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Microscopic, biochemical and physiological assessment of the effect of methylene bisthiocyanate on the sapstain fungus *Ophiostoma floccosum*

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Abstract

In vitro effects of methylene bisthiocyanate (MBT) on hyphal morphology and ultra-structure of *Ophiostoma floccosum* were examined using differential interference contrast, epifluorescence and transmission electron microscopy (TEM). To understand the mode of action of MBT, experiments were undertaken to measure potassium ion (K^+) leakage from cells, oxygen consumption, glucose and ATP levels. Differential interference contrast microscopy indicated that MBT caused rapid changes in *O. floccosum* hyphae resulting in extensive vaculoation and accumulation of granular materials within the cytoplasm. Epifluorescence microscopy provided evidence that MBT treatment causes a loss in the permeability properties of the plasma membrane. TEM showed retraction of the plasma membrane from the cell wall, aggregation of cytoplasmic contents, vesiculation of membranous components, a dramatic increase in vacuolation, and eventually a complete loss in the integrity of organelles. There was a rapid efflux of intracellular K^+ ions from cells, a substantial loss in K^+ ions occurring within the first 5 min of MBT treatment. The rate of K^+ leakage was MBT concentration treatment-time dependent. The study also showed that the effect of lower concentrations of MBT (0.01 and 0.1 mM) on respiratory activity was negligible. However, at the same concentrations, glucose consumption and ATP production were affected. Taken together, these observations suggest that the target site of MBT in *O. floccosum* alters membrane properties and uncouples oxidative phosphorylation from the respiratory chain.

Introduction

Methylene bisthiocyanate (MBT) is the main active ingredient in some biocide formulations used in various industrial applications, including paper production, water treatment in cooling towers, oilfield water injection systems and leather tanning (Galloway and Cooper, 1974; Goldstein, 1988). MBT has also been used in the forestry sector to protect unseasoned sapwood timber from fungal degradation (Butcher, 1973). A key attribute of MBT is its ability to diffuse below the wood surface of unseasoned wood following treatment (Williams et al., 1985; Kennedy and Woods, 1996), which is important for effective protection of unseasoned timbers from microbial invasion. Recent research in New Zealand has further investigated the mobility of MBT and has led to the development of an antisapstain product that has proved highly successful in arresting fungal pre-infections in radiata pine export logs (Eden et al., 1999; Kreber et al., 2001).

The mode of action of MBT against wood-inhabiting fungi is not well understood. Previous research has largely focused on efficacy studies evaluating the use of antisapstain products

containing MBT against fungal degrade (Butcher, 1973; Williams, 1990). Williams and Eaton (1988) determined *in vitro*, the minimum inhibitory concentration (MIC) for MBT towards selected mould and sapstain fungi. The authors showed that *Trichoderma viride* tolerated the highest level of MBT, which is in agreement with the results of a related study (Singh et al., 2001). Singh et al. (2001, 2004) also found that established mycelium of the test fungi is more tolerant to MBT in liquid culture and on wood than their respective propagules. Recent studies showed that the vapours released from MBT also had an inhibitory effect on fungi (Kreber and Chittenden, 1999/2000; Singh et al., 2004).

The present *in vitro* study was undertaken to characterise the mode of action of MBT on an important sapstain fungus *Ophiostoma floccosum*, using a range of microscopic, biochemical and physiological techniques. The results obtained indicated that the cell membrane may be the primary target of MBT, while uncoupling of oxidative phosphorylation from the respiratory chain may also occur.

Materials and methods

Test fungus

Ophiostoma floccosum isolate # 925 (from Ensis, SCION, New Zealand culture collection) was grown on malt agar (2% malt extract, 1% agar) and incubated at 25 °C and 75% relative humidity (RH) in the dark. After 7 days of incubation, 2 ml of sterile distilled water (SDW) was added to each Petri dish which were gently shaken to release spores off the mycelium into suspension. Spore concentrations were measured using a haemocytometer and adjusted to 10^5 spores ml^{-1}.

Fungicide

For microscopic analysis, three concentrations of MBT, 0.1, 1 and 10 mM were prepared by dissolving appropriate amounts of MBT in methanol (Sigma). Five MBT concentrations ranging from 0.01 to 100 mM were used to measure the effect of MBT on potassium concentration, oxygen consumption and glucose depletion rate in the fungal cells. For ATP measurements, concentrations of MBT tested were 0.01, 0.1 and 1 mM.

Differential interference contrast microscopy

Sterile glass coverslips (40×20 mm) were immersed for 1–2 s in autoclaved, molten water agar (20 g agar in 1 l of water) and allowed to drain until dripdry. Each coverslip was then laid singly on a glass plate. After the agar film had solidified, 10 µl of *O. floccosum* spore suspension containing 10^5 spores ml^{-1} were placed in the centre of the agar film. Inoculated coverslips were incubated for 2 days at 25 °C and 75% RH in the dark. After 2 days incubation, a small cavity was made in the agar, 3 mm beyond the margin of the colony and 10 µl MBT was placed into the cavity. Three replicates were prepared for each MBT concentration. Additionally, inoculated coverslips were treated with 10 µl of SDW to serve as controls. After 30 min, each coverslip was inverted on an observation chamber consisting of a large (70 × 25 mm) microscope slide with a rectangle of glass spacers (2 mm high), which was sealed to the slide with vaseline to prevent rapid drying (Laing and Deacon, 1991). After 2, 4, 8 and 24 h of incubation differential interference contrast microscopy was used to quantify the number of healthy and injured hyphal tips from all treatments. The length of hyphal tip monitored was 50 µm from the apex. At each assessment time, to ensure uniform selection of field of view, 20 hyphal tips selected at random from each of four different regions of the fungal colony were examined and defined as healthy or injured. The tips showing cytoplamic granulation and/or vacuolation were considered to be injured. For each MBT concentration, the mean percentage of healthy fungal tips was calculated for each coverslip based on 80 hyphal tips examined. The overall percentage of healthy tips was then determined using the means from three replicate coverslips, and then plotted over incubation time for each MBT concentration used.

Statistical analysis

A two-way ANOVA was conducted and LSD tests were used to compare the mean numbers of total healthy hyphal tips at different incubation times and for different treatment concentrations. Differences were considered to be significant at $P \leq 0.05$.

Epifluorescence microscopy

Microscope slides were coated with a thin (500 µl) layer of water agar (2% agar). Inoculation of the test fungus and introduction of MBT was performed as described above. A live/dead viability kit, which utilised a mixture of two nucleic acid stains, SYTO 9 green-fluorescent stain and the red-fluorescent stain, propidium iodide, was used to determine the integrity of the plasma membrane of *O. floccosum* (Molecular Probes, 1995, Eugene, OR, USA). Using this material, microorganisms with an intact plasma membrane stain fluorescent green and those with a damaged membrane stain fluorescent red. The methods supplied in the Molecular Probes Kit Cat # L-7012 were followed. Both reagents were prepared in a solution of anhydrous dimethylsulfoxide. A mixture of SYTO 9 dye and propidium iodide was prepared in an Eppendorf tube. The dyes were vortex mixed for a few seconds and then incubated at room temperature in the dark for 15 min. A small amount (10 µl) of the stain mixture was placed over the fungal colony. After 15 min incubation at room temperature, *O. floccosum* was examined under blue light excitation, using a 470 nm excitation and 490–700 nm emission, filter set 9, using a Zeiss Axioplan 2 light microscope. The live/dead stain mixture was used in repeated experiments with *O. floccosum* exposed to different concentrations of MBT. *Ophiostoma floccosum*, which had not been exposed to MBT, was used as a control.

Transmission electron microscopy

Erlenmeyer flasks (100 ml) containing 9 ml of yeast–malt (0.2% yeast extract and 1.5% malt extract) were inoculated with 100 µl of *O. floccosum* inoculum (10^5 spores ml^{-1}). After 5 days incubation at 25 °C (75% RH) in the dark, either 0.1, 1.0 or 10 mM of MBT were added. For each concentration, an appropriate amount of MBT stock solution was added to distilled water to make up a total volume of 900 µl. This was subsequently added to the culture solution to obtain a total volume of 10 ml. For the controls, SDW was included instead of the MBT stock solution. After 24 h incubation, 100 µl of culture was taken out from both treated and untreated flasks. Fungal hyphae were fixed in 3% glutaraldehyde (prepared in cacodylate buffer, pH 7.4) at 4 °C for 4 h, and then post-fixed in 1% osmium tetroxide in the same buffer overnight at 4 °C. After dehydration in acetone, the samples were embedded in a Spurr's resin. Ultrathin sections (ca. 80 nm) were cut with a diamond knife on a RMC MTX ultramicrotome. The sections were sequentially stained with uranyl acetate and lead citrate prior to examination with a JEOL 1010 transmission electron microscope (TEM).

Potassium leakage

Ophiostoma floccosum was grown in liquid media (containing 0.2% yeast extract and 1.5% malt extract) for 5 days in the dark at 25 °C. The fungal biomass was harvested and K^+ leakage monitored using the method described by Rui and Morrell (1994) with some modification. After 5 days incubation, mycelium was filtered through a coarse glass filter paper and washed with 50 mM maleic–NaOH buffer (pH 5.5). The mycelium was resuspended in 200 ml of the same buffer and homogenised for 5 s in a blender. The suspension was filtered through a cheesecloth to remove larger fragments, then centrifuged for 20 min at 4500 rpm. The supernatant was discarded and pelleted hyphal fragments were used for the assay. Pelleted hyphal fragments (0.2 g) were suspended in 50 mM maleic–NaOH buffer (pH 5.5) containing 1 mM glucose and MBT at various levels, ranging from 0.01 to 100 mM. The suspension contained 4.8 mg oven dry weight ml^{-1}. The mixtures were incubated for 0, 5, 15 and 30 min at 28 °C. After each incubation time, 1.5 ml of mixture was centrifuged for 5 min at 10,000 rpm and the supernatant was removed. The pellet was extracted in 1 ml of 6 N HNO_3 for 30 min at 95 °C. The solution was centrifuged for 5 min at 10,000 rpm; then 0.8 ml of supernatant was diluted with 0.8 ml of SDW and 0.8 ml of 3 N HNO_3. The diluted supernatant was analysed for potassium using an atomic absorption spectrophotometer with an air-acetylene flame and a wavelength of 324.8 nm. Three replicates per sample were analysed. Control samples were run without MBT. The intracellular level of K^+ ion was expressed in µM mg^{-1} oven dry weight of fungi and plotted against time.

Statistical analysis
A two-way ANOVA was conducted and LSD tests were used to compare the means of total K^+ level recorded at different incubation times and for treatment concentrations. Differences were considered to be significant at $P \leq 0.05$.

Oxygen consumption

The culture was grown and fungal biomass harvested as described earlier. The pelleted hyphal fragments (0.2 g) were dissolved in 10 ml of 50 mM Na–maleate buffer (pH 5.5). An oxygen probe was used to measure oxygen consumption. The probe chamber contained the fungal suspension (4.8 mg dry weight ml^{-1}). The chamber was air-sealed and a magnetic stirrer was placed inside the chamber. When fully aerated the solution contained 9.1 mg ml^{-1} of dissolved oxygen. A 1 ml aliquot of 10 mM glucose was added with a micro-syringe through the side arm of the chamber as well as enough MBT solution to produce concentrations of 0.01 to 100 mM. The final concentration of glucose in the solution was 1 mM and the total volume in the probe chamber was 10 ml. Control samples were run with methanol instead of MBT. The course of oxygen uptake was recorded over time and the experiment was repeated three times.

Statistical analysis
A two-way ANOVA was conducted and LSD tests were used to compare means of total oxygen consumed at different incubation times and treatment concentrations. Differences were considered to be significant at $P \leq 0.05$.

Glucose depletion

Pelleted hyphal fragments (0.2 g) were dissolved in 10 ml of 50 mM Na–maleate buffer. Fungal suspension of 3.84 ml was combined with 80 µl of 55 mM glucose and 80 µl of appropriate MBT stock solution, to produce MBT concentrations ranging from 0.01 to 100 mM. Control samples were run without MBT. The mixture was incubated at 25 °C and glucose levels were determined at different time intervals. At each assessment time, 0.5 ml of solution was withdrawn and centrifuged for 8 min at 10,000 rpm. The supernatant was then analysed for residual glucose using high performance liquid chromatography (HPLC). The experiment was repeated three times.

Estimation of ATP content

Fungal suspension containing glucose was prepared as described above. An appropriate MBT stock solution was added to produce MBT concentrations ranging from 0.01 to 1 mM. After 4 h incubation of MBT-treated mycelium at 25 °C, the ATP was extracted from the mycelium using cold 4% trichloroacetic acid (TCA) for 20 h. The method described by Nieto et al. (1997) was followed with some modifications (Promega Corporation, USA; protocol # TB267). ATP in cell extracts was estimated using a Polar Star Galaxy luminometer (BMG Lab Technologies, GmbH, Germany) equipped with a fully automated microplate reader. Internal standards were used to determine the ATP content of the samples. The experiment was repeated three times.

Results

Differential interference contrast microscopy

The interference contrast microscopy showed that MBT caused changes to the morphology of *O. floccosum*. The overall effect of different MBT concentrations on health of fungal tips is presented in Figure 1. It is apparent that there was a close relationship between the health of fungal tips and the MBT concentrations, with 1 and 10 mM concentrations being most detrimental ($P \leq 0.05$). After 24 h incubation, overall percentages of healthy tips were 85% for the control, 70% for 0.1 mM MBT, 30% for 1 and 10 mM MBT. While a reduction in the percentage of healthy tips was MBT concentration-dependent upto a value of 1 mM MBT, a further 10-fold increase in the MBT concentration did not cause an additional reduction. Over the entire exposure period, although 0.1 mM MBT was least effective it still had a significant effect on fungal tips ($P \leq 0.05$) over time. After 8 h incubation about 30% of fungal tips examined had granules and large vacuoles within the cytoplasm (Figure 2), which was not seen in the control (Figure 3).

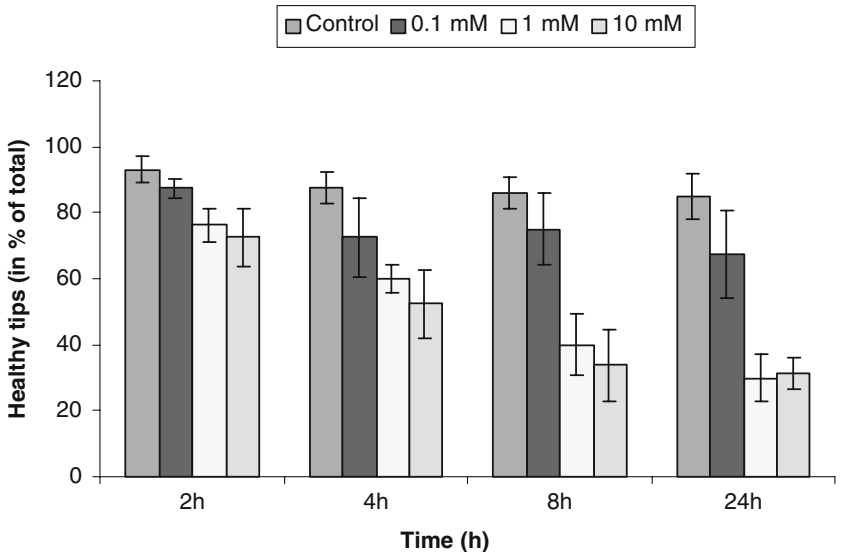

Figure 1. Average percentage of healthy tips determined during exposure to different MBT concentrations ($n = 240$ tips).

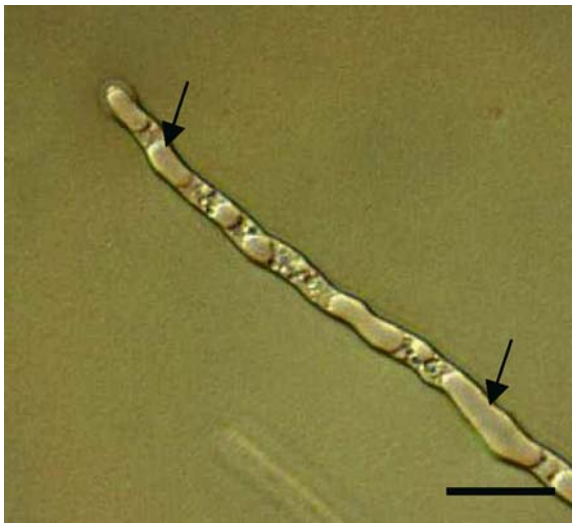

Figure 2. Extensive vacuolation in a hypha after 8 h incubation with 0.1 mM of MBT (arrows). Bar = 20 μm.

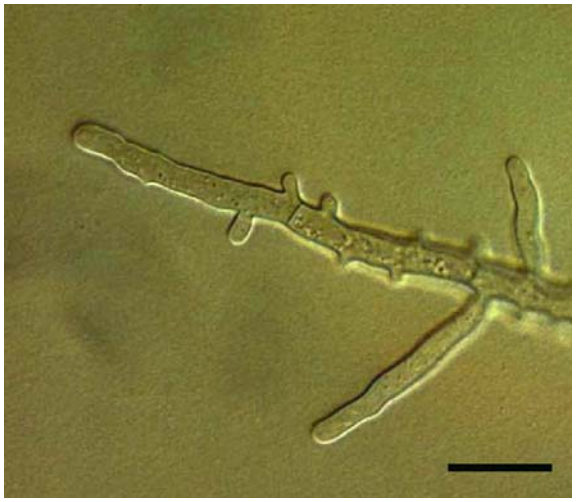

Figure 3. Lack of vacuolation in hyphae after 8 h of incubation without MBT (untreated). Bar = 20 μm.

Epifluorescence microscopy

The untreated hyphae emitted intense green fluorescence immediately after the application of the mixture of SYTO 9 and propidium iodide (Figure 4a). In contrast, treated hyphae emitted red fluorescence as a result of the entry of propidium iodide stain into the cytoplasm (Figure 4b). However, in some instances, especially when 0.1 mM MBT was used, the intensity of red fluorescence associated with *O. floccosum* was low and localised (Figure 4c).

Transmission electron microscopy

The TEM micrographs prepared from untreated hyphal masses and those which had been exposed to varying concentrations of MBT provided evidence of cellular changes from disruption in the plasma membrane to almost complete loss of integrity in the cytoplasm and cell wall, depending

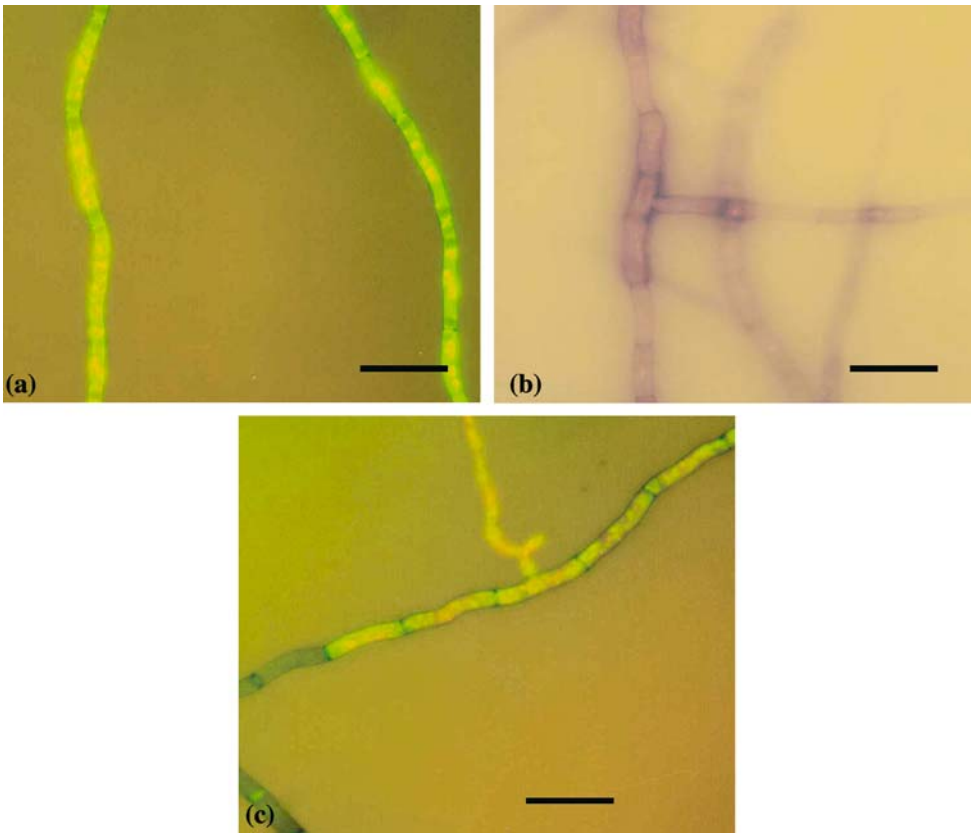

Figure 4. Hyphae of *O. floccosum* after application of the mixture of SYTO 9 and propidium iodide. Scale bar = 20 μm. (a) Untreated hyphae showing green fluorescence. (b) Hyphae treated with 1 mM MBT showing red fluorescence. (c) Hyphae treated with 0.1 mM MBT showing a mixture of green and red fluorescence.

upon the MBT concentrations used. The untreated *O. floccosum* hyphae appeared dense, with the cell wall and the cytoplasmic integrity intact. The cells did not appear to be plasmolysed and the plasma membrane was intact (Figure 5a). Marked changes in the density and integrity of the cytoplasm were visible in the hyphae, which had been treated with 0.1 mM MBT (Figure 5b); the plasma membrane had retracted from the cell wall and there was an appreciable loss in density of hyphae. After treatment with 1 mM MBT, fungal hyphae completely lost cytoplasmic integrity, the plasma membrane was severely disrupted and the cytoplasm was aggregated into irregular masses (Figure 5c). After treatment with 10 mM MBT, the fungal cell lost its total integrity and none of the cell components including the cell wall could be distinguished; much of the cytoplasm had disintegrated, and the residues that remained were visible as dense, compact masses (Figure 5d).

Potassium leakage

There appeared to be no K^+ leakage at 0.01 mM concentration of MBT. However, significant K^+ leakage occurred at 0.1 mM concentration of MBT within only 5 min of treatment. The leakage in K^+ increased with increasing concentrations up to 10 mM MBT, which resulted in the maximum leakage (Figure 6). Thirty minutes after treatment, intracellular level of potassium at 10 mM MBT was 1.25 μm mg^{-1} dry weight, which was about 69% of the original K^+ concentration, indicating that 31% of K^+ leaked out at this concentration of MBT. At the highest concentration of 100 mM of MBT, there was a noticeable decrease in the leakage of total K^+ relative to 10 mM concentration of MBT. The leakage of K^+ reached a maximum value within 5 min of exposure of fungi for all effective MBT concentrations, and there was no further leakage beyond this time period.

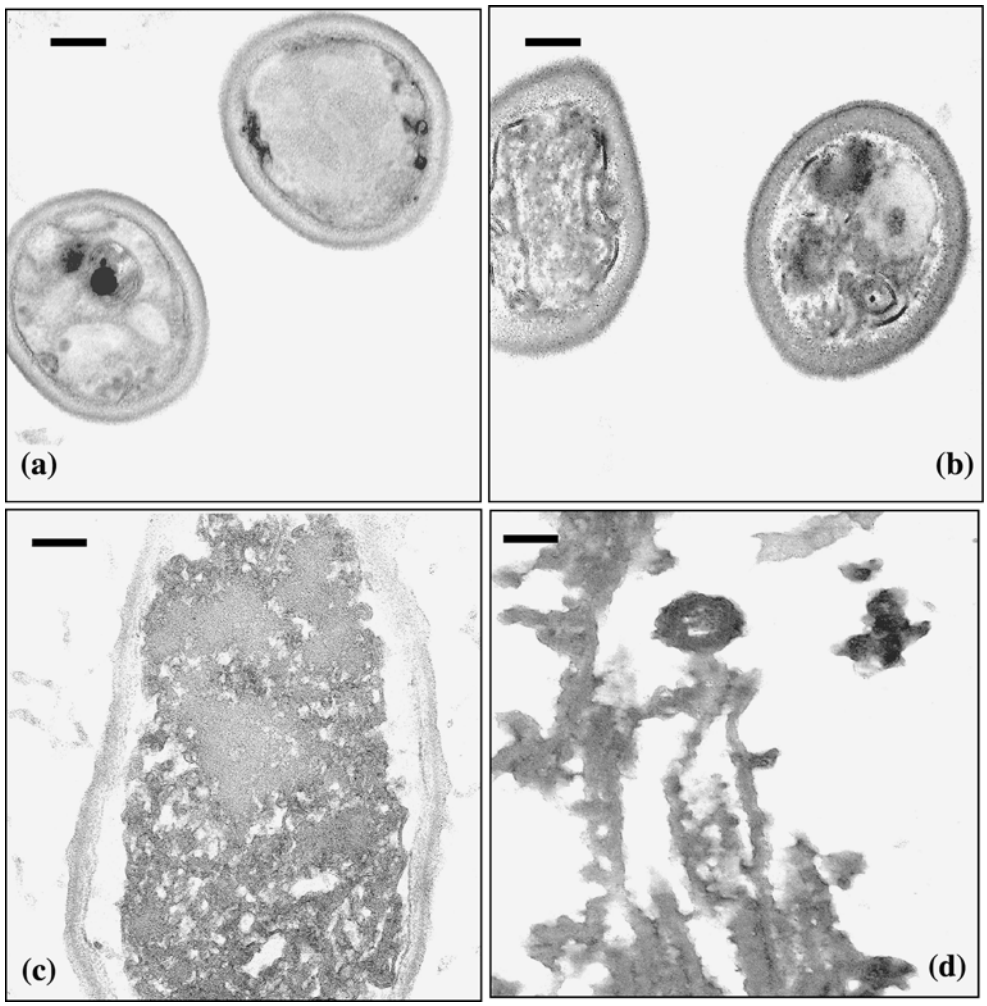

Figure 5. (a–d) TEM micrographs of *O. floccosum* hyphae. Scale bar = 200 nm. (a) Untreated hyphae showing an intact plasma membrane. (b) Hyphae treated with 0.1 mM MBT. The plasma membrane disrupted, cytoplasm degenerated. (c) Hyphae treated with 0.1 mM MBT. The cytoplasm is degenerated and cells are filled with vesicles and other residues. (d) Hyphae treated with 1.0 mM MBT. Cell components including cell wall cannot be distinguished.

Oxygen (O_2) consumption

For the first 5 min of exposure, there was no effect of MBT on oxygen consumption ($P \leq 0.05$) but after this period, the consumption of O_2 generally decreased with increasing MBT doses (Figure 7). Both control and 0.01 mM-treated *O. floccosum* were able to consume all the available oxygen within 24 h. For the 0.1 mM MBT treatments, the rate of O_2 consumption was slowed but the fungus was still able to consume all the available oxygen within 48 h. However, for the 1 mM and higher concentrations of MBT, the uptake of O_2 was similar to the control ($P \leq 0.05$) for the first 5 min of incubation, but the uptake slowed down during 5–30 min of treatment and almost stopped after 1 h. Fungi treated with 1, 10 and 100 mM MBT were only able to consume about 45%, 30% and 10% of available oxygen, respectively, after 48 h incubation.

Glucose depletion

The untreated fungus consumed limited amounts of glucose within 48 h (Figure 8). The effect of 0.01 mM MBT on glucose consumption showed a stimulation of respiratory rate for the first 4 h ($P \leq 0.05$), but the fungus was unable to consume

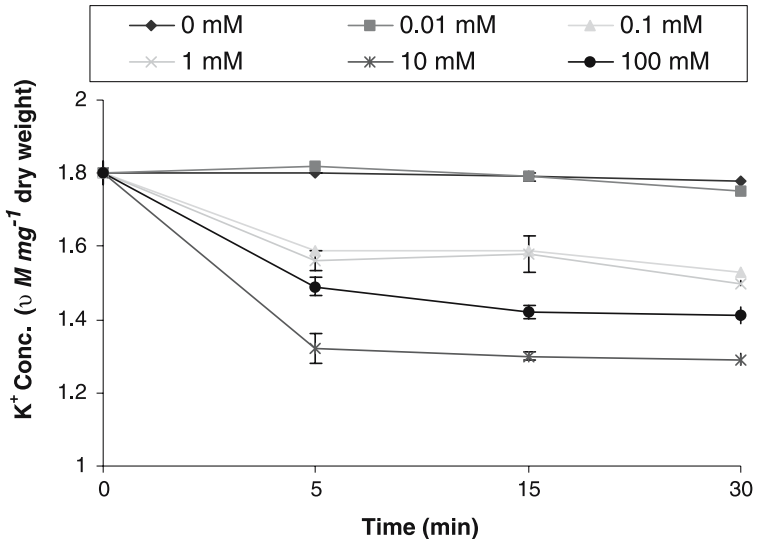

Figure 6. Levels of K^+ in *O. floccosum* after exposure to different concentrations of MBT ($n = 3$; error bars represent standard deviations of the means).

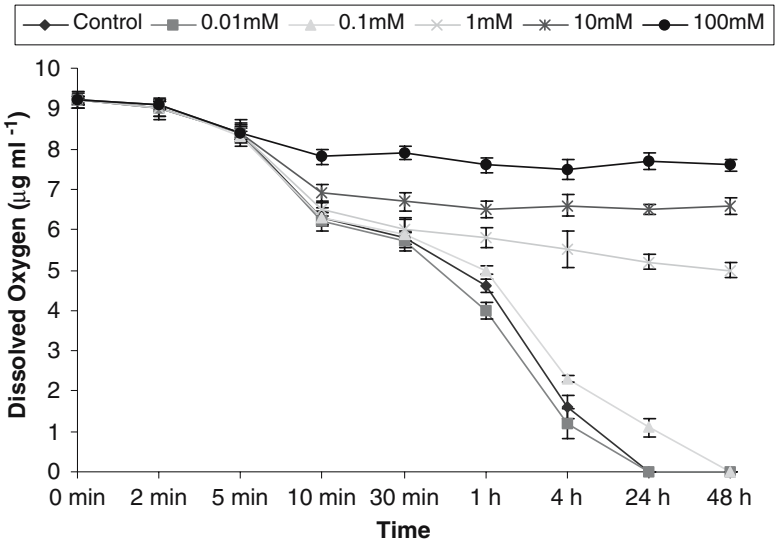

Figure 7. Effect of different MBT concentrations on oxygen uptake of *O. floccosum* after different time intervals ($n = 3$; error bars represent standard deviations of the means).

all the available glucose even after 48 h. At 0.1 mM of MBT, the consumption of a limited concentration of glucose was very slow between 4 and 48 h, with only 7% of glucose depleted. For the 1 mM and higher concentrations of MBT, there was no consumption of glucose. After 48 h of incubation, the concentration of glucose was still about 0.2 mg ml^{-1}

Estimation of ATP content

The luminescent assay showed that the control *O. floccosum* mycelium had 3.7×10^{-8} moles of intracellular ATP (Figure 9). Samples treated with 0.01 mM MBT for 4 h had only 4×10^{-15} moles of ATP, which was further reduced to 3.3×10^{-16} moles in the mycelium treated with 0.1 mM MBT.

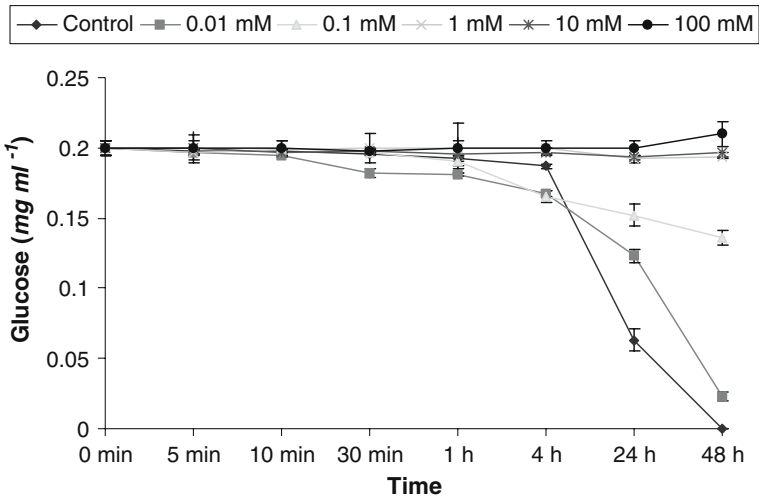

Figure 8. Effect of different MBT concentrations on glucose consumption by *O. floccosum* after different time intervals ($n = 3$; error bars represent standard deviations of the means).

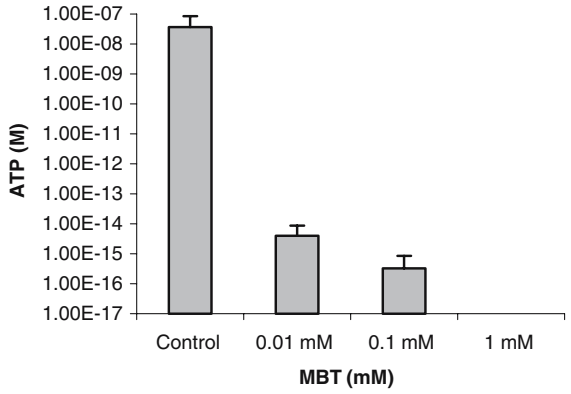

Figure 9. ATP yields of *O. floccosum* treated with different MBT concentrations ($n = 3$; error bars represent standard deviations of the means).

ATP level was below the detection limit for the samples treated with 1 mM MBT.

Discussion

The evidence from the microscopic and K^+ leakage studies described here suggests that in *O. floccosum* the membranes are the likely target of MBT action, and that K^+ leakage may be a consequence of an alteration in the properties of the plasma membrane. K^+ leakage has been reported to be a good measure of permeability changes in the plasma membrane in some other studies (Lambert and Hammond, 1973; Rodriguez-Navarro, 2000; Cox et al., 2001) where the leakage in K^+ ion was related to membrane damage following exposure to membrane-active agents. Maintaining proper concentration and balance (homeostasis) of ions, such as K^+, is important for normal functioning of metabolic processes. We propose that the imbalance in physiological concentrations of K^+ resulting from MBT treatments disrupt the normal metabolic functions eventually leading to disorganisation and disintegration of the cytoplasmic constituents, as electron microscopy has shown. This proposal is based on the following considerations. Firstly, K^+ leakage was significant and rapid, occurring within only 5 min of exposure of hyphae to MBT. Secondly, the use of a live/dead viability kit in combination with fluorescence microscopy provided evidence of alteration in the permeability of the plasma membrane within 15 min of exposure. The viability test using this kit is based on the property of the plasma membrane in normally functioning microbial cells to permit the entry of green fluorescing SYTO 9 and exclude the entry of red fluorescing propidium iodide (Molecular Probes, 1995; Boulos, et al., 1999). Propidium iodide readily penetrates through damaged plasma membrane, and subsequent binding of this reagent to nucleic acid causes an intense red fluorescence (Lopez-Amoros et al., 1995). Thus, in this study, the intense red fluorescence suggests extensive damage to the fungal plasma membrane and lighter

red fluorescence less severe damage from MBT treatments. Thirdly, TEM observations provided confirmation that plasma membrane integrity was indeed affected by MBT treatments for concentrations as low as 0.1 mM. Higher MBT concentrations caused more severe cell damage, such as aggregation of cytoplasmic residues, a feature which was also noted in other earlier studies that investigated the effect of chitosan (Benhamou, 1992) and pyrazole-pyrimidines (Mares et al., 2000) on fungi.

The data provided indicate a relationship between MBT concentrations used and K^+ leakage, as leakage was appreciably greater at 10 mM compared to 1 mM, and slightly higher at 1 mM relative to 0.1 mM. However, an exception to this trend occurred when the fungal biomass was treated with 100 mM MBT, which resulted in a decrease in K^+ leakage relative to the value obtained at the 10 mM concentration of MBT. The maximum K^+ leakage within the first 5 min at all effective concentrations of MBT suggested that the response of fungal cells to toxic MBT concentrations was rapid. The assumption is that the plasma membrane becomes leaky soon after MBT treatment, and the extent of damage to this membrane increases with an increase in the concentration of MBT and the time of treatment upto a period of 5 min. The reason why there was no further proportional loss in K^+ from cells beyond this period may be that K^+ becomes tightly bound to the aggregating masses of degenerating cytoplasm, thus becoming trapped (Sikka et al., 1974). This may also explain why K^+ is not completely lost to the extracellular medium even after prolonged MBT treatment. With the 100 mM MBT treatment, precipitation may also have occurred along the plasma membrane because of the high amount of MBT present in the cells, which is likely to result in the blockage of pores in the damaged membrane (Lambert and Smith, 1976).

Further work using cation ion channel blockers, such as tetraethylammonium which is a known K^+ channel blocker, could provide an insight into the mechanism responsible for K^+ leakage. If, K^+ leakage was prevented using an ion channel blocker then involvement of ion channel activation is likely. Likewise, if ion channel blockers failed to affect K^+ leakage, then this would indicate that the MBT is affecting the integrity of the lipid bilayer.

Contrary to K^+ leakage, where leakage occurred within the first 5 min of MBT exposure, there was no major effect of MBT on oxygen consumption until after this time period; however the degree of inhibition increased with time. The delayed cellular response to MBT treatment with regard to oxygen uptake and glucose utilisation is understandable, if we assume that the effect of MBT on the cell membrane is rapid and direct, and that inhibition of respiration is a more general response reflecting disorganisation of vital cellular compartments, such as mitochondria.

The effect of lower concentrations of MBT (0.01 and 0.1 mM) on glucose consumption showed stimulation in total glucose consumed for the first 4 h of incubation, which suggests that lower MBT concentrations may stimulate attendant metabolic processes. A progressive inhibition of glucose consumption at higher MBT concentrations (1–100 mM), may indicate cellular disruptions being too extensive for fungal hyphae to effectively oxidise glucose. The same low MBT concentration which stimulated glucose consumption, dramatically inhibited ATP production, which can be taken to indicate that MBT may act an energy uncoupler (Hugo and Bloomfield, 1971; Skulachev, 1998; Norman et al., 2004).

Uncoupling is defined as a condition in which the rate of electron transport is no longer regulated by the intact chemiosmotic gradient as it uncouples the processes of oxidation and phosphorylation (Skulachev, 1998). Uncoupling proteins, which are located in the inner mitochondrial membrane, dissipate the proton electrochemical gradient built up by respiration producing heat instead of ATP (Megharaj et al., 1991). Although uncouplers abolish the H^+ gradient, effectively shutting down ATP synthesis, they do not stop oxidation/the electron transport chain (Winkler and Klingenberg, 1994). Some compounds may act as both inhibitor and uncoupler (Norman et al., 2004), for example pentacholorophenol, which is a powerful uncoupler of oxidative phosphorylation and inhibits electron transport at the level of Complex II (Ravanel and Tissut, 1986). The present study indicates that MBT is acting as an energy uncoupler. However, understanding if MBT also acts as an inhibitor and if so, elucidating its site of inhibition on the electron transport chain will form the focus of future work.

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