Decreased Permeability as a Mechanism of Resistance to Methyl Benzimidazol-2-yl Carbamate (MBC) in *Sporobolomyces roseus*

By A. NACHMIAS AND I. BARASH

Department of Botany, The Dr George S. Wise Center for Life Sciences,
Tel Aviv University, Tel Aviv, Israel

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SUMMARY

Mutants of *Sporobolomyces roseus* resistant to benzimidazole fungicides varied in their responses to 2-(thiazol-4-yl)benzimidazole (thiabendazole, TBZ), methyl 1-(butylcarbamoyl)-benzimidazol-2-yl carbamate (benomyl) and methyl benzimidazol-2-yl carbamate (carbendazim, MBC). Incorporation of [^{14}C]MBC into trichloroacetic acid extracts of the sensitive strain s4 increased during a 2 h incubation period, whereas incorporation into the resistant mutant M55 was unchanged. [^{14}C]MBC uptake by s4 cells was five times higher than that by M55. MBC was identified as the main radioactive compound inside the s4 cells and reached a level of $2.4\ \mu\text{g}/100\ \text{mg}$ dry wt.

The compound MBC enters the cells of *Sp. roseus* by a temperature-, energy-, pH- and concentration-dependent transport system which may be specific for compounds containing a benzimidazole nucleus. It is suggested that tolerance of M55 to MBC is due to decreased permeability of the cell to this compound.

INTRODUCTION

Systemic benzimidazole fungicides are effective against a wide range of plant pathogenic fungi (Evans, 1971) and provide disease control for agricultural and horticultural crops (Byrde, 1970; Erwin, 1973; Evans, 1971). However the usefulness of these fungicides has been reduced by the frequent appearance of resistant strains (Bollen, 1971; Erwin, 1973; Schroeder & Provvidenti, 1969). These strains show cross-resistance to all toxic benzimidazole derivatives (Erwin, 1973). The phenomenon precludes substitution among the various benzimidazole fungicides and endangers their further use in agriculture. Despite the isolation of numerous resistant strains during the past few years, information on their mode of resistance to the fungicides is scanty.

The two most prominent benzimidazole fungicides are benomyl and thiabendazole (TBZ). Benomyl breaks down rapidly in aqueous solutions to form MBC (Clemons & Sisler, 1969), and the latter is probably the toxic material in the plant at sites removed from the points of application. MBC inhibits cytokinesis or mitosis by interference with spindle formation (Davidse, 1973; Hammerschlag & Sisler, 1972).

The epiphytic *Sporobolomyces roseus* is highly sensitive to benzimidazole fungicides and has been used for *in vivo* and *in vitro* bioassays (Gross & Kenneth, 1973). Our study was undertaken to determine cellular processes which might explain the mode of resistance of *Sp. roseus* to benzimidazole fungicides.

METHODS

Organisms. *Sporobolomyces roseus* isolate 2224 was kindly provided by R. Kenneth of the Department of Plant Pathology, Hebrew University, Rehovot, Israel. A subculture made from a single cell of the original isolate sensitive to 0.1 p.p.m. MBC was designated as s4. Strain s4 was used for mutagenesis with ethyl methanesulphonate (EMS) according to the procedure described by Lindegren *et al.* (1965). The treated organisms were centrifuged, resuspended in 2 % sucrose and plated on selective media. Strains M24 and M34 were mutants selected on media containing TBZ and benomyl respectively. Strain M55 was a positive spontaneous mutant of s4 selected on a medium containing benomyl.

Culture methods. The strains were maintained at 25 °C on plates of YEG agar medium containing: yeast extract (5 g), dextrose (10 g) and agar (20 g) in distilled water (1 l). Similar YEG agar media, containing 50 p.p.m. of either TBZ or benomyl, were used for selecting resistant mutants. The compounds were added in dimethylsulphoxide (final concentration less than 0.1 % v/v). Toxicity studies were carried out in 250 ml Erlenmeyer flasks containing 50 ml of YEG liquid medium. Flasks were incubated on a reciprocal shaker at 28 °C.

For dry weight determinations, 1 ml suspensions were filtered through preweighed nitrate-cellulose membranes (0.45 µm, Sartorius membrane filters). After washing with distilled water, the filters were dried for 4 h at 90 °C, stored in a desiccator for an additional 24 h and reweighed. A linear relationship was established between absorbance at 540 nm and dry weight. Growth was expressed as the increase in dry weight during the exponential phase of growth, determined from the absorbance at 540 nm, and from this the generation time was calculated.

Uptake of [¹⁴C]MBC. The compound [2-¹⁴C]MBC, specific activity 11.2 mCi mmol⁻¹, was used in uptake studies. Exponential phase cells from a YEG culture were washed in 0.1 M-phosphate buffer, pH 7.2, by centrifuging at 5000g for 10 min. Samples of 7 mg dry wt were transferred to 5 ml of GNP medium containing 0.67 % yeast nitrogen base (Difco), 0.3 % Bacto-peptone (Difco) and 1 % glucose, in 50 ml Erlenmeyer flasks. Flasks were incubated on a reciprocal shaker for 20 min at 28 °C, and then [2-¹⁴C]MBC was added to give a final concentration of 0.2 µCi ml⁻¹. Incubation was continued for additional periods of time, until terminated by centrifuging at 10000g for 10 min.

The cells were washed six times in 0.2 M-phosphate buffer, pH 7.2 (5 ml) containing 0.4 M-KCl, 0.01 M-2-mercaptoethanol, 0.1 M-EDTA and 10 p.p.m. of non-labelled MBC. This washing procedure removed any weakly adsorbed radioactive compounds. The residue was extracted once with 5 ml cold 5 % (w/v) trichloroacetic acid (TCA). Radioactivities were measured in all the above fractions, including the cell residue.

The radioactivity of each fraction was determined in a Packard Tricarb Model 2003 liquid scintillation counter by counting 0.1 ml of the filtrate in 5 ml of Bray's counting solution containing: 2,5-diphenyloxazole (8 g), 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (0.6 g), naphthalene (150 g), methanol (100 ml), ethylene glycol (20 ml) and 1,4-dioxane (to make 1 l). All counts were corrected for background and quenching by the external standard method.

Thin-layer chromatography (t.l.c.) was employed to resolve radioactive components in the washings and TCA extract. Samples were spotted on 0.25 mm thick silica gel (G-254, Merck) and the t.l.c. plates were developed in benzene-acetone (7:1, v/v) and ethyl acetate-methanol (10:1, v/v).

Chemicals. The [2-¹⁴C]MBC was obtained from International Chemical and Nuclear

Fig. 1. Growth rate of strains of *Sp. roseus* at different concentrations of (a) TBZ, (b) benomyl, (c) MBC. Organisms were grown in YEG agar medium as described in Methods. ○, s4; ●, M24; □, M34; ■, M55.

Corporation; recrystallized MBC, 2-amino benzimidazole and benomyl from Dupont; and TBZ from Merck. All chemicals or solvents were either analytical reagent grade or spectral grade.

RESULTS AND DISCUSSION

Toxicity studies

Dosage response plots for strains of *Sp. roseus* that are resistant and sensitive to TBZ, benomyl and MBC are shown in Fig. 1. The generation time of strain s4 increased from 0.5 to 5 h in the presence of either 3 p.p.m. benomyl or MBC whereas 10 p.p.m. TBZ were required to obtain similar inhibition. All the mutant strains exhibited a complete tolerance to TBZ up to a concentration of 50 p.p.m. (Fig. 1a). In contrast, variations were detected in the sensitivity of the mutants to benomyl and MBC. Thus mutant M24 (selected on TBZ-containing medium) revealed only a slight resistance to MBC and a higher resistance to benomyl (Figs. 1b, c); but mutant M55 (selected on benomyl-containing medium) was highly resistant to both benomyl and MBC. The data presented indicate that MBC and benomyl are more effective toxicants than TBZ to *Sp. roseus*. Strain M55 was used for further studies on the nature of resistance to MBC.

Uptake of [¹⁴C]MBC by sensitive and resistant strains of *Sp. roseus*

The incorporation of [¹⁴C]MBC into TCA extracts of strains s4 and M55 was measured after various incubation times. The amount of labelled compounds in s4 increased substantially during the first hour and reached saturation after approximately 3 h (Fig. 2). A significant amount of radioactivity was also detected in a TCA extract of M55, but this remained constant throughout the incubation period. After 2 h incubation, the radioactivity in s4 was five times higher than that in M55. At this time, 90 to 94 % of the radioactivity remained in the medium, about 5 % was removed from the cells by washing, and 5 and 1 % respectively were detected in the TCA extracts of s4 and M55; the cell residue contained only traces of radioactivity. Under similar conditions *Ustilago maydis* removed approximately 1 % MBC from the medium (Clemons & Sisler, 1971), whereas spores of *Pithomyces chartarum* achieved almost complete removal of TBZ and other benzimidazole compounds (Stutzenberger & Parle, 1972). These major differences in adsorption capacity between *Sp. roseus* or *U. maydis* and *P. chartarum* could be attributed to variations in the adsorptive sites on the cell's surface.

Fig. 2

Fig. 3

Fig. 2. MBC uptake by strains s4 and M55 as a function of time. Incubation was carried out in GNP medium, pH 6.5, containing 3.4 p.p.m. [^{14}C]MBC ($0.2 \mu\text{Ci ml}^{-1}$) as described in Methods. $\circ$, s4; $\blacksquare$, M55.

Fig. 3. Effect of pH on uptake of [^{14}C]MBC. Cells of s4 and M55 were incubated for 2 h under the conditions described for Fig. 2. $\circ$, s4; $\blacksquare$, M55.

The main radioactive component in all the different fractions was identified as MBC by t.l.c. The amount of MBC in the TCA extract of s4 was calculated to be $2.4 \mu\text{g}/100 \text{ mg dry wt}$ of cells. The observation that MBC is taken up in very small quantities by cells of *Sp. roseus* is similar to that reported for *U. maydis* and *Neurospora crassa* (Clemons & Sisler 1971). Nevertheless there were differences between the incorporation of [^{14}C]MBC into TCA extracts of sensitive and resistant strains (Fig. 2). The presence of radioactivity in the cold TCA extract could result from transport of [^{14}C]MBC into the soluble pool of the cells or by adsorption on to their outer surface. The following experiments were designed to distinguish between these two possibilities.

pH- and temperature-dependence of [^{14}C]MBC uptake

Results in Fig. 3 show a distinct pH optimum for MBC uptake by strain s4. The maximum uptake by s4 at pH 6.5 was more than five times higher than that by M55. The pK_a of MBC is 4 and at pH 6.5 it exists predominantly as an uncharged species (Aharonson & Kafafi, 1975). Thus, the decrease in uptake at high pH could reflect the effect of pH on some cell components rather than on the ionic form of the substrate. The decrease in uptake at low pH could result from an effect on cell components as well as ionization of the molecule. It is interesting that the pH optimum for adsorption of benzimidazole compounds by *P. chartarum* conidia is 4.5 (Stutzenberger & Parle, 1972).

The temperature-dependence of [^{14}C]MBC uptake is shown in Fig. 4. The high Q_{10} (greater than 2 between 20°C and 30°C) and activation energy of 606 kJ mol^{-1} , as calculated by the Arrhenius equation, are characteristic of an active, carrier-mediated process. However, the latter results should be interpreted with care since, as shown below, MBC uptake is probably energy dependent. Thus, the results shown in Fig. 4 may reflect the temperature-dependence of energy production rather than the temperature-dependence of the transport system itself.

Effect of extracellular MBC concentration

Figure 5 shows the rate of [^{14}C]MBC uptake as a function of MBC concentration. A saturation curve was obtained with a maximal uptake at approximately 2 p.p.m. ($12 \mu\text{M}$) MBC.

Fig. 4

Fig. 5

Fig. 4. Effect of temperature on [¹⁴C]MBC uptake by s4 cells. Incubation conditions were as for Fig. 2.

Fig. 5. Effect of MBC concentration on [¹⁴C]MBC uptake. Incubation was carried out under the conditions described for Fig. 2. Uptake was terminated after 2 h incubation period.

Table 1. *Effect of various metabolic inhibitors on MBC uptake*

Organisms were pre-incubated aerobically for 15 min in GNP medium containing 10⁻³ M-inhibitor, before [¹⁴C]MBC was added. MBC uptake was measured for 2 h under conditions described in Methods.

Strain	Addition	Relative uptake rate (% of control)
S4	None (control)	100
S4	Sodium azide	20
S4	2,4-Dinitrophenol	18
S4	<i>p</i> -Chlorophenol	18
S4	Sodium cyanide	13
M55	None (control)	100
M55	Sodium azide	75

Table 2. *Effect of benzimidazole derivatives and other compounds on MBC uptake by strain s4*

Organisms were incubated under the conditions described in Table 1. The compounds tested were added simultaneously with the labelled MBC.

Addition	Concentration (p.p.m.)	Relative uptake rate (% of control)
None (control)	—	100
TBZ	5	68
TBZ	25	45
2-Aminobenzimidazole	25	38
Histidine	10	100
Guanine	10	100
Yeast extract	0.3 %	14

Energy-dependence and specificity of MBC uptake

The uptake of MBC was inhibited by 80 % or more after a brief pre-incubation of s4 cells with known respiratory inhibitors (Table 1). These results suggest that MBC transport is energy dependent. The uptake inhibition of 25 % observed for strain M55 in the presence of sodium azide (Table 1) suggests that although the permeability of resistant cells to MBC was significantly inhibited (Fig. 2), it was not entirely excluded. However, the accumulation of the toxicant in the tolerant strain is not sufficient to show a fungistatic action.

The specificity of the MBC transport system was investigated by determining the effect of structurally-related compounds on [14 C]MBC uptake (Table 2). Derivatives of benzimidazole such as TBZ or 2-amino-benzimidazole acted as effective inhibitors of MBC transport. Guanine and histidine, which contain a purine and an imidazole nucleus respectively, were ineffective. The highest inhibition, caused by yeast extract, is presumably due to the presence of benzimidazoles or structurally-related compounds which compete with MBC for the transport system.

The results suggest that the incorporation of [14 C]MBC into the TCA extract is mediated by an active transport system. Such a system may be specific for compounds containing a benzimidazole nucleus and is dependent on energy, pH, temperature and MBC concentration. A part of the [14 C]MBC in the TCA extract could result from adsorption of the fungicide on to the cell surface, as reported for other fungi (Clemons & Sisler, 1971; Stutzenberger & Parle, 1972). The observed differences in tolerance and MBC uptake between strains s4 and M55 (Figs. 1c and 2) can be attributed to an impairment of the MBC uptake system in the latter strain. If a similar resistance mechanism existed in other tolerant strains, cross-resistance (Figs. 1a, b, c) could depend on decreased permeability to benzimidazole fungicides.

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