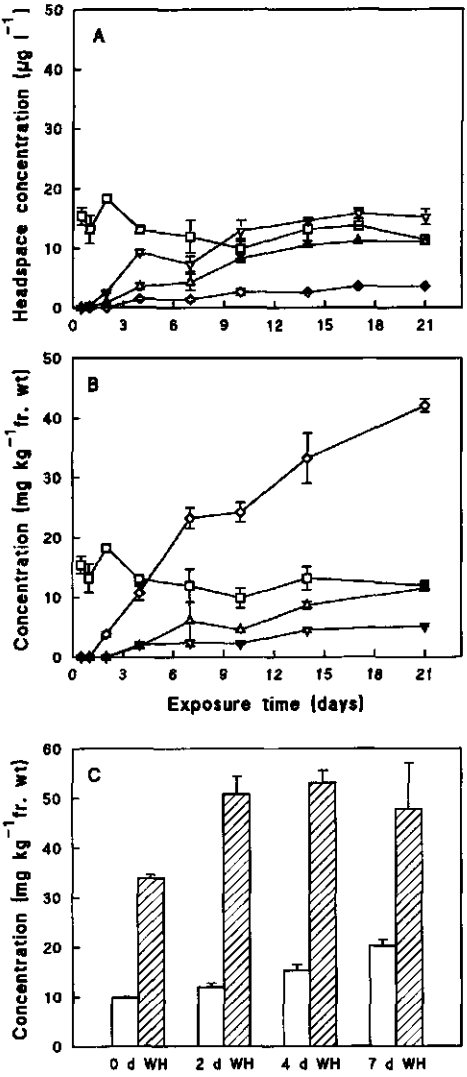

Fig. 7.1
A. Concentration of S-carvone and its bioconversion products in the headspace of the containers.

B. Bioconversion of S-carvone in wounded potato tuber tissue, which has been exposed to S-carvone. Extracts were made from two independent experiments, and determinations were performed in duplicate per extract. Results are expressed as means ± SD. □ = S-carvone; △ = dihydrocarvone; ▽ = isodihydrocarvone; ◊ = neoisodihydrocarveol.

C. Concentration of S-carvone (open bars) and S-carvone including its chloroform-soluble bioconversion products (hatched bars) in wounded potato tuber tissue, which has been exposed to S-carvone for 7 days, after a previous healing period of 0, 2, 4 and 7 days. For statistics see Fig. 7.1B.

wound tissue (Figs 7.1A and 7.1B respectively). As a consequence the concentrations of neoisodihydrocarveol and isodihydrocarvone in the tuber tissue and those of isodihydrocarvone and dihydrocarvone in the headspace increased during the treatment (Figs 7.1A and 7.1B).

After 2 days S-carvone treatment the wound tissue contained about 15 mg kg^{-1} fr. wt, which gradually disappeared again from the tissue within 7 days after ending the S-carvone exposure (Fig. 7.2A). Neoisodihydrocarveol was the predominant conversion product of S-carvone. Its concentration increased after 2, 4 and 7 days of S-carvone treatment (Fig. 7.2B); ending the exposure to S-carvone led to a decrease in the neoisodihydrocarveol content, but its decline was not as fast as the decrease of S-carvone (Fig. 7.2A).

Exposure of healing potato tuber tissue to S-carvone (2, 4 and 7 days) after the wounding, resulted in increasing S-carvone concentrations in the tissue after 7 days of treatment (Fig. 7.1C). However, the total amount of S-carvone and its bioconversion products was not influenced by the time period of wound healing.

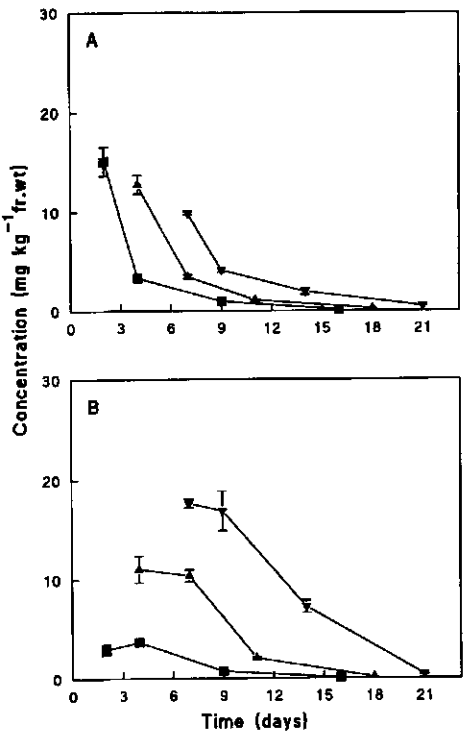

Fig. 7.2
Concentration of S-carvone (A) and neoisodihydrocarveol (B) in wounded potato tuber tissue which has been exposed to S-carvone for 2 (■), 4 (▲) and 7 (▼) days. For statistics see legend of Fig. 7.1B.

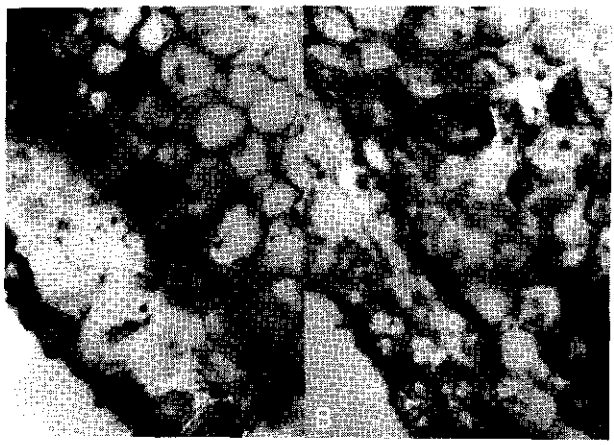

Fig. 7.3
Suberin stain of potato tuber wound periderm of control (A) and S-carvone treated (B) tissue 14 days after wounding.

The formation of a suberized cell layer was visible after 4 days in control wound tissue. A cambium layer was present after 10 days. The formation of wound periderm, i.e. a layer consisting of both suberized cells and a cambium layer, was completed after 14 days (Fig. 7.3A). S-carvone treatment led to a delayed suberin formation. After 14 days, most of the tubers (about 70%) had formed a suberin layer, but only few wounded tubers showed the regular layers of suberin just below the wound area as found in the control (Fig. 7.3B). After 21 days, all the S-carvone treated tubers showed suberization. About 50% of the tissues had a more or less regular, normal suberin layer and 50% showed abnormal suberin formation, i.e. irregular clusters of suberized cells or suberized cells deep in the tissue (Fig. 7.3B). At day 21 only 10% of the wound tissue showed cambium layer formation following S-carvone exposure.

In order to investigate the effect of S-carvone on healing wound tissue, tubers were wounded and were subsequently allowed to proceed a normal wound reaction for 2, 4 or 7 days, whereafter S-carvone was applied for 7 days. Control tubers showed suberized cell layers after 4 days. Only 30% of the tuber tissue had a suberized cell layer after 2 days wound healing followed by 7 days S-carvone treatment; the extent of suberization was only marginal and the site of suberization was 3-4 cell layers deep, whereas control tissue (9 days) was completely suberized just below the wounded surface as shown in Fig. 7.3A. A 4 days wound healing period followed by 7 days S-carvone resulted in 50% of the tubers containing suberin, and 7 days wound healing followed by 7 days S-carvone resulted in more than 95% of the tubers showing a normal suberin layer.

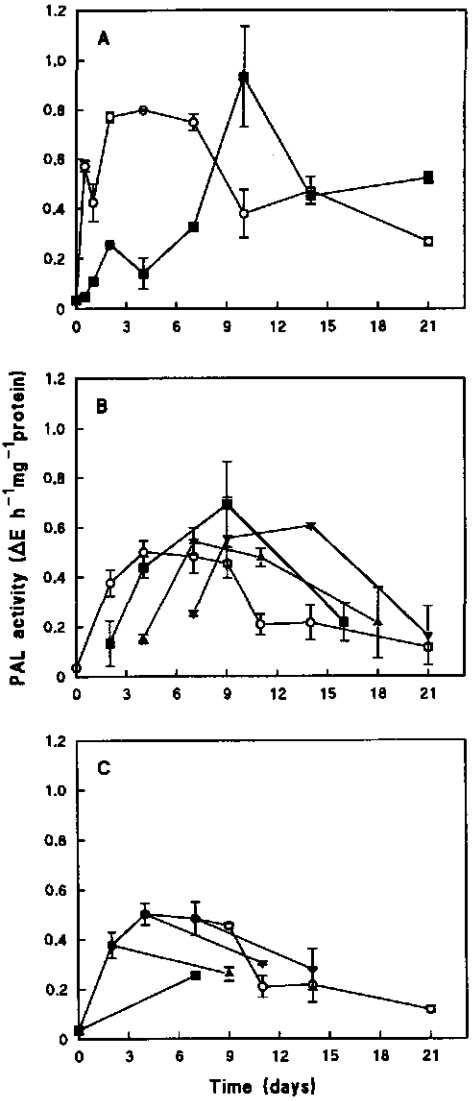

Fig. 7.4
Phenylalanine ammonia lyase activity in wound tissue of potato tuber after various treatments.

A. Continuous exposure to S-carvone (■); control: ○;

B. Exposure to S-carvone for 2 (■), 4 (▲) and 7 (▼) days after which periods the treatment was finished; control: ○;

C. Exposure to S-carvone for 7 days, started after 0 (■), 2 (▲), 4 (▼) and 7 (◆) days of wound healing; control: ○.
Extracts were made from two independent experiments, and determinations were performed in duplicate per extract. Results are expressed as means ± SD.

The activity of PAL in control wound tissue was strongly induced within 12 h after wounding and reached a constant high level between 2 and 7 days whereafter the activity declined (Fig. 7.4A). Exposure of wound tissue with S-carvone led to a different PAL activity pattern: instead of a rapid increase within 12 h, a continuous increase was observed. At day 10, the activity reached its maximum and thereafter it decreased (Fig. 7.4A). The specific activity reached the same level as in control tissue. Fig. 7.4B shows that ending the S-carvone exposure after 2, 4 or 7 days led to an immediate

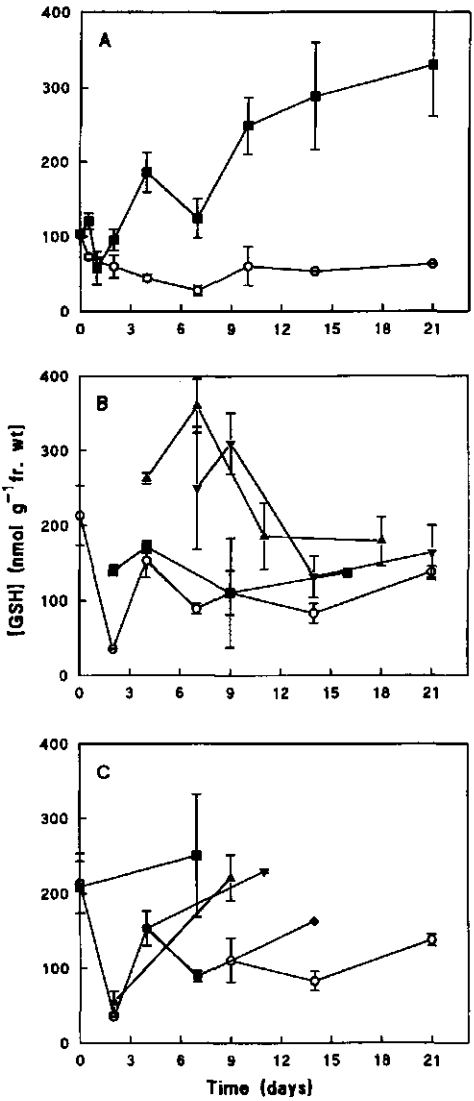

Fig. 7.5
Levels of GSH in wound tissue of potato tuber after various treatments.

A. Continuous exposure to S-carvone (■); control: O;

B. Exposure to S-carvone for 2 (■), 4 (▲) and 7 (▼) days after which periods the treatment was finished; control: O;

C. Exposure to S-carvone for 7 days, started after 0 (■), 2 (▲), 4 (▼) and 7 (◆) days of wound healing; control: O.
For statistics see legend of Fig. 7.4.

increase in PAL activity. Exposure of tuber tissue to S-carvone after 2 days of wound healing resulted in a lower PAL activity compared with control tissue at day 9 (Fig. 7.4C). Wound healing for 4 and 7 days followed by 7 days S-carvone treatment led to comparable activities as the controls after 11 and 14 days respectively. This indicates that S-carvone does influence the extent of the induction of PAL, but it does not influence PAL activity after it has reached its highest activity.

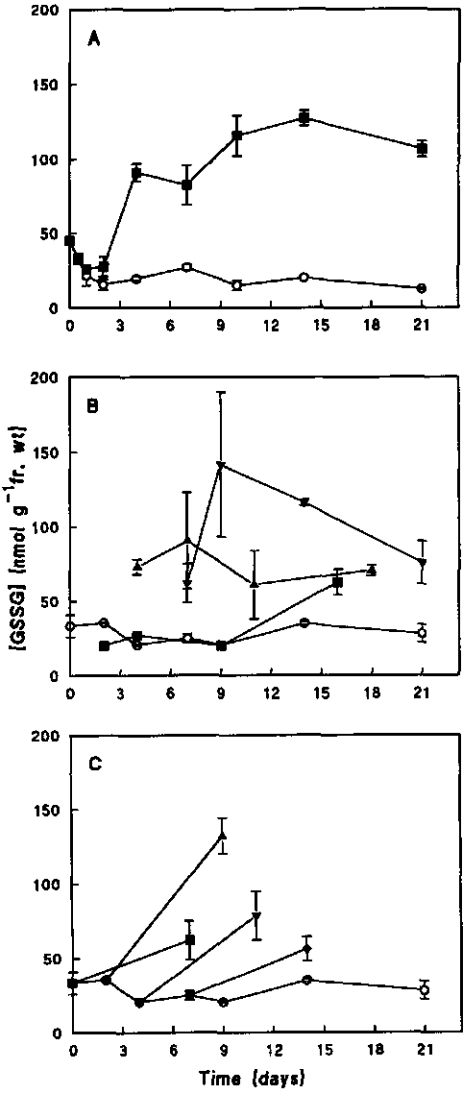

Fig. 7.6
Levels of GSSG in wound tissue of potato tuber after various treatments.

A. Continuous exposure to S-carvone (■); control: O;

B. Exposure to S-carvone for 2 (■), 4 (▲) and 7 (▼) days after which periods the treatment was finished; control: O;

C. Exposure to S-carvone for 7 days, started after 0 (■), 2 (▲), 4 (▼) and 7 (◆) days of wound healing; control: O.
For statistics see legend of Fig. 7.4.

Wounding led to an initial decrease of the GSH content in control tissue, from 100 nmol g^{-1} fr. wt to a constant level of about 60 nmol g^{-1} fr. wt (Fig. 7.5A). S-carvone treated wound tissue showed also a decrease, but at day 3 the GSH content started to increase leading to a level of 325 nmol g^{-1} fr. wt after 21 days (Fig. 7.5A). The amount of GSSG in S-carvone treated tissue rose six-fold from 20 nmol g^{-1} fr. wt at day 2 to 120 nmol g^{-1} fr. wt (Fig. 7.6A); however, the ratio GSH/GSSG remained in favour of GSH. The GSSG content in control tissue did not change.

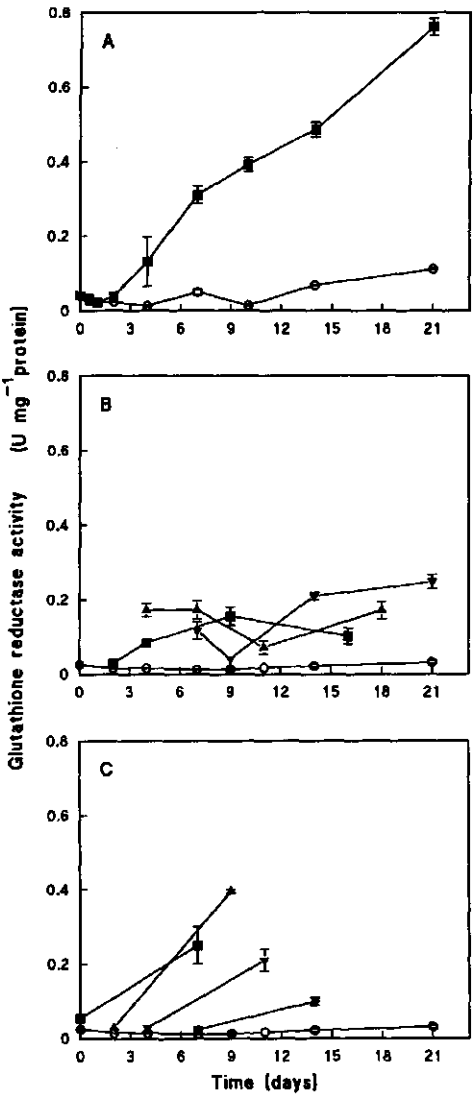

Fig. 7.7
Glutathione reductase activity in wound tissue of potato tuber after various treatments.

A. Continuous exposure to S-carvone (■); control: O;

B. Exposure to S-carvone for 2 (■), 4 (▲) and 7 (▼) days after which periods the treatment was finished; control: O;

C. Exposure to S-carvone for 7 days, started after 0 (■), 2 (▲), 4 (▼) and 7 (◆) days of wound healing; control: O.
For statistics see legend of Fig. 7.4.

The exposure to S-carvone for 2 days and then finishing the treatment did not have an effect on the glutathione metabolism, but 4 and 7 days treatments did alter the glutathione metabolism significantly: an increased level of GSH as well as of GSSG was observed (Figs 7.5B and 7.6B). After the treatment was stopped, a further increase in GSH and GSSG was measured with a maximum at 2-3 days after finishing the S-carvone exposure and a subsequent decrease to about the same GSH and GSSG contents as the control at day 21 (Figs 7.5B and 7.6B).

Application of S-carvone to healing wound tissue resulted in an increased GSH and GSSG content (Figs 7.5C and 7.6C). However, the extent of the GSH and GSSG increase was dependent on the time period of wound healing.

GR activity strongly increased during a permanent S-carvone exposure (Fig. 7.7A), whereas the control tissue only showed a slight increase after 10 days. A 2 days S-carvone treatment did not lead to an increased GR activity, but after stopping the exposure, an increased GR activity was observed (Fig. 7.7B). An S-carvone treatment for 4 and 7 days did also lead to an increased GR activity. Finishing the S-carvone treatment led to a decreasing activity, but surprisingly it increased again a few days later (Fig. 7.7B).

Exposure of 2, 4 and 7 days healing wound tissue to S-carvone resulted in increased GR activities, but the extent of the increase was inversely related to the length of the period of wound healing (Fig. 7.7C).

7.4 Discussion

Chapter 5 dealt with the effect of S-carvone on the wound healing of potato tuber. The inhibitory effect of S-carvone was only temporary and we suggested that the eventual low concentration of S-carvone in the tissue, mainly due to bioconversion processes, enabled suberization of the tissue. In this chapter we show that also when the concentration of S-carvone in the tissue remains at a level of 10-15 mg kg^{-1} fr. wt, 95% of the wounded tubers developed clearly suberized cell layers after 21 days of treatment, indicating that the suberization process is not impossible in the presence of S-carvone. Apart from the fact that the suberin formation was delayed for about 10 days, the character and localization of the suberization also changed. In S-carvone treated tissue, the suberin layer was located 3-4 cell layers below the wounded surface, whereas control tubers contained a suberin layer just below the wound surface (Fig. 7.3). Addition of S-carvone to 7 days healing wound tissue showed a continuous suberization at normal sites, indicating that the tissue had escaped the S-carvone treatment. So once the process has been developed for 4-7 days, S-carvone does no longer influence the site of suberization.

The development of a cambium layer was inhibited during the S-carvone treatment, since at day 21 only 10% of the wound tissue had more or less regular cambium layers. Soon after the S-carvone exposure was stopped the cambium layer formation started. This implies that cell division is largely inhibited by S-carvone. The formation of a cambium layer and the suberization are not strictly correlated processes. Apparently, suberization can take place without the development of a cambium layer. Addition of S-carvone and cycloheximide, a *de novo* protein synthesis inhibitor, to potato sprouts resulted in similar effects: the same protein composition of microsomal membranes after 5 days of treatment, and an activity loss of HMGR (Chapter 4). However, S-carvone did not inhibit the overall protein synthesis as was determined with *in vitro* translation assays. Furthermore, the induction of GR (Fig. 7.7) and glutathione S-transferase (Chapter 8) indicates that also induction of protein synthesis by S-carvone is possible.

The appearance of suberin was preceded by an induction of PAL which makes PAL a good marker for studies on suberization processes. S-carvone treatment of wound tissue led to a delayed increase of PAL activity. A short term S-carvone treatment led to an immediate increase in PAL activity after removal of S-carvone (Fig. 7.4B) indicating that S-carvone is the stressor that blocks the development of PAL activity. However, S-carvone did not act directly on the PAL enzyme; when tissue with a high PAL activity (after 4 days wound healing) was exposed to S-carvone, PAL was not much influenced by this treatment (Fig. 7.4C): the activity remained almost the same and in spite of S-carvone PAL was still active after 7 days. So, S-carvone must influence other factors that in turn influence the induction or activation of PAL.

Concomitantly with the rise of PAL activity (Fig. 7.4A), the GSH level also increased (Fig. 7.5A). PAL activity is known to be modulated by GSH (Wingate *et al.* 1988), and the rise of GSH in the tuber tissue might play a role in the adaptation of the tissue to S-carvone. An argument against this hypothesis is that the induction of PAL after 2 days S-carvone treatment is observed without any effect of the GSH metabolism (Fig. 7.5B), indicating that the PAL induction is not directly related to GSH.

In Chapter 5 an increase of the concentrations of both GSH and GSSG after a single dosage of S-carvone was described. Here we show that a 2 days S-carvone treatment of tuber wound tissue did not result in increased levels of both GSH and GSSG, whereas 4 and 7 days treatments did lead to

increased levels. Also GR was strongly induced during the S-carvone treatment. GR showed a significant increase in activity already after only 2 days S-carvone exposure, although the concentrations of GSH and GSSG were not changed. Smith *et al.* (1985) proposed that the oxidation of GSH to GSSG may have been the trigger for the increased glutathione synthesis in photosynthetic tissue, possibly by releasing the feedback inhibition of the pathway through an initial decrease in GSH. However, according to our data, the initial decrease in GSH is observed for both control and S-carvone treated tissues. Only the S-carvone treated tissue reacted with an increasing GSH synthesis, and so the overall synthesis of glutathione is not just related to initially decreased GSH levels.

There is a lag phase of 2 days for the induction of GR and the increased glutathione metabolism, and this correlates with the bioconversion of S-carvone, which starts about 2 days after the start of the S-carvone treatment (Fig. 7.1B). Furthermore, reduction of S-carvone into neoisodihydrocarveol needs oxygen and NADPH, and is most probably catalysed by cytochrome P450-reductases (Donaldson and Luster 1991). This mono-oxygenase type reaction can lead to the production of free radicals, and the increasing glutathione metabolism could thus be related to the bioconversion of S-carvone.

The concentration of S-carvone and its conversion products in wound tissue healing for a few days is higher than in the tissue immediately after wounding (Fig. 7.1C). It is possible that S-carvone binds to suberin.

S-carvone, applied to wounded potato tubers, inhibited the wound response since a cambium layer was not formed and the formation of a normal suberin layer was delayed. The results obtained strongly suggest that the delayed suberization is related to the decreased activity of PAL due to the application of S-carvone.

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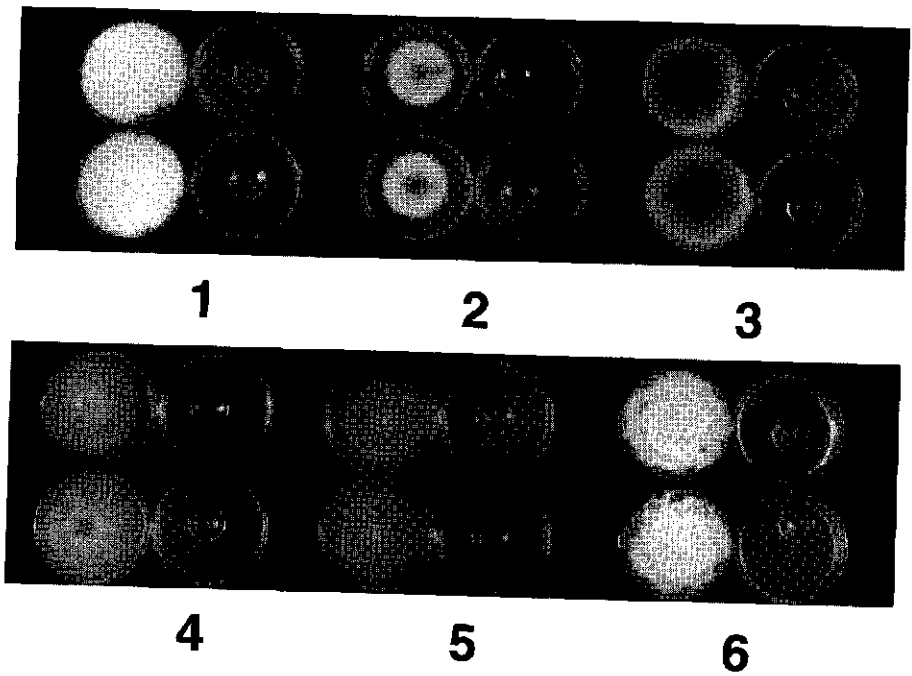

Growth inhibitory effect of S-carvone towards six storage pathogens. At time zero a small part of an agar plate containing good growing fungi was placed on each Petri dish. Two Petri dishes were supplied with 10 µl S-carvone whereas two others served as controls. This figure shows the growth inhibitory effect of S-carvone on the fungi.

1 = *Fusarium sulphureum*
2 = *Fusarium solani*
3 = *Sclerotium cepivorum*
4 = *Helminthosporium solani*
5 = *Botrytis aclada*
6 = *Phytophtora erythroseptica*

CHAPTER 8

Bound forms of S-carvone and the induction of glutathione S-transferase

Summary Wounded potato tubers exposed to S-carvone converted this compound mainly into neoisodihydrocarveol, isodihydrocarvone and dihydrocarvone. In addition to free S-carvone, a conjugated form of carvone was found in considerable amounts in the potato tissue. Carvone was detected in an aqueous phase, obtained after a chloroform-methanol extraction, after treatment with HCl. After 7 days exposure to S-carvone, the amount of conjugated carvone in wounded tuber tissue was higher than that of free S-carvone. ß-Glucosidase treatment could explain only 10-15% of the S-carvone detected. It is possible that S-carvone is also indirectly conjugated to glutathione (GSH), because there is an induction of the activity of a glutathione S-transferase (GST) and a rise in the level of GSH.

8.1 Introduction

Detoxification processes are well known in the plant kingdom. Especially the detoxification of xenobiotics has been extensively studied. Detoxification reactions are oxidation, hydrolysis, sugar conjugation, glutathione (GSH) conjugation, amino acid conjugation, malonyl conjugation and reduction (Lamoureux and Rusness 1993 and references therein). Conjugation of xenobiotics with GSH is very important because it is utilized to detoxify electrophilic alkylating agents. The conjugation of a xenobiotic with GSH requires a sufficient amount of GSH, the presence of an electrophilic site in the xenobiotic (or it must be metabolized to an intermediate that contains such a site) and the activity of a glutathione S-transferase (GST) enzyme to catalyse the reaction.

Not only synthetic pesticides are compounds that have to be detoxified, but also cinnamic acid, a natural compound, has been found to be conjugated to GSH (Diesperger and Sandermann 1979). The GST involved is described as GSCT (glutathione S-cinnamoyl transferase), that has been partially

characterized from cell suspension cultures of French bean, *Phaseolus vulgaris* (Edwards and Dixon 1991). Lipid peroxidation products may also be detoxified by the action of a GST enzyme (Williamson and Beverley 1987).

GST enzymes occur in a soluble as well as a microsomal bound form (Diesperger and Sandermann 1979). The intracellular localization has not been studied in detail, but the enzymes are assumed to be cytoplasmic. In general, several isoforms of GST are described. In pea, two isozymes are active with regard to *trans*-cinnamic acid, whereas a third is active with regard to fluorodifen (Diesperger and Sandermann 1979). Maize showed a more complex pattern: eight isozymes are described which vary significantly in their selectivity towards different substrates (Dean *et al.* 1991).

Using ^{13}C-labelled carvone, water-soluble forms of S-carvone, or derivatives thereof, were found in potato tuber tissue (Chapter 3). The present chapter describes that low concentrations of S-carvone in wounded potato tuber tissue caused an induction of GST and a rise in the level of GSH. S-carvone is also present in a conjugated form, and we speculate that S-carvone might be indirectly conjugated to GSH.

8.2 Materials and methods

Plant material and S-carvone treatment After harvest, potato tubers (*Solanum tuberosum* L. cv. Bintje) were stored at 4°C without sprout suppressants until further use. Tubers were cut in halves and ten half tubers were placed in a 20 l container as described in Chapter 5. The potato tubers were exposed to S-carvone (250 µl S-carvone, 2 Petri dishes with 125 µl S-carvone each) for 2, 4 and 7 days, and then S-carvone was removed by opening the containers for 4 h. Subsequently, 2 or 3, 7 and 14 days after finishing the S-carvone treatment, tissue samples were taken for various analyses. Control samples (no exposure to S-carvone) were prepared in the same way.

Sampling At each sampling date, tissue samples of the wound layer, of about 3 mm thickness, were taken in the dark with a potato peeler and immediately frozen in liquid N_2; the samples were stored at -30°C until further use.

Cell fractionation About 2.5 g frozen material was homogenized twice in an Ultra Turrax mixer for 30 s with 5 ml homogenization medium: 0.2M potassium phosphate buffer (pH 7.5), 0.35 M sorbitol, 5 Mm $MgCl_2$ and 10 mM EDTA. Prior to use, 2-mercaptoethanol (20 mM, final concentration), insoluble PVPP (50 g l^{-1}) and PMSF (0.1 mM, final concentration) were added. The slurry was squeezed through two layers of cheese cloth, and then centrifuged at 1,200 *g* for 8 min. The supernatant was centrifuged at 16,000 *g* for 40 min. The resulting supernatant was used for enzymatic analysis.

GST The assay was carried out according to Bilang *et al.* (1993), with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate.

GSH determination GSH was essentially determined according to the specific enzyme method of Brehe and Burch (1976) as described in Chapter 5.

Determination of free monoterpenes The amounts of S-carvone and its conversion products in the tissue and in the headspace were determined as described in Chapter 3.

Determination of "bound carvone" The aqueous layers obtained during the extraction were subjected to acid hydrolysis by adding 2 ml 5 M HCl to 50 ml of the aqueous extract (pH 0.8) and incubated for 2 h at 80°C. After cooling, the pH was adjusted to 5.5 with 5 M NaOH. This solution was extracted twice with 50 ml hexane, containing a known amount of an internal standard (naphthalene). The volume of the hexane extract was reduced using a rotary evaporator and analysed by GC. Another 50 ml of the aqueous layer was treated with ß-glucosidase (100 U from almond, Boehringer and Sigma) for 48 h at 30°C. The extract was buffered with sodium citrate (0.1 M final concentration, pH 5.5). Extraction and analysis of carvone and derivatives thereof were as described in Chapter 3.

The activity of ß-glucosidase in the aqueous extracts was checked by the addition of 3 mM 4-nitrophenyl-ß-D-glucopyranoside (Boehringer), a synthetic substrate for ß-glucosidase. The formation of the coloured breakdown product due to enzymatic activity was determined spectrophotometrically. To a sample from the aqueous extract of 1 ml, 143 µl 0.7 M

Na_2CO_3 was added, the mixture was diluted ten-fold with water and measured at 420 nm.

8.3 Results

S-carvone was taken up by the wounded potato tubers and then it was rapidly converted into mainly neoisodihydrocarveol and dihydrocarvone (Fig. 8.1). Traces of isodihydrocarveol and hydroxylated carvones were also detected, but this accounted for less than 5% of the total amount of free monoterpenes. The concentration of free monoterpenes reached a level of about 35 mg kg^{-1} fr. wt after 7 days S-carvone exposure. The treatment was stopped after 7 days, and during the next 14 days the concentration of S-carvone and its derivatives decreased until there was almost nothing left in the tubers (Fig. 8.1). However, treatment of the aqueous layer, obtained after the chloroform-methanol extraction, with acid produced a considerable amount of carvone (Figs 8.2A-C). After 2 days S-carvone exposure, only a small amount of water-soluble carvone was present (Fig. 8.2A) which increased after prolonged S-carvone exposure (Fig. 8.2C). The conjugated carvone remained longer in the tissue than the free S-carvone, but 14 days after 7 days of S-carvone treatment, most of the conjugated carvone also had disappeared.

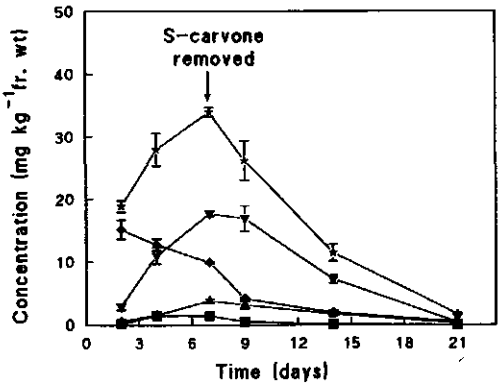

Fig. 8.1
Bioconversion of S-carvone in wounded potato tuber tissue, which has been exposed to 250 µl S-carvone (air concentration: 5-10 µg l^{-1}) for 7 days after which period the treatment was finished.
★ = sum of all non-bound monoterpenes; ◆ = S-carvone; ▼ = neoisodihydrocarveol; ▲ = dihydrocarvone; ■ = isodihydrocarvone.

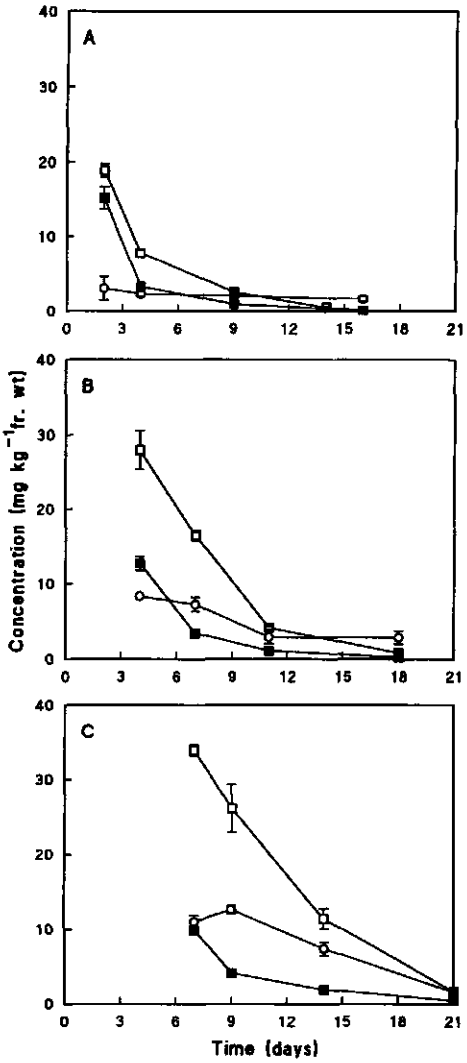

Fig. 8.2
Bioconversion products in wounded potato tuber tissue following 2 (A), 4 (B) and 7 (C) days of exposure to S-carvone after which periods the treatment was finished; the treatment started at day zero.
□ = sum of all non-bound monoterpenes;
■ = S-carvone;
○ = conjugated carvone (detected after treatment with HCl).

Only 10-15% of the conjugated carvone was found after treatment with ß-glucosidase. The activity of ß-glucosidase in the aqueous extract was checked with the addition of a synthetic substrate, 4-nitrophenyl-ß-D-glucopyranoside, which was completely hydrolysed within 24 h indicating that ß-glucosidase remained active in the potato aqueous extract.

A 2 days S-carvone treatment led to a strong induction of the activity of a GST enzyme (Fig. 8.3). The activity remained high for at least 14 days after the 7 days of S-carvone treatment, although the amount of free S-carvone was almost zero after one week (Fig. 8.2C).

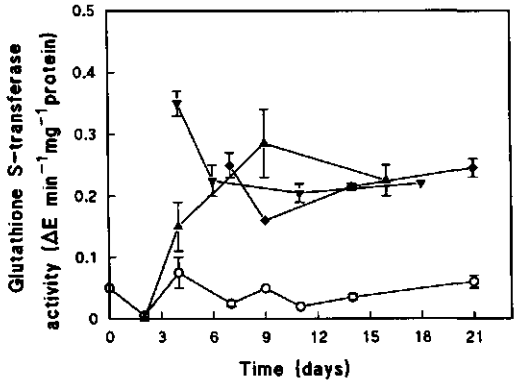

Fig. 8.3
Induction of glutathione S-transferase in wounded potato tuber tissue following 2 (▲), 4 (▼) and 7 (◆) days of exposureto S-carvone, after which periods the treatment was finished.
○ = control.

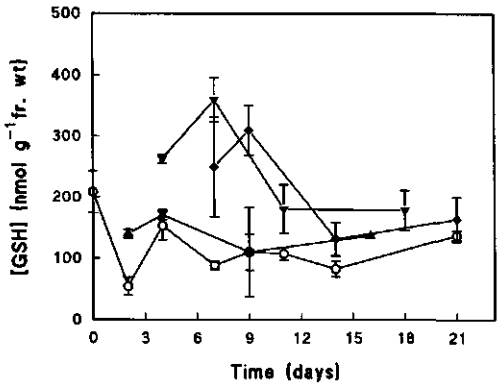

Fig. 8.4
GSH levels in wounded potato tuber tissue following 2 (▲), 4 (▼) and 7 (◆) days of exposure to S-carvone, after which periods the treatment was finished.
○ = control.

Exposure of the tubers to S-carvone for 4 or 7 days did not enhance the GST activity compared with the 2 days exposure. Along with the induction of GST, the amount of GSH was increased after a 4 or 7 days period of S-carvone exposure. Figure 8.4 shows that after an initial drop of the GSH content after 2 days, an increased amount of GSH was measured. A 2 days exposure to S-carvone did not lead to significantly higher GSH levels. On a molar basis there is a sufficient amount of GSH for the conjugation with S-carvone or its derivatives even without an S-carvone treatment (Table 8.1).

8.4 Discussion

S-carvone was taken up by the potato tuber and then it was converted into reduced compounds as was also described in Chapter 3. Using [^{13}C]S-carvone, it was found that water-soluble carvone derivatives were formed in the sprouts. In this chapter this finding was confirmed, and the results indicated that S-carvone might be conjugated to GSH. S-Carvone was found in considerable amounts after addition of HCl to the aqueous extracts of the potato tuber tissue. ß-Glucosidase could only release 10-15% of the conjugated carvone, although the enzyme remained active in the extracts because a synthetic substrate was completely hydrolysed within 24 h.

Table 8.1 The amount of free and conjugated S-carvone and reduced glutathione (GSH) in wounded potato tuber tissue (in µmol kg^{-1} fr. wt) following different exposure times.

	free [carvone]	conjugated [carvone]	[GSH]
0 days			214
2 days S-carvone	100	20	140
4 days S-carvone	85	56	263
7 days S-carvone	66	73	250
7 days S-carvone + 2 days	28	84	310
7 days S-carvone + 7 days	13	49	132
7 days S-carvone + 14 days	3	10	164
control 14 days after wounding			105

All the requirements for the conjugation of S-carvone to GSH are present: an electrophilic site at S-carvone, a sufficient amount of GSH, and the (induced) activity of a GST enzyme.

Detoxification processes that utilize GSH are described with respect to xenobiotics. To our knowledge, this is the first report that gives indications for the conjugation of GSH with a monoterpene, S-carvone, in plants. In pea, the conjugation of cinnamic acid, a phenylpropanoid, with GSH was described (Diesperger and Sandermann 1979). In mice, GST was induced after feeding S-carvone. A structure-activity relationship between different carvone-like compounds showed that the α,ß-unsaturated ketone group appeared to be

critical for the observed high enzyme activity (Zheng *et al.* 1992). The amount of GSH decreased following S-carvone feeding in mice, whereas we found an elevated concentration of GSH. The availability of GSH never was limiting for the putative indirect conjugation of S-carvone with GSH.

For wounded potato tuber tissue, S-carvone is a toxic compound that needs to be detoxified. However, the tissue was not capable to detoxify the amount of S-carvone that was present in the tissue. A considerable amount of S-carvone existed in the free form, and part of the S-carvone was converted into compounds without a strong electrophilic site which cannot be conjugated. These compounds, mainly neoisodihydrocarveol and dihydrocarvone, together with free S-carvone might exert toxic effects since the process of suberin formation and cambium layer development in wound healing potato tubers, was inhibited by these compounds (Chapter 5).

The finding that GST was induced in carnation following an ethylene treatment suggests a natural function for GST (Meyer *et al.* 1991). This is further confirmed by the observation that a soluble auxin-binding protein from *Hyoscyamus muticus* turned out to be a GST. Auxins might become conjugated to GSH either for temporary storage or for direct modulation of hormone activity as was speculated by Bilang *et al.* (1993).

It seems attractive to speculate that the action of monoterpenes with an α,β-unsaturated ketone group - having the highest anti-germinating effect (Reynolds 1987) - is related to the action of GST. The amount of auxins or the activity of GST might be influenced by the monoterpenes thereby inhibiting germination.

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CHAPTER 9

General discussion

Dutch Caraway Research Programme

In order to stimulate the cultivation of alternative crops for the production of fibres from hemp, lubricants and fuels from oil seeds, and essential oil from caraway, research on these topics was stimulated by the Dutch government. Main goal of these research activities was to develop new applications for the products of the crops in order to provide good opportunities for farmers to include these crops within the traditional crop rotation (potatoes, sugar beets and wheat).

The Dutch Caraway Research Programme included research on the cultivation and pests of caraway as well as on the use of the essential oil from the seeds. This programme aimed at finding new applications for the essential oil. The possible applicability of S-carvone, the main component of the oil, in the enantioselective synthesis of interesting biologically active compounds was investigated by Versteegen-Haagsma (1994). Another possible new use of the oil involves the application of S-carvone as a natural potato sprout inhibitor. This thesis deals with several aspects of the use of S-carvone as a potato sprout inhibitor, including the mechanism via which S-carvone acts on potato sprout growth and the consequences of the application for the physiology of the potato tuber.

Potato tuber sprout inhibition on a practical scale

The presence of a low, but stable headspace concentration of S-carvone can inhibit the sprouting of potato tubers. S-carvone appeared to be a very effective sprout inhibitor on a laboratory (Chapter 3) as well as on a practical scale (Hartmans *et al.* 1995). In a parallel programme, the use of S-carvone as a potato tuber sprout inhibitor on a practical scale was optimized. The results of this programme were so successful that in July 1994 a line of natural sprout inhibitors, with S-carvone as active ingredient, was registered and brought on the market under the tradename Talent® by Luxan (Hartmans *et al.* 1995). Talent® can be applied as a volatile using the swing fog, an apparatus also used for (automatic) application of liquid (C)IPC. Since S-

carvone is a reversible sprout growth inhibitor, it has to be applied regularly. Using such a regime, S-carvone shows storage results that are as good as or sometimes even better than (C)IPC (Hartmans *et al.* 1995).

Talent® is now being sold in The Netherlands, and a provisional registration was given in Switzerland, end of 1994. A development programme in other West European countries is underway.

Although Talent® is more expensive than the traditionally used sprout inhibitors IPC and CIPC, there is a growing market for the new product. Export of potatoes with a good quality but without (C)IPC residues to Scandinavian countries, for instance, is possible by the use of Talent®. Also the so-called "green market" takes advantage of this sprout inhibitor in order to inhibit sprouting of MBT grown potatoes [MBT = Milieu-Bewuste Teelt (environmentally-friendly cultivation)]. Furthermore, farmers who have built new storage rooms which are not solely meant for the storage of consumption potatoes, may use Talent® instead of (C)IPC. Application of (C)IPC during the storage of consumption potatoes can give problems with respect to the germination of grass seeds or grains and the sprouting of seed potatoes if these products are stored in the same storage room during the next year. This means that once a storage room is used for consumption potatoes treated with (C)IPC, it cannot be used for some other crops.

Infection of seed potatoes with silver scurf (*Helminthosporium solani*) is a large problem, because it leads to quality and weight losses of the product. At this moment some strains of silver scurf have become resistant to synthetic pesticides normally used to reduce their growth. S-carvone was found to reduce the growth and development of silver scurf on seed potatoes to a great extent, and therefore Talent® is also used as an antifungal agent. To study the use as an antifungal agent towards other (potato) storage pathogens, a follow-up of the research programme was started in January 1995.

Although there are no clear indications that (C)IPC has adverse effects on human health, the registration of (C)IPC is not guaranteed because the toxicity file is not complete. Since the product forms only a small market for the producer, the chance is small that the toxicity file will ever be completed. As the governmental policy is directed towards the reduction of the residue levels of synthetic pesticides in food, S-carvone may play an important role as an alternative, environmentally friendly sprout inhibitor.

Sprout elongation blocked by S-carvone

S-carvone inhibits the sprouting of potato tubers and the elongation of the sprouts. Addition of S-carvone to growing sprouts blocks the elongation within 1-2 days. Sprouts do not become necrotic if the headspace concentration remains below 10-15 mg m^{-3}. Above this concentration, sprouts become necrotic but the eyes (tuber buds) retain their viability; if the sprouts are brought into an atmosphere without S-carvone, a normal growth of the sprouts from the buds is observed. This phenomenon might be used in storage practice: if sprouts develop on the tubers too early, a single high dosage can kill the sprouts thereby preventing quality loss due to evaporation of water.

Wound healing inhibited by S-carvone

The curing or wound healing period immediately after the harvest of potatoes is the period in which the wounds, that occur during harvest, are healed. This is an essential phase of potato storage since wounds are ideal entries for pathogens and because water can easily evaporate from the wounded tubers. S-carvone inhibits the process of wound healing temporarily (Figs 5.1 and 7.3) by reduction of the suberization and the cambium layer formation. Suberization as a process is not impossible in the presence of S-carvone, since S-carvone-containing tissue develops clearly visible suberin layers after 10-14 days, i.e. after a delay of about 10 days. Initially, S-carvone inhibits the induction of PAL (Fig. 7.4) but after about 10 days, the PAL activity increases and suberin is formed.

This implies that the application of S-carvone-containing sprout inhibitors should not be performed at a high dosage before the end of the curing period, because this would lead to a delayed wound healing. In practical trials, a low dosage immediately after harvest has not shown a negative effect on wound healing, on pathogen attack or on weight loss (Hartmans *et al.* 1995).

It is likely that S-carvone itself is the most important inhibitory compound since the growth inhibition of the sprouts and the inhibition of wound healing already occur within 1-2 days, the period in which only small amounts of conversion products are formed. Furthermore, the conversion products of S-carvone possess less inhibitory activities.

Bioconversion of carvone

S-carvone is converted by potato sprout and tuber tissues into more reduced compounds (Fig. 9.1). The enzymes involved in the conversion are induced since cell-free extracts of non-treated potato sprouts (or tuber tissue) are not able to convert S-carvone, whereas those of S-carvone treated tissue convert S-carvone into isodihydrocarvone, dihydrocarvone and neoisodihydrocarveol (unpublished results). The enantiomer of S-carvone, R-carvone, is also converted by potato sprout and tuber tissues. The conversion is stereoselective: R-carvone is mainly converted into neodihydrocarveol, whereas S-carvone is mainly converted into neoisodihydrocarveol. Minor chloroform-soluble products that are formed during the bioconversion are the other dihydrocarveol isomers, hydroxylated carvones and carvyl acetates (Fig. 9.1).

In addition to chloroform-soluble compounds also water-soluble, carvone derived compounds were found in sprouts and in wound healing tuber tissue. Labelling studies suggested the presence of these water-soluble compounds, but using NMR analysis the signals could not be designated to particular compounds (Chapter 3). Carvone was found after addition of HCl, indicating that there must be a sort of a binding between (a derivative of) carvone and a conjugating molecule. Carvyl-ß-D-glucosides are found in spearmint (Shimizu *et al.* 1990). It is possible that also in potato tissue carvone is partly bound to sugars. Wound healing potato tuber tissue may also incorporate carvone in the matrix of suberin. Another explanation might be that carvone is indirectly conjugated with glutathione (Fig. 9.1). The induction of GST is an indication that such a detoxification may take place (Chapter 8). Further studies must reveal the chemical identity of the conjugates.

Bioconversion of chemical compounds is often referred to as detoxification if the cells metabolize the compounds in such a way that they are less toxic to cell metabolism, either by reduction or by conjugation (Lamoureux and Rusness 1993). In fact, this seems also true for S-carvone since the products of the conversion, mainly dihydrocarvone and neoisodihydrocarveol, are less toxic to potato sprouts and to potato cell suspension cultures (Chapter 3).

S-carvone applied to whole tubers is mainly found on and in the skin where it is not converted, as bioconversion takes only place in metabolically active tissue (Chapter 2). This implies that bioconversion is not important in the practical use of S-carvone. Applied as a temporary sprout inhibitor, S-

carvone will be converted rapidly by the sprouts. Thus an increased dosage of S-carvone is needed to inhibit the growth of the sprouts, but on the other hand it provides the advantage of being a reversible sprout inhibitor.

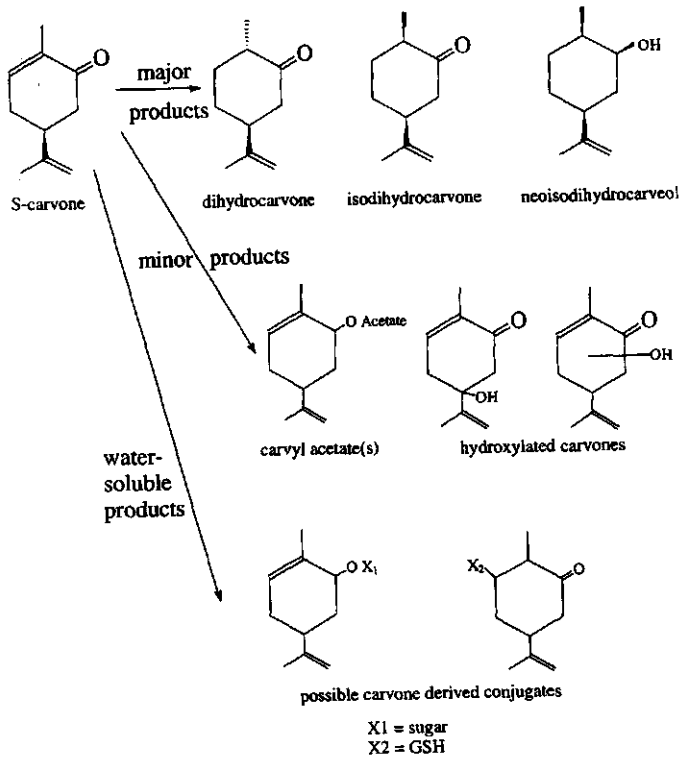

Fig. 9.1 Bioconversion products of S-carvone found in potato tuber tissue.

Induction of stress related enzymes by S-carvone

Induction of stress related enzymes takes place within 2 days of an S-carvone treatment. This coincides with the accumulation of conversion products in the tissue. The conversion of S-carvone is most probably performed by cytochrome P-450 reductases. In general, cytochrome P-450 dependent mono-oxygenase systems are capable of catalysing oxidation reactions of a variety of endogenous substrates (Porter and Coon 1991); the hydroxylation of camphor is, seemingly, the first step in the metabolism of this monoterpene ketone (Funk and Croteau 1993). Furthermore, the monoterpenes nerol and geraniol were shown to be substrates for avocado

cytochrome P-450 reductase (Hallahan *et al.* 1992). These reactions are known to produce free radicals (Karuzina and Archakov 1994), that are toxic for normal cell functioning. Free radicals are detoxified by scavenging enzyme systems, such as catalase, superoxide dismutase and the glutathione pathway as proposed by Foyer and Halliwell (1976). The free radicals are converted into hydroxyl radicals whereafter hydrogen peroxide is formed. As a consequence of the bioconversion of S-carvone, glutathione related stress enzymes may be induced in order to withstand the toxic effects of the free radicals.

Mechanism of sprout growth inhibition

Growing sprouts exposed to S-carvone are blocked in their sprout growth already after 1-2 days. The question arises whether S-carvone blocks cell division, cell elongation or both processes. Due to the treatment the sprouts stop growing and become thicker, indicating that at least the cell elongation is blocked. It is questionable whether cell division processes are also blocked. By microscopic analysis, using CSLM, SEM and light microscopy, of treated and untreated sprout and root tissues, all stages of cell division were observed. Furthermore, addition of S-carvone to cell suspension cultures of potato and tobacco did not lead to a block of the cell cycle, as analysis of the DNA content using flow cytometric techniques revealed (Fig. 3.2). Microscopic analysis of treated cell suspension cultures also showed all stages of cell division. On the other hand, wound tissue exposed to S-carvone showed almost no cambium layers after 14-21 days, and the formation of a cambium layer needs cell division. This might indicate that (the initiation of) cell division is blocked due to the S-carvone exposure.

The generally accepted hypothesis that lipophilic compounds, such as monoterpenes, interfere with membranes thereby exerting their toxic effects by desintegration of the energy generating system (Sikkema 1993) could not be confirmed. Maximum S-carvone levels in the tissue corresponded to 0.1 mM. If membrane interference is involved in the action of S-carvone on potato tuber sprout growth, the mitochondrial respiration, a membrane bound process, might be impaired. However, S-carvone did not have an effect on the mitochondrial O_2 uptake in the concentration range which is found in tuber and sprout tissue (Fig. 2.1). On the contrary, the O_2 uptake was stimulated upon S-carvone treatment of wound tissue (Fig. 2.2). Only at S-carvone

concentrations of 1 mM and higher, the O_2 consumption of the mitochondria was inhibited to some extent (Fig. 2.1). Therefore, other mechanisms are probably involved in the growth inhibitory effect of S-carvone.

Inhibition of HMGR activity by S-carvone

The inhibition of elongation following S-carvone treatment correlates strongly with the decreasing activity of HMGR (Figs 4.1 and 4.2), a key enzyme in the mevalonate pathway (Fig. 1.2). This effect seems rather specific since enzymes of the citric acid cycle, such as isocitrate dehydrogenase and malate dehydrogenase, were not affected whereas the activity of glutathione related enzymes was stimulated (Chapters 5 and 7). PAL was also inhibited following the S-carvone treatment, but the inhibition was temporary (Fig. 7.4). After about 10 days the activity of PAL increased, although the concentration of S-carvone remained constant in the tissue. Along with the increased PAL activity suberization took place. When sprouts are grown in the dark they develop a purple colour due to the formation of anthocyanins, and PAL is the first step in the pathway leading to anthocyanin formation. The observed bleeching of the sprouts during the S-carvone exposure can be explained by an inhibition of PAL activity, similar to that observed in healing wound tissue (Fig. 7.4).

The mevalonate pathway provides several important compounds, such as hormones, vitamins, carotenoids, membrane components etc., necessary for growth and development (Fig. 1.2). The (lack of) HMGR activity may already explain the reduced sprout growth. Inhibitor studies with mevinolin showed the essential role of HMGR in the root and sprout elongation of radish seedlings (Bach and Lichtenthaler 1983). Although the mRNA level remained at a high level, the presence of the HMGR protein could not be detected, using Western blotting techniques, in extracts from sprout tissue exposed to S-carvone for 3 days. This implies that the protein synthesis of HMGR is blocked or that the protein is rapidly degraded. However, protein synthesis in general is not blocked, since *in vitro* experiments have shown that translation as a process is not inhibited by S-carvone. Furthermore, the activity of several enzymes is induced in tuber tissue following an S-carvone treatment, probably together with an increased synthesis of these enzymes, e.g. glutathione reductase, glutathione-S-transferase, dehydroascorbate reductase, S-carvone converting enzymes etc.

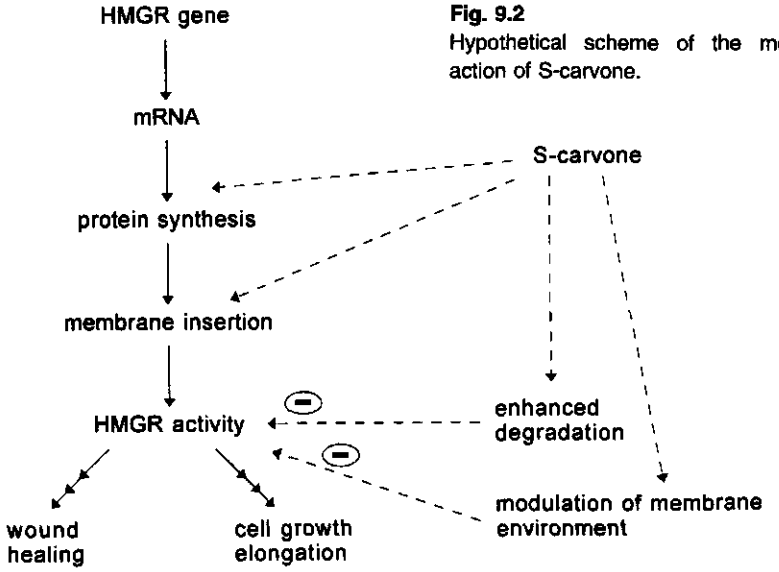

Fig. 9.2
Hypothetical scheme of the mode of action of S-carvone.

HMGR is a membrane bound enzyme that, in animal tissue, is synthesized on membrane bound polysomes. HMGR is then co-translationally inserted into microsomes, and the insertion is dependent on the presence of a signal recognition particle (Bach 1987, and references therein). In plants, HMGR is also membrane bound, but the process of HMGR insertion in membranes has not been established yet. If plant HMGR is synthesized similar to that occurring in animal tissue, then a hypothesis may be that S-carvone interferes with the co-translational insertion of HMGR in the membranes thereby preventing the translation of active HMGR from the messenger. In sprouts, the synthesis of HMGR is then prevented, and due to normal degradation processes - HMGR has an estimated half-life of about 3 h in etiolated pea seedlings (Brooker and Russell 1979) -, already present HMGR will disappear from the membrane fractions as was indicated in the Western blotting experiments (Fig. 6.1). Such a hypothesis is in line with the observation that a cycloheximide (an inhibitor of protein synthesis) treatment of sprouts leads to a similar loss of HMGR activity as observed for an S-carvone treatment. In wound healing tuber tissue S-carvone may also affect membrane insertion, thereby preventing HMGR induction and activity (Chapter 5, Fig. 5.3).

The lipophilic nature of S-carvone may also lead to specific interactions with membrane processes such as co-translation, or with modulation of the membrane environment. S-carvone is taken up rapidly by potato tissue, and it is possible that specific interactions with membranes occur. Davis and Poznansky (1987) showed that alteration of the fluidity of membranes affected the activity of HMGR in animal tissue.

It is interesting to note that the (specific) effect of S-carvone on potato HMGR is similar to that of oxygen-containing monoterpenes on the HMGR activity in animal tissue (Clegg *et al.* 1980, 1982). The conclusion of Clegg *et al.* was that the rate of degradation of HMGR was increased in terpene treated rats, which was responsible for the decreased HMGR activity. In view of recent knowledge of the regulation of HMGR activity, the degradation of HMGR is a well described regulatory mechanism in animal tissue (Gil *et al.* 1985; Nakanishi *et al.* 1988; Chun *et al.* 1990; Hampton and Rine 1994; Correll *et al.* 1994). It is hypothesized that it functions as a feed-back regulation mechanism, in which farnesol is the main trigger leading to enzymatic HMGR degradation (Correll *et al.* 1994) that takes place in the endoplasmatic reticulum (Chun *et al.* 1990; Hampton and Rine 1994). It is possible that a specific degradation is also involved in the disappearance of HMGR from microsomal and organelle membranes of potato tissue. It is an interesting hypothesis that S-carvone acts as an intermediate leading to an enhanced degradation, analogous to the effect of farnesol on HMGR in animal tissue.

Since HMGR is a key enzyme of processes leading to cell division, it is very likely that the decreased HMGR activity in sprouts is responsible for the growth inhibition upon S-carvone treatment. Furthermore, the lack of HMGR activity in wound healing tissue seems to be the main reason for the fact that almost no cambium layers developed, not even after prolonged exposure to S-carvone (up to 3 weeks). The effect of S-carvone on HMGR is therefore different from that on PAL: S-carvone inhibits the HMGR activity, whereas the PAL activity can be induced although S-carvone is present.

Conclusion

S-carvone can be used as a natural, environmentally friendly potato sprout inhibitor (Hartmans *et al.* 1995) and several aspects of the use of S-carvone are described in this thesis. The results show that S-carvone has no

permanent, negative effects on potato tubers as long as the potatoes are allowed to finish the wound healing period before application of the compound. Potato sprouting as well as sprout elongation is blocked if a low, but stable S-carvone headspace concentration is present around the tubers. This is a disadvantage for the use on consumption potatoes because it has to be applied regularly. On the other hand, the reversible effect of S-carvone makes it useful as a temporary sprout inhibitor for seed potatoes. The inhibitory effect of S-carvone on the development of several potato pathogens, e.g. silver scurf, is an important additional advantage.

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Summary

After harvest, potato tubers are usually stored at a temperature of 6-8°C in combination with the application of a synthetic sprout inhibitor. Frequently used sprout inhibitors are isopropyl N-phenyl-carbamate (propham or IPC), isopropyl N-(3-chlorophenyl)carbamate (chlorpropham or CIPC) or a combination of both compounds. There are several reasons for the development of alternative, natural sprout inhibitors. First, the Scandinavian market, for example, requires potato tubers free of (C)IPC residues, and the so-called "green" market, for which no or very little synthetic chemicals are allowed, does not yet have alternative sprout inhibitors. Secondly, governmental policy is directed towards a reduction of the amount of synthetic pesticides used in agricultural practice (Meerjarenplan Gewasbeschermingsmiddelen, MJPG).

Natural potato sprout inhibitors were already used in the ancient Inca cultures. After harvest, the potato tubers were stored in boxes or bins together with the twigs of muña plants (*Minthostachys* species). Treating the tubers in this way controlled sprouting as well as insect attack during a prolonged storage. Volatiles emanating from the muña leaves during the storage were responsible for the insect repellent and sprout inhibitory effects.

The monoterpene S-carvone is a related volatile compound which can be isolated from the seeds of caraway (*Carum carvi* L.) or dill (*Anethum graveolens* L.), for example; also this compound has good potato sprout growth inhibitory effects. Application of S-carvone, derived from caraway seed, as a potato sprout inhibitor can stimulate the demand for caraway and therefore the need to grow it. This can be beneficial for Dutch growers, since cultivation of caraway is suitable on heavy clay soils in which crop rotation is limited to only a few crops. The research described in this thesis has been performed within the Dutch Caraway Research Programme in which nine research groups were amalgamated with the objective to reduce the problems with respect to the cultivation of caraway and to stimulate possible new applications of its essential oil or of S-carvone.

S-carvone inhibits the sprouting of potato tubers and the sprout growth reversibly: removal of S-carvone allows sprouting and regrowth of the individual sprouts. A high dosage leads to necrosis, but the side buds remain their viability and they start to sprout again when the concentration of S-carvone in

the atmosphere comes below a threshold value. The enantiomer of S-carvone, R-carvone, can be isolated from spearmint (*Mentha spicata* L.) and possesses almost the same sprout growth inhibitory properties as S-carvone. Current research is focussed on the practical application of S-carvone to seed potatoes as a reversible sprout growth inhibitor.

In addition to the inhibitory effects just mentioned, the growth of several storage pathogens is also reduced by S-carvone. However, the susceptibility of fungi to S-carvone, e.g. *Fusarium* species that cause dry-rot, differs between (sub)species. *F. solani* var. *coeruleum* is able to grow on tubers treated with S-carvone, whereas *F. sulphureum* cannot withstand it. This difference was not found *in vitro*; both fungi were susceptible to the same range of S-carvone concentrations, they were both able to convert S-carvone with the same rate, and into almost the same conversion products. Therefore, the difference in susceptibility *in situ* must be found in, for example, a specific interaction of the fungi with the potato tubers.

Carvone is stereoselectivily converted into other compounds by potato tissue: R-carvone mainly into neodihydrocarveol, and S-carvone into neoisodihydrocarveol. The bioconversion only takes place in easily accessible tissues, such as sprouts and tuber wound tissue. More than 90% of the amount of S-carvone found in intact tubers, is located on or in the skin. In addition to the chloroform-soluble bioconversion products, water-soluble carvone derived compounds were detected in potato tissue, using ^{13}C-labelling studies. The identity of the conjugated compounds has not been established yet, but S-carvone is found after addition of HCl to the aqueous phase containing the conjugates. The induction of glutathione S-transferase may point to the conjugation of S-carvone to glutathione. Conjugation to saccharides may be an alternative explanation.

The sprout growth inhibition is correlated strongly with a decreasing 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity, a key enzyme providing building blocks for the synthesis of various essential plant metabolites. Using specific potato HMGR antibodies, it was found that the decrease of activity correlated well with the disappearance of HMGR protein signals on Western blots derived from samples of proteins from organelle fractions and microsomal membranes.

S-carvone inhibits the healing of wounded tubers temporarily; in particular, suberization is delayed for about 10 days. The formation of a cambium

layer is almost completely inhibited, which indicates that S-carvone interferes with cell division processes during the healing of wounded tissue. The suberization is correlated with the activity of phenylalanine ammonia lyase (PAL). This enzyme catalyses the first step that leads to the synthesis of suberin, and in S-carvone treated wound tissue, the induction of PAL is delayed for about 10 days. This implies that tuber wound tissue is able to adapt to the exposure to S-carvone.

In conclusion, based on the research described in this thesis, it can be stated that S-carvone is a compound with a great potential because of its sprout growth inhibitory effect, possibly partly due to an inhibition of HMGR. Since S-carvone inhibits sprouting reversibly, it may also be useful as a temporary inhibitor of seed potatoes. In addition, S-carvone reduces the development and growth of several storage pathogens. These effects make the chances of an application of S-carvone as a natural potato sprout growth inhibitor even better.

Samenvatting

Consumptie-aardappelen worden na de oogst gewoonlijk opgeslagen in speciale bewaarschuren en bewaard bij een temperatuur van 6-8°C met toepassing van een spruitremmingsmiddel. Veel gebruikte middelen zijn isopropyl N-fenyl-carbamaat (propham of IPC), isopropyl N-(3-chlorofenyl)carbamaat (chlorpropham of CIPC) of een mengsel van beide stoffen. Er is een aantal redenen aan te geven waarom er gezocht wordt naar alternatieve spruitremmingsmiddelen. Allereerst vanwege marketingtechnische overwegingen: export van consumptie-aardappelen zonder IPC/CIPC-residuen aan bijvoorbeeld de Scandinavische markt, en levering aan de zogeheten "groene" markt waarvoor het gebruik van synthetische middelen niet gewenst is. In de tweede plaats worden van overheidswege maatregelen genomen die gericht zijn op ontmoediging van het gebruik van gewasbeschermingsmiddelen, in het kader van het Meerjarenplan Gewasbeschermingsmiddelen (MJPG), waarbinnen tevens het gebruik van (C)IPC valt.

De oude Inca-culturen maakten al gebruik van spruitremmingsmiddelen. Na de oogst werden de aardappelen opgeslagen in kuilen of kisten waarbij rondom de aardappelen twijgen van muñaplanten (*Minthostachys*-soorten) werden aangebracht. Een dergelijke opslag had een aanzienlijke reductie van de spruiting tot gevolg en het leidde tevens tot een verminderde aantasting door insecten. Dit gebruik berustte op de aanwezigheid van vluchtige stoffen (etherische olie) welke vrijkomen uit de muñablaadjes tijdens de opslag. Deze methode wordt tot op de dag van vandaag door de bewoners van het Andesgebied toegepast.

Een verwante vluchtige verbinding, S-carvon, komt onder anderen voor in de zaden van karwij (*Carum carvi* L.) en dille (*Anethum graveolens* L.). Deze verbinding blijkt ook een uitstekende spruitremmende werking te hebben. Toepassing van S-carvon, gewonnen uit karwijzaad, kan de vraag naar karwijzaad en dus de teelt ervan, stimuleren. Met name voor de Nederlandse landbouw kan dit een gunstige uitwerking hebben, omdat karwij een gewas is dat bij uitstek goed gedijt op de wat zwaardere gronden waar de vruchtwisseling beperkt is. Het onderzoek beschreven in dit proefschrift is uitgevoerd in het kader van het Nationaal Karwij Onderzoeksprogramma, een programma waarbij een negental onderzoeksgroepen hun krachten heeft gebundeld om de

problemen rond de karwijteelt te verminderen en de mogelijke (nieuwe) toepassing van carvon of van karwij-olie te stimuleren.

S-carvon remt de spruiting en de spruitgroei van aardappelen reversibel: verwijdering van S-carvon leidt tot hergroei van de afzonderlijke spruiten. Een hoge dosis geeft necrose van de spruit, maar de zijspruiten in aanleg (in de okselknoppen) blijven in staat tot spruiting hetgeen blijkt wanneer S-carvon niet langer aanwezig is. De enantiomeer van S-carvon, R-carvon, komt voor in *Mentha spicata* L. (aarmunt; "spearmint") en heeft praktisch dezelfde spruitremmende eigenschappen als S-carvon. De reversibele remming van de spruitgroei maakt S-carvon tot een interessante stof die ook toepasbaar zou kunnen zijn als tijdelijke spruitgroeiremmer van pootgoed. Deze mogelijkheden worden momenteel nader onderzocht.

S-carvon remt tevens de ontwikkeling en de groei van diverse schimmels die problemen kunnen vormen tijdens de bewaring van aardappelen. Niet alle schimmels zijn echter in gelijke mate gevoelig voor S-carvon. *Fusarium*-soorten zijn de veroorzakers van droog-rot. Onder een regime van S-carvone wordt *F. sulphureum* in zijn groei op de aardappel geremd, terwijl *F. solani* var. *coeruleum* zich normaal kan ontwikkelen. *In vitro* reageren beide schimmels echter gelijk op S-carvon: ze zijn beide in dezelfde mate en in een gelijk concentratie-traject gevoelig, en ze zijn in staat S-carvon met dezelfde snelheid om te zetten in (praktisch) dezelfde verbindingen. Het verschil in gevoeligheid *in situ* moet wellicht worden gezocht in een specifieke interactie van de schimmels met de aardappelknollen.

Carvon wordt stereoselectief omgezet door aardappelweefsel: R-carvon voornamelijk in neodihydrocarveol, en S-carvon in neoisodihydrocarveol. De bioconversie van carvon vindt alleen plaats in weefsel dat gemakkelijk toegankelijk is voor carvon zoals spruiten en verwond aardappelweefsel. De hoeveelheid carvon die aangetroffen wordt in intacte knollen, zit voor meer dan 90% in of op de schil. Naast de chloroform-oplosbare bioconversieprodukten zijn er door middel van ^{13}C-labellingstudies tevens van carvon afgeleide water-oplosbare stoffen aangetoond. Hierbij is carvon al dan niet direct geconjugeerd aan nog niet nader geïdentificeerde verbindingen waaruit het kan worden vrijgemaakt door een toevoeging van zoutzuur. De aangetoonde inductie van glutathion-S-transferase zou kunnen wijzen op een conjugatie van carvon aan glutathion. Een alternatief zou de vorming van glucosidische verbindingen kunnen zijn.

De remming van de spruitgroei is in sterke mate gecorreleerd met een afnemende activiteit van 3-hydroxy-3-methylglutaryl-coenzym A-reductase (HMGR), een sleutelenzym in de mevalonzuurroute die leidt tot de synthese van diverse essentiële celbestanddelen. Studies met antilichamen, opgewekt tegen aardappel-HMGR, hebben uitgewezen dat de vermindering in activiteit samenhangt met het verdwijnen van HMGR-signalen in "Western blots", gemaakt van eiwitmonsters van microsomale membranen en organelfracties van spruitweefsel.

De natuurlijke wondheling van aardappelen wordt geremd door S-carvon, zij het tijdelijk; het wondhelingsproces, en met name de suberisatie, wordt ongeveer 10 dagen vertraagd. De vorming van een cambiumlaag wordt praktisch geheel geblokkeerd, hetgeen erop wijst dat S-carvon de celdeling in verwond weefsel remt. De suberisatie is nauw gecorreleerd met de activiteit van fenylalanine-ammonia-lyase (PAL). Dit enzym is de eerste stap in de route die leidt tot de vorming van suberine, en in S-carvon behandelde, wondhelende, knollen is de inductie van PAL ongeveer 10 dagen vertraagd. Dit betekent dat het weefsel zich aanpast aan de blootstelling aan S-carvon. De verhoogde expressie van enzymen van het glutathionmetabolisme draagt hier hoogstwaarschijnlijk aan bij.

Concluderend kan, op basis van het in dit proefschrift beschreven onderzoek, worden gesteld dat S-carvon een veelbelovende verbinding is vanwege zijn spruitremmende effect, waarschijnlijk mede als gevolg van een blokkering van HMGR. Omdat S-carvon de spruitgroei reversibel remt, kan het mogelijkerwijs tevens dienen als tijdelijk spruitremmingsmiddel voor pootgoed. De verbinding remt bovendien de ontwikkeling en groei van bepaalde schimmels tijdens de bewaring. Deze effecten maken dat de kansen voor de toepassing van S-carvon als natuurlijk spruitremmingsmiddel nog verder worden versterkt.

Publications

The thesis is based on the following papers:

Oosterhaven, K., K.J. Hartmans and H.J. Huizing (1993). Inhibition of potato (*Solanum tuberosum*) sprout growth by S-carvone: reduction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity without effect on its mRNA level. J. Plant Physiol. 141: 463-470.

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Other publications:

Van der Leij, F.R., R.G.F. Visser, K. Oosterhaven, D.A.M. van der Kop, E. Jacobsen and W.J. Feenstra (1991). Complementation of the amylose-free starch mutant of potato (*Solanum tuberosum*) by the gene encoding granule bound starch synthase. Theor. Appl. Genet. 82: 289-295.

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Curriculum vitae

Jacobus (Koos) Oosterhaven werd geboren op 4 februari 1963 te Gaast, gem. Wonseradeel in Friesland. Na het doorlopen van de HAVO te Sneek werd het diploma van de Hogere Landbouwschool te Leeuwarden behaald (1984), met als specialisaties bodemkunde en plantenteelt. In augustus 1985 werd begonnen met de studie biologie aan de Rijksuniversiteit Groningen. In september 1989 studeerde hij af in de richtingen cel- en plantengenetica (bij prof. dr. ir. W.J. Feenstra) en biochemie (bij prof. dr. B. Witholt). Vanaf november 1989 is hij in dienst van het Agrotechnologisch Onderzoek Instituut (ATO-DLO) te Wageningen. In de periode november 1989 tot en met december 1994 werd onderzoek verricht in het kader van het Nationaal Karwij Onderzoeksprogramma. Dit proefschrift is de schriftelijke weergave van dat onderzoek. Momenteel is de auteur werkzaam als afdelingshoofd van de afdeling Interactieve Bewaarsystemen bij ATO-DLO.

Nawoord

Het karwei is grotendeels geklaard: een proefschrift over de effecten van S-carvon, een component van de etherische olie van de zaden van karwij, op de spruitgroei van aardappelen. Deze woordspeling is mij jarenlang door velen voorgehouden en het lijkt mij toepasselijk om deze uitspraak hier, ongerefereerd, te plaatsen.

De beschreven onderdelen van het karwei, aan karwij, is mede tot stand gekomen door de inzet van velen. Een aantal wil ik noemen. In de eerste plaats moet Klaasje Hartmans hier vermeld staan. Haar praktische kennis en haar stimulerende suggesties voor de experimenten waren onmisbaar. De heer Kasper Hamster, grote inspirator van het Karwij Onderzoek Programma wil ik noemen omdat er zonder de inspanningen van de heer Hamster geen onderzoek naar de teelt van karwij en de toepassingen van karwijolie zou hebben plaatsgevonden. Mede dankzij de heer Hamster heb ik 4 jaar onderzoek kunnen doen aan de kiemremmende werking van S-carvon. Henk Huizing wil ik noemen voor de vrijheid die ik kreeg om het onderzoek binnen zijn hoofdafdeling uit te voeren. Janny en Sarah wil ik bedanken voor het uitvoeren van aan aantal analyses en Aart van Amerongen voor de organisatie bij de antilichamen. Het spijt mij erg dat ik Henk Plasman, die een wezenlijke bijdrage aan het onderzoek naar de HMGR-antilichamen heeft geleverd, niet meer persoonlijk kan bedanken omdat hij in het voorjaar van 1995 verongelukt is. Theo de Rijk, GC-MS specialist, wil ik noemen voor zijn bijdrage in het onderzoek naar de omzettingen van monoterpenen door aardappelweefsels. Antonio Leitao uit Lissabon, voor het onderzoek naar de effecten van S-carvon op de *Fusarium* schimmels.

Ongetwijfeld doe ik een aantal mensen onrecht aan door ze niet te noemen, zoals al mijn voormalige labgenoten, de mensen van moleculaire biologie, de NMR-specialisten, de hulpvaardige handen bij de celcultures en al die anderen, zowel binnen als buiten het ATO, die mij daadwerkelijk (verder) hebben geholpen.

Welnu, het promotiekarwei is af, maar er komen er weer vele nieuwe aan. Daarom, genoeg gedankt, weer aan het werk.....