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Antifungal Activity of the Essential Oil of Hyssop (*Hyssopus officinalis*)

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Abstract

The antifungal and fungicidal effects of hyssop (*Hyssopus officinalis*) oil and its individual components were studied in a series of *in vitro* and *in vivo* experiments. Mycelial growth of the plant pathogenic fungi *Pyrenophora avenae* and *Pyricularia oryzae* was completely inhibited by 0.4% hyssop oil. Volatile components diffusing from agar medium containing 0.4% hyssop oil also completely inhibited the growth of these two fungi. Various components of hyssop oil (L-bornyl acetate, isopinocampheol and pinocamphone), used individually, reduced growth of *P. avenae* and, where combinations of individual components were used, any mixture containing isopinocampheol completely inhibited fungal growth. Growth of *P. oryzae* was less affected by individual components of the oil. Hyssop oil reduced germination of *Botrytis fabae* conidia and uredospores of *Uromyces viciae-fabae*, but in contrast to the data from *in vitro* experiments, its effects on pathogen infection were less clear cut. Thus, although 0.05% hyssop oil reduced rust infection of broad bean when applied 1, 2 or 3 days before, or 1 or 2 days after inoculation, its effects against barley powdery mildew and apple powdery mildew were variable. It is suggested that this variability might be the result of the volatile components of the oil diffusing away from leaf surfaces, thus reducing the concentration of active components on the leaf surface.

Introduction

Essential oils represent very complex mixtures of compounds, mainly monoterpenes and sesquiterpenes. Although in some plant species one main constituent of the oil may predominate, in many species no single compound predominates and instead, there is a balance of various components (Svoboda and Hampson, 1999).

Essential oils are known to possess a variety of biological properties (Maruzzella and Robbins, 1961), including antimicrobial activity. Most of the work on the antimicrobial effects of essential oils has been performed on human pathogens, spoilage micro-organisms, and dermathophytes, with very little research on phytopathogenic micro-organisms. Moreover, in spite of the encouraging results of *in vitro* experiments on phytopathogenic bacteria (Maruzzella et al., 1963) and fungi (Maruzzella and Liguori, 1958; Maruzzella and Baltei, 1959; Maruzzella, 1963; Yegen et al., 1992), the *in vivo* fungicidal activity of the oils was not tested. In fact, most reported work on the use of plant extracts against plant pathogens *in vivo* has focussed on seed treatments (e.g. Asthana et al., 1989) or control of post-harvest pathogens (e.g. Grover and Aulakh, 1968). Awuah (1994) examined the effects of crude steam distillate from *Ocimum gratissimum* on infection of cocoa pods by the black pod pathogen *Phytophthora palmivora*, but little information exists on the *in vivo* effects of plant essential oils on plant pathogens.

The present study examined the effect of hyssop oil and its components on phytopathogenic fungi in both *in vitro* and *in vivo* experiments, the aim of which was also to identify the individual components of hyssop oil with greatest fungicidal activity.

Materials and Methods

Extraction of essential oil from hyssop

Hyssop (*Hyssopus officinalis*) was grown in the herb garden at SAC, Auchincruive, harvested and dried at 35°C. Volatile oils were extracted by hydrodistillation and analysed by gas chromatography as described by Svoboda and Hampson (1999). The main components of the hyssop essential oil were β-pinene (4.0–8.9%), iso-pinocamphone (47.2–57.7%), pinocamphone (11.1–22.1%), bornyl acetate (3.2–4.3%) and borneol (4.2%) (the numbers in brackets represent percentage of the total oil).

Effects of hyssop oil, its individual components and hyssop oil vapour on *in vitro* fungal growth

The effects of hyssop oil, the volatiles of hyssop oil and the individual components of hyssop oil, were tested on growth of *Pyrenophora avenae* and *Pyricularia oryzae* (*Magnaporthe grisea*) in *in vitro* experiments. A study of the effects of hyssop oil on germination of *Botrytis fabae* conidia and *Uromyces viciae-fabae* uredospores was also undertaken.

Pyrenophora avenae and *P. oryzae* were grown on potato dextrose agar (PDA) Petri dishes, 8 cm in diameter. For inoculation, mycelium was taken from the periphery of stock cultures. Plugs of mycelium were removed with a 10 mm cork borer, inverted and placed in the centre of each Petri dish, with the mycelium facing the medium. Essential oils are considered to be sterile (Zaika, 1988) and therefore were added to 150 ml flasks of PDA immediately prior to use, to obtain final concentrations of 0.01, 0.05, 0.1 and 0.4% hyssop oil. Thereafter, 20 ml of the medium amended with the essential oil was added to each Petri dish. The concentrations of individual components of the essential oil that were used were determined from their relative proportions in the whole essential oil, as determined previously by gas chromatography (M. P. Letessier and D. R. Walters, unpublished results).

The effects of volatile components of the oil were tested using three compartment Petri dishes, two of which contained PDA (one compartment was inoculated with *P. avenae* and the second with *P. oryzae*), whereas the third compartment contained 7 ml of PDA containing hyssop oil. In this way, the fungi were not in direct contact with the oil and any effect on growth of the mycelium could be attributed to vapour activity.

In all the experiments, fungal growth was assessed on the sixth day after inoculation for *P. avenae* and on the tenth day after inoculation for *P. oryzae*. The colony radius was measured in millimetres, excluding the plug. An average was taken of three measurements made on each Petri dish. Three replicate Petri dishes were used for all treatments and experiments were repeated twice.

For spore germination studies, *B. fabae* was induced to sporulate by growth in the medium described by Leach and Moore (1966). A suspension of spores in distilled water (DW) was passed through four layers of muslin to remove mycelial debris (Doherty and Preece, 1978). Thereafter, a suspension of 10^{15} spores/ml (checked with an improved Neubauer Haemocytometer; Gordon-Keeble Laboratory Products, Barton Mills, England, UK) was used in the experiments. Uredospores of *U. viciae-fabae* were collected from stock plants. Because these spores are clumped they require special treatment to release self inhibitors. Approximately 50 mg of uredospores were floated on 100 ml sterile DW as a monosporic layer at 4°C for 16 h. They were then washed with DW and suspended in DW at a concentration of 5×10^4 spores/ml. The oil suspension was prepared by adding oil to DW containing 0.01%

Tween 20 to obtain concentrations of 0, 0.1 and 0.4%. A 15 μl droplet of the spore suspension was mixed with a 30 μl droplet of oil suspension on a glass slide. The slide was suspended on a glass rod in a Petri dish containing moist filter paper. The Petri dishes were placed in polythene bags at 20°C and left for 20 h. Each slide was then stained with lactophenol cotton blue containing Tween 20, to stop further germ tube formation and growth. The Tween 20 was added to the lactophenol to facilitate the action of lactophenol, as the oil had formed a barrier around the spores. To assess the percentage germination, the number of germinated spores out of 100 was counted.
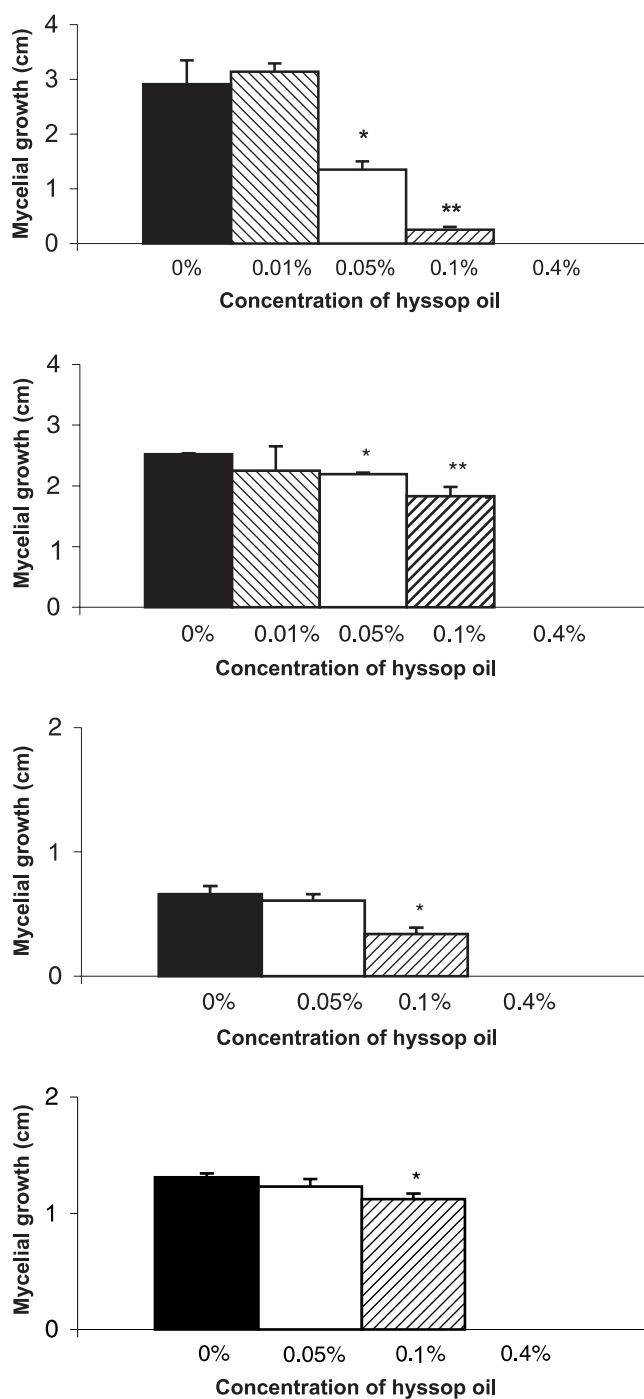
In vivo fungicidal effects of hyssop oil

Barley (*Hordeum vulgare* L. cv. Delibes), broad bean (*Vicia faba* L. cv. Bunyards Exhibition) and apple (*Malus bitenfelder*) seedlings were grown in the glasshouse and inoculated, respectively, with *Blumeria graminis* f. sp. *hordei* (powdery mildew), *Uromyces viciae-fabae* (bean rust) and *Podosphaera leucotricha* (apple powdery mildew) as described previously (Havis et al., 1994). Barley and apple seedlings were used when they had three and four leaves, respectively, whereas broad bean seedlings were used when the second pair of leaves had fully formed. Different concentrations of hyssop oil were obtained by suspending the oil in DW containing 0.01% Tween 20. Suspensions of crystalline components of hyssop oil were prepared by dissolving 1 g in 1 ml of ether. Distilled water containing 0.01% Tween 20 was then added to obtain the desired final concentration. Controls for the whole essential oil contained Tween 20, whereas controls for the individual components of the oil contained the same volume of ether used to dissolve the components.

The oil suspensions were sprayed onto all leaves of barley and apple seedlings and onto the upper surfaces of the second pair of leaves of broad bean. The suspension was carefully mixed, before and during the application and was sprayed to run-off using a Shandon spray unit (Sigma Chemical Company, Poole, Dorset, UK). Powdery mildew infection of barley seedlings was assessed 6 days after inoculation by estimating the percentage area of the second leaf that was covered with powdery mildew. For broad bean rust, infection intensity was assessed 17 days after inoculation by counting the number of pustules per cm^2 of leaf. For apple powdery mildew, the percentage area of the third leaf covered by the fungus was assessed 17 days after inoculation.

Results
Effects of hyssop oil on *in vitro* fungal growth
Although 0.01% hyssop oil had no effect on mycelial growth of *P. avenae* (Fig. 1) and little effect on growth of *P. oryzae* (Fig. 2), growth of both fungi was substantially reduced by exposure to 0.05 and 0.1% of hyssop oil, and was completely inhibited by 0.4% of the oil.

Figs 1–4 Effect of various concentrations of hyssop oil on mycelial growth of *Pyrenophora avenae* (Figs 1, 3) and *Pyricularia oryzae* (Figs 2, 4). Figures 3 and 4 refer to volatile components of hyssop oil. All values are the mean of three replicates. Significant differences are shown at *P = 0.05 and **P = 0.01

Effect of volatile components of hyssop oil on *in vitro* fungal growth

Four days after exposure, the volatile components diffusing from the medium containing 0.4% of hyssop oil completely inhibited mycelial growth of *P. avenae* (Fig. 3) and *P. oryzae* (Fig. 4). Fungal growth was not affected by volatile components diffusing from medium containing 0.05% of hyssop oil (data not shown).

Effects of individual components of hyssop oil on growth of *P. avenae* and *P. oryzae*

Various components (isopinocampheol, pinocamphone, L-bornyl acetate, β-pinene) of hyssop oil were used alone at various concentrations or in combination to test for effects on fungal growth *in vitro* (Table 1). When used individually, L-bornyl acetate, isopinocampheol and pinocamphone reduced growth of *P. avenae*. The largest reductions in fungal growth were obtained with 0.08% L-bornyl acetate, 0.3% isocampheol and 0.13% pinocamphone. Where combinations of individual components were used, any mixture containing isopino-campheol completely inhibited fungal growth (Table 1).

The growth of *P. oryzae* was less affected by the individual components of hyssop oil than *P. avenae* (Table 1). Interestingly, although isopinocampheol completely inhibited the growth of *P. oryzae* when used alone, the only combination containing isopinocam-pheol to inhibit fungal growth was that containing all four components (Table 1).

Effects of hyssop oil on spore germination

Hyssop oil, used at 0.1% and 0.4% of concentrations, produced small but significant reductions in germination of *B. fabae* conidia (Fig. 5) and more substantial reductions in the germination of *U. viciae fabae* uredospores (Fig. 6).

Effect of hyssop oil on infection of barley, broad bean and apple seedlings

In an initial experiment, suspensions containing 0.1% to 3.2% hyssop oil were used to treat barley, broad bean and apple seedlings. Phytotoxicity was observed with the oil used at 0.2% on barley seedlings and 0.32% on apple seedlings. Some signs of phytotoxicity were observed on broad beans 2 weeks after treatment. As a result of this phytotoxicity, a range of lower concentrations of the oil was used in experiments.
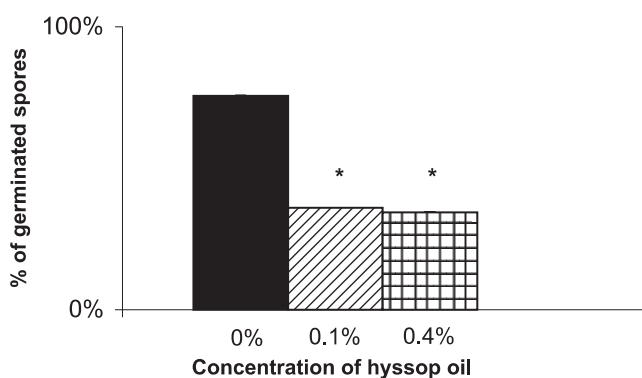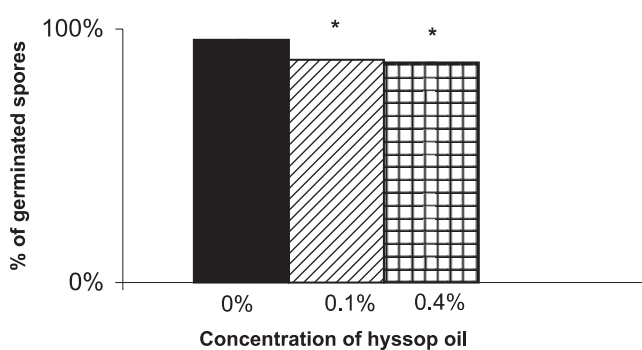
Application of 0.05% hyssop oil to barley seedlings 1 day or 3 days prior to inoculation actually increased powdery mildew infection, whereas curiously, application of the oil 2 days prior to inoculation reduced infection (Fig. 7). In contrast, post-inoculation treatment with the oil reduced powdery mildew infection, with the greatest reduction occurring when the treatment was applied 1 day post-inoculation.

Application of 0.05% hyssop oil to broad bean seedlings, 1, 2 or 3 days prior to inoculation or 1 or 2 days post-inoculation led to significant reduction in rust infection. Rust infection was significantly increased when the oil was applied 3 days after inoculation (Fig. 8).

The effect of hyssop oil, used at 0.05%, on powdery mildew infection of apple seedlings was variable (Fig. 9). Thus, when applied as a pre-inoculation treatment, it reduced infection (although not significantly), whereas when applied as a post-inoculation treatment, it increased mildew infection (Fig. 9).

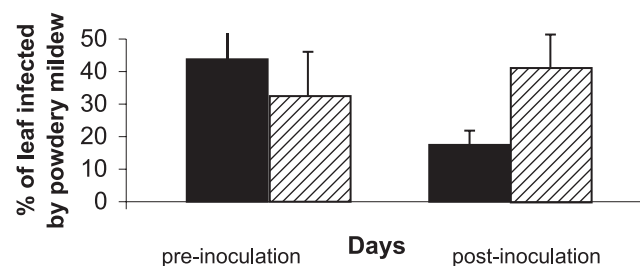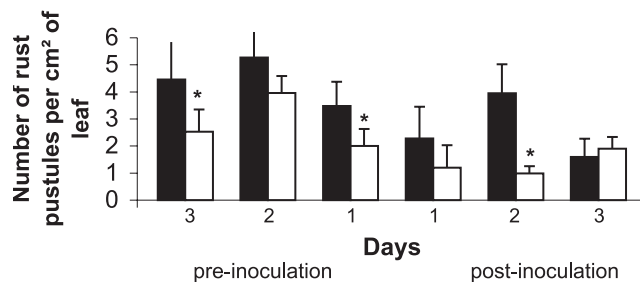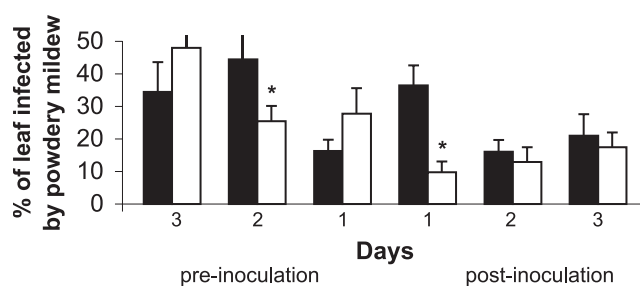| Components	Mycelial growth (cm)	
	P. avenae	*P. oryzae*
Control	2.67 ± 0.330	2.20 ± 0.000
β-pinene 0.004%	3.42 ± 0.029	2.19 ± 0.010
β-pinene 0.008%	3.13 ± 0.120	2.35 ± 0.023
Control	1.99 ± 0.290	1.80 ± 0.000
L-bornyl acetate 0.01%	1.23 ± 0.180*	1.84 ± 0.013
L-bornyl acetate 0.08%	0.35 ± 0.070**	1.37 ± 0.130*
Control	3.34 ± 0.072	1.65 ± 0.013
Isopinocampheol 0.05%	0.79 ± 0.107**	1.16 ± 0.049*
Isopinocampheol 0.3%	0.00 ± 0.000**	0.00 ± 0.000**
Pinocamphone 0.02%	2.94 ± 0.129	1.62 ± 0.010
Pinocamphone 0.13%	0.70 ± 0.404**	1.07 ± 0.062**
Pinocamphone + isopinocampheol	0.24 ± 0.046**	1.25 ± 0.029*
Pinocamphone + *β*-pinene	2.92 ± 0.107	1.59 ± 0.010
Isopinocampheol + L-bornyl acetate	0.00 ± 0.000**	0.55 ± 0.294**
Isopinocampheol + *β*-pinene	0.00 ± 0.000**	1.07 ± 0.057**
Control	3.02 ± 0.029	2.13 ± 0.066
Pinocamphone + L-bornyl acetate	2.83 ± 0.049	2.21 ± 0.010
Pinocamphone + *β*-pinene + L-bornyl acetate	2.89 ± 0.020	2.20 ± 0.040
Isopinocampheol + *β*-pinene + pinocamphone	0.23 ± 0.186**	1.86 ± 0.033
Pinocamphone + isopinocampheol + L-bornyl acetate	0.00 ± 0.000**	1.18 ± 0.312**
Isopinocampheol + *β*-pinene + L-bornyl acetate	0.00 ± 0.000**	0.62 ± 0.302**
Isopinocampheol + *β*-pinene + pinocamphone + L-bornyl acetate	0.00 ± 0.000**	0.00 ± 0.000**

Table 1
Effects of individual components of hyssop oil, used alone at various concentrations or in combination, on mycelial growth of *Pyrenophora avenae* and *Pyricularia oryzae*. Values are the mean of three replicates. Significant differences are shown at *P = 0.05 and **P = 0.01

Figs 5, 6 Effect of hyssop oil on germination of conidia of *Botrytis fabae* and uredospores of *Uromyces viciae-fabae*. All values are the mean of three replicates. Significant differences are shown at *P = 0.05 and **P = 0.01

Figs 7–9 Effect of hyssop oil on infection of barley with powdery mildew (Fig. 7), broad bean with rust (Fig. 8) and apple with powdery mildew (Fig. 9). Values are the means of 10 replicates (barley) or four replicates (broad bean and apple). Significant differences are shown at *P = 0.05. ■, control; □, 0.05% hyssop oil; ▨ 0.08% hyssop oil

The effects of individual components of hyssop oil on infection of barley with powdery mildew

L-bornyl acetate, isopinocampheol and *β*-pinene, when applied individually to barley seedlings, reduced powdery mildew infection compared with controls not

containing ether. However, when compared with controls containing ether, these differences disappeared. Indeed, ether applied to control plants post-inoculation led to much lower levels of mildew than those obtained with the oil treatments (data not shown).

Discussion

Very different results were observed in our *in vitro* and *in vivo* experiments. The *in vitro* studies showed clearly the efficacy of hyssop oil against fungal pathogens, whereas the results of the *in vivo* studies were less clear cut. Although some information exists on the *in vitro* effects of essential oils on phytopathogenic fungi, there is a paucity of data on the *in vivo* effects of essential oils in plant/pathogen interactions. The data presented here show that hyssop oil reduced germination of *B. fabae* conidia and *U. viciae-fabae* uredospores, whereas a suspension of 0.4% hyssop oil completely inhibited *in vitro* mycelial growth of both *P. avenae* and *P. oryzae*. The volatile components of the oil were probably responsible for this inhibitory activity. Thus, Noleyan and Narasimham (1986) observed that the inhibitory activity of the compounds incorporated into agar was in fact due to the volatile components that accumulated above the medium. In contrast, Zaika (1988) stressed that the volatiles could be a disadvantage because the inhibitory substances could disappear via evaporation following prolonged incubation, allowing the micro-organisms to resume growth.

After examining the effect of hyssop oil on fungal growth and fungal infections, it was decided to test the effect of components of hyssop oil. *Pyrenophora avenae* was sensitive to a greater number of components of hyssop oil than was *P. oryzae*. Isopinocampheol, the main component of hyssop oil, appeared to have an important role in the antifungal effects of hyssop oil observed in the present work. Indeed, Hitokoto et al. (1980) also found that the inhibitory effects of three powdered spices were largely accounted for by the main component of the essential oils in each sample (eugenol from cloves, anethol from star anise seeds, thymol from thyme). However, the addition of the other components to isopinocampheol was also effective. Thus, the strongest antifungal effect was achieved when the mixture of components was closest to the composition of hyssop oil. Indeed, Carlton et al. (1992) suggested that separate components of an essential oil have differing modes of actions which complement one another in the whole oil. They suggested that a complete oil would probably present a greater barrier to pathogen adaptation than would a relatively simple mixture.

In contrast to the *in vitro* results, the results of the *in vivo* work were variable and inconclusive. This was especially true for the work on barley and apple powdery mildew, where treatment with the oil led to either increased or decreased mildew infection. The data for broad bean rust, however, are more consistent, with all but one treatment reducing infection, although not always significantly.

From the above discussion, it is clear that hyssop oil was active against germination of fungal spores and it is possible that its volatile components were responsible for the antifungal effects observed. The fact that the components which were active against mycelial growth and possibly also spore germination are volatile may explain the difference between the *in vitro* and *in vivo* results. The volatiles were confined in the *in vitro* experiments, whereas they were able to diffuse away from the plant in the *in vivo* experiments. For this reason, even though the same concentration was used in each case, the effective concentration was likely to be different in the *in vivo* and *in vitro* experiments. The application of higher oil concentrations in *in vivo* experiments was impossible due to phytotoxicity. Indeed, the phytotoxicity could explain the increase in infection observed in some treatments: the oil could have injured the cuticle of the leaf and facilitated penetration by the pathogens.

In conclusion, the essential oil of hyssop has been shown to possess significant antifungal activity. It would be useful to determine the mode of action of the oil, since this may provide leads for the development of new antifungal agents. A useful starting point may be to use differential display technology to study the effects of the oil and its components on gene expression in plant pathogenic fungi.

Literature

Asthana, A., K. Dixit, N. N. Tripathi, S. N. Dixit (1989): Efficacy of *Ocimum* oil against fungi attacking chilli seeds during storage. Trop. Sci. **29**, 15–20.

Awuah, R. T. (1994): *In vivo* use of extracts from *Ocimum gratissimum* causing black pod disease of cocoa. Ann. Appl. Biol. **124**, 173–178.

Carlton, R. R., S. G. Deans, P. G. Watermann (1992): Antifungal activity of the leaf gland volatile oil of sweet gale (*Myrica gale*). Chemoecology **3**, 55–59.

Doherty, M. A., T. F. Preece (1978): *Bacillus cereus* prevents germination of uredospores of *Puccinia allii* and the development of rust disease of leek, *Allium porrum*, in controlled environments. Physiol. Plant Pathol. **12**, 123–132.

Grover, R. K., K. S. Aulakh (1968): Oospora rot of tomato fruits and its control by oils. Ind. Phytopath. Soc. Bull. **4**, 37–42.

Havis, N. D., D. R. Walters, S. A. Foster, W. P. Martin, F. M. Cook, D. J. Robins (1994): Fungicidal activity of the synthetic putrescine analogue (E)-1,4-diaminobut-2-ene, and derivatives. Pest. Sci. **41**, 61–69.

Hitokoto, H., S. Morozumi, T. Wauke, S. Sakai, H. Kunata (1980): Inhibitory effects of spices on growth and toxin production of toxigenic fungi. App. Environ. Microbiol. **39**, 818–822.

Leach, R., K. G. Moore (1966): The effect of 6-benzyl-aminopusine (benzyladenine) on senescence and chocolate spot (*Botrytis fabae*) of winter beans (*Vicia faba*). Ann. Appl. Biol. **61**, 67–76.

Maruzzella, J. C. (1963): The effect of perfume oil on the growth of phytopathogenic fungi. Plant Dis. Reptr. **47**, 756–757.

Maruzzella, J. C., J. Baltei (1959): The action of essential oils on phytopathogenic fungi. Plant Dis. Reptr. **43**, 1143–1147.

Maruzzella, J. C., L. Liguori (1958): The *in vitro* antifungal activity of essential oils. Am. Pharm. Assoc. **47**, 250–254.

Maruzzella, J. C., A. L. Robbins (1961): Effects of essential oils on seed germination. Natürwissenschaften **48**, 383.

Maruzzella, J. C., C. C. Kleinberg, C. J. Urso (1963): Effects of vapors of aromatic chemicals on phytopathogenic bacteria. Plant Dis. Reptr. **47,** 1067–1070.

Noleyan, V., P. Narasimham (1986): Antifungal activity of some essential oil components. Food Microbiol. **3** (4)**,** 331–336.

Svoboda, K. P., J. B. Hampson (1999): Bioactivity of essential oil of selected temperature aromatic plants: antibacterial, antioxidant, anti-inflammatory and other related pharmacalogical activities. In: Speciality Chemicals for the 21st Century. ADEME/IENICA

Int Seminar, 16–17 September 1999, pp. 43–49. ADEME, Paris, France.

Yegen, O., B. Bergen, R. Heitefuss (1992): Untersuchungen zur fungitoxischen Wirkungden Extriakte sechs ausgewählten Pflanzen aus der Türkei auf Phytopathogene Pilze. Z. Pflkr. U. Pflsch. **99,** 349–359.

Zaika, L. L. (1988): Spices and herbs: their antimicriobial activity and its determination. J. Food Safety **9,** 97–118.