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FEMS Microbiology Letters 231 (2004) 205–209

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Modification of malachite green by *Fomes sclerodermeus* and reduction of toxicity to *Phanerochaete chrysosporium*

Víctor L. Papinutti *, Flavia Forchiassin

Laboratorio de Micología Experimental, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, BA, Argentina

Received 8 July 2003; received in revised form 26 August 2003; accepted 14 December 2003

First published online 14 January 2004

Abstract

Malachite green (MG) is a triphenylmethane dye used as a fungicide but also possesses a high toxicity to mammalian cells. The toxicity of MG to *Fomes sclerodermeus* and *Phanerochaete chrysosporium* was assessed. *P. chrysosporium* was highly sensitive to the dye and it was unable to grow on solid media containing 64 μM of MG, lower concentrations caused a delay in growth. The radial growth of *F. sclerodermeus* was not affected at this concentration and up to 128 μM. In liquid media both fungi were more sensitive. *F. sclerodermeus* not only was able to grow in the presence of high concentrations of MG, but also it was able to decolorize and detoxify the dye. MG treated with supernatants containing high laccase activity in the presence or absence of 1-hydroxybenzotriazole (1-HBT) gave a colorless product (DMG) that was not toxic to *P. chrysosporium* and other white rot fungi tested. On the basis of the data of maximal absorbance, it is probable that the mechanism involved in the modification of the dye was different if 1-HBT was added to the reaction.

Keywords: *Phanerochaete chrysosporium*; *Fomes sclerodermeus*; Ligninase; White rot fungus; Laccase

1. Introduction

White rot fungi are organisms able to efficiently mineralize lignin. The enzymes involved in the first steps of the process are at least three: lignin peroxidase (LiP) [1,2], manganese-dependent peroxidase (MnP) [3,4] and laccase. LiP is a heme protein with high oxidation potential, this enzyme can oxidize phenolic and non-phenolic substrates [5–8]. MnP oxidizes Mn^{2+} to Mn^{3+}, which binds to chelators secreted by the fungus and then oxidizes a wide range of substrates [9]. Laccase belongs to a family of multicopper oxidases, which oxidize preferentially phenolic lignin dimers [10]. Laccase is able to oxidize lignin model compounds if appropriate redox mediators like 2,2′-azinobis(ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (1-HBT) [11], or violuric acid [12] are present. In these conditions laccase can oxidize substrates that were formerly restrictive to LiP activity, like veratryl alcohol [11] or polycyclic aromatic hydrocarbons (PAHs) [13,14]. Rarely are these three enzymes present in the same organism, and different combinations of them can operate.

White rot fungi have been demonstrated to be capable of transforming and mineralizing a wide range of organopollutants, and ligninases have been implicated in this process [15]. Pointing [16] listed the diversity of known substrate compounds that were shown to be degraded by white rot fungi, among them polychlorinated biphenyls (PCBs), PAHs, pentachlorophenol, organochlorines, organophosphates and synthetic dyes (including those of azo, triphenylmethane or heterocyclic/polymeric structure).

Malachite green (MG) is a triphenylmethane dye used as a fungicide and antiseptic in fish cultures (The Merck Index, 12th edition), its high toxicity to bacteria and mammalian cells was also demonstrated [17]. It promotes malignant cell formation in hamster embryos by decreasing the sensitivity to apoptosis [18]. Because of the toxicity of MG to major microorganisms, and the health hazard to humans, organisms able to grow in the presence of the dye and able to degrade it are of particular importance. In previous reports the production of laccase and MnP by *Fomes sclerodermeus* was shown [19,20]. The objective of this work was to evaluate the toxicity of MG to *F. scle-*

* Corresponding author. Tel.: +54 (11) 4576 3300; Fax: +54 (11) 4576 3384.
E-mail address: leandru@bg.fcen.uba.ar (V.L. Papinutti).

0378-1097/04/$22.00
doi:10.1016/S0378-1097(03)00957-1

rodermeus and the ability of the fungus to degrade it, in particular focused on laccase activity. The toxicity of the colorless reaction products, in this work named decolorized malachite green (DMG), by laccases from *F. sclerodermeus* was assessed in *Phanerochaete chrysosporium* and other white rot fungi.

2. Materials and methods

2.1. Microorganisms

F. sclerodermeus BAFC 2752, *Coriolus versicolor* f. *antarcticus* BAFC 266, *P. chrysosporium* BAFC 246, *Pleurotus lindquistii* BAFC 2102, *Spongipellis fissilis* BAFC 170, *Trametes extenuata* BAFC 270, all of them belonging to the culture collection of the Faculty of Exact and Natural Sciences (University of Buenos Aires), were used in these experiments. Stock cultures were maintained on malt extract (1.2%) agar (2%) slants at 4°C.

2.2. Basal culture medium

Basal solid medium (MEA) was 2% agar, 1.2% malt extract, glucose 1% and basal liquid medium (MEL) was 1.2% malt extract, glucose 1%. Final pH: 5.6. An aqueous solution of MG 10 mM was prepared and maintained as stock and it was added to reach the concentrations indicated in the figures. Agar plates with 20 ml of medium were inoculated with a 0.25-cm^2 agar plug cut from the advancing margin of a 5-day-old colony grown on MEA. Incubation was carried out at 28°C (liquid cultures were incubated under static conditions). Radial growth was measured in two perpendicular directions from the edge of the inoculum to the advancing margin of the colony. For liquid cultures mycelial mats were separated from the culture fluids by filtration through a filter paper at reduced pressure. Mycelial dry weights were determined after overnight drying at 80°C. Values given in the figures represent the mean from triplicate independent experiments.

2.3. Enzyme source and assays

To obtain the enzymatic crude, *F. sclerodermeus* was cultivated in defined liquid medium containing (per liter): glucose 15 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.6 g; $CuSO_4 \cdot 5H_2O$, 0.5 g; H_3BO_3, 0.07 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamine hydrochloride, 0.1 mg; asparagine monohydrate, 6 g. Final pH was 6.4. Erlenmeyer flasks were incubated at 28°C under static conditions. All chemicals were of analytical grade and used without further purification.

At 12 days post inoculation the cultures were harvested, filtered and the supernatants were used as the enzyme source. Under these conditions MnP and LiP were not detected in the supernatants. All enzymes were determined spectrophotometrically at 30°C in a total volume of 1 ml. Laccase activity was determined by using 5 mM ABTS as a substrate, the reaction product was monitored at 420 nm ($\varepsilon_{420} = 36\ mM^{-1}\ cm^{-1}$) [21]. The measurements were made in 0.1 M sodium acetate buffer (pH 3.5). Laccase activity in the plates was determined by incubating 50 mg of medium obtained from the decolorization halo [22]. In this case, the activity was expressed as U g^{-1} of medium. Manganese peroxidase activity was determined by using 0.01% phenol red as a substrate and the reaction product was monitored at 610 nm ($\varepsilon_{610} = 22\ mM^{-1}\ cm^{-1}$) [3]. The measurements were made in 50 mM sodium dimethylsuccinate buffer (pH 4.5). The reaction was initiated by addition of hydrogen peroxide (0.1 mM final concentration). Lignin peroxidase activity was determined by oxidation of veratryl alcohol to veratraldehyde ($\varepsilon_{310} = 9300\ M^{-1}\ cm^{-1}$). The reaction mixture contained 0.1 M sodium tartrate buffer (pH 3), 2 mM veratryl alcohol, 0.4 mM H_2O_2 and the appropriate volume of enzyme [2]. One enzyme unit (U) was defined as 1.0 μmol of product formed per minute under the assay condition. Enzyme activity was expressed as U ml^{-1} of culture filtrate.

2.4. Decolorization and detoxification assays

The reactions were carried out at 30°C. The decolorization of MG was monitored spectrophotometrically at 618 nm [23] and the values were expressed as A_t/A_0 where: A_t = absorbance at time t and A_0 = absorbance at time 0. The reaction mixture for decolorization experiments contained MG 32 μM and 1-HBT 10, 2.5 and 1.25 mM. The reaction was carried out in sodium acetate buffer 50 mM pH 3.5, and 1 (with 1-HBT) or 10 (without 1-HBT) U of laccase in a total volume of 3 ml. For the detoxification assays, 0.02, 0.1 and 0.4 mM MG was incubated in sodium acetate buffer pH 3.5, 5 mM 1-HBT and 1 U of laccase activity in a total volume of 2 ml. After a decolorization of at least 95%, the content of the reaction tube was mixed with 18 ml of MEA or MEL medium. The absorbances were measured in samples diluted in the same buffer. Reaction tubes with the same mixture but without MG were the controls. To determine the toxicity of DMG, the plates or Erlenmeyer flasks containing MEA or MEL medium with 2, 10 and 40 μM DMG were inoculated with the fungi to be tested. The radial growth was measured daily in plates while in liquid cultures the biomass was measured at 12 and 18 days.

3. Results

Fig. 1 shows the time course of the growth in solid media of both fungi in the presence of MG. *P. chrysosporium* (Fig. 1a) was highly sensitive to the dye and was unable to grow on media containing 64 μM of MG. Concentrations of 32 μM and below affected its growth and a

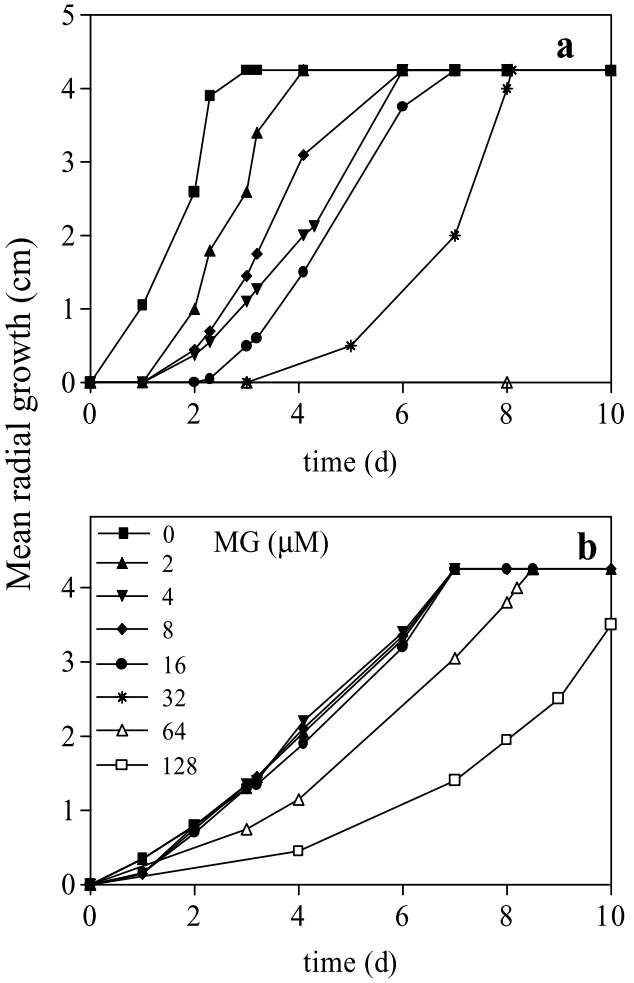

Fig. 1. Mean radial growth of *P. chrysosporium* (a) and *F. sclerodermeus* (b) on MG-amended MEA. Standard error of values is less than 10% of the mean for triplicate cultures.

slower kinetics was observed than in the control without the dye. *P. chrysosporium* grown in MEA filled the plate in 60 h while the addition of 2 μM of the dye caused a 24-h delay in the growth and the plate was filled in 84 h. At 64 μM the radial growth was totally inhibited. The radial growth of *F. sclerodermeus* (Fig. 1b) was less affected, at 128 μM the fungus filled half the plate in 9 days and was unaffected at concentrations of 32 μM or lower, in which cases the covering of the plate occurred after 7 days. Both fungi were able to decolorize the dye but showed a different pattern: while in *F. sclerodermeus* the halo was coincident with the advancing margin of the colony, in the case of *P. chrysosporium* the decolorization halo was delayed with respect to the growth.

Both fungi cultured in liquid media (Fig. 2) showed higher sensitivity to the concentration of the toxin than that observed in plates. Growth of *P. chrysosporium* was affected at 4 μM while *F. sclerodermeus* was more resistant and a concentration of 16 μM was necessary in order to observe a delay in the growth (1-HBT at 5 mM was not toxic to any fungus tested).

Laccase activity in solid and liquid media both containing or not containing MG was detected in *F. sclerodermeus*. There were no significant differences in the laccase production by *F. sclerodermeus* in MG-amended media compared to that obtained in basal culture medium. The maximum activity reached was 0.1 ± 0.01 U g^{-1} ($n = 3$) in plates and 1.2 ± 0.07 U ml^{-1} ($n = 3$) in liquid cultures. On

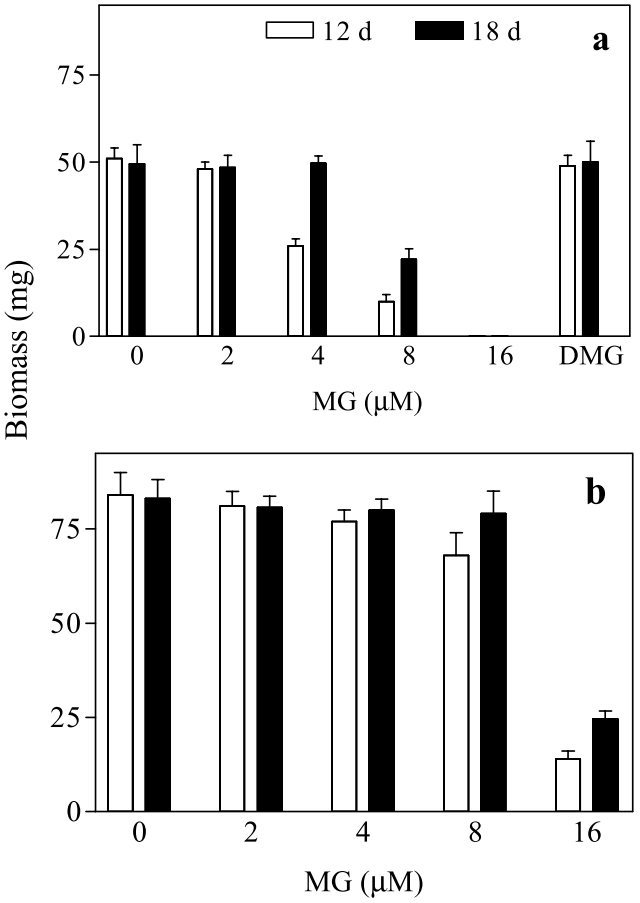

Fig. 2. Biomass production by *P. chrysosporium* on MEL medium. Flasks were amended with MG at the concentrations shown in the figure or with DMG (40 μM). Values shown are the means ± S.E.M. for triplicate cultures.

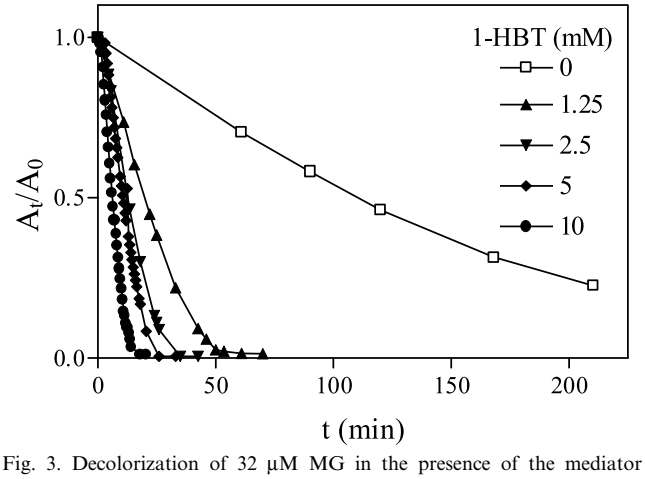

Fig. 3. Decolorization of 32 μM MG in the presence of the mediator 1-HBT and 1 U ml^{-1} laccase or 10 U ml^{-1} in the absence of the mediator. Standard error of values is less than 5% of the mean for triplicate determinations.

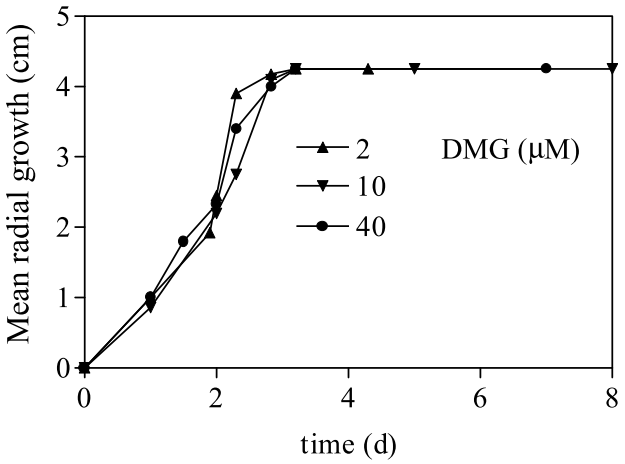

Fig. 4. Mean radial growth of *P. chrysosporium* on DMG-amended MEA. Standard error of values is less than 10% of the mean for triplicate cultures.

the other hand, *P. chrysosporium* did not produce laccase in any of the culture media tested.

Decolorization kinetics is shown in Fig. 3. The presence of 1-HBT increased the rate of decolorization by 200-fold. At 30°C only 13 and 20% of the initial laccase activity was recovered following 24 h reaction in the presence of 1-HBT 10 or 2.5 mM respectively, while in the absence of the mediator the enzyme was completely stable. MG showed its maximal absorption at 618 nm, although after 100 min of incubation in the presence of 1-HBT (0.16 mM) it shifted to 610 nm. In the case of the reaction without the mediator the absorbance at 618 nm decreased, while an increase was observed at 445 nm. In both cases, in the reaction tubes left overnight a dark precipitate was observed.

Plates containing basal culture media mixed with DMG from reaction tubes both containing and not containing 1-HBT (2, 10 and 40 μM DMG final concentration) were non-toxic for *P. chrysosporium* (Fig. 4). The fungus showed a growth kinetics similar to that observed in MEA (Fig. 1a). This fungus cultured in liquid DMG-amended medium showed the same kinetics of biomass production as that observed in the control medium (Fig. 2a).

The toxicity of MG and DMG was assessed in other white rot fungi (Table 1). Results were very similar to those observed in *P. chrysosporium*, except for *C. versicolor*, which showed similar delayed values of radial growth in the presence of MG or DMG.

4. Discussion

The tolerance of *F. sclerodermeus* and *P. chrysosporium* to MG was assessed across various concentrations of the dye. In solid medium, growth of *P. chrysosporium* was affected at concentrations of 32 μM and below and a slower growth rate than the control was observed, 64 μM was completely inhibitory. The faster diffusion of the enzymes in liquid media compared to that in plates explains the differences observed in tolerance to the fungicide in both fungi.

Although *P. chrysosporium* has the enzymatic machinery to degrade the lignin, it exhibited very low tolerance to the dye. Likely, the fact that this fungus produces ligninases in idiophasic cultures could be a contribution to the delay in the growth and delayed halo in plates in the presence of the toxic. By contrast, *F. sclerodermeus* produces laccase and MnP in trophophasic cultures [24]. On the other hand, previous reports showed that the non-ligninolytic fungus *Cunninghamella elegans* was able to grow in the presence of MG at a concentration of 81 μM [23]. Therefore, possessing the ligninolytic enzymes is not sufficient to tolerate toxic compounds that could be degraded and detoxified by these enzymes, the physiological state in which they are secreted being important. Conversely, non-ligninolytic fungi were able to grow in the presence of MG and it was suggested that cytochrome P450 could be involved in the modification of the dye [23].

The shift in the absorbance maximum (618 to 610 nm) in the presence of 1-HBT is in agreement with previous results where it was proposed that the shift was due to *N*-demethylation reactions [23]. In the case of the reaction without the mediator the absorbance at 618 nm decreased, while an increase was observed at 445 nm. This could be due to a difference in the mechanism of action of laccase in the presence of the mediator, as it was demonstrated for laccases from *Trametes versicolor* [11]. The precipitate formed in the reaction tubes in the presence or absence of 1-HBT could be caused by the polymerization of the dye units. Leaving the mixture reaction overnight led to the formation of a dark precipitate suggesting that the products are further oxidized, possibly leading to the formation of dimers or higher oligomers. These results suggest that at least two reactions could occur to MG and that these modifications render the dye less toxic to other white rot fungi, which in turn could degrade the products of these reactions. Similar results were observed with ferul-

Table 1
Effect of MG 16 μM and DMG 40 μM on mean radial growth at 7 days post inoculation of four white rot fungi[a]

	Radial growth without MG (cm)	Radial growth with MG (cm)	Radial growth with DMG (cm)
C. versicolor f. *antarcticus*	4	2.2	2.5
P. lindquistii	4	1.4	3.9
S. fissilis	4	0	4
T. extenuata	4	0.8	2.6

[a]Mean values are from triplicate determinations; separate values do not differ more than 10%.

ic acid in the presence of laccase or lignin peroxidase [25] and with 2,6-DMP which was oxidized to the dimer coerulignone by the laccase of *Pleurotus eryngii* [26].

All the organisms tested in this work are white rot fungi that are therefore able to degrade lignin. This character is related to the ability to attack aromatic substances such as dyes, PAHs and PCBs [27], although a primary problem is the establishment of the organism on contaminated environments. One solution to this problem would be to screen not only for organisms able to perform the detoxification but also for the ability of the organism to adapt to higher concentrations of these compounds. Another solution to this problem would be to use the enzymes involved in the process of detoxification. In this work, we demonstrated the ability of *F. sclerodermeus* to detoxify MG by two possible pathways. The fungus was not only able to grow in the presence of high concentrations of MG, but it was also able to decolorize and detoxify the dye. MG treated with supernatants containing high laccase activity with or without the addition of 1-HBT gave a colorless substance (DMG), which was not toxic to *P. chrysosporium* along with other white rot fungi.

Acknowledgements

This work was supported partly by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) Argentina, and the Universidad de Buenos Aires.

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