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ORIGINAL PAPER

In vitro efficacy of *Hyptis suaveolens* L. (Poit.) essential oil on growth and morphogenesis of *Fusarium oxysporum* f.sp. *gladioli* (Massey) Snyder & Hansen

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Abstract *Hyptis suaveolens* L. (Poit.) essential oil was tested in vitro on the growth and morphogenesis of *Fusarium oxysporum* f.sp. *gladioli* (Massey) Snyder & Hansen, which causes *Fusarium* corm rot and yellows in various susceptible cultivars of *gladiolus*. The fungitoxicity of the oil was measured by percentage radial growth inhibition using the poisoned food technique (PF) and volatile activity assay (VA). The mycelial growth of the test fungus was completely inhibited at 0.998 and 0.748 μg ml^{-1} concentration of oil in PF and VA, respectively. Essential oil was found to be fungicidal in nature at 1.247 and 0.998 μg ml^{-1} concentration of oil in PF and VA, respectively. Determination of conidial germination in the presence of oil was also carried out and it was found that the oil exhibited 100% inhibition of conidial germination at 0.450 μg ml^{-1} concentration. The effect of essential oil on the yield of mycelial weight was observed and it was found that at 0.873 μg ml^{-1} concentration no mycelium was recorded and 100% inhibition was observed. The fungitoxicity of oil did not change even on exposure to 100°C temperature or to autoclaving, and the oil also retained its fungicidal nature even after storage of 24 months. The main changes observed under light microscopy after oil treatment were a decrease and loss of conidiation and anomalies in the hyphae such as a decrease in the diameter of hyphae and granulation of cytoplasm. The treatment of the oil also showed highly reduced cytoplasm in the hyphae, showing clear retraction of the cytoplasm from the hyphae and ultimately in some areas hyphae without cytoplasm were also found. GC-MS studies of the essential oil revealed that the oil consisted of 24 compounds with 1,8-cineole as major component accounting for 44.4% of the total constituents.

Keywords *Fusarium oxysporum* f.sp. *gladioli* · *Hyptis suaveolens* · GC-MS · Fungitoxicity

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Introduction

Fusarium oxysporum is a ubiquitous soil-borne fungus that includes pathogenic and non-pathogenic members. The pathogenic members are best known for causing *Fusarium* wilt diseases of many economically important horticultural crops. One such crop *gladiolus* (*Gladiolus* spp.) is affected by a special form of the pathogen known as *Fusarium oxysporum* f.sp. *gladioli* (Massey) Snyder & Hansen, which is responsible for causing *Fusarium* corm rot and yellows in various susceptible cultivars of *gladiolus* (McClellan and Pryor 1957). Application of synthetic fungicides has been considered one of the cheapest and most common approaches for the control of plant diseases. These chemicals usually take long timelines to be degraded completely, causing heavy toxicity to human beings, domestic animals, etc. (Lingk 1991). However, due to the development of new physiological races of pathogens, many of these synthetic chemicals are becoming ineffective (Spotts and Cervantes 1986).

Accordingly, there is an urgent need to work towards the development of safer antifungal agents which can be expected to be renewable, non-petrochemical, naturally eco-friendly and easily available. A further technology with increasing use is that of essential oils, where a number of

plants have been reported to show antimicrobial activity against a wide array of plant pathogens. The antifungal activity of essential oils is well documented previously, as a potential source of antifungal compounds (Reuveni et al. 1984; Meepagala et al. 2002). Particularly, essential oils were found to exert good antifungal activities both in vitro and in vivo conditions against a wide range of pathogens (Baruah et al. 1996; Bhaskara Reddy et al. 1998; Caccioni et al. 1998; Delespaul et al. 2000; Tripathi et al. 2004; Holley and Patel 2005; Sharma and Tripathi 2006, 2008; Sharma et al. 2007).

Because of their potency as antimicrobial agents, these compounds are increasingly more utilized outside their natural source in disease control. Effective phytocompounds are expected to be far more advantageous than synthetic pesticides, as they are easily decomposable, not environmental pollutants and posses no residual or phytotoxic properties (Bishop and Thornton 1997).

However, no extensive work has been carried out so far to explore the antifungal activity of the volatile oil from the *Hyptis suaveolens* (L.) Poit. and its effect on growth and morphogenesis of *Fusarium* oxysporum f.sp. *gladioli*. Earlier, Pandey et al. (1982) demonstrated the fungitoxicity of *H. suaveolens* oil against *Helminthosporium oryzae* causing leaf spot disease of rice. The antifungal activity of *H. suaveolens* oil against soil-borne fungi (Singh and Handique 1997) and storage mycoflora (Sharma et al. 2007) has also been reported. These findings, and the need to reduce the use of pesticides, emphasize the necessity to develop alternative means of controlling pathogen causing *Fusarium* corm rot and yellows of *gladiolus*.

The aim of this study was to determine the in vitro efficacy of essential oil of *Hyptis suaveolens* by different antifungal efficacy measurements to control the growth of *Fusarium oxysporum* f.sp. *gladioli.* The study also explored the possible mode of action of essential oil and its effect on the morphogenesis of the test fungus.

Materials and methods

Fungal strain

Fusarium oxysporum f.sp. *gladioli* strain MPPLU 01 from the collection of Mycology and Plant Pathology Division, Botany Department, University of Lucknow was used. The fungus was maintained on Potato Dextrose Agar (PDA) at 25 ± 1°C.

Plant material

Hyptis suaveolens (L.) Poit. (Family: Lamiaceae, Common name: Ganga tulsi, Wilayati tulsi) is a rigid, sweetly aromatic, annual herb, which attains a height of about 180–210 cm and is found as a weed in the tropics and subtropics of India. Voucher specimens (no. BDLU 77/03) of collected plants were deposited in the Herbarium of Botany Department, Lucknow University.

Extraction of essential oil

During the months of September to December, leaves of *Hyptis suaveolens* (L.) Poit. were collected from the left bank of the river Gomti, behind New Hyderabad, Lucknow, India and used for extracting oil by hydro distillation for 5 h using a Clevenger type apparatus (Guenther 1948). In the Clevenger type apparatus a clear, light yellow-colored, oily layer with a pleasant smell was obtained on the top of the aqueous distillate, which was separated from the latter and dried with anhydrous sodium sulfate. The extracted essential oil was kept in air-tight sealed glass vials, covered with aluminum foil at 4°C until further analysis.

GC-MS analysis of essential oil

GC-MS analysis was done on a Shimadzu QP-2000 instrument at 70 eV and up to 250°C. The GC column was as follows: ULBON HR-1 was equivalent to OV-1; the size of fused silica capillary was 0.25 mm × 50 M with film thickness of 0.25 μ. The GC-MS was operated under the following conditions: the initial temperature was 60°C for 5 min and then heated at the rate of 5°C per min to 250°C. Carrier gas (helium) flow was 2 ml per min.

Identification of components was based on comparison of their mass spectra fragmentation patterns with those of the Mass Spectrometry Data Centre, the Royal Society of Chemistry, UK (Eight Peak Index of Mass Spectra, 3rd Ed. 1983) and those reported in the literature (Adams 1995).

In vitro antifungal efficacy measurements

Poisoned food technique

The fungal toxicity of the essential oil was evaluated against the test fungus by the poisoned food technique (PF) of Grover and Moore (1962). PDA (20 ml) was poured into sterilized Petri dishes and a measured amount of oil was added to give desired concentrations 0.031–1.372 μg ml^{-1}. In medium 0.05% Tween-80 was also added for even distribution of the oil in the medium. For control sets, the medium was supplemented with the same amount of distilled water instead of oil and 0.05% Tween-80. Plates were incubated at 25 ± 1°C. The growth of the test fungi was recorded for 7 days and percentage inhibition was computed after comparison with the control. Fungitoxicity was expressed in terms of percentage of mycelia growth inhibition and calculated as per formula of Pandey et al. (1982).

Percentage of mycelial growth inhibition = $[(d_c - d_t)/d_c \times 100]$, where d_c = average diameter of fungal colony in control and d_t = average diameter of fungal colony in treatment.

Volatile activity assay

Tests for the volatile activity (VA) of oil were carried out by the inverted Petri plate method in 90 mm Petri plates (Borosil) containing 20 ml of solidified PDA. A 5 mm diameter disc of the test fungus, cut from the periphery of an actively growing culture, was placed on the agar in each Petri plate and the plates were kept in inverted position. A sterilized cotton swab was placed on the upper lid of each inverted plate. Different concentrations (0.031–1.372 μg ml^{-1}) of oil were pipetted on to the cotton swab and were sealed by parafilm to check the release of volatile oil. For each corresponding control, an equal amount of water was pipetted on to the sterilized cotton swab. The dose of essential oil was calculated as μg ml^{-1} volatile present in air in the Petri plate [Total volume of 90 mm Petri plate was 95.46 cm^3, in this 20 ml (20 cm^3) PDA was added so the volume of air left was 75.46 cm^3]. The inverted Petri plates were incubated at 25 ± 1°C for 7 days. Fungitoxicity was expressed in terms of percentage of mycelial growth inhibition and calculated according to the formula of Pandey et al. (1982).

Percentage of mycelial growth inhibition = $[(d_c - d_t)/d_c \times 100]$, where d_c = average diameter of fungal colony in control and d_t = average diameter of fungal colony in treatment.

Determination of mycelial weight

To determine the effect of essential oil on the dry weight of the test fungus different concentrations of oil in Potato Dextrose Broth (PDB) medium were prepared in Erlenmeyer flasks and inoculated with 10^6 spores/ml of *Fusarium oxysporum* f.sp. *gladioli*. The spore population was counted using a haemocytometer. In the corresponding control an equal amount of distilled water was added. After 15 days dry weight of mycelium was determined. Flasks containing mycelia were filtered through Whatman filter no. 1 and then washed with distilled water. The mycelia were allowed to dry at 60°C for 6 h and then at 40°C overnight. The filter paper containing dry mycelia were weighed. Percent growth inhibition based on dry weight was calculated as:

$$[(\text{Controlweight} - \text{Sampleweight})/\text{Controlweight}] \times 100.$$

Spore germination assay

Thirteen concentrations of oil (0.031–1.372 μg ml^{-1}) were tested for conidial germination of the test fungus. Fungal conidia obtained from ten-day-old cultures were taken and placed on glass slides in triplicate. Slides containing the conidia were incubated in a moist chamber at 25 ± 1°C for 24 h. Each slide was then fixed in lactophenol-cotton blue and observed under the microscope for conidial germination. About 200 conidia were counted and the number of conidia germinated was scored using haemocytometer to calculate the percentage of conidial germination (Surender et al. 1987). The conidium was found considered germinated when the length of germ tube was apparently equal to that of conidia.

Nature of toxicity of the essential oil

The fungitoxicity (fungistatic/fungicidal) of the essential oil was tested by using the technique of Thompson (1989). Experiments were carried out to ascertain the nature of the toxicity of the oil at its minimum inhibitory concentration (MIC), and hyper-MIC doses by the poisoned food technique and the volatile activity assay. On day 7, the inhibited fungal discs of treatment sets were taken out, washed with sterilized water and reinoculated, separately into petriplates containing fresh medium. The plates were incubated similarly and the observation for the revival of growth of the reinoculated fungal discs was recorded on day 7 as presence or absence of mycelial growth.

Effect of temperature and autoclaving on toxicity of oil

Experiments were performed to determine the thermostable or thermolabile nature of the oil. Different glass vials containing 5 ml oil each were subjected to different temperature treatments for 3 h in incubators already adjusted to 40, 60, 80 and 100°C. Antifungal activity of oil was also tested after autoclaving it at 121°C for 15 min. The glass vials were then allowed to cool down to room temperature and the fungitoxicity of the treated oil from each set was tested at its MIC against the test fungus using the poisoned food technique and volatile activity assay.

Effect of storage or self-life of the oil

Experiments were undertaken to ascertain the duration for which the oil can be stored without losing its fungitoxicity. Five milliliter of essential oil was stored in an air tight glass vial at room temperatures (20–38 ± 2°C). The fungitoxicity of the stored oil at its MIC was tested at regular intervals of 2 months using the poisoned food technique and volatile activity assay.

Mechanism of toxicity of essential oil: light microscopy

A sample of mycelium was taken from the periphery of the colony grown on PDA with 0.450 and 0.748 μg ml^{-1}

treatments of oil after 4 days of incubation. The samples were fixed in lactophenol-cotton blue and examined under the microscope (Nikon ECLIPSE E200, Japan) at 400× to examine structural abnormalities. Samples from control plates without oil were also stained and observed. Photographs were taken with the help of computer attached Samsung Color Camera SAC-410PA.

Results

Hyptis suaveolens essential oil and its components

The steam distillation of the leaves of *H. suaveolens* for 5 h yielded 0.46% pale yellow essential oil. The oil consisted of 24 compounds with 1,8-cineole accounting for 44.4% of the total constituents. Out of 24 components, 21 were identified with a typical library search match exceeding 90%. Besides 1,8-cineole, the other major compounds present in the oil were β-pinene (11.7%) and β-caryophyllene (10.0%). Significant amounts of other components like camphene (5.7%), β-myrcene (5.3%), α-farnesene (2.8%), α-pinene (2.5%) and terpinen-4-ol (2.2%) were also detected (Table 1). A typical GC-MS chromatogram of essential oil was shown in Fig. 1, in which peak number 6 indicates the major component 1,8-cineole.

Table 1 Components of *Hyptis suaveolens* (L.) Poit. essential oil identified by GC-MS analysis

Peak no.	Components	Percentage in total oil
1	α-Thujene	0.8
2	β-Carene	1.1
3	α-Pinene	2.5
4	β-Pinene	11.7
5	β-Myrcene	5.3
6	**1,8-Cineole**	**44.4**
7	Linalool	1.7
8	Camphor	1.1
9	Camphene	5.7
10	Terpinen-4-ol	2.2
11	α-Cubebene	0.3
12	β-Gurjunene	1.6
13	β-Elemene	1.2
14	β-Caryophyllene	10.0
15	α-Farnesene	2.8
16	β-Selinene	0.9
17	Longifolene	0.4
18	γ-Humulene	0.5
19	α-Copaene	0.6
20	γ-Codinene	0.3
21	α-Bergamotene	1.7
22	Unidentified	1.6
23	Unidentified	0.8
24	Unidentified	0.7

Matter in bold indicating major component of the oil

Inhibitory effect of the essential oil on the test fungus under in vitro conditions

The fungitoxicity of the oil was measured by percent radial growth inhibition using the PF and VA tests. In the PF 0.998 μg ml^{-1} oil was found to be completely inhibit the growth of the fungus after 7 days of incubation. Radial growth of *F. oxysporum* f.sp. *gladioli* was significantly reduced in response to various concentrations of *Hyptis* oil ranging from 0.031 to 1.372 μg ml^{-1}. In the PF test at 1.247 μg ml^{-1} oil showed its fungicidal nature by killing the fungus completely (Table 2).

In the VA test at 0.748 μg ml^{-1} of the essential oil concentration, fungal development was completely inhibited after 7 days of incubation. Studies on nature of toxicity of oil revealed that the oil was fungicidal at 0.998 μg ml^{-1} (Table 2). As is evident from the Table 2, the volatile activity assay was more promising that of the poisoned food technique. In the PF test, the MIC was 0.998 μg ml^{-1} whereas in VA the MIC was 0.748 μg ml^{-1}. So it is evident that oil is more fungitoxic in its volatile phase.

Determination of conidial germination in presence of oil was also done and it was found that the oil exhibited 100% inhibition of conidial germination at 0.450 μg ml^{-1} concentration (Table 2). It was also observed that those spores which germinated in presence of low concentrations of oil produced small germ tube as compared to the control.

Determination of mycelial weight

Effect of essential oil on the yield of mycelial weight was observed in liquid medium Potato Dextrose Broth and it was found that insignificant growth occurred at 0.748 μg ml^{-1} concentration. At 0.873 μg ml^{-1} concentration no mycelium was recorded and 100% inhibition was observed (Table 3). On the basis of per cent inhibition of dry weight it was found that the oil was more effective in liquid medium than solid medium (Tables 2, 3).

Effect of temperature and storage on the shelf-life and toxicity of the oil

The thermostability of the oil was tested under PF and VA at MIC and it was found that at temperature ranging from 40 to 100°C and even after autoclaving (121°C for 15 min) the oil, its activity was not altered in both the techniques

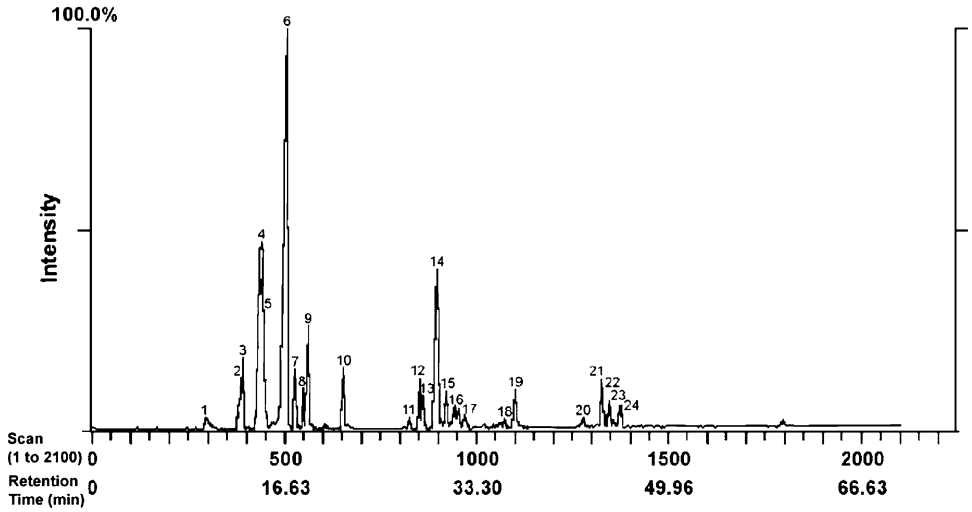

Fig. 1 GC-MS chromatogram of *Hyptis suaveolens* (L.) Poit. essential oil, *peak 6* showing major component 1,8-cineole

Table 2 In vitro efficacy of different concentrations of *Hyptis suaveolens* oil on per cent radial growth inhibition, nature of toxicity and per cent conidial germination of *Fusarium oxysporum* f.sp. *gladioli* after 7 days of incubation at 25 ± 1°C

Conc. of oil (μg ml^{-1})	Percent radial growth inhibition (PF)	Nature of toxicity (reinoculated) (PF)	Percent radial growth inhibition (VA)	Nature of toxicity (reinoculated) (VA)	Percent conidial germination
Control	–	–	–	–	100.0
0.031	12.5	NT	19.4	NT	85.2
0.062	20.6	NT	25.6	NT	69.6
0.125	25.8	NT	35.2	NT	48.3
0.249	32.4	NT	46.1	NT	23.8
0.374	50.1	NT	59.2	NT	11.5
0.450	63.8	NT	71.6	NT	0.0
0.624	76.4	NT	85.3	NT	0.0
0.748	88.7	NT	100.0	86.4 (S)	0.0
0.873	98.3	NT	100.0	94.8 (S)	0.0
0.998	100.0	93.8 (S)	100.0	100.0 (C)	0.0
1.122	100.0	97.6 (S)	100.0	100.0 (C)	0.0
1.247	100.0	100.0 (C)	100.0	100.0 (C)	0.0
1.372	100.0	100.0 (C)	100.0	100.0 (C)	0.0

All values are means of three replications per treatment of two experiments

Abbreviations: *PF* poisoned food technique, *VA* volatile activity assay, *NT* not tested, *S* Fungistatic toxicity of essential oil allowing the fungus to regain its vitality on fresh medium. *C* Fungicidal toxicity of essential oil not allowing the fungus to regain its vitality on fresh medium

tested and 100% inhibition of mycelial growth of test fungus was found.

The efficacy of essential oil was also determined in terms of percentage inhibition of mycelial growth after various storage periods using PF and VA at the MIC, and it was observed that the oil retained its fungitoxicity even after storage of 24 months. The efficacy of oil was first tested after storing it for 6 months and then after regular interval of 2 up to 24 months and it was found that complete mycelial growth inhibition occurred during all storage periods.

Mechanism of toxicity of essential oil: light microscopy

The observations of *F. oxysporum* f.sp. *gladioli* examined under light microscope at 400× magnification, after treatment with different concentrations (0.450 and 0.748 μg ml^{-1}) of *Hyptis suaveolens* essential oil are presented in Fig. 2. In addition to inhibited growth, mycelial colonies grown in the presence of essential oil exhibit distinct morphological changes when compared to the control. These variations included lack of conidiation or decreased density of conidia and visible loss of pigmentation. In

Table 3 Effect of different concentrations of *Hyptis suaveolens* oil on dry weight (after 15 days) of *Fusarium oxysporum* f.sp. *gladioli* at 25 ± 1°C

Conc. of oil (μg ml^{-1})	Dry weight (mg ± SE)	Per cent growth inhibition on the basis of dry weight
Control	325.23 ± 2.13	–
0.031	296.21 ± 1.19	8.92
0.062	253.54 ± 3.21	22.04
0.125	176.87 ± 1.58	45.61
0.249	150.17 ± 2.15	53.82
0.374	126.45 ± 2.35	61.11
0.450	90.32 ± 3.02	72.22
0.624	50.21 ± 1.27	84.56
0.748	19.17 ± 1.65	94.10
0.873	00.00 ± 00.00	100.00
0.998	00.00 ± 00.00	100.00
1.122	00.00 ± 00.00	100.00
1.247	00.00 ± 00.00	100.00
1.372	00.00 ± 00.00	100.00

All data shown are averages and standard errors from three determinations of experiment

microscopic examination of untreated mycelium, hyphae had homogenous, clear cytoplasm and profuse conidiation and healthy conidia (Fig. 2a, b) while treatment with 0.450 μg ml^{-1} concentration essential oil clearly showed reduction of conidiation or conidia having smaller and very low cytoplasm. Mycelium treated with 0.450 μg ml^{-1} essential oil showed anomalies in the hyphae, diameter of hyphae decreased, the cytoplasm started leaving the cell wall and the cytoplasm became granulated. The conidia also showed a decrease in size, having low cytoplasm detached from the conidial wall (Fig. 2c, d). On increasing the concentration of essential oil (0.748 μg ml^{-1}) mycelium showed highly segmented granulated cytoplasm in hyphae and it was of lesser diameter in comparison to control. There was also recorded a complete absence of conidiation (Fig. 2e). In some observations it was also found that the hyphae were highly thin and showed bulbous anomalous structure (Fig. 2f). Treatment with 0.748 μg ml^{-1} essential oil also showed highly reduced cytoplasm in the hyphae, which illustrated clear retraction of the cytoplasm from the hyphae and ultimately in some areas, hyphae without cytoplasm were also found (Fig. 2g).

Discussion

The *Hyptis suaveolens* oil used in the present study consisted of 24 components having 1,8-cineole as the major component, accounting 44.4% of the total components. Interestingly, it was also observed that the percentage of this compound was more from the plants growing in Lucknow when compared with those from Bangalore (31.51%) and Hyderabad (35.30%) by Mallavarapu et al. (1993) and Australia (32%) by Peerzada (1997). Azevedo et al. (2002) reported that in plants of *Hyptis suaveolens* the latitude seems to be the most important environmental factor influencing the percentage and components of oil. The pattern of geographic variation in essential oil composition indicated that sesquiterpenes are mainly produced in the samples grown at lower latitudes and altitudes. The change in percentage of various components in various geographical regions of the world should be responsible for the differing antimicrobial activity of *H. suaveolens,* which may be due to a change in the synergistic action of the components of the essential oil.

The most important feature of essential oils is that all components work in a synergistic way, which is reported by various workers in different contexts (Dubey and Kishore 1987; Pandey and Dubey 1997; Lachowicz et al. 1998; Fyfe et al. 1998; Hummelbrunner and Isman 2001; Tripathi et al. 2000, 2003). Synergistic activity of 1,8-cineole and camphor against some bacteria has already been reported (Cosentino et al. 1999; Viljoen et al. 2003) and in the present study the oil itself contains 44.4% 1,8-cineole and 5.7% camphene, so suggesting the synergistic activity of essential oil.

The inhibitory activity of essential oils has been attributed to their most abundant components (Griffin et al. 1999; Giamperi et al. 2002; Guynot et al. 2003; Rota et al. 2004) and in the present study the most abundant component is 1,8-cineole. Dubey and Kishore (1987) demonstrated that combinations of oils of *Lippia alba*, *Ocimum canum* and *Chenopodium ambrosioides* were more fungitoxic to the mycelial growth of *Rhizoctonia solani* than the individual oils, suggesting a synergistic effect between components of the different oils. Jedlickova et al. (1992) also studied the antibacterial properties of Vietnamese cajeput oil and *Ocimum* oil in combination and found that the activity is due to synergistic action of both which was proved by in vitro tests. The essential oil of *Cinnamomum camphora* consists of compounds, linalool, limonene and camphor and these compounds were reported to be toxic, repellent or fumigant against insects and it was proved that they acted synergistically (Hummelbrunner and Isman 2001; Tripathi et al. 2000, 2003). Recently, Kiran and Devi (2007) reported that the essential oil from *Chloroxylon swietenia* has mosquitocidal activity. The sesquiterpenes isolated from this was the major active principles responsible for the potent mosquitocidal activity, but the synergistic action of other constituents of the oil cannot be disregarded, as the whole oil was more effective than the individual compounds. The present study showed that besides 1,8-cineole as major component, the other

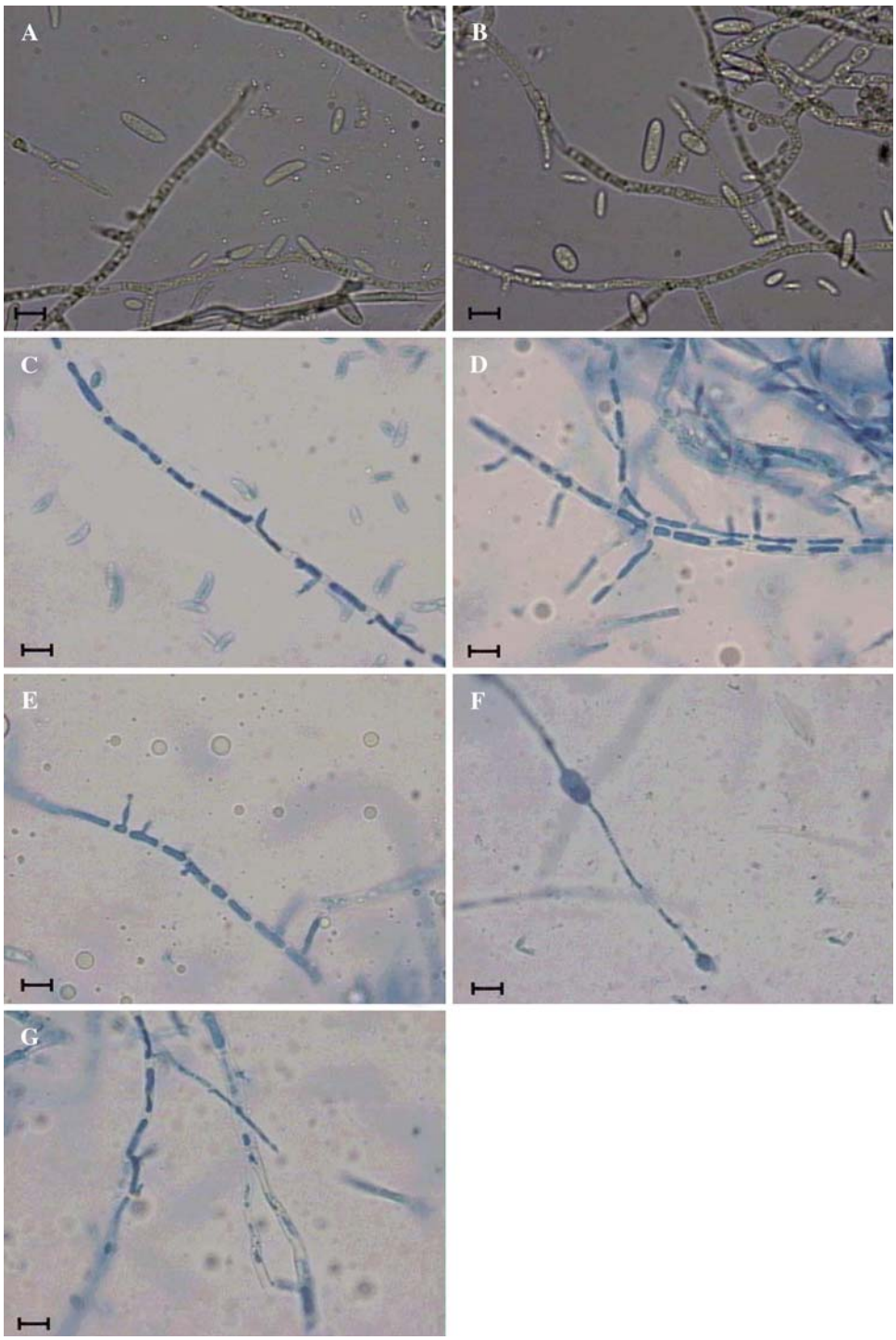

Fig. 2 Microphotographs of *Fusarium oxysporum* f.sp. *gladioli* hyphae grown on PDA with or without *Hyptis suaveolens* essential oil after 4 days of incubation at 25 ± 1°C. **a**, **b** *F. oxysporum* f.sp. *gladioli* control hyphae, structure is homogenous, hyphae full of cytoplasm, profuse conidiation and healthy conidia. Bar 10 μm. **c**, **d** Treatment with 0.450 μg of essential oil ml^{-1} showing anomalies in the hyphae, diameter of hyphae decreased, cytoplasm started leaving the cell wall and cytoplasm become granulated, conidia also showing decrease in size, having low cytoplasm detached from the conidial wall. Bar 10 μm. **e**, **f** and **g** Treatment with 0.748 μg of essential oil ml^{-1} showing highly segmented granulated cytoplasm in hyphae, hyphae is of lesser diameter in comparison to control, complete absence of conidiation (**e**), highly thin hyphae showing bulbous anomalous structure (**f**), highly reduced cytoplasm in the hyphae showing clear separation of cytoplasm from cell wall, some parts of hyphae clearly devoid of cytoplasm (**g**). Bar 10 μm

compounds present more than 10% in the oil were β-pinene, and β-caryophyllene. Significant amounts of other components like camphene, β-myrcene, α-farnesene, α-pinene and terpinen-4-ol were also detected and it could be concluded that the antifungal activity of the oil is due to synergistic activity of all the components of the oil with the major component 1,8-cineole.

There are also reports that besides the major component, minor components have also a critical part to play in bio-activity of essential oil, possibly by producing a synergistic effect between other components (Lattaoui and Tantaoui-Elaraki 1994; Paster et al. 1995; Marino et al. 1999, 2001). Some studies have concluded that whole essential oils have a greater antibacterial activity than the major components

mixed (Gill et al. 2002; Mourey and Canillac 2002), which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence.

A few workers have also reported the antagonistic activity of different components of essential oil. Essential oils of *Origanum vulgare*, *Coridothymus capitatus* and *Satureja thymbra* as well as the compounds thymol and carvacrol present in the three extracts, were tested for insecticidal and genotoxic activities on *Drosophila melanogaster* (Karpouhtsis et al. 1998). The toxic effect of carvacrol and thymol did not correspond with their relative presence in the oils and mixtures of the two compounds at levels resembling their content in the three extracts showed that the toxicity of carvacrol was reduced in the presence of thymol, suggesting an antagonistic effect.

The results reported in the present study indicate the mycotoxic potential of the essential oil of *H. suaveolens* and provide detailed information on the antifungal activity against *F. oxysporum* f.sp. *gladioli*. The antifungal and antimicrobial activity of *H. suaveolens* oil has been reported by Iwu et al. (1990) and Pandey et al. (1982). In the present study its minimum inhibitory concentration in the poisoned food technique and the volatile activity assay was 0.998 and 0.748 μg ml^{-1}, respectively, which is lower than many of essential oils previously tested by different workers (Pandey et al. 1982; Beg and Ahmad 2002; Nguefack et al. 2004). The fungicidal nature of the oil strengthens its exploitation as an economical source of fungitoxicant and there was no report of its use as a fungitoxic fumigant. Keeping in view the residual toxicity and the side effects of synthetic fungicides, the essential oil of *H. suaveolens* may be exploited as fungitoxicant to control the growth of *F. oxysporum* f.sp. *gladioli*.

The fungicidal concentrations of essential oil of *H. suaveolns* in the poisoned food technique and volatile activity assay were 1.247 and 0.998 μg ml^{-1}, respectively for *F. oxysporum* f.sp. *gladioli*, which is less than many of essential oils previously tested by different workers (Singh et al. 1980; Pandey et al. 1982; Zambonelli et al. 1996; Antonov et al. 1997; Beg and Ahmad 2002; Daferera et al. 2003; Nguefack et al. 2004). It was found that *Hyptis* oil was more effective in volatile phase, as evident by the decreased MIC values in volatile activity assay. This property was utilized in concentrating the oil in treatment of diseases by fumigation technique. Based upon the present study it could be concluded that fungitoxic activity of volatile oil from *H. suaveolens* is worth exploiting for management of *gladiolus* diseases.

The effect of *H. suaveolens* oil on the percentage inhibition of spore germination indicates that 0.450 μg ml^{-1} concentration is sufficient to check the conidial germination in *F. oxysporum* f.sp. *gladioli*. This concentration is lower than many other essential oils previously tested by various workers (Antonov et al. 1997; Beg and Ahmad 2002). A number of workers reported the inhibition of spore germination using different testing methods (Jain 1977; Begum et al. 1993; Pattnaik et al. 1996). Soliman and Badeaa (2002) reported complete inhibition of *Aspergillus flavus*, *A. parasiticus* and *A. ochraceus* by the oils of thyme and cinnamon (<500 ppm), marigold (<2,000 ppm), spearmint, basil (3,000 ppm).

In vitro light microscopic study of the test fungus revealed the mechanism of action of *H. suaveolens* essential oil. It was found that mycelium treated with oil showed alterations in the morphology of hyphae, which appeared severely collapsed, loss of conidiation and degradation of the cytoplasm. The synergistic antifungal property of various components found in *H. suaveolens* essential oil against *F. oxysporum* f.sp. *gladioli* could involve inhibition of extracellular enzyme synthesis and the disruption of the cell wall structure resulting in lack of cytoplasm, damage of integrity and ultimately mycelial death. There are reports that the antifungal property of essential oils possibly leads to structural and metabolic changes in the fungus like cytoplasm granulation, cytoplasm membrane rupturing, cytoplasm hyperacidity, breakdown of the electron transport chain, H^+-ATPase and channel inhibition (Lopez-Diaz et al. 2002; Burt 2004). To our knowledge no comparative studies has been carried out on growth, conidiation and microscopy of *Fusarium oxysporum* f.sp. *gladioli* on an essential oil-containing medium. Similar types of results were also reported by Sharma and Tripathi (2008) against *Aspergillus niger* with treatment of *Citrus sinensis* oil. *Cymbopogon nardus* oil was also used previously to investigate the growth, conidiation and ultrastructure of *A. niger* (de Billerbeck et al. 2001). Our observations can also be compared to the results presented by *Cymbopogon nardus* oil, but we found that *H. suaveolens* essential oil caused severe damage and alterations to vegetative hyphae leading to complete loss of cytoplasm from the hyphae. Carmo et al. (2008) also reported the effect of *Cinnamomum zeylanicum* essential oil on growth and morphogenesis of *A. niger*. The main morphological changes observed by Carmo et al. (2008) under light microscopy were decreased conidiation, leakage of cytoplasm, loss of pigmentation and disrupted cell structure, indicating fungal wall degeneration. The mode of action of *C. zeylanicum* oil is more or less similar to that of *H. suaveolens* against *F. oxysporum* f.sp. *gladioli*. These reports clearly indicate the possible mode of action of essential oil of *H. suaveolens* against *F. oxysporum* f.sp. *gladioli* and this knowledge could be exploited for the study of mode of action of essential oil at molecular level.

Conclusions

The present work confirmed the fungitoxicity of *Hyptis* oil against the *Fusarium oxysporum* f.sp. *gladioli* and also showed the possible mode of action and resulted morphological alterations against the test fungus. Based upon the present study it could be concluded that essential oil from *H. suaveolens* possess fungitoxic activities inhibiting the growth of *F. oxysporum* f.sp. *gladioli* leading to irreversible deleterious morphological alterations and thus it is worth exploiting for the biomanagement of *F. oxysporum* f.sp. *gladioli.*

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