

Screening of Natural Plant Volatiles to Control the Potato (*Solanum tuberosum*) Pathogens *Helminthosporium solani*, *Fusarium solani*, *Phoma foveata* and *Rhizoctonia solani*

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Abstract The potential utility of natural volatiles in various essential oils (EOs) from plants as fumigants to control potato tuber (*Solanum tuberosum* L.) pathogens was assessed. The antifungal effects of the volatiles at various concentrations were studied at 10 °C both in vitro using conidial suspensions of *Helminthosporium solani*, *Fusarium solani* var. *coeruleum* and *Phoma foveata* plated on agar, and in vivo by inoculating potato tubers. The effects of the volatiles on the mycelial growth of *Rhizoctonia solani* were also studied, but only in vitro. Vapours of many of the EOs tested exhibited some fungicidal activity but volatiles of garlic, *Allium sativum*, were, with few exceptions, most effective on all four pathogens in all experiments. An exposure time of at least 2 weeks was usually required for good control of disease development in vivo. Vapours of *A. sativum* never stimulated conidial germination, as was observed with some other oils, or damaged tubers, but those of *Armoracia rusticana* caused tuber collapse. Volatiles from thyme (*Thymus vulgaris*) EO showed antifungal activity in vitro on all four pathogens, but did not control *F. solani*, *P. foveata* or *H. solani* in vivo. In contrast, sage (*Salvia officinalis*) EO was ineffective against *P. foveata* and *H. solani* in the in vitro system, but controlled disease development in vivo at similar doses. The sometimes conflicting results obtained in the two test systems show that screening in vitro only is insufficient for evaluation of potent antifungal substances to be used in practice.

Keywords *Allium sativum* · *Thymus vulgaris* · *Salvia officinalis* · Antifungal · Essential oils · Fungicidal

Abbreviations

cfu colony-forming units
EO essential oil

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Introduction

In accordance with increasing global concerns for the protection of public health and the environment, in Sweden there is growing public and governmental pressure to minimise the use of synthetic pesticides in food production and to increase the acreage of organic farming. Consequently, alternative methods to control pests and diseases are urgently needed. Since antiquity, aromatic plants have been used to improve the storability of food and feed. For example, in the Andes, leaves of Muña plants (*Minthostachys* and *Satureja* spp.) have been traditionally placed in potato (*Solanum tuberosum* L.) tuber storage chambers to repel insects and prevent the tubers from sprouting (Aliaga and Feldheim 1985). More recently, the potential utility of natural volatile products in potato production has also been explored, but mainly as sprout suppressants of tubers (Meigh 1969; Vaughn and Spencer 1993; Vokou et al. 1993; Hartmans et al. 1995; Oosterhaven et al. 1995a, b, c). Their antimicrobial activities against potato pathogens have not been extensively studied to date. In an in vitro trial, the effects of caraway, cassia, cumin, dill and spearmint essential oils (EOs) at 160 ppm on the radial growth of the tuber pathogens *Fusarium solani* var. *coeruleum*, *F. sulphureum* (also known as *F. sambucinum*), *Phoma exigua* var. *foveata* and *Helminthosporium solani* were limited (Gorris et al. 1994). Mayton et al. (1996) have observed fungicidal actions of volatile compounds released from fresh, macerated *Brassica juncea* cv. Cutlass leaves in vitro on the mycelial growth of the potato pathogens *Verticillium dahliae*, *V. albo-atrum*, *Pythium ultimum*, *Colletotrichum coccodes* and *Rhizoctonia solani*. In vivo trials have generally focused solely on the effects of single compounds. For instance, Vaughn and Spencer (1994) found that tuber decay caused by two different strains of *F. sambucinum* (*F. sulphureum*) was reduced by vapours of both cineole and menthol. In addition, carvone purified from caraway seeds significantly reduced the silver scurf (*H. solani*) index in naturally infected potato tubers when they were exposed to vapours under semipractical storage conditions (Hartmans et al. 1995). Rotting was also prevented in tubers artificially inoculated with *F. sulphureum* or *Phoma foveata*, but not in those inoculated with *F. solani*, when exposed to carvone vapours in studies by Gorris et al. (1994) and Hartmans et al. (1995).

The aim of the work presented here was to study the potential utility of volatiles released from various EOs or macerated aromatic plant material, as natural postharvest fumigants to control potato tuber pathogens. Four pathogenic fungi, with varying infection strategies, were included in the experiments: *H. solani* Dur. et Mont, which actively penetrates the tuber skin and causes silver scurf; the two wound parasites *F. solani* var. *coeruleum* (Sacc.) Booth and *P. foveata* Foister, which cause dry rot and gangrene of potatoes, respectively; and *R. solani* Kühn, which produces superficial sclerotia, ‘black scurf’, on the surface of tubers. Preferably, one plant species would be identified that would be effective against all four pathogens and could be routinely and conveniently used to control infections in organic potato seed production. This is the first report of antifungal activity by volatiles of a broad array of plant EOs on conidia of *H. solani*, *F. solani* and *P. foveata* in vitro and diseases caused by these pathogens in vivo and, in addition, on mycelial growth of *R. solani*, most probably AG3, in vitro.

Materials and Methods

To resemble practical conditions during the first few weeks of tuber storage, the most critical period for disease development, a treatment temperature of 10 °C and relatively short exposure periods were used in all experiments. The effects of varying (1) the duration of the exposure to the EOs, (2) the doses of the EOs and (3) the number of applications of the EOs were studied both *in vitro* (using inoculated agar in Petri dishes) and *in vivo* (using artificially inoculated potato tubers) in successive experiments. As the aim was to find something that could be practically useful, the results of preceding experiments sometimes led to adjustments of the experimental plans in those that followed.

Essential Oils

Most of the EOs used were imported ones, bought from CreArom, Gammelstad, Sweden, although some were gifts from other sources listed in the tables and “Acknowledgements”. The plant species from which the EOs were derived are shown in the tables. According to the suppliers, all of the EOs were produced by steam distillation. All of the EOs were stored in the dark at 5 °C and handled in a fume cupboard.

The initially and subsequently added (as appropriate) liquid volume of the oils introduced into the treatment chambers is indicated as the dose used in the experiments. However, owing to leakage in the experimental systems the concentrations of the vapours during treatments were not constant. To facilitate comparisons between systems, the amounts of oil used are presented in ppm, i.e. the volume of EO added divided by the volume of the treatment chamber (the headspace above the agar surface in the Petri dishes or the volume of the empty boxes used in the *in vivo* studies).

Pathogen Inocula

All isolates used to prepare inocula originated from naturally infected tubers and were cultured at room temperature (20–22 °C): *F. solani*, *P. foveata* and *R. solani* on a previously described sucrose agar medium amended with streptomycin sulphate (Bång 1989), and *H. solani* on a medium specifically developed to accelerate the growth rate of this slow-growing organism, consisting of 39 g l⁻¹ potato dextrose agar from Oxoid with 2 g l⁻¹ of both yeast extract and bacteriological peptone, from Difco, adjusted to pH 6.6–6.7.

Conidial suspensions of *H. solani* and *F. solani*, and pycnidiospore suspensions of *P. foveata* were mostly prepared from mixtures of strains, occasionally only one, by repeatedly washing cultures with sterile tap water and gently rubbing their surfaces with a sterile plastic loop, followed by filtration through a thin layer of sterile cotton wool. The *P. foveata* cultures were flooded with water 30 min prior to harvesting to stimulate the release of pycnidiospores from pycnidia. The age of the cultures used varied from 3 to 5 weeks for *H. solani* and from 4 to 16 weeks for *F. solani* and *P. foveata*, respectively. The suspensions were counted using a Bürker haemocytometer and diluted with sterile tap water so they contained

appropriate amounts of conidia per ml. In experiments with *R. solani* in vitro mycelial plugs from 1–2-week-old cultures were used.

In Vitro Experiments: Inoculation and Treatment Procedures, Scoring of Fungal Growth

The technique used to assess the effects of the EO vapours on the pathogens in vitro was based on the microatmosphere method of Kellner and Kober, described by Benjilali et al. (1984). Suspensions of fungal propagules were evenly plated on agar in 9-cm Petri dishes. The suspensions were applied using sterile plastic 10- μ l loops in amounts varying between 20 and 40 μ l. After the agar had been allowed to absorb the suspensions for 1–2 h the plates were inverted and the EO to be tested was added to a 10-mm-diameter filter paper disc in the middle of the cover. In experiments with *R. solani*, agar plugs (4 mm diameter) with actively growing mycelia were cut out using a sterilised cork borer and inserted into holes of the same diameter made in new agar dishes. These inoculated dishes were handled as described above.

The dishes were sealed with Parafilm and replicates of the same treatment (the number of which varied among experiments; see later) were placed in a plastic bag, which was sealed. Dishes were stored at 10 °C in the dark during the EO treatments, the length of which varied among experiments; see later. The covers containing the EO-amended filter paper were then removed and replaced by new sterile covers, after which the plates were stored at room temperature without any sealing to permit ventilation of the residual volatiles. The numbers of colony-forming units (cfu) of *H. solani*, *F. solani* and *P. foveata* developing on the agar and the radial growth of *R. solani* were continuously recorded for at least 5 weeks, making assessments of the fungistatic versus fungicidal actions of the EOs possible. Registrations started from the time the EOs were removed and ended when there was no further colony development or when the number had reached 200, as the agar surface in the Petri dishes was then fully covered. The maximum radial growth of *R. solani* was 85 mm.

In Vivo Experiments: Disinfection of Tubers, Inoculation and Treatment Procedures

Tubers to be inoculated were gently washed in running tap water, surface-sterilised by immersion in 2% sodium hypochlorite for 2 min, rinsed for 5 min in running tap water, immersed in 0.5% sodium thiosulphate for 2 min to inactivate excess chlorite and then rinsed for 15 min in running tap water (Löschenkohl 1979). The tubers were subsequently air-dried and rested at 10 °C for 2–5 days in the dark and then inoculated.

Tubers to be inoculated with *F. solani* or *P. foveata* were wounded by swinging a pendulum bearing a circular metal disc with nine sharp 2-mm points at them from an angle of 60° (modified after Olsson 1988). Each tuber was hit twice, once on each side, and a single drop (approximately 30 μ l) of conidial or pycnidiospore suspension (as appropriate) was applied at each wounding site using a Pasteur pipette.

In order to prevent movement of the tubers to be superficially inoculated with *H. solani*, their downward side was cut flat. On the upper surface, they were then

inoculated with conidial suspension at sites, gently marked using a cork borer and waterproof ink to facilitate observations of pathogen development, by means of a sterile plastic loop dipped in the conidial suspension.

After inoculation, tubers were allowed to rest in darkness for 4 h at 10 °C before being placed in treatment chambers (3.25-l plastic boxes) that had been prepared by placing the bottom of a 9-cm glass Petri dish, containing the desired amount of the EO to be tested, on a paper towel that had been wetted by adding 50 ml of tap water to maintain high levels of relative humidity, on the floor of each chamber, and covering the dish with a centred 16×16 cm square of plexiglass. The plexiglass in each case was perforated with a 14-mm-diameter hole in its centre and eight peripheral 8-mm holes, each approximately 2 cm from its edges to promote even distribution of the vapours. The inoculated tubers were gently placed on the perforated plexiglass, avoiding covering the central hole, so that passage of vapours within the box would not be impeded. The boxes were closed with a plastic lid. During the treatment period, the boxes were ventilated by removing the lid once or twice a week for 2 min, to keep the oxygen and carbon dioxide concentrations at approximately atmospheric levels. Controls were handled and stored likewise, but without plant volatiles. All *in vivo* experiments were carried out at 10 °C in the dark. The plastic treatment boxes, which absorbed some components, were carefully marked and reused with the same EO in successive experiments.

After the EO treatments, tubers were gently transferred to plastic trays with wetted paper towels on the bottom and loosely covered with a plastic sheet. Trays were stored at 10 °C in the dark until pathogen development was scored.

In Vivo Experiments: Scoring of Disease Development

The number of *H. solani* conidiophores developing at each inoculated site on the tubers was non-destructively observed at 25–40-fold magnification on at least two occasions, the first within 15 days after the last EO treatment when conidia were already abundant on controls, and the last 3 weeks later. Infection was evaluated as the number of conidiophores per inoculation site and expressed as an index, ranging from 0 to 10, as follows: 0, no conidiophores; 1, one to ten conidiophores; 2, 11–20 conidiophores; 3, 21–30 conidiophores; 4, 31–40 conidiophores; 5, 41–50 conidiophores; 6, 51–60 conidiophores; 7, 61–70 conidiophores; 8, 71–80 conidiophores; 9, 81–90 conidiophores; and 10, 91 or more conidiophores. Occasionally, some inoculation sites had more than 200 conidiophores, probably owing to spontaneous infections. They were also given a score of 10. Index scores from all inoculation sites in each replicate were summed and divided by the total number of inoculation sites to obtain the mean infection index per replicate.

Tubers inoculated with *P. foveata* or *F. solani* were scored 7 weeks after inoculation, a period encompassing exposure to the EOs and the following storage period. Each tuber was sliced through both inoculation sites and visually rated for both the width (w_i) and depth (d_i) of the rots. For w_i a scale of 1 corresponding to no rotting and 2, 3, 4 and 5 corresponding to rots with diameters of 13 mm or less, 13–26 mm, 26–39 mm and more than 39 mm was used. Values for d_i were assigned as follows: 1, no extension; 2, superficial lesion restricted by the vascular bundle;

and 3, lesion extending deep into the tuber. An index of attack was calculated using the formula

$$\text{Attack index} = \frac{1}{n} \sum_{i=1}^n (w_i \times d_i),$$

where n is the number of inoculation sites in a sample. The rot index could thus have values between 1 and 15.

Treatments that were only initially significantly suppressive, and gave scores that were close to control values and not significantly different from them at the final reading, were deemed to be fungistatic, while those resulting in significantly fewer colony-forming units or significantly less disease on the final registration date were deemed to be fungicidal.

In Vitro and In Vivo Experiments Including Exposures of 1, 2 or 3 Weeks

In the first in vitro studies, 50 μl of EO, listed in Table 1, was added to the filter paper discs in the lids of each inoculated Petri dish, giving an initial concentration of around 800 ppm (volume of EO divided by volume of air). The treatments lasted for 1, 2 or 3 weeks, after which the oil-amended filter paper discs were removed as described earlier. In each of these experiments there were two dishes per pathogen and treatment duration. *F. solani* and *H. solani* experiments were repeated after some time, resulting in four replicates, but regarding *P. foveata* there was one experiment, i.e. two replicates. In a following in vitro experiment of a similar kind with *H. solani*, 10 μl oil was used in each Petri dish, resulting in an initial concentration of 160 ppm. EOs of *Allium sativum*, *Carum carvi*, *Lavandula angustifolia*, *Mentha arvensis*, *M. piperita*,

Table 1 Relative values of colony-forming units, compared with untreated controls, of *Fusarium solani* var. *coeruleum*, *Phoma foveata* and *Helminthosporium solani* after exposure of conidia plated on agar for 1, 2 or 3 weeks to vapours of the essential oils listed at 800 ppm. Data refer to final registration 5 weeks or more after each respective treatment had stopped

Essential oil exposure period (weeks)	<i>Fusarium solani</i> ($n=4$)			<i>Phoma foveata</i> ($n=2$)			<i>Helminthosporium solani</i> ($n=4$)		
	1	2	3	1	2	3	1	2	3
Control	117	110	104	101	156	174	129	124	121
<i>Allium sativum</i>	0*	0*	0*	0*	0*	0*	0*	0*	0*
<i>Anethum graveolens</i>	70	15*	19*	35*	0*	0*	25*	0*	0*
<i>Carum carvi</i>	64*	23*	0*	28*	10*	0*	27*	0*	0*
<i>Mentha piperita</i>	89	86	97	228*	91	49*	81	73*	67*
<i>Mentha spicata</i>	34*	0*	0*	1*	0*	0*	24*	0*	0*
<i>Ocimum basilicum</i>	92	97	96	70	25*	8*	80	74*	73*
<i>Populus balsamifera</i> ^a	0*	0*	0*	0*	0*	0*	0*	0*	0*
<i>Tagetes glandulifera</i>	88	84	94	110	107	32*	NT	NT	NT
<i>Tagetes mexicana</i> ^a	19*	38*	39*	103	42*	21*	18*	14*	28*
<i>Tagetes rosola</i> ^a	47*	35*	17*	111	29*	17*	21*	8*	0*
<i>Thymus vulgaris</i>	65*	48*	51*	31*	0*	0*	24*	0*	0*
<i>Thymus vulgaris</i> ^a	68*	50*	47*	53*	0*	0*	53*	0*	0*

NT not tested

* Means significantly different from the control mean at $P \leq 0.05$

^a Essential oil obtained from Lars Björk, Swedish University of Agricultural Sciences, Balsgård, Sweden

M. spicata and *Minthostachys verticillata* received from Vincenzo De Feo, University of Salerno, Italy, *Populus balsamifera* received from Lars Björk, Swedish University of Agricultural Sciences, Balsgård, Sweden, *Rosmarinus officinalis*, *Salvia officinalis* and *Thymus vulgaris* were then included. There were two Petri dishes (replicates) per EO and treatment duration. Suspensions used for inoculations in these experiment contained between 7×10^3 and 12×10^3 conidia per ml.

The influence of treatment durations as described above was also studied in in vivo experiments with *F. solani*, *P. foveata* and *H. solani*, respectively. An amount of 250 μl was then added to the 3.35-l plastic treatment boxes, resulting in an initial concentration of 77 ppm. The same EOs as those used in in vitro studies, listed in Table 1, were used except for *Tagetes mexicana*, which was not included. In the *F. solani* experiment, performed in October, there were two replicates per EO treatment. Each treatment box contained nine inoculated (8.5×10^4 conidia per ml) tubers, cv. King Edward. Three tubers were picked out of the boxes after 1 and 2 weeks, respectively, when the tubers were ventilated, leaving three tubers for the 3-week treatment period. Consequently there were six inoculations (three tubers with two inoculations each) per replicate. In the *P. foveata* experiment, performed in March, there were six replicates per EO treatment, and the cultivar used was Sabina, inoculated using a suspension with 7.8×10^4 conidia per ml. Otherwise the procedure was as described for *F. solani*.

Two in vivo experiments were performed with *H. solani*, one in November, using cultivar Sabina, and one in January, using cultivar Bintje. In the November experiment, each tuber was inoculated (10×10^4 conidia per ml) at five marked sites as earlier described, and there were nine tubers in each treatment box, three for each exposure as described above. Consequently, each replicate consisted of 15 inoculations in this experiment. In the January experiment, there were three inoculation sites per tuber (7.5×10^4 conidia per ml). Instead of picking tubers out at intervals as in the previous experiment, there were two boxes, each containing ten tubers, for each EO and exposure. Consequently, each of the two replicates consisted of 30 inoculations in the January experiment.

In Vitro and In Vivo Experiments Including Single and Split Doses

The effects of single and split doses on fungal developments were compared in vitro and in vivo. In an *H. solani* in vitro experiment, the single doses were obtained by initially adding 5 or 10 μl oil to the filter paper disc in each Petri dish, giving 80 and 160 ppm, respectively. In a twofold split dose 5 μl was added initially and another 5 μl was added to the same filter paper disc after 1 week, and in a threefold split there was 4 μl (60 ppm) initially, 3 μl (50 ppm) was added after 1 week and an additional aliquot of 3 μl was added after 2 weeks. The total exposure lasted 5 weeks for all treatments (2 weeks after the last addition of oil in the threefold split). There were five dishes per dose.

In a parallel in vivo experiment, potato tubers, cv. Bintje, were inoculated with *H. solani* at three marked sites as described above at the beginning of April. In the single doses, 260 μl or 520 μl was added to the treatment boxes, resulting in 80 and 160 ppm, respectively, the twofold split consisted of 260 μl (80 ppm) plus 260 μl that was added after 1 week, and in the threefold split 195 μl (60 ppm) plus 162.5 μl

(50 ppm) after 1 week plus 162.5 µl after 2 weeks was used to obtain concentrations comparable to those used in the in vitro study. The total exposure lasted 5 weeks for all treatments (2 weeks after the last addition of oil in the threefold split). There were three boxes per EO and dose containing eight tubers each, resulting in 24 inoculations per replicate. The same original suspension was used for inoculations in both in vitro and in vivo experiments, starting the same day, having 4.5×10^3 and 10×10^3 conidia per ml, respectively.

In two other in vivo experiments, the effect of a single dose of 77 ppm was compared with a twofold split of 38.5 ppm initially plus 38.5 ppm after 11 days using Ukama tubers inoculated with *F. solani* in February and Bintje tubers, each inoculated at three marked sites with *H. solani*, in March. In addition to the EOs listed in Table 2, *L. angustifolia*, *M. spicata* and *M. verticillata* received from Vincenzo De Feo, *P. balsamifera* received from Lars Björk and *T. vulgaris* (*H. solani* experiment, only) were included. In the *F. solani* experiment there were five replicates per EO and dose, and in the *H. solani* experiment there were two replicates per EO and dose, each comprising eight tubers. Exposures were 3 weeks in both experiments.

In Vitro and In Vivo Experiments Including Various Doses in Single Applications

The effects on *P. foveata* of various oils, listed in Table 3, at concentrations between 10 and 320 ppm were investigated in an in vitro experiment, and in vivo at concentrations between 10 and 160 ppm. *A. sativum* EO was the only one used at 10 and 20 ppm and it was not included at 160 or 320 ppm. Appropriate amounts of

Table 2 Relative values of *Helminthosporium solani* colony-forming units, and infection index, in in vitro and in vivo experiments, respectively, compared with untreated controls, after exposing conidia plated on agar in Petri dishes (in vitro) or inoculated potato tubers (in vivo) to vapours from the essential oils listed in single or split applications, as indicated. In vitro experimental data from two occasions are presented: on removal of the essential oil (*date 1*), and 5 weeks thereafter (*date 2*)

	In vitro (n=5)								In vivo (n=3)			
	Date 1				Date 2				Index 3.5			
Control values	102 cfu				113 cfu							
Amount of essential oil added initially (ppm)	80	160	80	60	80	160	80	60	80	160	80	60
After 1 week (ppm)			80	50			80	50			80	50
After 2 weeks (ppm)				50				50				50
<i>Allium sativum</i>	0*	0*	0*	0*	0*	0*	0*	0*	29*	53*	15*	15*
<i>Carum carvi</i>	4*	5*	1*	0*	80	26*	57*	28*	77	76	54*	57*
<i>Mentha arvensis</i> var. <i>sachalinensis</i> ^a	0*	0*	0*	0*	116	104	114	114	105	107	104	123
<i>Mentha piperita</i>	0*	0*	0*	0*	78	77	60*	60*	73	44*	29*	41*
<i>Rosmarinus officinalis</i>	106	78	106	101	103	77	102	93	95	61	83	79
<i>Salvia officinalis</i>	122	72*	37*	9	121	90	95	114	115	84	86	64*

cfu colony-forming units

*Means significantly different from the control mean at $P \leq 0.05$

^aEssential oil obtained from T.H. Iversen, University of Trondheim, Norway

Table 3 Relative values of *Phoma foveata* colony-forming units, and infection index, in in vitro and in vivo experiments, respectively, compared with untreated controls, after exposing conidia plated on agar in Petri dishes (in vitro) or inoculated potato tubers (in vivo) to vapours from the essential oils listed, for 2 or 3 weeks, respectively, at the doses indicated. In vitro experimental data from two occasions are presented: on removal of the essential oil (*date 1*), and 5 weeks thereafter (*date 2*)

Control values	In vitro (<i>n</i> =5)										In vivo (<i>n</i> =3)		
	Date 1					Date 2					Index 10.3		
	200 cfu					200 cfu							
Amount of essential oil added (ppm)	10	40	80	160	320	10	40	80	160	320	10	40	160
<i>Allium sativum</i>	0 ^a	0*	0*	–	–	0 ^a	0*	0*	–	–	68*	35*	–
<i>Carum carvi</i>	–	49*	2*	0*	0*	–	100	100	66*	0*	–	87	100
<i>Lavandula angustifolia</i>	–	97	81*	29*	0*	–	100	100	100	97	–	–	–
<i>Mentha arvensis</i>	–	–	–	–	–	–	–	–	–	–	–	81*	70*
<i>Mentha piperita</i>	–	76*	12*	0*	0*	–	100	100	95	85*	–	86	72*
<i>Mentha spicata</i>	–	72*	24*	0*	0*	–	100	100	71*	0*	–	82*	77*
<i>Ocimum basilicum</i>	–	29*	3*	0*	0*	–	73*	36*	8*	0*	–	93	86
<i>Origanum majorana</i>	–	92	74*	66*	12*	–	100	100	100	91	–	105	85
<i>Salvia lavandulafolia</i>	–	99	100	87	22*	–	100	100	100	100	–	70*	43*
<i>Salvia officinalis</i>	–	87	38*	42*	19*	–	100	100	100	100	–	85	61*
<i>Thymus vulgaris</i>	–	14*	0*	0*	0*	–	62*	11*	0*	0*	–	89	89

*Means significantly different from the control mean at $P \leq 0.05$

^aData not included in statistical analyses

oils were added as described earlier. In the in vitro experiment, there were five Petri dishes per EO and dose and the exposure lasted 2 weeks. In the in vivo experiment King Edward tubers were inoculated in February and exposures lasted 3 weeks. Per EO and dose there were three boxes, each containing eight tubers, leading to 16 inoculations per replicate. The same original conidial suspension was used for both experiments, which started the same day. For inoculation of tubers it contained 6.2×10^4 conidia per ml, and for plating on agar it was diluted tenfold.

Similar types of parallel in vitro and in vivo experiments were performed with *F. solani* at 40–160 ppm over 3 weeks. The conidial suspension contained 7.5×10^4 conidia per ml when inoculating tubers, cv. Escort, and it was diluted tenfold for use in in vitro experiments. *A. sativum*, *C. carvi*, *M. piperita* and *S. officinalis* oils were included.

In addition, two *P. foveata* in vitro experiments were repeated twice with the same EOs as those listed in Table 3, except that *L. angustifolia*, *O. majorana* and the *Salvia* species were not included and the lowest dose of *A. sativum* used was 20 ppm (included in one of these experiments). Also there were three more *P. foveata* in vivo experiments including various oils at concentrations between 40 and 277 ppm.

In Vitro *R. solani* Experiments Including Various Doses

The effects on the radial growth of *R. solani* of various oils, listed in Table 4, at concentrations between 40 and 320 ppm were investigated in two similar in vitro experiments. In each of these there were five Petri dishes per EO and dose and exposures lasted 2 weeks.

Table 4 Relative radial growth, compared with untreated controls, of *Rhizoctonia solani* after exposure of mycelial plugs plated on agar in Petri dishes to vapours of the essential oils listed, at doses between 40 and 320 ppm, for 2 weeks. Data from two occasions are presented: on removal of the essential oil (*date 1*), and 5 weeks thereafter (*date 2*). The data are means of ten observations (five replicates each in two experiments) per essential oil and dose unless otherwise stated. Mean control values were 44.9 mm at date 1 and 85 mm (the maximum) at date 2

Amount of essential oil added initially (ppm)	Date 1				Date 2			
	40	80	160	320	40	80	160	320
<i>Allium sativum</i>	0*	0*	0*	0*	13*	0*	0*	0*
<i>Anethum graveolens</i>	23*	18*	0*	0*	100	100	70*	25*
<i>Carum carvi</i>	17*	6*	0*	0*	100	100	40*	0*
<i>Lavandula angustifolia</i>	40*	10*	0*	0*	100	100	100	80
<i>Mentha arvensis</i> var. <i>sachalinensis</i> ^a	0*	0*	0*	0*	100	100	100	0*
<i>Mentha piperita</i>	17*	0*	0*	0*	100	100	87	33*
<i>Mentha spicata</i>	42*	5*	0*	0*	100	100	70*	0*
<i>Ocimum basilicum</i>	25*	0*	0*	0*	100	100	60*	0*
<i>Origanum majorana</i>	79*	30*	0*	0*	100	100	100	68*
<i>Pogostemon patchouly</i> ^b	87	82	84	81	100	100	100	100
<i>Rosmarinus officinalis</i>	51*	30*	13*	5*	100	100	70*	80
<i>Salvia lavandulafolia</i>	107	82	30*	0*	100	100	100	100
<i>Salvia officinalis</i>	81	51*	17*	0*	100	100	100	90
<i>Thymus vulgaris</i>	0*	0*	0*	0*	0*	0*	2*	0*
<i>Thymus vulgaris</i> ^c	12*	0*	0*	0*	100	100	40*	0*

*Means significantly different from the control mean at $P \leq 0.05$

^a Essential oil obtained from T.H. Iversen, University of Trondheim, Norway, one experiment only, five replicates

^b One experiment only, five replicates

^c Essential oil obtained from Lars Björk, Swedish University of Agricultural Sciences, Balsgård, Sweden, one experiment only, five replicates

In Vivo *P. foveata* Experiment Including Delayed Start of EO Treatments After Inoculation

The effects of applying vapour treatments some time after inoculation with *P. foveata* were studied in an experiment starting in November using cv. Bintje. Various oils, indicated in Fig. 2, were added to the boxes 0, 4, 8 or 12 days after inoculation with a suspension having 9×10^4 conidia per ml. The amount of oil used was 300 μ l, 92 ppm, and the exposure lasted 3 weeks. Per EO and time interval there were three boxes, each containing eight tubers, leading to 16 inoculations per replicate. Gangrene indexes were scored 8 weeks after inoculations in this experiment.

In Vivo *P. foveata* and *F. solani* Experiments Including Vapours of Macerated Plant Material

In two experiments the influence of vapours from garlic (*A. sativum*) or onion (*A. cepa*) bulbs or horseradish (*Armoracia rusticana*) roots on dry rot and gangrene development in artificially inoculated tubers was studied. Material was macerated using an electric household mincer (Fig. 3a). In the *P. foveata* experiment, tubers of cv. Bintje were inoculated in December (7.6×10^4 conidia per ml) and in the

F. solani experiment, tubers of cv. Escort were inoculated in January (7.5×10^4 conidia per ml). Treatments lasted 3 weeks and were performed in 5.5-l glass desiccators wherein 50 g macerated plant material (in the *F. solani* experiment only 10 g of *A. rusticana* was used) was placed on the bottom and the inoculated tubers were rested above on the perforated ceramic disc. The dose thus achieved for the *Allium* species was 9.1 g l^{-1} . There were three desiccators per treatment.

In the third of this type of experiments, performed in February, *A. sativum* bulbs were macerated manually using the finest side of a handhold grater, put in wide glass bowls at doses of 0.2, 0.04 or 0.02 g l^{-1} in 25-l plastic boxes for 3 weeks (Fig. 3b). There were three boxes per dose, each containing 20 King Edward tubers, inoculated with *P. foveata* (6.2×10^4 conidia per ml), which rested on a frame constructed of plastic mosquito netting.

Statistical Analyses

Statistical calculations were performed using GLM-ANOVA as implemented in NCSS, copyright Jerry L. Hintze, Kaysville, UT, USA. Since many treatments applied in the in vitro experiments resulted in zero values, while others resulted in large numbers of colony-forming units per dish, these skewed data sets were square-root-transformed according to the formula $\sqrt{(x + 0.5)}$ before the calculations, where x is the number of colony-forming units per plate. The disease indexes obtained in the in vivo trials were used without further transformation. With very few exceptions, analysis of variance (ANOVA) revealed significant interactions between the factors *oil* and *exposure time*, or *oil* and *concentration* when all data from a trial were analysed by two-factorial ANOVA. Partly for this reason, and partly because the separate means for each treatment are of greater interest than the overall mean values, the data were partitioned and analysed by one-way ANOVA. In some cases, data from two experiments with the same experimental plan were combined and the experiment was then treated as for a random blocking factor. To facilitate the interpretation and comparisons of results from trials with differing units, treatment means of untransformed data are expressed relative to controls in all in vitro and most in vivo trials.

Results

In Vitro and In Vivo Experiments Including Exposures of 1, 2 or 3 Weeks

The results from in vitro experiments including exposures at 800 ppm of *F. solani*, *P. foveata* and *H. solani* conidial suspensions plated on agar for 1, 2 or 3 weeks are presented in Table 1. Many treatments significantly reduced the number of colony-forming units, in most cases more efficiently so with increasing treatment periods. None of the pathogens grew after any of the treatment periods using vapours of *A. sativum* or *P. balsamifera*. After a minimum of 2 weeks of exposure, *M. spicata* completely prevented growth of all three fungi too, while *T. vulgaris* and *Anethum graveolens* were then only effective against *P. foveata* and *H. solani*.

After a treatment period of at least 3 weeks using *C. carvi* none of the organisms produced any colonies; for *H. solani* only 2 weeks was needed for complete control. Significantly more colonies of *P. foveata* developed when conidia were exposed to an atmosphere of *M. piperita* for 1 week, but not if the treatment was longer.

In an in vitro experiment with *H. solani* at a lower dose, 160 ppm, for 1, 2 or 3 weeks only a few colonies developed after the shortest treatment time with *A. sativum*, which was otherwise completely fungicidal, as was *T. vulgaris* oil in 3-week treatments (data not shown). Most of the other oils used were merely fungistatic under these experimental conditions, but the *C. carvi* oil significantly stimulated conidial germination in exposures of 1 week (relative value 213, $P \leq 0.001$) or 2 weeks (relative value 194, $P \leq 0.001$).

In Fig. 1 selected results from three in vivo experiments with *F. solani*, *P. foveata* and *H. solani*, respectively, examining the effects of exposures for 1–3 weeks at 77 ppm, are presented. *F. solani* and *P. foveata* disease developments were significantly reduced when *A. sativum* treatments lasted at least 2 weeks, while the *H. solani* disease index was reduced after a treatment period of 1 week. *P. balsamifera* EO significantly reduced the *P. foveata* disease index after just 1 week of exposure and the *F. solani* index after at least 2 weeks, but it only transiently reduced *H. solani* conidiophore development after 2 weeks (data not shown). *M. piperita* EO reduced the rot incidence of *F. solani* after 2 and 3 weeks of treatment and the disease index of *H. solani* after 3 weeks (data not shown). All oils included in the *P. foveata* experiment except *A. sativum* and *P. balsamifera* increased the gangrene incidence significantly (data not shown).

In Vitro and In Vivo Experiments Including Single and Split Doses

The results from in vitro and in vivo experiments with *H. solani*, in which the effects of single and split doses were compared, are presented in Table 2. *A. sativum*

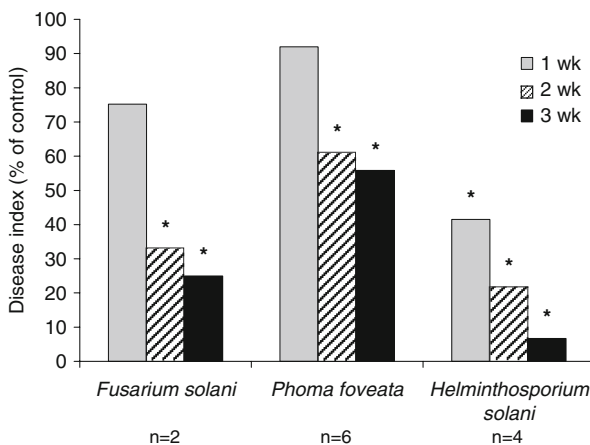


Fig. 1 Relative values of disease caused by *Fusarium solani*, *Phoma foveata* and *Helminthosporium solani*, compared with untreated controls, having mean disease indexes of 6.2, 9.7 and 4.0, respectively, in artificially inoculated potato tubers after exposure to volatiles of *Allium sativum* essential oil at 77 ppm for 1, 2 or 3 weeks. Data selected from three experiments, one pathogen each, performed on different occasions, using different cultivars of potato

vapours completely prevented growth of the fungus in vitro and significantly reduced disease development in vivo at all doses. *C. carvi* significantly reduced disease in vivo at the split doses and the number of colony-forming units in vitro at single and split doses summing up to 160 ppm. *M. piperita* oil significantly controlled *H. solani* development in vivo at all 160-ppm doses and in vitro at split doses. For the threefold split, *S. officinalis* EO significantly reduced the *H. solani* disease index in vivo but had merely a fungistatic action in vitro for the 160 and 80+80 ppm applications. *M. arvensis* var. *sachalinensis* had a fungistatic action in vitro and was not effective in vivo, whereas the fungus was indifferent to all *R. officinalis* treatments.

The two in vivo *F. solani* and *H. solani* experiments including 77 ppm or 38.5+38.5 ppm applications again confirmed that *A. sativum* effectively controlled disease development of both pathogens, the relative infections compared with control values after single and split applications being significantly reduced to 41 and 37, and 11 and 17, for the two pathogens, respectively (data not shown). Also, *S. officinalis* significantly reduced the *F. solani* rot index to 79% of the control value at a single dose and the *H. solani* index to 30% for the split application, whereas *P. balsamifera* significantly increased the *H. solani* infection index to relative values of 227 and 167 for single and split applications (data not shown).

In Vitro and In Vivo Experiments Including Various Doses in Single Applications

The results from *P. foveata* experiments with EO doses between 10 and 320 ppm (in vitro) or 10 and 160 ppm (in vivo) are presented in Table 3. *A. sativum* EO completely prevented growth of the fungus in vitro at all doses (also at 20 ppm; data not shown) and significantly reduced gangrene development in vivo, even at the lowest dose of 10 ppm. Both *Salvia* species controlled disease development in tubers significantly although their actions in vitro were merely fungistatic or non-significant. *T. vulgaris* and *Ocimum basilicum* EOs on the other hand were not fungicidal in vivo but greatly and significantly reduced the numbers of colony-forming units in vitro. The *Mentha* species reduced gangrene to 70–77% of the index in control tubers at 160 ppm.

In parallel in vitro and in vivo *F. solani* experiments at 40–160 ppm, *A. sativum* oil was nearly completely fungicidal in vitro and significantly reduced dry rot development in vivo (data not shown). *O. basilicum* oil caused significantly increased dry rot development but had no visible stimulatory effect on conidia (data not shown). Across all additional in vivo *P. foveata* experiments of the single-dose type, *A. sativum* was most effective and significantly reduced rot indexes at all doses included, while *C. carvi*, *O. basilicum*, *P. balsamifera* and *T. vulgaris* showed inconsistent and weak, although sometimes significant, gangrene reduction (data not shown).

In Vitro *R. solani* Experiments Including Various Doses

The effects of 40–320 ppm of a range of EOs on mycelial growth of *R. solani* in vitro are presented in Table 4. *A. sativum* volatiles almost completely prevented the radial growth of *R. solani* mycelium; limited outgrowth occurred only at the lowest

dose used. At the highest dose, 320 ppm, the *C. carvi*, *M. arvensis* var. *sachalinensis*, *M. spicata*, *O. basilicum* and *T. vulgaris* oils were fungicidal and at lower doses most of them were fungistatic. The domestic thyme oil was less effective than the imported one. *A. graveolens* and *M. piperita* EOs strongly prevented normal growth of the fungus at the highest dose and were fungistatic at lower ones. *R. officinalis*, *L. angustifolia*, *S. officinalis* and *S. lavandulafolia* EOs were merely fungistatic at higher doses, while *Pogostemon patchouli* EO did not influence the growth of *R. solani* at all.

In Vivo *P. foveata* Experiment Including Delayed Start of EO treatments After Inoculation

The results of an experiment in which tubers were inoculated with *P. foveata* and immediately exposed to vapour (equivalent to 92 ppm) or where exposure was delayed for up to 12 days after inoculation are presented in Fig. 2. When *A. sativum*, *C. carvi* and *S. officinalis* oils were used, the greatest reductions in gangrene severity occurred when tubers were concurrently inoculated with *P. foveata* and exposed to EO vapour. However, volatiles of *M. piperita* oil were as effective as *A. sativum* and *S. officinalis* oils if tubers were exposed 4–8 days after inoculation, allowing wounds to first heal for that period. The *O. basilicum* oil treatments resulted in slight, but significant reductions in gangrene development, compared with the controls, if tubers were exposed 4 or 12 days after inoculation. The *T. vulgaris* oil treatments did not decrease rotting of tubers at all.

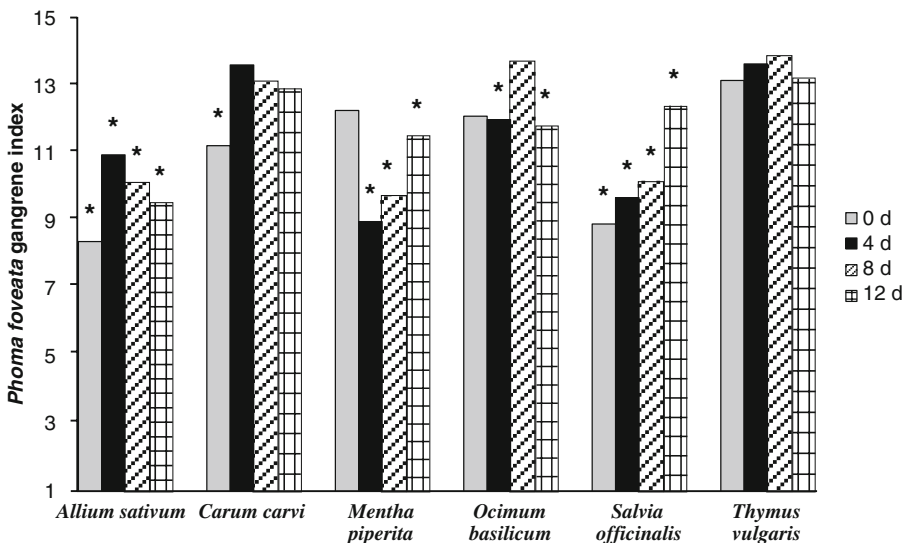


Fig. 2 Gangrene (*Phoma foveata*) index in tubers, cv. Bintje, after exposure to volatiles from essential oils for 3 weeks at 92 ppm, added 0, 4, 8 or 12 days after inoculation. Means of three replicates. Asterisks indicate that means are significantly different from the control mean (index 14.3) at $P \leq 0.05$

In Vivo *P. foveata* and *F. solani* Experiments Including Vapours of Macerated Plant Material

Figure 3a illustrates the results of experiments in which tubers were inoculated with *P. foveata* or *F. solani* and exposed to the volatiles from macerated plant material at 9.1 g l^{-1} of *Allium sativum* and *A. cepa*. The mean disease indexes of unexposed control tubers were 13.5 and 5.2, respectively. Macerated *A. sativum* significantly reduced the severity of the diseases caused by both pathogens, while vapours of macerated *A. cepa* also greatly reduced the extent of rotting by both pathogens, but only gangrene significantly. Tubers exposed to the vapours of macerated *A. rusticana* at 9.1 g l^{-1} completely collapsed, precluding observations of their potential antifungal disease activities.

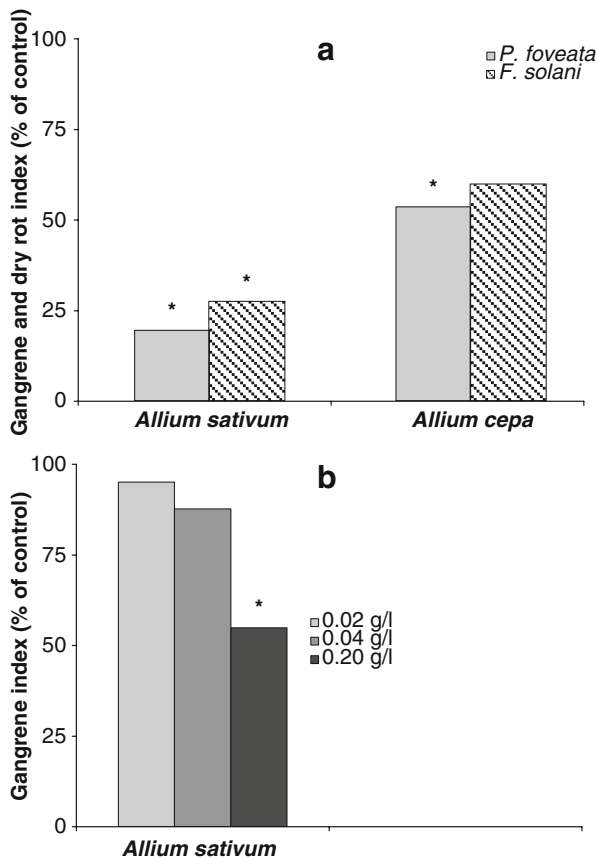


Fig. 3 Relative incidence of gangrene caused by *Phoma foveata*, and dry rot caused by *Fusarium solani* var *coeruleum*, compared with unexposed controls, in artificially inoculated potato tubers after being exposed to vapours of macerated bulbs of onion (*Allium cepa*) or garlic (*A. sativum*) at 9.1 g l^{-1} (a) and the doses indicated (b) for 3 weeks. Data from three experiments, each with three replicates. Asterisks indicate that the means are significantly different from the control mean at $P \leq 0.05$

Figure 3b illustrates the effect of macerated *A. sativum* at doses of 0.02, 0.04 and 0.2 g l⁻¹ on gangrene development. The mean disease index of the unexposed control was 9.1. Only the highest dose caused a significant reduction in gangrene severity.

Discussion

The effects of EO volatiles on fungal potato tuber pathogens and disease development were investigated. Information on the main constituents of the oils used was provided by the suppliers. However, the major components of the oil may not be the principal substances in the headspace (Benjlali et al. 1984), and they may consequently not be those responsible for the effects observed in an experiment using volatiles. As the headspace in the treatment chambers was not analysed in the experiments reported here, the discussion will focus on the observed potential of EOs (rather than specific constituents) of various plant species for use as antifungal agents.

The results of the in vitro experiments clearly show that vapours of many plant extracts have fungicidal action on the pathogens studied provided that there is a sufficiently high concentration and the duration of exposure is sufficiently long. Even at a very high dose, 800 ppm, a 1-week treatment was too short for many of the EOs used to exert fungicidal effects on *F. solani*, *P. foveata* and *H. solani* (Table 1), and at a lower dose (160 ppm) 2- and 3-week treatments with the most potent oils (*A. sativum* and *T. vulgaris*) were, respectively, needed to completely prevent conidial germination of *H. solani* (data reported but not shown). These results were corroborated by data from in vivo experiments, showing that an exposure for 1 week is too short for good control of disease development, even when using the very effective *A. sativum* EO (Fig. 1).

In some of the in vitro experiments, a short exposure to EOs resulted in increases in the numbers of colony-forming units at the final reading; for instance a 1-week exposure of *P. foveata* to volatiles of *M. piperita* oil at 800 ppm (Table 1), and either 1-week or 2-week exposures of *H. solani* to caraway vapours at 160 ppm (data reported but not shown). Over a longer exposure period (3 weeks) this stimulatory influence was not observed and in another experiment with a similar dose and an exposure period of 5 weeks the caraway oil strongly reduced the numbers of *H. solani* colony-forming units (Table 2). In these cases stress on the fungi caused by a sublethal dose seems to have stimulated the subsequent germination of conidia. Similarly, initial inhibition of growth and toxin production of toxin-producing moulds was followed by increased fungal activity after treatment with citral, citronellal and eugenol in studies by Mahmoud (1994). In addition, increases in the incidence of disease caused by *P. foveata*, *H. solani* and *F. solani* were observed after vapour treatments of inoculated tubers using several EOs (data reported but not shown).

Interpretation of data from the in vivo system is, however, less straightforward. Increases or decreases in the severity or frequency of attacks, compared with controls, could be due to influences of volatiles on the pathogen, or on the host, or interactions between these variables. If the wound healing or defence activities of the

host are impeded in any way by the volatiles, the ability of the pathogens to cause diseases would be expected to be higher in their presence than in unexposed controls. Figure 2 shows data from an experiment in which tubers were exposed to EOs up to 12 days after inoculation with *P. foveata*. The results from this experiment do not indicate that the defence or wound healing reactions were impaired in the prevailing experimental conditions. None of the treatments increased the gangrene index in this experiment. However, with an interval of at least 4 days between inoculation and start of exposure, the *M. piperita* oil at 92 ppm, which had not previously displayed strong activity against *P. foveata* in vivo in other experiments at similar doses, was as effective as *A. sativum* and *S. officinalis* oils applied concurrently with inoculation, although both the wound healing and the infection processes had progressed for 4 days before the exposure. Such responses might occur if a very volatile antifungal compound is accumulated in the host tissues at the site of pathogen attack, thus becoming more effective. In support of this hypothesis, Oosterhaven et al. (1995b) found higher amounts of (*S*)-carvone, and its metabolic products, in tissues that had been wounded and allowed to heal for 2 days or more before exposure than in tissues exposed directly after wounding. Split applications of *M. piperita* EO (Table 2) had stronger antifungal effects on *H. solani* in vitro than single initial application of the same total amount, indicating that the biologically active compounds in the steam phase of this oil is very volatile, quickly escaping from the experimental systems used.

T. vulgaris oil showed strong antifungal potential in the in vitro system against all pathogens included (Tables 1, 3, 4) but repeatedly failed to control gangrene development in inoculated tubers in vivo (Fig. 2, Table 3). Different results may be due to the use of different batches of EO since the composition of the oils may depend (inter alia) on the cultivation site and the cultivar of the source plants, harvest dates and handling procedures (Hay 1993). Differences may also be due to variations in the sensitivity of the isolates used, as shown for *F. sambucinum* by Vaughn and Spencer (1994). However, in the parallel *P. foveata* experiments (Table 3), similar doses, the same oil batches and the same original conidial suspensions were used. In accordance with results presented here, *T. vulgaris* EO has been reported to give diverging results in vitro and in vivo against *Phytophthora infestans* (Quintanilla et al. 2002), and Guynot et al. (2003) found it to have potent fungicidal activity on bread spoilage fungi in vitro, but only weak effects in tests with sponge cake analogues. In addition, Arras and Usai (2001) found the effects of *T. capitatus* oil against *Penicillium digitatum* on orange fruits to be weaker than expectations based on in vitro results. Some of the cited authors have argued that the lipophilic compounds in EOs may interact with food components, such as proteins and lipids, thereby decreasing their activity. This seems to be a plausible explanation, especially if the substrate is not a living host. If so, however, another possibility is that the biologically active compounds may be converted to less toxic substances by the host. Bioconversion of volatiles has been demonstrated in studies with strawberries (Hamilton-Kemp et al. 1996; Archbold et al. 1997), and Oosterhaven et al. (1995b, c) found that (*S*)-carvone was converted (mainly to neoisodihydrocarveol) by wounded potatoes.

In contrast to *T. vulgaris* oil, oils from *Salvia* species did not appear to be an effective natural fungicide against *P. foveata* when evaluated in the in vitro system,

but significantly reduced gangrene in vivo at similar doses in parallel experiments using the same batch of oil and inoculum source (Table 3). Also, in vivo *S. officinalis* was as effective as *A. sativum* oil at 92 ppm (Fig. 2). Similarly, conflicting data were obtained in investigations of the activity of *S. officinalis* oil on *H. solani*. Neither single nor split doses showed fungistatic activity in vitro, but a threefold split used in vivo significantly reduced disease development in parallel experiments (Table 2). It also reduced dry rot development, although not significantly so, but had no visible effect in vitro on *F. solani* (data not shown). Such disparities could occur if the host converts volatiles in the EO into substances with greater toxicity to the pathogen, or if the treatment triggers an effective defence reaction in the host. Stronger antifungal effects of an EO in vivo than in vitro have also been observed by Quintanilla et al. (2002), who found that hyssop (*Hyssopus officinalis*) oil had very limited antifungal activity in plate assays against *P. infestans*, but strongly inhibited disease when sprayed as a dilute solution on potato plants that were challenge-inoculated with a zoospore suspension of *P. infestans* 3 days later. Quintanilla et al. (2002) speculate that the differences in results between in vivo and in vitro tests might be due to induced resistance of the host.

A. sativum EO showed the strongest effects against all four pathogens in vitro and in vivo, consistently giving significant reductions in pathogen development and disease compared with controls. Gangrene development was significantly reduced at 10 ppm (Table 3). Vapours from *A. sativum* EO have also been found to have potential against bread spoilage fungi in a study by Nielsen and Rios (2000), although they were less effective than mustard oil at 16 ppm (applications of 1 µl per Petri dish). In addition, volatiles from macerated bulbs controlled gangrene development (Fig. 3), indicating a possible practical use in small-scale organic farming systems. Clearly, a potential disease control agent should not inflict damage on the host itself. In contrast to the strong reaction of potato tubers to horseradish (*A. rusticana*) volatiles, *A. sativum* EO did not cause any visible damage to tubers in any of the experiments presented here. Thus, it stands out as the most promising plant EO for use as a natural fungicide of tuberborne diseases. The effects of treating seed tubers with vapours of *A. sativum* and other EOs on sprouting, disease development and yield have been studied in field trials, the results of which will be presented elsewhere.

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