The photodynamic effect of 5-(4-hydroxy-1-butinyl)-2,2′-bithienyl on dermatophytes

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The thiophene 5-(4-hydroxy-1-butinyl)2,2′-bithienyl (BBTOH) strongly inhibited *in vitro* eight different dermatophytes. *Epidermophyton floccosum* proved most sensitive to all doses of BBTOH when applied in conjunction with uv-A irradiation. BBTOH also proved quite active against *Nannizia cajetani*, the only dermatophyte which was also strongly inhibited when treated (50 μg ml^{-1}) and kept in the dark. For this reason, *N. cajetani* was chosen as the test organism for TEM and SEM aimed at determining what treatment-induced ultrastructural and morphological modifications had occurred. TEM revealed that the photoactive mechanism of BBTOH was similar to that of 2,2′:5′,2″-terthienyl (α-T). SEM, on the other hand, showed that early culture aging resulted from treatment.

Thiophene chemical properties and biological activity have been widely studied (Bohlmann, 1988). These natural compounds are chemically classified as polyacetylene sulphurates and are known for their photo-dynamic effect on viruses, bacteria, fungi, nematodes, insects and other organisms (Hudson & Towers, 1991). The most thoroughly studied member of this family of compounds is 2,2′:5′,2″-terthienyl (α-T) (Table 1) which can produce photodermatitis in humans (Rampone *et al.*, 1986). This compound also exhibits strong dermatophyte inhibition (Mares, Fasulo & Bruni, 1990).

For this reason, during the course of an ongoing, in-depth study on thiophene biological activity, our group focused attention on one of the lesser studied compounds: 5-(4-hydroxy-1-butinyl)-2,2′-bithienyl (BBTOH) (Table 1). Although not frequently studied, large amounts of this compound are present in some plant extracts of pharmacological interest. The purpose of the present work has been to test BBTOH to determine potential biological activity and possible clinical use against dermatophytes and, if possible, to determine whether its mode of action is comparable to that of the more thoroughly studied α-T.

MATERIALS AND METHODS

Chemicals

5-(4-hydroxy-1-butinyl)-2,2′-bithienyl (BBTOH) was kindly supplied by Prof. R. Rossi, University of Pisa, Italy. The standard was checked for purity by HPLC and GLC--MS, and spectroscopically identified (IR, uv, NMR).

Micro-organisms and growth media

The following fungi were tested: *Trichophyton mentagrophytes* var. *mentagrophytes* (C. P. Robin) R. Blanch. (CBS 160.66), *T. rubrum* (Castell.) Sabour. (CBS 494.62), *Microsporum cookei* Ajello (CBS 189.46), *Epidermophyton floccosum* (Hartz) Langeron & Miloch. (CBS 214.63), purchased from CBS, Baarn, the Netherlands; *T. tonsurans* Malmsten (strain number 1285), *Nannizzia cajetani* Ajello (strain number 3441), *M. canis* E. Bodin (strain number 4727), *M. gypseum* (Bodin) Guiart & Grigoraki (strain number 3999) purchased from the Institute of Hygiene and Epidemiology-Mycology Laboratory (IHME),

Table 1. Formulae and related names of the thiophenes

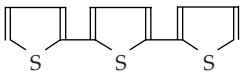
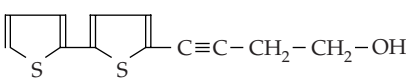
Formula	Related name	Code
	2,2′:5′,2″-terthienyl	α-T
	5-(4-hydroxy-1-butinyl)2,2′-bithienyl	BBTOH

Table 2. Growth (% of control) of eight dermatophytes on the 8th day of dark or light treatment with BBTOH

	Dark			Light		
	5 μg ml^{-1}	10 μg ml^{-1}	50 μg ml^{-1}	5 μg ml^{-1}	10 μg ml^{-1}	50 μg ml^{-1}
T. mentagrophytes	128	100	95	89	72	41
T. rubrum	159	100	97	90	73	38
T. tonsurans	120	100	98	90	80	45
E. floccosum	117	100	95	35	17	0
M. cookei	126	100	96	95	74	35
M. canis	90	85	80	80	65	28
M. gypseum	124	100	90	79	65	30
N. cajetani	80	70	20	62	42	4

Brussels, Belgium. Cultures were grown on Sabouraud agar medium (SDA, Difco).

Antifungal activity

For antifungal evaluation, BBTOH was dissolved in dimethylsulphoxide (DMSO) at the appropriate concentrations and aseptically added to the growth medium. The DMSO concentration in the final solution was adjusted to 0·1%.

For the experiments, cultures were incubated at 28 ± 1 °C in the dark and grown until they had reached the mid-log phase (from 5 to 10 d). Subsequently, they were transferred to Petri dishes, some of which contained the thiophene compound (5, 10, 50 μg ml^{-1}). The mycelia were kept in contact with the drug for 24 h in the dark. After this initial period, half of the Petri dishes were irradiated with uv-A (320–400 nm) for 90 min while the remainder was kept in the dark. From this moment on, the diameter of the colonies was measured at 24 h intervals throughout the 8 d of the experiment. Three replicate plates were used for each fungus at each BBTOH concentration. Control tests were performed keeping some plates in the dark, and irradiating others with uv-A; 0·1% solvent (DMSO) was added to the control plates. All tests were run in triplicate.

The uv-A source was a black light blue fluorescent lamp (Sylvania, F20, T 12-BLB) with a light intensity of 0·5 mW cm^{-1} at the treatment site (peak intensity 350 nm).

Fluorescence microscopy

Control and treated specimens of young *Nannizzia cajetani* hyphae were directly observed under uv light with a Zeiss Axiophot microscope, equipped with an incident fluorescence condenser and the filter set (BP 385, FT 395, LP 397) appropriate for thiophenes emitting fluorescence (Zechmeister & Sease, 1947).

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

For TEM and SEM the youngest *N. cajetani* hyphae were chosen from untreated mycelia and from mycelia treated for 24 h with 10 μg ml^{-1} of BBTOH; some of the latter were irradiated while others were kept in the dark. Outer mycelia cells were harvested immediately after the uv-A irradiation and routinely fixed with 6% glutaraldehyde (GA) in 0·1 M sodium cacodylate buffer, pH 7·2, for 3 h at 4°. After rinsing in the same buffer, the fungi for TEM were post-fixed for 20 h at 4° 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 Zeiss EM 109 electron microscope at 80 kV.

For SEM the fungi were fixed in 6% GA in a sodium cacodylate buffer, briefly post-fixed for 1 h at 4° 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 at an accelerating voltage of 20 kV.

RESULTS

Table 2 gives the effect of BBTOH application, with or without uv-A irradiation, on mycelial growth in the eight dermatophytes studied. The values are reported as a percentage of the growth achieved in the controls, all measurements being taken on the eighth day after uv-A irradiation. Good growth inhibition was observed in all dermatophytes treated with the highest dose (50 μg ml^{-1}) as long as treatment was followed by uv-A irradiation. Only *Epidermophyton floccosum* was completely inhibited at this dose. The same 50 μg ml^{-1} dose gave only a modest inhibition when the experiment was conducted in the dark.

At the lowest dose used, 5 μg ml^{-1}, treatment in the dark had an unusual effect: growth increased. On the other hand, when treated with 10 μg ml^{-1} and kept in the dark, the colony growth was the same as in the controls. When kept in the dark, only *N. cajetani* and *Microsporum canis* showed a slight inhibition after the 5 and 10 μg ml^{-1} treatments. On the contrary, when the 5 and 10 μg ml^{-1} treatments were followed by uv-A irradiation, growth of all fungi was reduced and the extent of this reduction was dose dependent.

The organism which proved most sensitive to BBTOH plus uv-A irradiation was *E. floccosum*, with growth 35% and 17% that of the controls when treated with 5 and 10 μg ml^{-1}, respectively. On the other hand, *N. cajetani*, which showed a greater growth than the previous fungus at the same doses (62% and 42%) was the only dermatophyte nearly totally

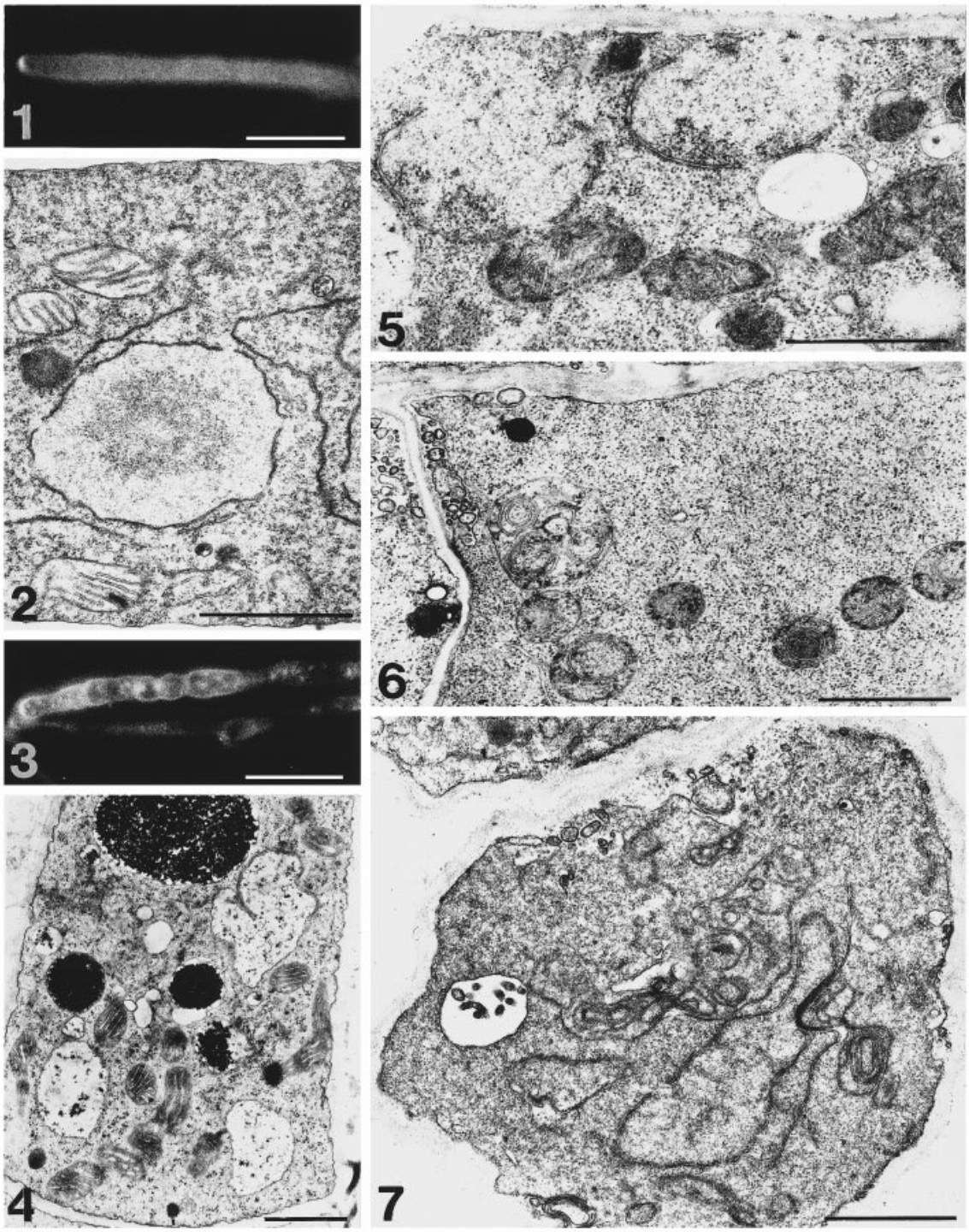


Fig. 1. Control *N. cajetani* hyphae under fluorescence microscopy. A widespread blue fluorescence can be seen, most likely originating in the wall. Bar, 10 μm. **Fig. 2.** Control *N. cajetani* hyphae under TEM. The cytoplasm shows the typical organule arrangement with normally structured membranes. Bar, 1 μm. **Figs 3–7.** Hyphae of *N. cajetani* treated with BBTOH (10 μg ml^{-1}). **Fig. 3.** Kept in the dark. Numerous fluorescent spots can be seen under fluorescent microscopy. Bar, 10 μm. **Fig. 4.** Kept in the dark. TEM shows granular, electron-dense material corresponding to the photodetector; this material is segregated in vacuoles of varying size. Bar, 1 μm. **Fig. 5.** With uv-A irradiation. Nuclei and mitochondria having severely altered casings can be seen. Bar, 1 μm. **Fig. 6.** With uv-A irradiation. The mitochondria show internal membranes with varying degrees of disorganization. Bar, 1 μm. **Fig. 7.** With uv-A irradiation. Several vesicles and plasmalemma fragments can be seen near the wall. The organules in the cytoplasm can hardly be recognized and are fully involuted. Bar, 1 μm.

inhibited (growth 20%) by the 50 μg ml^{-1} treatment in the dark. Because of its greater sensitivity under all treatment conditions, *N. cajetani* was chosen at test organism in the ultrastructural evaluation of the BBTOH action mechanism.

UV-microscopy of the control *N. cajetani* hyphae, irradiated or not, showed a weak blue fluorescence; this is most likely due to parietal auto-fluorescence (Fig. 1). Under TEM the cytoplasm of the young hyphae revealed normal organization

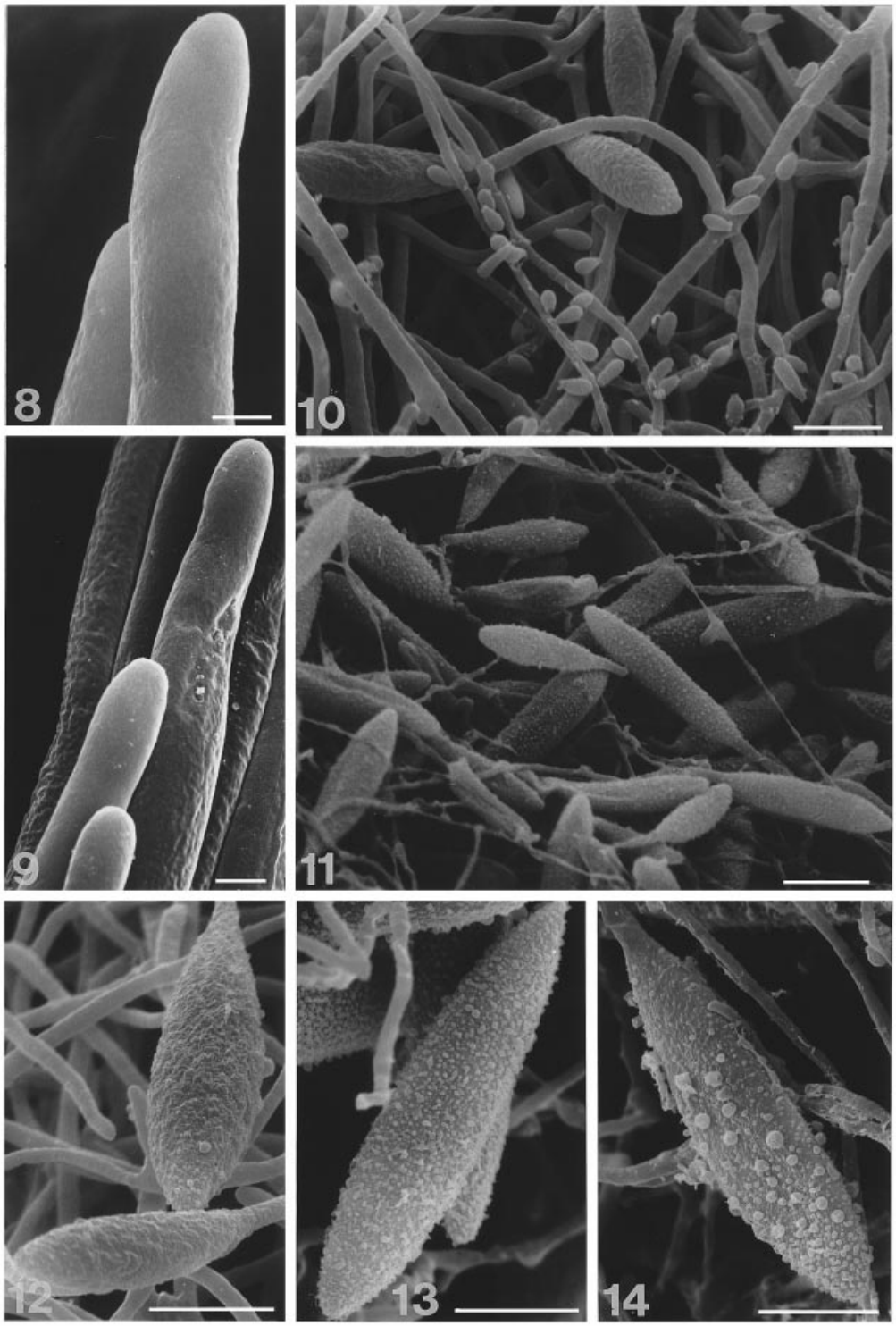


Fig. 8. Control *N. cajetani* hyphae (SEM). The typical straight shape and normally smooth surface can be seen. Bar, 2 μm. **Fig. 9.** Hyphae of *N. cajetani* treated with BBTOH (10 μg ml^{-1}) plus uv-A irradiation (SEM). While the shape appears practically normal, the surface is wrinkled by numerous folds of varying depths. Bar, 2 μm. **Fig. 10.** Young mycelium portions of control *N. cajetani* with few macroconidia and numerous microconidia. Bar, 10 μm. **Fig. 11.** Young mycelium portions of *N. cajetani* treated with BBTOH (10 μg ml^{-1}) in the dark. Note the absence of microconidia and the significant number of macroconidia. Bar, 10 μm. **Fig. 12.** Macroconidia of control *N. cajetani* with the typical elongated shape and rough surface. Bar, 10 μm. **Fig. 13.** Macroconidia of *N. cajetani* treated with BBTOH (10 μg ml^{-1}) in the dark. The entire surface appears covered with numerous small bulges. Bar, 10 μm. **Fig. 14.** Macroconidia of *N. cajetani* treated with BBTOH (10 μg ml^{-1}) plus uv-A irradiation. The surface bulges appear larger and are concentrated in the distal portion of the macroconidia. Bar, 10 μm.

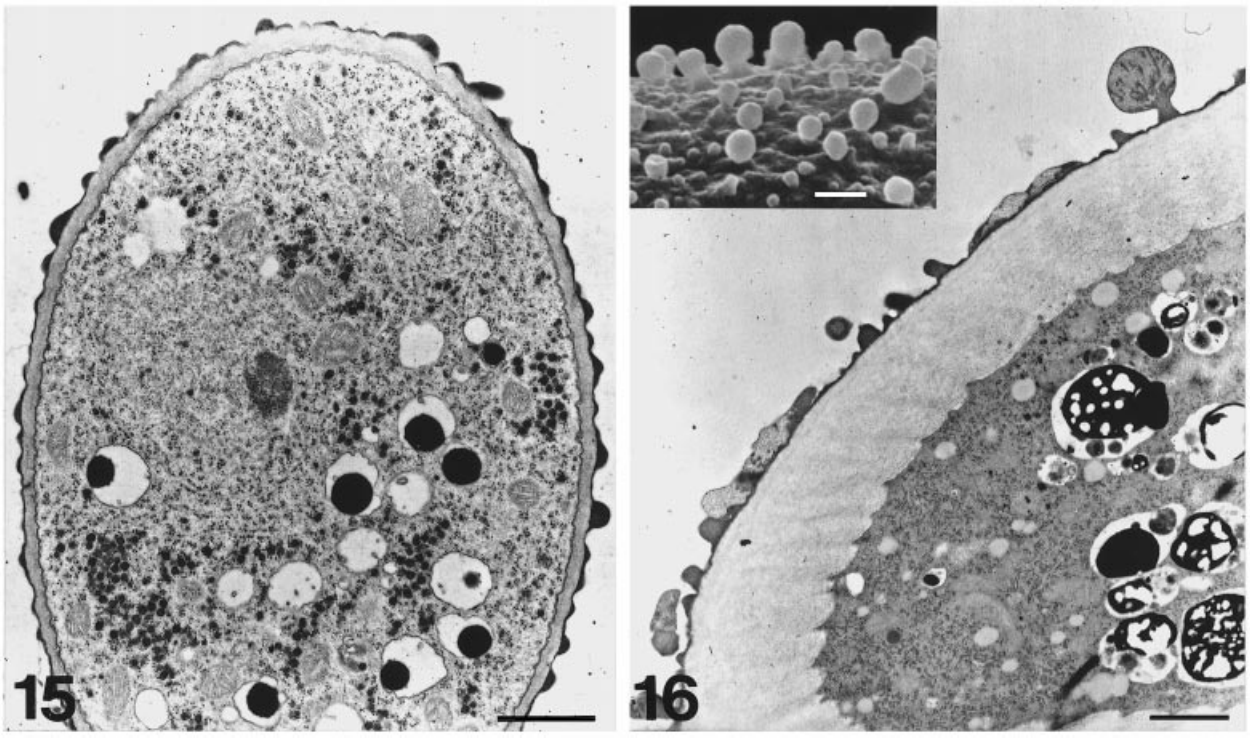


Fig. 15. Transverse section of control *N. cajetani* macroconidia (TEM). The entire circumference of the outermost, electron-dense layer of the wall appears slightly corrugated. **Fig. 16.** Detail of macroconidia of *N. cajetani* treated with BBTOH (10 μg ml^{-1}) plus uv-A irradiation. A marked increase in wall thickness and the presence of a lumpy outer layer: some of these bulges are rather large and can clearly be seen. The inset at the upper left shows the same portion of the macroconidia observed under SEM. Bars, 1 μm.

with mitochondria, an abundance of ribosomes and many small nuclei (Fig. 2). After treatment with BBTOH (10 μg ml^{-1}) in the dark the hyphal protoplasts presented aggregates emitting a strong blue fluorescence (Fig. 3). The TEM findings were analogous to those obtained with fluorescence microscopy: the cytoplasm of the non-irradiated, treated samples showed numerous vacuoles of varying sizes containing electron-dense material. These vesicles had the same localization as the fluorescent aggregates (Fig. 4). The other organelles (i.e. nuclei, mitochondria and hyphae walls) were unaffected by the treatment and subsequent incubation in the dark. On the other hand, when the treatment was followed by uv-A irradiation, significant ultrastructural anomalies were encountered. All cell membrane structures were damaged in some way: the nuclear envelopes showed large holes allowing the content of the nucleus to come into direct contact with the cytoplasm (Fig. 5); the mitochondria, often difficult to recognize, appeared as flattened sacculae or had an unusual shape, at times containing convoluted cristae (Fig. 6). The plasma membrane was broken at several points showing numerous invaginations with various sized vesicles located between the wall and the cytoplasm (Fig. 7). All these modifications brought about a generalized disorganization of the cytoplasm, destroying the entire cell architecture.

Under SEM the young control *N. cajetani* mycelium (Fig. 8) revealed hyphae similar in shape to those treated in the dark or irradiated, the sole textural difference being that the irradiated samples showed some wrinkling, thickening and hollows in the wall (Fig. 9). In addition, the mycelium of the young controls also showed numerous obovate microconidia and a few echinulate macroconidia (Fig. 10). It is worth noting the disappearance of microconidia and the increase in macroconidia occurred in the treated mycelia, whether kept in the dark or irradiated (Fig. 11). The macroconidial surface in the controls showed a fine roughness typical of young mycelia (Fig. 12) while in the dark-treated cultures the surface was full of minute bulges covering the entire spore (Fig. 13). In the light-treated samples, there were fewer of these bulges but they were larger and localized at the tip of the macroconidia (Fig. 14). Sectioning spores taken from control cultures and subjecting them to TEM clearly showed a two-layer wall: an internal, irregular, electron-transparent layer and an outer, electron-opaque layer extending to create numerous small, irregular excrescences (Fig. 15).

All treated samples, whether kept in the dark or light, showed a highly thickened macroconidial wall, as much as five times the thickness found in the controls. This increase in wall thickness was most evident in the macroconidial taken from the treated, irradiated cultures and it appeared due to a thickening of the intermediate layer of the wall which became progressively less opaque to electrons. On the other hand, the outermost layer formed voluminous tree-like bulges with average electron opacity (Fig. 16).

DISCUSSION

The experimental results confirm that, following uv-A irradiation, BBTOH has a photoactive effect on dermatophytes. The data are in agreement with the results of previous studies using α-T on the same, or similar, organisms (Mares *et*

al., 1990, Romagnoli *et al*., 1994). The affinity of the growth trends for α-T or BBTOH-treated fungi (whether kept in the dark or irradiated), and the ultrastructural similarities observed after such treatments under both experimental conditions, lead one to hypothesize that the same action mechanism operates in both cases. In fact, like α-T, BBTOH also penetrates the fungus followed treatment in the dark, but at lower doses (5 and 10 μg ml^{-1}), leading to a non-toxic accumulation in the vacuoles. On the other hand, after irradiation, this thiophene is activated, thus attacking the entire dermatophyte endomembrane system. This appears to follow the mechanism described by Hudson & Towers (1991) leading to widespread disorganization of the entire fungal cell and, in turn, inhibiting growth.

The effect of BBTOH treatment on fungi, however, also shows some interesting differences from α-T. At the highest treatment dose (50 μg ml^{-1}) growth of *N. cajetani* was severely inhibited, even in the dark. This would suggest that, at this concentration, BBTOH does not require light to be toxic for *N. cajetani*. Because all other dark-treated fungi grew almost normally, however, it is possible that this fungus is the most sensitive to BBTOH among those tested.

Another peculiar aspect of BBTOH activity, not found in α-T, was seen in those fungi treated at the lowest dose and kept in the dark. Under such treatment conditions, six strains showed a significant increase in growth as compared to the controls, thus suggesting that, at low doses, this thiophene can stimulate dermatophyte growth. This is not unusual; other compounds such as protoanemonin or plant hormones stimulate cell growth at low doses while inhibiting it at higher doses (Mares, 1987).

Still another interesting observation regards the presence of the typical asexual spores of *N. cajetani* and their surface morphology. In the present experiments, thiophene treatment in the dark gave rise to significant macroconidial surface wrinkling. After BBTOH treatment plus uv-A irradiation, the macroconidia take on the appearance typical of old cultures (between 8 and 15 d, in agreement with Vasquez, Riesco & Pasqual, 1990); i.e. there are large, non-homogeneously distributed bulges located, above all, in the distal portion of the macroconidia. Since TEM and SEM were performed on treated, irradiated fungi in 5-day-old cultures (young mycelium in agreement with Vasquez *et al*., 1990), it can be assumed that BBTOH induces abnormal, early aging of the mycelium in *N. cajetani*.

This consideration is in agreement with the data on the thiophene action mechanism involving the generation of oxygen radicals (Rampone *et al*., 1986). Although these reactive molecules most likely play an important role in fungal differentiation (Schreck, Albermann & Baeverle, 1992; Frese & Stahl, 1992), they potentially threaten life as they can destroy DNA, membranes and proteins. In normal metabolism there is a delicate balance between the production and removal of free radicals which, for the most part, operate in close proximity to their site of production (i.e. the mitochondrial membrane). Since the mitochondria are, at the same time, the main producers and victims of such oxygen reactive species, they can become the target of choice when stress causes an increase in the production of free radical (Foote, 1976). Since the regulation of many metabolic systems depends on mitochondrial membranes and on an intact endoplasmic reticulum, any alteration in these structures will lead to mycelial ageing and/or death. Thus the aberrations in the endomembrane system found in the fungi subsequent to BBTOH treatment plus uv-A irradiation, the increased thickness of the macroconidial walls and the formation of bulges on the macroconidia can all be interpreted as aspects of mycelial aging. In conclusion, the senescence encountered subsequent to thiophene treatment would suggest that in *N cajetani* aging is the price to be paid for a last resort attempt to adopt to environmental conditions incompatible with life.

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