0971-4693/94 US $ 5.00
Allelopathy Journal 23 (2): 357-368 (2009) *International Allelopathy Foundation* 2009
Tables: 3, Figs : 2

Antifungal and allelopathic effects of *Asafoetida* against *Trichoderma harzianum* and *Pleurotus* spp.

P. ANGELINI*, R. PAGIOTTI, R. VENANZONI and B. GRANETTI

Dipartimento di Biologia Applicata, Borgo XX Giugno, 74 - 06121 Perugia, Italy
E. Mail: paolaangelini@ymail.com

(Received in revised form: November 8, 2008)

ABSTRACT

Methanol extract (MeOH) of *Asafoetida oleogum*-resin was assayed for its *in-vitro* ability to control *Trichoderma harzianum*. The thirty-two components of MeOH-extracted resin were identified by GC-MS analysis. The antifungal and allelopathic effects of the MeOH extracts concentrations against *T. harzianum* and *Pleurotus* spp., were investigated in dual culture experiments on an agar-based medium. MeOH extract showed fungistatic and fungicidal properties against *T. harzianum* strains and *Pleurotus* spp. at higher concentrations. In dual culture, all strains of *T. harzianum* were antagonistic to *Pleurotus* spp. than in control. When MeOH extracts concentrations was added to the substrate culture, the antagonistic activity of *T. harzianum* against the *Pleurotus* spp. was moderate (0.625 µg/ml of MeOH extract) or weak (1.25 µg/ml of MeOH extract) against the *Pleurotus* spp. that either completely or partially replaced *T. harzianum*. TEM observations revealed that fungal growth inhibition from the MeOH extract was accompanied by marked morphological and cytological changes.

Key words: Antagonism, GC/MS, green moulds, MIC, MFC, transmission electron microscopy.

INTRODUCTION

Oyster mushroom spp. belonging to *Pleurotus eryngii* spp.-complex [*P. eryngi* (DC:Fr.) var. *eryngii*, *P. eryngi*(DC:Fr.) var. *ferulae* Lanzi, *P. nebrodensis* (Inzenga) Quél., and *P. hadamardii* Costantin] are well known for their fruiting bodies that have excellent organoleptic qualities (31). In recent years, the improved edible fungus cultures have become of great interest due to economic importance of mushroom production (7). Mycelial growth of *Pleurotus eryngii* spp.-complex is fast and various lignocellulosic waste products can be used as culture substrate (32). The aim of commercial mushroom substrate preparation is to produce a substrate that is optimal and selective for vegetative mycelial growth (23,30).

Trichoderma harzianum Rifai (filamentous soil fungi), is an antagonist that causes extensive losses in *Apiaceae* oyster mushroom production. *Pleurotus* compost or casings infected with *T. harzianum* do not produce mushrooms and the crop loss is proportional to the infected area and this infection is called green mould disease (19). *Pleurotus eryngii* spp.-complex, colonise the roots and stems of some Umbelliferous aromatic plants (*Apiaceae*), rich in active materials (resins and essential oils), that plays

*Correspondence author

important role in plant-plant, plant-animal and plant microbe interactions and are primary source of potential allelochemicals (2, 28). The *Ferula* genus of *Apiaceae* family is rich source of oleogum-resin (8). *Ferula assa-foetida* (*Asafoetida*) grows in Kashmir, Iran and Afghanistan. It is an herbaceous, perennial plant of 2 m height (15). Ethanolic extract of *Asafoetida oleogum*-resin has shown antifungal activity against *Mucor dimorphosphorous*, *Penicillium commune* and *Fusarium solani* (24).

In this *in-vitro* study, the potential effects of *Asafoetida oleogum*-resin extract to inhibit *T. harzianum* strains encountered in *Apiaceae* oyster mushroom cultivation has been investigated. The possible allelopathic dose of *Asafoetida oleogum*-resin extract was investigated in dual culture to inhibit the *T. harzianum* and stimulation of *Pleurotus* spp. mycelium growth. Transmission electron microscopy techniques were used to observe the action of *Asafoetida oleogum*-resin extract on the ultrastructure of *T. harzianum* and *Pleurotus* spp. fungal cells.

MATERIALS AND METHODS

T. harzianum strains, A, B, and C, were isolated from a naturally-contaminated *Apiaceae* oyster mushrooms straw-based cultivation substrate in 2002. *P. eryngii* var. *ferulae* strains 1 and 2, were isolated from basidiocarps in Tarquinia (VT, Italy) in 2001; *P. eryngii* var. *eryngii* strains were isolated from basidiocarps in Senise (MT, Italy) in 2001; *P. nebrodensis* strains 529 and 193 were obtained from the Mycothèque du Museum National d'Histoire Naturelle de Paris), *P. hadamardii* strain was isolated from basidiocarp in Predazzo (TN, Italy) in 2002. All fungi were maintained on malt extract agar (MEA; 2% malt extract and 1.5% agar) and stored at 4±1°C in the dark. Voucher cultures are kept in the DBVBAZ culture collection (University of Perugia, Italy) and are accessible.

The hardened oleogum-resin of *Asafoetida* used in this study was supplied by Aboca Erbe, San Sepolcro, AR, Italy. Using 250 ml glass bottles with screw caps, a sample (30 g) of oleogum-resin was macerated with 100 ml of 96% (v/v) MeOH (methanol) at room temperature for 7 days. The sample was filtered and concentrated in a rotary evaporator under reduced pressure at 50° C. The dried MeOH extract was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Company) and used for antifungal assays.

GC and GC-MS analysis: The GC analyses were carried out using a Varian 3300 instrument equipped with a FID and an HP-InnoWax capillary column (30 m x 0.25 mm, film thickness 0.17 μm), starting at 60°C (3 min) and increasing to 210°C (15 min) at 4°C/min or an HP-5 capillary column (30 m x 0.25 mm, film thickness 0.25 μm) starting at 60°C (3 min) and increasing to 300°C (15 min) at 4°C/min; injector and detector temperatures, 250°C; carrier gas, helium (1 ml/min); split ratio, 1 : 10.GC-MS analyses were carried out using a Hewlett Packard 5890 GC-MS system operating in the EI mode at 70 eV. The operating conditions were the same as those reported in the GC analysis section. Injector and transfer line temperatures were 220°C and 280°C, respectively. Helium was used as the carrier gas, flow rate 1
ml/min. Split ratio, 1 : 10.

The components of MeOH extract of *Asafoetida oleogum*-resin were identified by matching the spectra with those from mass spectral libraries and the identity of each

component was confirmed by comparing the retention indices, relative to the C6-C22 *n*-alkanes from both columns, with those from the literature (1). When reported, coelution gas chromatography with reference compounds was used as an additional confirmation of the compound identity.

The percentage composition of the MeOH extract was obtained by the normalisation method from the GC peak areas, without using correction factors.

Antifungal assay: The antifungal activity of Asafoetida oleogum-resin extract was tested using the macrodilution technique (13). The mycelium growth inhibition (MGI), fungistatic and fungicidal oleogum-resin extract concentrations were determined against strains of T. harzianum and Pleurotus spp..

A known amount (0.625, 1.25, 2.5, 5, 10, 20, 30 and 40 µg/ml) of dried extract, mixed with dimethyl sulfoxide (DMSO, Sigma Chemical Company), was then added to Petri dishes containing 15 ml of Sabouraud Dextrose Agar (SDA, Oxoid) medium. The final concentration of DMSO in these assays was <1%. The growth medium was inoculated the next day at the centre of plates, using 5 mm cores taken from mycelial stock culture plates and incubated at 25° C for 21 days.

The mycelium growth inhibition (MGI) percentage was calculated as per following equation:

$$MGI = (dc-dt)/dc \times 100,$$

Where, dc is fungal colony diameter in control, dt is fungal colony diameter in treatment sets, 21 days after incubation.

The minimal inhibitory concentration (MICs) values of tested MeOH extract were the lowest concentrations that did not exhibit any visible growth of fungal mycelium, but which remained viable and grew when plated on SDA medium, after 21 days of incubation. The minimum fungicidal concentration (MFCs) values were determined by method of Garber and Huston (9). This was done by subculturing the inhibited fungal discs at MICs on SDA medium. Observations were recorded 7 days after incubation at 25°C. Fungal growth on day 7 was indicative of a fungistatic nature, while the absence of fungal growth denoted a fungicidal action of the MeOH extract.

Every experiment was done in triplicate.

Allelophatic assay: Allelophatic effects of MeOH extract on competitive interactions between *Pleurotus* spp. and *T. harzianum* were studied in dual-culture experiments (6). The *Asafoetida oleogum*-resin extract dissolved in DMSO, was added to autoclaved SDA at 0.625 µg/ml and 1.25 µg/ml and then poured into Petri dishes (9 cm dia) at 40–45 °C. Sterile double-distilled H2O alone was added to the SDA of control plates. In each dish, two 2-mm dia mycelial disks, one from *Pleurotus* spp. colony and one from *T. harzianum* were placed on the agar surface 30 mm apart. The *Pleurotus* spp. and *T. harzianum* strains were paired in all possible combinations. Three replicates were prepared for each pairing. Paired cultures were incubated on a laboratory bench at ambient room temperature of 26 ± 2 °C, for 21 days and examined daily under a stereomicroscope to study the interaction process. A rating scale with 3 types (A, B and C) and 4 sub-types (CA1, CB1, CA2 and CB2) of reactions was used for each fungus. Type A and B were deadlock (mutual

inhibition, in which neither organism was able to overtake the other) at mycelial contact (A), or at distance (B); type C replacement, able to over take without initial deadlock. The intermediate subtypes scored were: CA1 partial, and CA2 complete, replacement after initial deadlock with mycelial contact; CB1 partial, and CB2 complete, replacement after initial deadlock at a distance.

The following score was assigned to each type or sub-type of reaction:

A=1; B=2; C=3; CA1=3.5; CB1=4; CA2=4.5; CB2=5.

The antagonism index (AI) was calculated for each fungal spp. using the formula:

$$AI = \sum n \times i$$

Where, n: number (frequency) of each type or sub-type of reaction; i: corresponding score.

Transmission electron microscopy: After 21 and 74 days of *Asafoetida oleogum*-resin extract treatment (1.25 µg/ml) by macrodilution technique, small pieces of agar from the fungal colony edge were taken from the treated and control Petri dishes for transmission electron microscope (TEM) studies.

Samples from single cultures of each fungus were fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) with 1 mM CaCl and 1% (w/v) sucrose for 3 h at room temperature. Samples were then rinsed six times with the same buffer and postfixed with 1% (w/v) osmium tetroxide in the same buffer for 2 h at room temperature. After being rinsed thoroughly with 0.1 M cacodylate buffer (pH 7.4), samples were dehydrated in a graded ethanol series. Fully dehydrated samples were moved from absolute ethanol through a 1:1 mixture of ethanol and propylene oxide to pure propylene oxide. Samples were infiltrated through a series of Epon-Araldit-Mixture resin in propylene oxide, then embedded in blocks with fresh 100% resin and polymerised at 65°C for 36 h. More than three replicate experiments were performed. Ultrathin sections, cut with a glass knife, were collected on formvar-coated slot grids. After drying, the grids were contrasted with uranyl acetate and lead citrate and examined with an EM 10 CR electron microscope (Zeiss, Oberkochen, Germany) at 60 KV.

Statistical analyses: The effects of antifungal activity of *Asafoetida oleogum*-resin extract were analysed by two factorial analysis of variance (ANOVA), followed by LSD post hoc determinations ($p \leq 0.05$). All computations were done using the statistical software SuperAnova for Mac Plus (1989-90, Abacus Concepts, Inc).

RESULTS AND DISCUSSION

GC-MS was used to identificaty and determine the percentage composition of compounds found *Asafoetida oleogum*-resin extract (Table 1). Thirty - two compounds were identified, representing 79.75 % of the extract. The main constituents were: sec-butyl propenyl disulfide (19.53%), vinil-4-guaiacol (17.43%), ferulic acid (11.18%) and *ß*-pinene (6.17 %).

Table 1. Composition of MeOH extract of *Asafoetida oleogum*-resin (%).

Constituents	Relative peak area (%)
sec-Butyl propenyl disulfide	19.53
Vinil-4-guaiacol	17.43
Ferulic acid	11.18
β-Pinene	6.17
Acetaldehyd diethyl acetal	5.08
α-Pinene	3.11
Guaiol	2.05
Vanillin	1.23
Elemol	1.09
Cariophyllene oxide	0.87
α-Cariophyllene	0.83
Farnesil acetate	0.64
cis-β-Ocimen	0.62
Sativene	0.58
Methyl chavicol	0.53
trans-Pinocarvyl acetate	0.53
trans-β-Ocimen	0.52
Aristolen	0.50
β-Eudesmol	0.47
Farnesol	0.30
di-sec-Butyl disulfide	0.23
Limonene	0.21
α-Terpineol	0.21
Pinocarveol	0.20
Propyl sec-butyl disolfide	0.20
Fenchol	0.13
Myrcen	0.12
2,3,4-Trimethylthiophene	0.06
Isovalerianic acid	0.05
Canphene	0.05
p-Cimene	0.02
α-Fenchene	0.01
Others	25.25

Antifungal effects of *Asafoetida oleogum*-resin extract on *T. harzianum* and *Pleurotus* spp.

The mycelium growth inhibition, fungistatic and fungicidal activity values of MeOH extract against the tested fungi are reported in Table 2 and Figure 1. *T. harzianum* strains exhibited fungal growth inhibition at the lowest concentrations of MeOH extract (0.625 – 1.25 µg/ml), while *Pleurotus* spp. showed mycelial growth stimulation at the same concentrations.

The MeOH extract of *Asafoetida oleogum*-resin showed fungistatic and fungicidal properties against *P. nebrodensis* 529, *P. eryngii var. ferulae* 1 and 2 and *P. eryngii var. eryngii* 1 at the highest concentrations used, i.e. 30 µg/ml MIC and 40 µg/ml MFC. The MeOH extract had fungistatic activity against *P. nebrodensis* 193 and

Table 2. Mycelial growth inhibition, fungistatic and fungicidal activity of asafoetida oleogum-resin extract on *Pleurotus* species

Asafoetida oleogum-resin extract (µg/ml)	*P. n.* 529	*P. n.* 193	*P. f.* 1	*P. f.* 2	*P. e.*1	*P. e.* 2	*P. h.*
0.625	- 46.7 ± 0.5 (b)	- 86.2 ± 0.7 (b)	- 33.4 ± 0.6 (b)	- 64.4 ± 1.1 (b)	-20.0 ± 0.6 (b)	-46.1 ± 0.6 (b)	-5,1 ± 0.1 (a)
1.25	- 60.0 ± 2.7 (a)	- 135.8 ± 0.6 (a)	- 48.9 ± 1.0 (a)	- 82.3 ± 0.6 (a)	-46.6 ± 0.3 (a)	-76.9 ± 0.2 (a)	-2,2 ± 0.1 (b)
2.5	-36.4 ± 0.2 (c)	- 56.7 ± 0.4 (c)	- 6.4 ± 0.2 (c)	-29.8 ± 0.5 (c)	2.2 ± 0.1 (c)	- 18.7 ± 0.6 (c)	21,2 ± 0.6 (c)
5	6.9 ± 0.4 (d)	- 2.3 ± 0.2 (d)	12.7 ± 0.4 (d)	10.2 ± 0.6 (d)	43.8 ± 1.1 (d)	8.5 ± 0.3 (d)	36.4 ± 0.5 (d)
10	32.8 ± 1.0 (e)	8.8 ± 0.6 (e)	41.3 ± 0.6 (e)	52.4 ± 1.1 (e)	78.6 ± 0.6 (e)	39.1 ± 0.6 (e)	46,7 ± 1.0 (e)
20	71.4 ± 0.5 (f)	47.6 ± 0.9 (f)	97.4 ± 1.1 (f)	92.3 ± 1.1 (f)	98.1 ± 0.6 (f)	78.2 ± 0.7 (f)	61,7 ± 0.4 (f)
30	100 (g) Fs	84.3 ± 1.1 (g)	100 (g) Fs	100 (g) Fs	100 (g) Fs	94.6 ± 1.1 (g)	69,6 ± 1.1 (g)
40	101 (g) Fc	100 (h) Fs	100 (g) Fc	100 (g) Fc	100 (g) Fc	100 (h) Fs	87.4 ± 0.6 (h)

P.n. - Pleurotus nebrodensis; P.f. - P. eryngii var. ferulae; P.e. - P. eryngii var. eryngii; P.h. - P. hadamardii. Data in the column followed by different letters in the parentheses are significantly different in LSD post hoc test ($p \leq 0.05$). The values are means of three repetitions ± standard error. Fs - Fungistatic activity; Fc - Fungicidal activity.

Table 3. Types and sub-types of hyphal reactions[a] between *Pleurotus* species and *Trichoderma harzianum* in pairings on PDA medium without asafoetida oleogum-resin extract (control), with 0.625 µg/ml and 1.25 µg/ml of asafoetida oleogum-resin extract

Pleurotus species	Control				Asafoetida oleogum-resin extract (0.625 µg/ml)				Asafoetida oleogum-resin extract (1.25 µg/ml)			
	T. h. A	*T. h.* B	*T. h.* C	Total A.I.[b]	*T. h.* A	*T. h.* B	*T. h.* C	Total A.I.[b]	*T. h.* A	*T. h.* B	*T. h.* C	Total A.I.[b]
P. nebrodensis 529	C_{A1}*	C_{A1}*	C_{A1}*	0	C_{B1}*	C_{B1}	C_{B1}	8	C_{B1}	C_{B1}	C_{B2}	13
P. nebrodensis 193	C_{A1}*	C_{A1}*	C_{A2}*	0	B	C_{B2}	C_{B2}	12	C_{B2}	C_{B2}	C_{B2}	15
P. eryngii var. *ferulae* 1	C_{A1}*	C_{A1}*	C_{B1}*	0	C_{B1}*	C_{B1}	C_{B1}*	4	C_{B1}	C_{B2}	C_{B2}	14
P. eryngii var. *ferulae* 2	C_{A2}*	C_{A1}*	C_{B1}*	0	C_{B1}*	C_{B1}	B	6	B	C_{B2}	C_{B1}	11
P. eryngii var. *eryngii* 1	C_{A2}*	C_{A2}*	C_{A1}*	0	C_{B2}*	B	B	4	C_{B2}	C_{B1}	C_{B2}	14
P. eryngii var. *eryngii* 2	C_{A2}*	C_{B1}*	C_{B1}*	0	C_{B1}*	C_{B1}	B	6	C_{B2}	C_{B2}	C_{B2}	15
P. hadamardii	C_{A1}*	C_{A1}*	C_{A1}*	0	C_{A1}*	A	A	2	C_{A1}*	A	C_{A1}*	1
Total A.I.[a]	27.5	23	27		26.5	3	11		5.5	1	3.5	

[a], A - deadlock, mutual inhibition, in which neither organism was able to overgrow the other after mycelial contact; B - deadlock at a distance without mycelial contact; C - replacement, overgrowth without initial deadlock; C_{A1} - Partial replacement after initial deadlock; C_{B1} - Partial replacement after initial deadlock at adistance; C_{A2} - Complete replacement after initial deadlock; C_{B2} - Complete replacement after initial deadlock at a distance. [b], A.I. = Antagonism Index. *, *T. harzianum* overgrew *Pleurotus* species. In the other replacement reactions *Pleurotus* species overgrew *T. harzianum*.

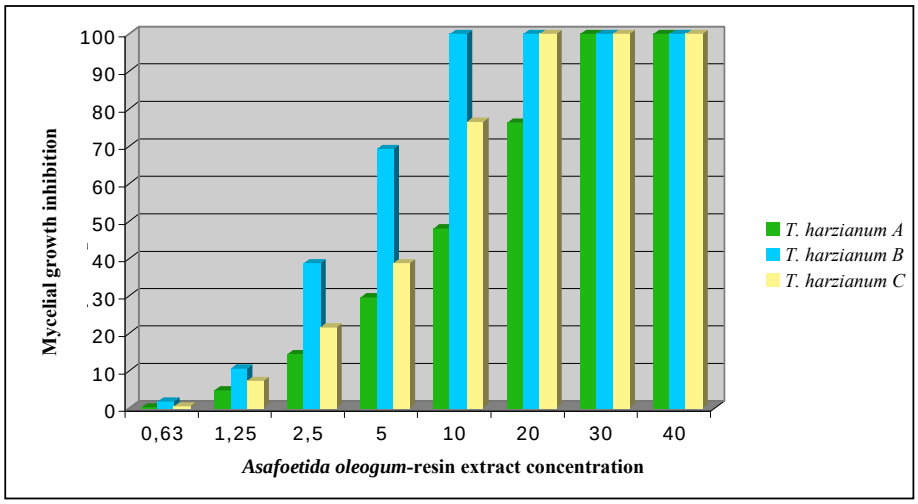

Figure 1. Mycelial growth inhibition of *Asafoetida oleogum*-resin extract on *T. harzianum* strains.

P. eryngii var. eryngii 2 at a concentration of 30 µg/ml (MIC). *P. hadamardii* was the least sensitive to the MeOH extract; no fungistatic or fungicidal properties were observed. *T. harzianum* A was the most resistant strain among the fungi tested with MIC and MFC values of 40 and >40 µg/ml, respectively, while *T. harzianum* B was the most sensitive strain with MIC and MFC values of 10 and 20 µg/ml, respectively. MIC and MFC value of MeOH extract against *T. harzianum* C were 20 and 30 µg/ml, respectively.

Our results showed that at the lowest concentrations, *Asafoetida oleogum*-resin MeOH extract exerts a semispecific antifungal effect on the growth of *T. harzianum* mycelium and stimulated the mycelial growth in *Pleurotus* spp. Although oleogum-resins/essential oils are well known antimicrobial agents, they stimulates some microorganisms and use them as carbon energy sources (18,29). Thus we suggest that the weak parasitism of *P. eryngii* spp.-complex on roots and stems of umbellifers (family *Apiaceae*, genera *Eryngium*, *Ferula*, *Ferulago*, *Cachrys*, *Laserpitium*, *Diplotaenia* and *Elaeoselinum*) is mediated by allelopathic interactions. The oleogum-resin/essential oils (or their components) shifts the microrganism balance in favour of those microrganisms (e.g. *Pleurotus* spp.) that can tolerate them. Some even use them as a carbon and energy source (4, 14).

Allelopathic effects of *Asafoetida oleogum*-resin extract on hyphal interactions between *Pleurotus* spp. and *T. harzianum* in dual-culture

The interactions between *T. harzianum* and *Pleurotus* spp. in dual-culture on PDA with *Asafoetida oleogum*resin MeOH extract are shown in Table 3. Three types of competitive reactions were observed: CB1, partial replacement after initial deadlock at a distance, CB2, complete replacement after initial deadlock at a distance and B, deadlock at a distance without mycelial contact. All *T. harzianum* strains were antagonistic to *Pleurotus* spp. in control, when the MeOH extract was not included in the culture

substrate. After the initial physical contact (4 to 7 days after culture), all *T. harzianum* strains overtook, sporulated on and completely inhibited the mycelial growth of *Pleurotus* spp.

When the MeOH extract was added to the culture substrate at 0.625 µg/ml, the *Pleurotus* spp. were divided into three groups based on the AI values: I – active (AI > 10): *P. nebrodensis* 193, II –moderately active (AI = 5 - 10): *P. nebrodensis* 529, *P eryngii* var. *ferulae* 2, *P eryngii* var. *eryngii* 2 and III – weakly active (AI < 5) *P eryngii* var. *ferulae* 1, *P eryngii* var. *eryngii* 1 and *P. hadamardii*. At the 1.25 µg/ml concentration *Pleurotus* spp. were divided as follows: I – active (AI > 10): *P. nebrodensis* 529, *P. nebrodensis* 193, *P eryngii* var. *ferulae* 1, *P eryngii* var. *ferulae* 2, *P eryngii* var. *eryngii* 1, *P eryngii* var. *eryngii* 2 and II – weakly active (AI < 5): *P. hadamardii.* In the presence of the MeOH extract, all strains of *T. harzianum* were moderately active (0.625 µg/ml of MeOH extract) or slightly active (1.25 µg/ml of MeOH extract) against the *Pleurotus* spp. that either completely or partially replaced *T. harzianum* (Table 3). *T. harzianum* A showed greater competitive activity. In most pairings, the fungus partially or completely overtook the Pleurotus spp. mycelium, after initial deadlock at a distance.

The antifungal and allelopathic activities of oleogum-resin/oils were correlated with their chemical structure (27), hence, it is necessary to isolate the active component. Ferulic acid (one of the main constituents of MeOH extract of *Asafoetida oleogum*-resin) is an allelochemical found in soil (16). Asiegbu *et al*. (5), reported that 5 g/l ferulic acid severely suppressed the growth of *T. harzianum,* while at 0.5 g/l, it slightly stimulated the growth of *Trametes versicolor* and *Pleurotus sajor-caju*. Granetti (10,11) and Angelini *et al.* (3) reported that ferulic acid did not effect the growth of some strains of *Umbelliferae* oyster mushrooms but increased their colony diameter and dry weight. In contrast higher concentrations of ferulic acid, inhibited and in same cases, totally blocked the growth of test spp.

Fine structural modifications of *T. harzianum* and *Pleurotus* spp. hyphae induced by *Asafoetida oleogum*-resin extract

Transmission electron microscopy (TEM) sections of *Pleurotus* spp. and *T. harzianum* control hyphae and hyphae treated with MeOH extract at a concentration of 1.25 µg/ml medium are shown in Figure 2. The cell wall of non-treated healthy mycelia is composed of a uniform layer, with a definite plasma-membrane and periplasm region with normal thickness. A septum, typical of ascomycetes and basidiomycetes, can be seen in Figure 2a and 2d, respectively. Treatment with the *Asafoetida oleogum*-resin MeOH extract disturbed the normal ultrastructure of the fungal cells. Cell structure alterations were observed in all fungal spp. 21 and 74 days after treatment with MeOH extract. After 21 days, the most frequent change in *T. harzianum* strains was an increase in number and size of vacuoles in cells of same age (Figure 2b). While after 74 days, the strains showed marked thickening of cell walls (up to twice of controls) and degeneration of hyphal cytoplasm (Figure 2c). No adverse effects were observed in *Pleurotus* spp., 21 day after treatment. However after 74 days, there was increased vacuolization and an alteration in the cytoplasmic membrane that was partially detached from the cell wall (Figure 2f).

The cytomorphological modifications (cell wall thickening and undulation of plasmalemma) of *T. harzianum* and *Pleurotus* spp., induced by MeOH extract of

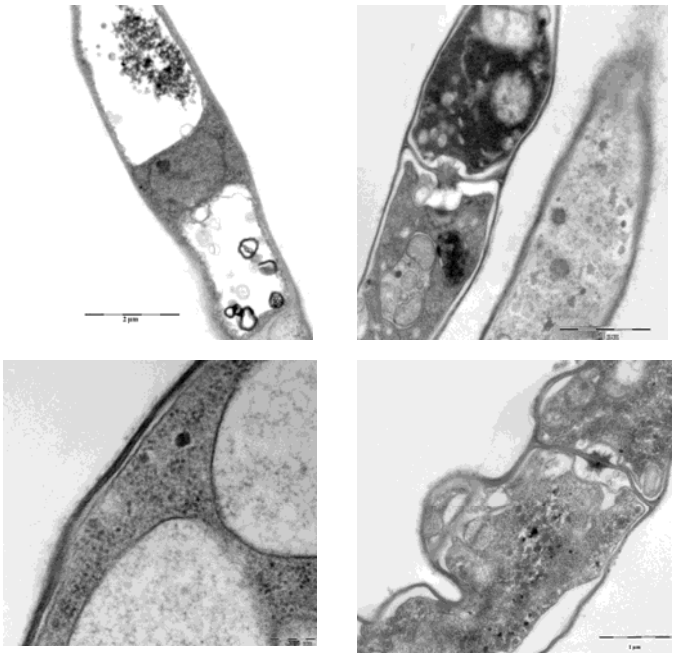

Figure 2. Transmission electron micrographs. *T. harzianum* C: (a) hyphae of control, bar 1 µm; (b) hyphae treated with *Asafoetida oleogum*-resin extract 0.125 µg/ml for 21 days showing increase in the number and size of the vacuoles in the cells, bar 2 µm; (c) hyphae treated with *Asafoetida oleogum*-resin extract 0.125 µg/ml for 74 days with a marked thickening in the cell walls and degeneration of hyphal cytoplasm, bar 2 µm. *P. nebrodensis* 193: (d) hypha of control, bar 1 µm; (e) particular of hypha treated with 0.125 µg/ml of *Asafoetida oleogum*-resin extract after 21 days showing no alterations of cell structure, bar 1 nm; (f) hypha treated with 0.125 µg/ml of *Asafoetida oleogum*-resin extract after 74 days showing undulation of the cytoplasmic membrane, bar 1 µm.

Asafoetida oleogum-resin, are similar to those observed in other fungal spp. during treatments with sterol-biosynthesis-inhibiting fungicides (12). Cell membrane alterations after treatment with fungitoxic products could be caused by change in the composition of the bi-lipid layer of same membrane (22). These alterations could, in turn, modify the activity of membrane enzymes involved in cell wall formation causing anomalous development.

CONCLUSIONS

The lower concentrations of MeOH extract of *Asafoetida oleogum*-resin, corresponding to the allelopathic concentrations tested, could be used to stop proliferation of green mould, which is currently being treated with other sanitizing agents. Solutions

and emulsions used as sprays with or without a carrier are the preferred forms in which the compounds can be applied with minimal effort to large areas of casing soil. Evaporation by heating should also be considered. Ingestion of *Asafoetida oleogum*-resin has not been associated with toxicity in adults (26). It is a potent antioxidant (21) and therefore ferulic acid has shown some promise as a chemopreventive agent (17); *Asafoetida* may offer some protection against carcinogenesis. Till now use of synthetic fungicides to control green mould is discouraged due to negative effects on food: carcinigenocity, teratogenicity, high and acute residual toxicity, longer degradation and side-effects in humans (20). One of the major problems related to the use of these synthetic chemicals is that the fungi can develop resistance. The use of higher concentrations of chemical, to overcome microbial resistence increases the risk of high levels of toxic residues in products. In contrast, the use of natural products to control green moulds does not seem to foster the development of resistance by the contaminants. This is due to the presence of a mixture of extract components which, apparently, have different mechanisms of antimicrobial activity (25).

Further studies are needed to determine the strategies that can be used for practical application. Consumer acceptance of mushrooms that have been treated with *Asafoetida oleogum*-resin MeOH extract must also be tested.

REFERENCES

1. Adams, R.P. (1995). *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*. Allured Publishing Corporation, Carol Stream, Illinois, USA.
2. Aliotta, G., De Napoli, L. and Piccialli, G. (1989). Inhibition of seedling growth by *Anagallis arvensis* extracts. *Giornale Botanico Italiano* **123**: 291-296.
3. Angelini, P., Granetti, B., Palanga, S. and Bellini M. (2006). Accrescimento *in-vitro* del micelio di alcuni ceppi di *Pleurotus* spp. in presenza di acido ferulico. *Micologia Italiana* **35**: 3- 9. (In Italian).
4. Angelini, P., Pagiotti, R. and Granetti, B. (2008). Effect of antimicrobial activity of *Melaleuca alternifolia* essential oil on antagonistic potential of *Pleurotus* spp. against *Trichoderma harzianum* in dual culture. *World Journal of Microbiology and Biotechnology* **24**: 197-202.
5. Asiegbu, F.O., Paterson, A. and Smith, J. E. (1996). Inhibition of cellulose saccharification and glycoligninattacking enzymes of five lignocellulose-degrading fungi by ferulic acid. *World Journal of Microbiology and Biotechnology* **12**: 16-21.
6. Badalyan, S.M., Innocenti, G. and Garibyan, G. (2002). Antagonistic activity of xylotrophic mushrooms against pathogenic fungi of cereals in dual culture. *Phytopathologia Mediterranea* **41**: 200-225.
7. Chang, S.T. (1999). World production of cultivated and medicinal mushrooms in 1997 with particular emphasis on *Lentinula edodes* (Berk.) Sing. in China. *International Journal of Medicinal Mushrooms* **1**: 291-300.
8. Fernch, D. (1971). Ethnobothany of the *Umbelliferae*. In: *The Chemistry and Biology of the Umberiferae*, (Ed., V.H. Heywood). pp. 285-412. Academic Press, London, UK.
9. Garber, R.H. and Houston, B.R. (1959). An inhibitor of *Verticillium alboatrum* in cotton seed. *Phytopathology* **49**: 449-450.
10. Granetti, B. (1987). Effetto stimolante dell'acido ferulico sulla crescita *in vitro* del micelio di alcuni ceppi di *Pleurotus eryngii* (D.C. ex Fr.) Quél, *P. ferulae* (Lanzi), *P. nebrodensis* Inzenga. *Annali della Facoltà di Agraria, Università di Perugia* **51**: 889-907.
11. Granetti, B. (1988). La fruttificazione dei *Pleurotus* delle Ombrellifere in presenza di acido ferulico. *Atti del Congresso della Società Italiana di Fitochimica*, Assisi, pp. 71-76. (In Italian).
12. Hippe. S. (1991). Influence of fungicides on fungal fine structure. In :Electron Microscopy of Plant Pathogens. (Eds., K. Mendgen and D.E. Lesemann). Springer-Verlag. Berlin.

13. Ishii, H. (1995). Monitoring of fungicide resistence in fungi: Biological to biochemical approaches. In: *Molecular Methods in Plant Pathology* (Eds., S.U. Singh and P.R. Singh). Lewis Publisher, Boca Raton.
14. Karamanoli, K., Menkissoglu-Spiroudi, U., Bosabalidis, A. M., Vokou, D. and Constantinidou, H. I. (2005). Bacterial colonization of the phyllosphere of nineteen plant spp. and antimicrobial activity of their leaf secondary metabolites against leaf associated bacteria. *Chemoecology* **15**: 59-67.
15. Kapoor, L.D. (1990). *Handbook of Ayurvedic Medicinal Plants*. CRC Press, Boca Raton, FL.
16. Lehman, M.E. and Blum, U. (1999) Evaluation of ferulic acid uptake as a measurement of allelochemical dose: effective concentration. *Journal of Chemical Ecology* **25**: 2585-2600
17. Mallikarjuna, G.U., Dhanalakshmi, S., Raisuddin, S. and Rao, A.R. (2003). Chemomodulatory influence of *Ferula asafoetida* on mammary epithelial differentiation, hepatic drug metabolising enzymes, antioxidant profiles and N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats. *Breast Cancer Research and Treatment* **81**: 1-10.
18. Misra, G., Pavlostathis, S.G., Perdue, E.M. and Araujo, R. (1996). Aerobic biodegradation of selected monoterpenes. *Applied Microbiology and Biotechnology* **45**: 831-838.
19. Ospina-Giraldo, M.D., Royse, D.J., Chen, X. and Romaine, C.P. (1999). Molecular phylogenetic analysis of biological control strains of *Trichoderma harzianum* and other biotypes of *Trichoderma* spp. associated with mushroom green mold. *Phytopathology* **89**: 308-313.
20. Roller, S. (2003). *Natural Antimicrobials for the Minimal Processing of Foods*. Woodhead Publishing Ltd, Cambridge, UK
21. Saleem, M., Alam, A. and Sultana, S. (2001). Asafoetida inhibits early events of carcinogenesis: a chemopreventive study. *Life Sciences* **68**: 1913-1921.
22. Sancholle, M., Dargent, R., Weete J.D., Rushing A.E. Miller, K.S. and Montant, C. (1988). Effects of triazoles on fungi. VI. Ultrastructure of Taphrina deformans, Mycologia **80** : 162-175.
23. Savoie, J.M., Iapicco, R. and Largeteau-Mamoun, M.L. (2001). Factors influencing the competitive saprophytic ability of *Trichoderma harzianum* Th2 in mushroom (*Agaricus bisporus*) compost. *Mycological Research* **105**: 1348-1356.
24. Thyagaraja, N. and Hosono, A. (1996). Effect of spice extract on fungal inhibition. *Lebensmittel-Wissenschaft und-Technologie* **29**: 286-288.
25. Tyler, V. E. (1992). Phytomedicines: Back to the future. *Journal of Natural Products* **62**: 1587-1592.
26. Unnikrishnan, M.C. and Kuttan, R. (1988). Cytotoxicity of extracts of spices to cultured cells. *Nutrition and Cancer* **11**: 251-260
27. Villar, A., Rios, J.L., Recio, M. C., Cortes, D. and Cave, A. (1986). Antimicrobial activity of Benzylisoquinoline alkaloids. II. Relation between chemical composition and antimicrobial activity. *Planta Medica* **6**: 556-557.
28. Vokou, D. (1992). The allelopathic potential of aromatic shrubs in phryganic (east Mediterranean) ecosystems. In: *Allelopathy*: *Basic and Applied Aspects* (Eds., S.J.H. Rizvi and V. Rizvi), pp. 303-320. Chapman & Hall, London.
29. Vokou, D. and Liotiri, S. (1999). Stimulation of soil microbial activity by essential oils. *Chemoecology* **9**: 41-45.
30. Zadrazil, F. (1978). Cultivation of *Pleurotus*. In: *Biology and Cultivation of Edible Mushrooms* (Eds., S. T. Chang and W. A. Hayes), pp. 521-558. Academic Press, New York.
31. Zervakis, G.I., Venturella, G. and Papadopoulou, K. (2001). Genetic polymorphism and taxonomic infrastructure of the *Pleurotus eryngii* spp.-complex as determined by RAPD analysis, isozyme profiles and ecomorphological characters. *Microbiology* **147**: 3183-3194.
32. Zervakis, G. I. and Venturella, G. (2002). Mushroom breeding and cultivation enhances ex situ conservation of Mediterranean *Pleurotus* taxa. In: *Managing Plant Genetic Diversity* (Eds., J.M.M. Engels *et al*.), pp. 351-358. CABI Publishing, UK.