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Antifungal activity of *Tagetes patula* extracts on some phytopathogenic fungi: ultrastructural evidence on *Pythium ultimum*

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Abstract

Methanol extract, obtained from *Tagetes patula* plant, was assayed against three phytopathogenic fungi: *Botrytis cinerea, Fusarium moniliforme* and *Pythium ultimum*. The antifungal activity was tested both in the dark and in the light, using two different lighting systems. The data showed that the extract proved to have a dose-dependent activity on all the fungi with a marked difference between treatments in the light than in the dark. Good growth inhibition was observed in fungi only when these were treated with the highest dose of the extract and irradiated, whereas the same dose gave only a modest inhibition when the experiment was conducted in the dark. At 5 and 10 μg/ml in the dark, growth increased. The results indicated that the presence of a luminous source enhances the antifungal activity, with small differences between UV-A and solar spectrum light. SEM and TEM observations on *Pythium ultimum* revealed that the *Tagetes patula* extract induced alterations on cell fungal membranes with a photoactivation mechanism possibly involving the production of free radicals and leading to a premature aging of the mycelium.

Introduction

Development of new and novel antifungal agents (natural or synthetic) can be useful in the control of infections caused by phytopathogenic fungi. With this aim, screening is oriented more and more towards molecules that possess a selective action against these fungi, without either being toxic to

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man or to ecosystem. The discovery of photosensitizers (molecules which express full toxicity in the presence of irradiation only) and their presence in numerous plant families, presents the possibility to examine the biological and ecological roles of these plants, with the further possibility of isolating new active molecules. This type of phenomenon has occurred, for example, with some furanocoumarins, currently being used in the phototherapeutic treatment of psoriasis (Simon et al., 2000), or in the agriculture field, where natural pesticides could assume an important role combating pests and diseases, maintaining, at the same time, the ecological balance (de Matos and Pinto Ricardo, 2003).

The objective of this paper is to verify, *in vitro*, the antifungal activity of the extract of *Tagetes patula*, a dicotyledon belonging to the family of the Asteraceae, on some phytopathogenic fungi. *T. patula* accumulates in its tissues high amounts of thiophenes, well known for their phototoxic activity towards bacteria, nematodes (Hudson and Towers, 1991) and also fungi (Romagnoli et al., 1994; Mares et al., 2002).

In this paper, the activity of the extract of *T. patula* was tested both in the dark and in the light, in order to evaluate the role carried out by the irradiation in the process of the photodynamic activation of the extract. In addition we used two different lighting systems: UV-A and Solar light spectrum lamps. The same experiments were also repeated using two pure standards namely alpha-terthienyl (α-T) and 5-(4-hydroxy-1-butinyl)-2,2′ -bithienyl (BBTOH), also present in the extract. The phytopatogenic fungi used as tests were *Botrytis cinerea, Fusarium moniliforme* and *Pythium ultimum*.

Finally, scanning and transmission electron microscopy was performed in order to evaluate the effect of extracts of *T. patula* on the ultrastructural morphology of the fungus *Pythium ultimum*, which actually proved to be the most sensitive. Thus, with the following series of experiments, we attempted to find out if a possible commercial use of this product in agriculture could exists instead of using synthetic pesticides.

Materials and methods

Plant material and extraction

The extract used in the experiments was obtained from *Tagetes patula* L. growing in an experimental field owned by "Mazzoni vivai" in Tresigallo (Ferrara, Italy). The methanol extraction was performed as reported by Tosi et al. (1991).

Test organisms

The three phytopathogenic fungi used during the growth experiments were as follows: *Botrytis cinerea* Person, strain no. 48339, *Pythium ultimum* Trow, strain no. 58812 and *Fusarium moniliforme* Sheldon, strain no. 36541, supplied by the American Type Culture Collection (ATCC, USA). The samples of mycelium necessary for the *in vitro* experiments, were taken from cultures grown in slants and kept at $26\pm2^{\circ}$C on Potato Dextrose Agar (PDA, Difco).

Media preparation with extracts of *T. patula*

The culture media for the treatments were prepared by adding different volumes of a solution of the extract of *T. patula* in dimethylsulphoxyde (DMSO) to each Petri plate (each still containing the PDA fluid), with the aim of obtaining the final concentrations of 5, 10, 50 μg/ml. Pure standards α-T and BBTOH were dissolved in DMSO and afterwards added to the Petri plates containing PDA in order to obtain the final concentrations of 5, 10, 50 μg/ml. The DMSO concentration in the final solution was 0.1%. Equivalent quantities (0.1%) of the solvent (DMSO) were added to the controls.

For each of the three fungi, two different experiments were prepared for the various types of luminous sources used during the phase of irradiation. Three replicates were used for each concentration and all experiments were repeated twice.

Solar spectrum light

For each fungus two series of plates treated with various doses were prepared. The first series was prepared for irradiation with solar spectrum light, the second was designed for incubation in dark conditions.

In the plates designed for light treatment, the inoculum was placed directly on the medium. The samples were then exposed to daily irradiation for 16 h by means of the solar spectrum light, using two lamps Biolux (Osram L 36 W/72, temperature heat 6500 K, flow total 1600 lumen/m^2 to 1 m of distance), kept at a constant distance of 50 cm from the Petri plates. Some samples treated with 50 μg/ml of extract were exposed to irradiation with solar spectrum light for 90 min, for TEM observation. The

second series was placed in dark conditions at 26±2°C.

Every 24 h the diameter of fungal colony on treated and control plates was measured. The total duration of the experiment was 5 days.

UV-A light

Two series of plates prepared in exactly the same way as those for the first experiment were prepared for each fungus. The fungi from the first series were kept in contact with the treated medium for 24 h in the dark, at 26±2°C. Subsequently the samples were irradiated with UV-A for 90 min, using a fluorescent lamp (black light blue fluorescent lamp, Sylvania, F 20, T 12-BLB) with a light intensity of 0.5 mW/cm^2, kept at a constant distance of 10 cm from the samples.

When the phase of irradiation was completed, the plates were placed again in the dark.

In this case also, the diameter of fungal colony was measured every 24 h for 5 days. The second series on the other hand, after the inoculum of the fungus, was placed in dark conditions at 26±2°C, for 5 days.

Experiment with the standards

The three fungi were treated with α-T and BBTOH standard following the same methods used with the extracts. All experiments were prepared in double quantity, and twice repeated.

Electron microscopy

Four experimental series were prepared: each one was composed of one control sample and one sample treated with 50 μg/ml of extract. For the first and the second series a sample treated with 10 μg/ml was also prepared, and every series was exposed to a different kind of treatment, as follows:

First series: control, treated with 10 and 50 μg/ml: kept at 26±2°C in the dark for 16 h.

Second series: control, treated with 10 and 50 μg/ml: irradiation with solar spectrum light (Biolux) for 16 h.

Third series: control, treated with 50 μg/ml: irradiation with UV-A for 90 min.

Fourth series: control, treated with 50 μg/ml: irradiation with solar spectrum light (Biolux) for 90 min and successive transfer to the dark at 26±2°C.

For the irradiation of the colonies, the same modalities and the same materials used in the growth experiments were also used. At the end of this phase the procedure of collection and sample preparation for observation by the TEM and the SEM was started.

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

The youngest *P. ultimum* hyphae were chosen from the margin of the mycelia; the samples were routinely fixed with 6% glutaraldehyde (GA) in 0.1 M sodium cacodylate buffer, pH 7.2, for 3 h at 4°C. After having been rinsed in the same buffer, the fungi which were to undergo TEM were post-fixed for 20 h at 4°C in 1% OsO_4 in the same buffer. They were then dehydrated in a graded series of ethanol solutions and embedded in Epon-Araldite resin. Sections were cut with an LKB Ultratome III, stained with uranyl acetate and lead citrate, and observed with a Hitachi H-800, at 100 kV.

For SEM the fungi were fixed in 6% GA in a sodium cacodylate buffer, briefly post-fixed for 1 h at 4°C in 1% OsO_4 in the same buffer and then dehydrated in acetone, critical point dried and gold coated with an S 150 Sputter coater (Edwards). SEM observations were performed with a Cambridge Stereoscan 360 scanning electron microscope at an accelerating voltage of 20 kV. Both microscopes are owned by the Electron Microscopy Center of Ferrara University.

Results

Botrytis cinerea

All the inhibition data related to this fungus are shown in Table 1. The treatment of *B. cinerea* with the extract of *T. patula* and irradiation with solar light showed a dose-dependent inhibition of mycelial growth, that reached the highest value (39.3%) in colonies treated with the maximum dose (50 μg/ml). At the same dose, irradiation with UV-A enhanced the action of the extract (57.4%) compared to irradiation with solar light. By comparison, the experimental series kept in the dark and treated with 50 μg/ml showed a lower inhibition (24.8%). At the inferior doses, the samples treated with the extract and irradiated with UV-A or kept in the dark, were grown even more than the control. Also, the responses of the two standards were very different if the fungi were kept in the dark or irradiated with UV-A. In fact, while α-T showed very low inhibition values at the three concentrations in these conditions, BBTOH showed higher values,

Table 1. Percentage inhibition rate after 5 days of treatment on *B. cinerea*

		Extract	α-T	BBTOH
Dark	Controls	0	0	0
	5 μg/ml	−16.7±0.5	7.1±1.4	15.7±2
	10 μg/ml	−15.6±0.9	8.1±1.4	36.0±1.5
	50 μg/ml	24.8±1.2	12.4±1.6	67.8±1.7
Solar spectrum light	Controls	0	0	0
	5 μg/ml	11.8±1.8	3.0±1.3	5.3±1.5
	10 μg/ml	18.6±2.8	16.6±1.6	13.3±1.7
	50 μg/ml	39.3±1.7	73.4±1.8	78.6±1.1
UV-A light	Controls	0	0	0
	5 μg/ml	−1.5±2.6	9.4±1.3	56.6±2.1
	10 μg/ml	−4.0±2.2	10.4±1.8	67.0±1.4
	50 μg/ml	57.4±2.0	14.4±0.9	85.3±0.5

All data shown are averages and standard errors from three determinations of two independent experiments.

Table 2. Percentage inhibition rate after 5 days of treatment on *F. moniliforme*

		Extract	α-T	BBTOH
Dark	Controls	0	0	0
	5 μg/ml	−23.2±1.1	3.7±0.7	27.7±1.7
	10 μg/ml	−2.0±0.2	4.2±1.0	33.9±0.9
	50 μg/ml	33.8±1.3	5.4±1.7	64.1±1.2
Solar spectrum light	Controls	0	0	0
	5 μg/ml	21.4±1.6	14.8±1.3	18.1±1.2
	10 μg/ml	37.0±1.2	16.1±1.3	34.0±1.3
	50 μg/ml	50.9±1.7	43.7±1.7	75.4±0.6
UV-A light	Controls	0	0	0
	5 μg/ml	6.7±1.3	29.8±1.1	44.7±1.5
	10 μg/ml	13.5±0.8	32.0±1.6	51.8±0.7
	50 μg/ml	47.3±1.0	36.2±1.4	73.2±1.1

All data shown are averages and standard errors from three determinations of two independent experiments.

that reached the maximum of inhibition (85.3%) at the highest dose, after irradiation with UV-A.

Fusarium moniliforme

The inhibition data on this phytopathogen are reported in Table 2. The first experiment, made using the extract of *T. patula* in combination with solar light, showed a marked inhibitory dose-dependent effect. The range of values started from 21.4% inhibition, for that treated with 5μg/ml reaching 50.9% for that treated with 50 μg/ml. The exposure to the UV-A lead to similar results, despite the fact that in the complex, the results were actually lower than those observed in the experiment done using the solar spectrum light.

On the other hand, in dark conditions, those treated with 5 and 10 μg/ml showed a growth increase, a phenomenon already observed on *B. cinerea*, whereas at the highest dose (50 μg/ml) the inhibition was 33.8%.

Among the standards used, the most active on *F. moniliforme*, after the irradiation with solar light, was BBTOH, which induced the highest inhibition value on those treated with 50 μg/ml (75.4%). This value was very similar to that obtained after irradiation with UV-A (73.2%). The samples treated with the lowest concentrations and kept in the dark or exposed to solar light showed similar values of inhibition, while those irradiated with UV-A showed higher values, as shown in Table 2.

Even if α-T, on the whole, was less active than BBTOH, it showed the highest inhibition values only after irradiation.

Pythium ultimum

The experiments on *P. ultimum* with the extract of *T. patula* both with the exposure to the solar light

and the UV-A exposure (Table 3) showed a percentage inhibition higher than that found on *B. cinerea* and *F. moniliforme*.

Also the samples treated with the highest dose (50 μg/ml) and kept in the dark showed high percentages of inhibition (51.4%), while those treated with the lower doses (5–10 μg/ml) grew more than the controls, as already observed with *B. cinerea* and *F. moniliforme*.

The treatment of *P. ultimum* with the extract of *T. patula*+UV-A demonstrated a dose-dependent inhibition, with smaller values however, compared to those observed after exposure to the solar light.

Following treatment with both the standards the data showed an inhibitory activity of BBTOH in all the experimental conditions (dark, solar light, UV-A) higher than that of α-T. The highest inhibition values were obtained however, after irradiation with UV-A.

P. ultimum resulted the most sensitive of the three tested fungi and for this reason this fungus treated with the extracts was chosen for scanning and transmission electron microscopy observations.

Pythium ultimum

SEM observations

The control (Fig. 1) showed the characteristic morphology of *P. ultimum*, with lengthened hyphae, of constant diameter, sub-parallel and with rounded or lightly tapering apex and smooth external surface. In the samples treated with 10 μg/ml of the extract, and kept in the dark (Fig. 2), the morphology reflects that of the controls, with the exception of the presence of undulations along the hyphal border. The irradiation with Biolux (Fig. 3) provoked rather, little swelling localized along the hyphae and at their extremities, and the formation of anomalous apex bifurcations.

In those treated with 50 μg/ml macroscopic morphologic alterations are evident, both in the samples irradiated and in those kept in the dark. In the latter, the apexes showed an irregular growth in dimension, with multiple ramifications in subapical expanded areas with irregular shape (Fig. 4). Also in the treated samples exposed to the solar light, several swellings of ovoidal or spherical shape are evident, localized in the subterminal position, near the apexes, or in the middle position along the hyphae (Fig. 5).

Pythium ultimum

TEM observations

Both the controls kept in the dark (Fig. 6), and those irradiated, maintained a normal morphology with mitochondria, nuclei, ribosomes and numerous granules of glycogen. The plasmalemma, adherent with the cellular wall, and the inner system of endomembrane, showed a normal morphology, indicating that they were not damaged by the irradiation. In hyphae treated with the highest dose of the extract (50 μg/ml) after growth in the dark, normal cells are present (Fig. 7) beside deeply altered cells, in an advanced necrotic condition (Fig. 8). In the first case (Fig. 7) there are not any evident alterations: the organules are integral and the endomembrane system is well developed. In the second case inner membranes are decaying and the organules are hardly recognizable, while the cell wall seems to be affected.

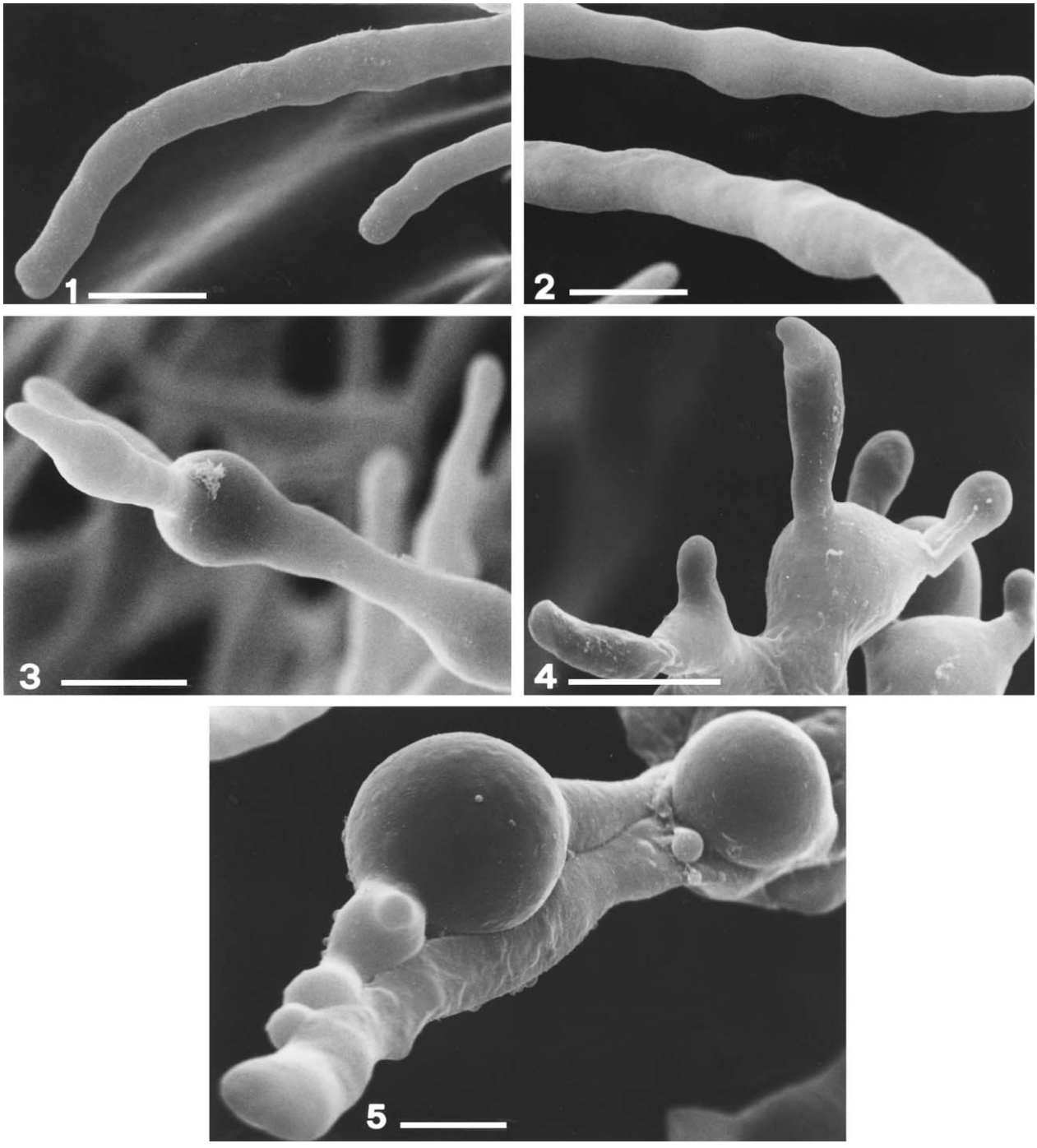
The extended irradiation (16 h) with solar spectrum light caused deep alterations in fungi treated with the same dose of extract (Fig. 9). An 80% of the cells appear to be in a state of partial or total necrosis, often with plasmolysed cytoplasm.

After shorter irradiation with solar spectrum light (90 min) the samples treated with 50 μg/ml of

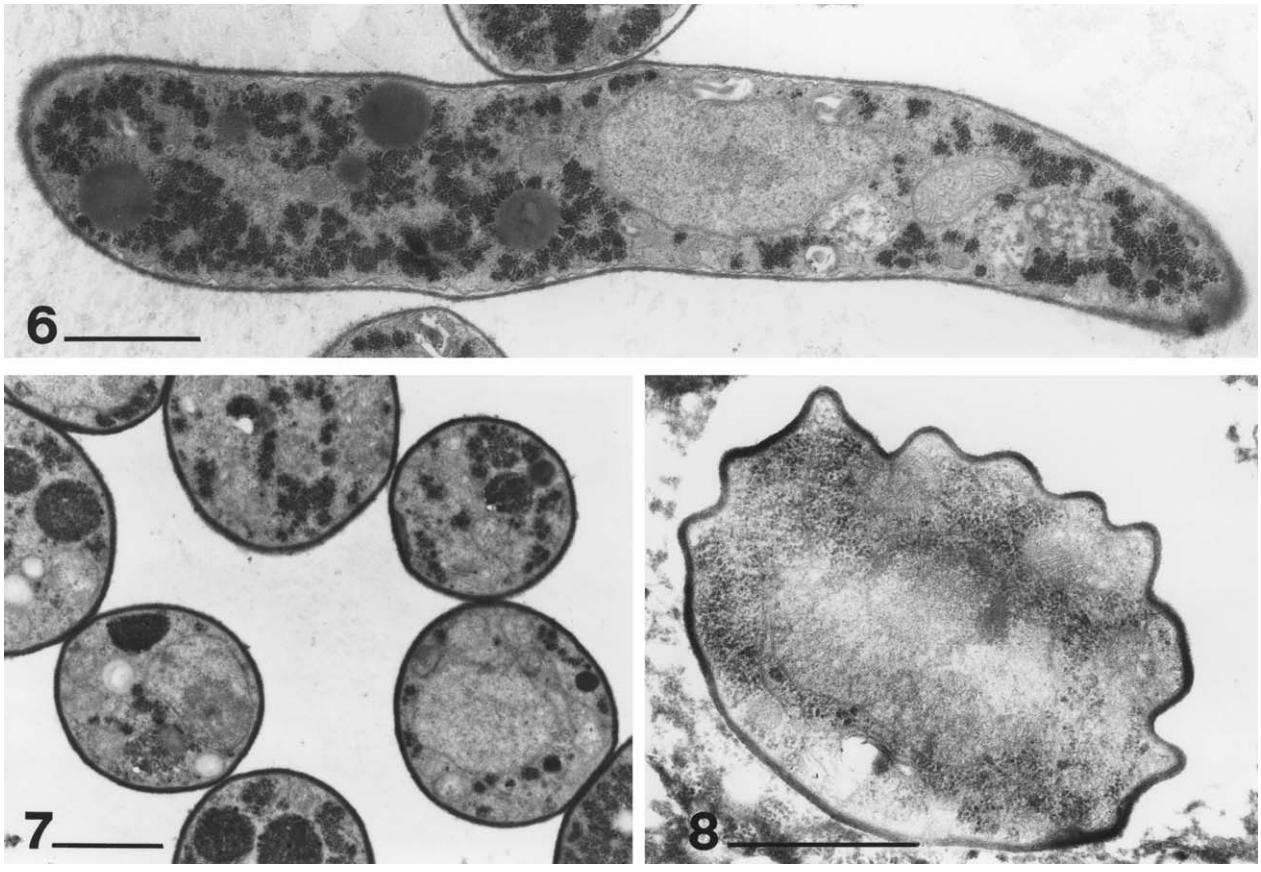
Table 3. Percentage inhibition rate after 5 days of treatment on *P. ultimum*

		Extract	α-T	BBTOH
Dark	Controls	0	0	0
	5 μg/ml	−21.3±0.8	17.8±1.0	20.4±1.3
	10 μg/ml	−28.1±1.3	22.0±1.5	32.7±1.1
	50 μg/ml	51.4±1.1	36.8±1.3	58.7±1.5
Solar spectrum light	Controls	0	0	0
	5 μg/ml	22.7±1.6	22.9±1.2	28.0±0.7
	10 μg/ml	40.2±1.3	44.1±1.0	46.6±0.5
	50 μg/ml	72.6±1.2	66.5±1.0	68.3±0.5
UV-A light	Controls	0	0	0
	5 μg/ml	18.6±1.2	37.0±1.1	45.1±1.3
	10 μg/ml	24.3±1.0	38.0±2.4	46.7±1.7
	50 μg/ml	62.7±1.2	41.4±1.7	85.9±0.6

All data shown are averages and standard errors from three determinations of two independent experiments.



Figures 1–5.1. Control of *P. ultimum*, observed at SEM, showing lengthened and subparallel hyphae with smooth surface and rounded apex. SEM; bar 5 μm. 2. Hyphae of *P. ultimum*, after treatment with 10 μg/ml of *T. patula* extract in the dark, maintain an almost normal morphology. The only difference is that the margin looses linearity, showing alternate diametral variations. SEM; bar 5 μm. 3. Hypha of *P. ultimum* after treatment with 10 μg/ml of extract and irradiated with Biolux for 16 h. Some anomalies such as little swellings along the hypha and apex bifurcations are visible. SEM; bar 5 μm. 4. In *P. ultimum*, after treatment with the extract at the highest dose (50 μg/ml) in the dark, apical swellings and multiple anomalous ramifications are evident. SEM; bar 5 μm. 5. Effects of irradiation with Biolux after treatment with extract at 50 μg/ml. The presence of several stressed swellings and a rough surface is worth noting. SEM; bar 5 μm.



Figures 6–8.6. Control hypha of *P. ultimum* observed at TEM. The regular disposition of organules and the integrity of the endomembrane system is evident. The nucleus, the plasmalemma, the mitochondria, glycogen granules and lipidic drops are clearly visible. TEM; bar 1 μm. 7–8. *P. ultimum* treated with 50 μg/ml of extract and kept in the dark. Normal cells with electron-opaque vacuoles (7), and deeply altered cells, with hardly recognizable organules (8) are visible. TEM; bar 1 μm.

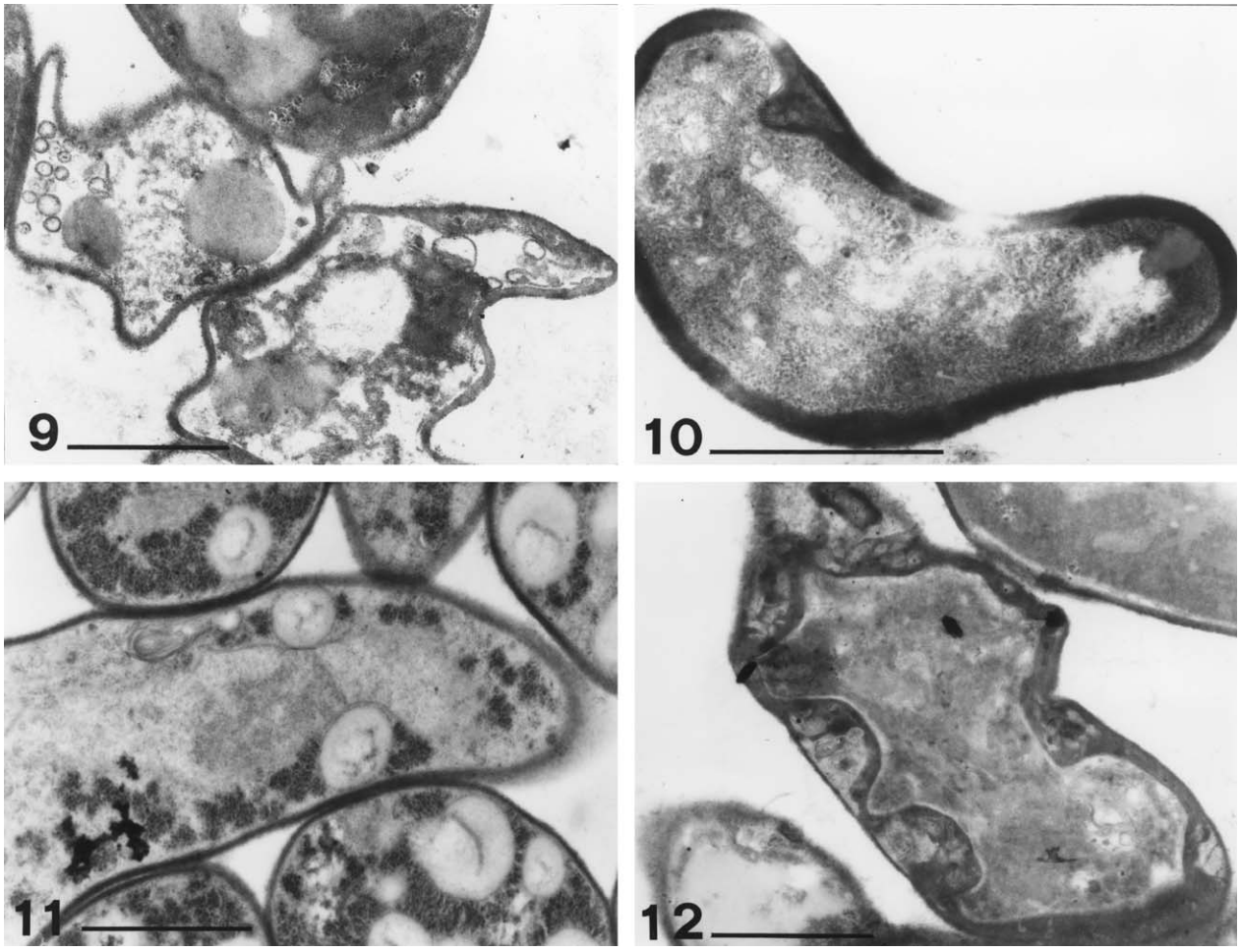
extract showed similar, but softer aspects (Fig. 10). The presence of alterations at cell wall level can be noticed, consisting of its thickening and moderate protrusions inside the cell cytoplasm. The irradiation with the same light for 16 h induced damage to the cells even in those treated with 10 μg/ml. There was damage to the membranes, as can be observed in Fig. 11, where the nuclear envelope appears to be partially destroyed.

The irradiation with UV-A for 90 min of the samples treated with 50 μg/ml, determined the death of the cells (Fig. 12). In those treated, moreover, complete cellular disorganization is accompanied by deep parietal alterations, formed by marked protrusions containing material incorporated in the wall.

Discussion

The extract of *T. patula* exercises a phototoxic dose-dependent action on all three tested phytopatogens. The comparison of the inhibition after treatment with the extract, both in the presence of irradiation and in the dark, shows a correlation between the toxicity and the process of photoactivation of the thiophenes present in the extract. In the dark, the extract inhibits the growth of the three fungi only at the highest dose, equal to 50 μg/ml, while at the lower doses of 5 and 10 μg/ml an increment of the mycelial growth compared to the controls is observed. This behaviour is not unusual when fungi were treated with compounds at subinhibitory concentrations. It was already observed with the thiophene BBTOH on the dermatophyte *Nannizia cajetani* (Romagnoli et al., 1998), and also with the extracts of ten different cultivars of *Tagetes* on the phytopathogens *B. cinerea* and *F. moniliforme* (Mares et al., 2002).

By comparing the results of UV-A and solar light it is evident that prolonged irradiation with solar light over a period of 5 days (even at low intensity) generally provokes greater damage on the treated fungi than those caused by a stronger irradiation with UV-A for a short time. This higher activity after



Figures 9–12.9. Hyphae of *P. ultimum* after treatment with 50 μg/ml of extract and irradiated with Biolux for 16 h. Plasmolysed cells with irregular cell wall outline are clearly visible. TEM; bar 1 μm. 10. *P. ultimum* treated with 50 μg/ml of extract and irradiated with Biolux for 90 min. The cytoplasm is almost completely disorganized: only membranous fragment are recognizable. A marked parietal protrusion is also worth noting. TEM; bar 1 μm. 11. The treatment with extract at 10 μg/ml and with solar spectrum light for 16 h, causes only partial damage to the membranes. A nucleus, with a little damaged envelope, is hardly recognizable. TEM; bar 1 μm. 12. Treated sample with 50 μg/ml of extract and irradiated with UV-A for 90 min. After UV-A irradiation, the extract determined the complete cellular degeneration. The cell wall shows numerous and deep protrusions. TEM; bar 1 μm.

irradiation with the solar spectrum light can be attributed also to the long light exposure that favours the drying of the medium further slowing down the mycelial growth.

P. ultimum has been shown to be, among the three phytopathogens, the most sensitive to the photodynamic action of the extract, especially at the lower doses, as demonstrated by the results obtained after irradiation with both the light sources. In fact the growth observed, in both the lighting conditions and at the same extract concentration, is generally lower than that of *B. cinerea* and *F. moniliforme*.

The observation at SEM on the most sensitive fungus *P. ultimum*, confirms the tendency noticed in the growth experiments, i.e. the entity of the morphological alterations correlates with extract concentration and depends on the irradiation. The fact that deepest alterations are observed in the irradiated samples suggests an involvement in the extract action of thiophenes, that possess phototoxic properties (Hudson and Towers, 1991). The observed light-independent fungitoxic activity of the extract and the standards is probably due to the presence of BBTOH which, in the dark, resulted more active than α-T. The strong antifungal activity of BBTOH at high concentrations, also in the absence of photoactivation, was also shown on other fungi, for example on dermatophytes (Romagnoli et al., 1998).

The ultrastructural examination of *P. ultimum* with TEM allows the formulation of a hypothesis on

the mechanism of the phototoxicity of the extract, a hypothesis supported by the analogy with results obtained in previous studies (Mares et al., 1990) that indicate the main target of thiophenes in the endomembrane system, and the cause of the structural alterations of the cells in the photactivation mechanism. In fact, in samples treated with 50 μg/ml and kept in the dark, necrotic cells can be observed alongside normal cells with electron-opaque bodies with granular aspect, encircled by one single membrane, indicating that they are vacuoles. The presence of analogous structures, is already documented for the dermatophyte *N. cajetani*, after treatment in the dark with 10 μg/ml of BBTOH (Romagnoli et al., 1998) and for *Microsporum cookei*, treated with α-T and kept in the dark, (Mares et al., 1990). In the latter, the existence of α-T inside vacuoles has been demonstrated with the optical microscope in fluorescence as these vacuoles, irradiated with the correct UV-A light (350 nm), emitted a characteristic blue fluorescence (Zechmeister and Sease, 1947). It appears therefore reasonable to assert that, after treatment with the extract of *T. patula*, the thiophenes present are segregated inside the vacuoles, where they remain inactive in dark conditions.

On the contrary, the same treated samples, after irradiation with UV-A or with solar light, demonstrated the presence of plasmolysed or at least strongly altered cells, in which the entire protoplasm appears to be in an advanced necrotic state, allowing only the recognition of fragments of membrane, belonging to the decaying organules inside.

The appearance of such alterations only after irradiation, indicates that the toxicity of the extract of *T. patula* is determined by the photo-induced activation of the thiophenes in its content. Even if the mechanism with which the thiophenes act on the cellular structures has not been completely clarified, it is possible to refer to the hypotheses formulated by several authors on the mechanism of action of the α-T (Towers, 1984; Hudson et al., 1986; Mares et al., 1990, 1994; Romagnoli et al., 1994) and of the BBTOH (Romagnoli et al., 1998). In these studies, the damage observed on membranes, indicates that the α-T acts, in the presence of UV-A, with a photodynamic mechanism that involves the formation of singlet oxygen or of free radicals (Hudson et al., 1986). This highly reactive chemical species, could cause, firstly the breaking of the tonoplast of vacuoles where the thiophenes are contained with the consequence, secondly, of the diffusion of lytic enzymes which cause finally the damage of the entire endomembrane system of the fungal cell (Mares et al., 1990).

The specific interaction with membrane proteins has been suggested by other authors (Hudson et al., 1986), that attribute the fast degradation of nuclear and mitochondrial membranes to the oxidation of amino acids, such as histidine, tryptophan and methionine, and to the oxidation of unsaturated compounds such as fatty acids present in the lipids of membranes.

The anomalous swellings in the apical or intercalary position, observed at the SEM on *P. ultimum* treated with the extract, are not easily interpretable: they can be considered as simply cell swellings or oogonia formations or beginning of sporangia. These latter survival structures also appear in normal conditions without treatment, when *P. ultimum* finds itself in adverse conditions, such as lack of food (Stanghellini and Hancock, 1971).

It can therefore be concluded that the extract of *T. patula* possesses a fungitoxic activity dose-dependent in presence of irradiation, independently from the kind of luminous source, succeeding to inhibit the growth of the three tested fungi already at very low doses. That can be correlated to the presence of thiophenes in the extract, in particular α-T and BBTOH that seem to act on the fungus with a mechanism of photoactivation involving, most likely, the formation of free radicals, causing deep damage at the level of the endomembrane system and production of sporangia as survival structures.

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