The effects of polyamine biosynthesis inhibitors on mycelial growth, enzyme activity and polyamine levels in the oat-infecting fungus *Pyrenophora avenae*

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The fungus *Pyrenophora avenae*, an important pathogen of oat crops, was grown on solid and liquid media containing the polyamine biosynthesis inhibitors difluoromethylornithine (DFMO), methylglyoxal bis(guanyldrazone) (MGBG), ethylmethylglyoxal bis(guanyldrazone) (EMGBG) +/− polyamines. All of the compounds inhibited mycelial growth of the fungus. MGBG and EMGBG were more effective than DFMO. The addition of putrescine and spermidine almost completely prevented inhibition of mycelial growth by DFMO. However, no such effect was observed for inhibition by MGBG or EMGBG. Neither the inhibitors nor exogenous polyamines had any significant effect on the size of the fungal cells. DFMO and MGBG, alone and in combination, reduced the activity of ornithine decarboxylase. Fungus grown in media containing EMGBG showed reduced activity of *S*-adenosylmethionine decarboxylase. Putrescine and spermidine concentrations decreased when the fungus was grown in media containing DFMO or DFMO/MGBG combined. MGBG reduced spermidine and spermine concentrations and EMGBG greatly reduced spermidine concentrations. All of the compounds reduced the concentration of cadaverine, which is a significant component of *P. avenae*. The respiration rate of the fungus decreased when grown in media containing MGBG or DFMO/MGBG combined.

**Introduction**

There is much evidence to suggest that polyamines are necessary for normal growth and development of higher plants and fungi (Slocum et al., 1984; Tabor & Tabor, 1985). In mammalian and fungal cells there appears to be only one route for synthesis of the diamine putrescine, the precursor for further polyamine synthesis; this is by the rate-limiting enzyme ornithine decarboxylase (ODC; EC 4.1.1.17) (Pegg & McCann, 1982; Tabor & Tabor, 1985). In plants and bacteria (Slocum et al., 1984) two pathways may lead to putrescine synthesis, either via decarboxylation of ornithine (as above) or indirectly via agmatine, the first product of arginine decarboxylation a reaction catalysed by arginine decarboxylase (ADC; EC 4.1.1.19). In addition, there are reports of biosynthetic ADC activity in a number of phytopathogenic fungi (Khan & Minocha, 1989a, b). The addition of aminopropyl groups from decarboxylated *S*-adenosylmethionine to putrescine results in the synthesis of the polyamines spermidine and spermine (Slocum et al., 1984).

The development of specific inhibitors of the main enzymes required for polyamine synthesis has not only provided evidence of the need for polyamines for growth, but has also helped in the understanding of polyamine metabolism and in the regulation of enzymes involved in this process. Studies using the inhibitor difluoromethylornithine (DFMO), which irreversibly inhibits ODC (Metcalfe et al., 1978), showed that DFMO was effective in controlling fungal growth *in vitro*. Reversal of inhibition was obtained by the addition of exogenous putrescine and spermidine (Birecka et al., 1986; Rajam & Galston, 1985).

Janne et al. (1985) suggested that by using an inhibitor which would block *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) activity, in conjunction with ODC inhibitors, complete depletion of the di- and polyamines putrescine, spermidine and spermine in mammalian cells should be possible. This should also apply to certain fungi providing they, like mammalian cells, possess only one pathway for putrescine synthesis. Such an approach has not hitherto been investigated for phytopathogenic fungi. Two such SAMDC inhibitors

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**Abbreviations:** DFMO, difluoromethylornithine; MGBG, methylglyoxal bis(guanyldrazone); EMGBG, ethylmethylglyoxal bis(guanyldrazone); ADC, arginine decarboxylase; ODC, ornithine decarboxylase; SAMDC, *S*-adenosylmethionine decarboxylase.

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are methylglyoxal bis(guanylylhydrazone) [MGBG] and ethylmethylglyoxal bis(guanylylhydrazone) [EMGBG].

In this paper we report the effects of DFMO, MGBG, EMGBG and exogenous polyamines on mycelial growth and polyamine levels in the oat pathogen Pyrenophora avenae. The activities of ODC and SAMDC and respiration rates were also measured. Carnitine was used in the presence of MGBG to assess its effectiveness against mitochondrial damage, as suggested by Nikula et al. (1984).

Methods

Growth of organisms. Stock cultures of Pyrenophora avenae Ito & Kuribayashi Apud Ito, obtained from the Commonwealth Mycological Institute, Kew, UK, were maintained on potato dextrose agar (PDA).

Growth on solid media. Filter-sterilized solution (10 ml) containing inhibitor was added to 140 ml of sterile PDA at 45-47 °C to obtain final concentrations of 0-1-2.0 mM. Putrescine or spermidine were added to obtain final concentrations of 0.1-2.0 mM to determine if they reversed inhibition by DFMO (0.5 mM), MGBG (0.5 mM), EMGBG (0.1 mM) or a mixture containing MGBG (0.5 mM) and DFMO (0.5 mM). Control plates contained culture medium only.

Sterile medium (20 ml) containing inhibitor was added aseptically to each 90 mm single-vent sterile plastic Petri dish. To obtain inoculum, a sterile 10 mm diameter cork borer was used to remove plugs of mycelium from the peripheral edge of stock cultures. The mycelial plugs were inverted and placed in the centre of each Petri dish. Inoculated plates were incubated in the dark at 24 °C.

Colonies were measured in mm, excluding the 10 mm plug of inoculum, 1, 3 and 6 d after inoculation. Three measurements were made from each Petri dish. Lengths and diameters of cells were measured 6 d after inoculation. All the results are the means of six replicates, and each experiment was repeated with similar results. Differences between means were analysed for significance using Student’s t-test. Standard errors were too low to be included in the Figures.

Growth in liquid media. Filter-sterilized solution (10 ml) containing inhibitor was added to 140 ml of sterile liquid PDA, in 250 ml flasks, to obtain the following concentrations: 1-0 mM-DFMO; 0-1 mM-MGBG; 0-1 mM-EMGBG; 0-1 mM-DFMO/MGBG combined; 0-1 mM-putrescine; 0-1 mM-spermidine. Each flask was inoculated with a 10 mm disc of mycelium and placed in a Gallenkamp orbital shaker (140 r.p.m.) at 24 °C. After 4 d the fungus was washed with distilled water through a fine-mesh sieve and centrifuged at 16000 g for 10 min. The pellet obtained was used for enzyme and polyamine analysis.

When measuring respiration rates, carnitine was added to each flask that contained inhibitor (concentrations as given above, except DFMO 0-1 mM) to obtain a final carnitine concentration of 1-0 mM.

Enzyme assays. Crude enzyme extracts were prepared by grinding the fungus in buffer (500 mg fungus per ml of buffer) using a prechilled pestle and mortar. Buffers used were as described by Stevens et al. (1976). The suspensions were sonicated using a Soniprep 150 for 10 cycles of 10 s on/20 s off. Test-tubes were kept on ice during sonication. Each sample was centrifuged at 24000 g for 15 min at 0 °C. For ODC assays, the supernatant (cytosolic fraction) was dialysed against 30 vols of buffer for 24 h in the dark. The pellet (nuclear fraction) was redissolved in the original volume of buffer and dialysed as above.

For SAMDC assays, 430 mg (NH₄)₂SO₄ was added to 1 ml of supernatant and redissolved pellet (cytosolic and nuclear fractions respectively). The suspensions were centrifuged at 24000 g for 20 min at 0 °C. The pellets obtained were redissolved in the original volume of buffer and dialysed as described above. Enzyme activities were assayed by measuring ¹⁴C0₂ released after incubation with [1-¹⁴C]ornithine and S-adenosylL-¹⁴C]methionine for ODC and SAMDC, respectively. Radioisotopes were obtained from Amersham.

The reaction mixtures used were as described by Stevens et al. (1976). Assays were done in 98 mm glass test-tubes fitted with silicone rubber stoppers (Vacutainer) and 35 mm-long, 22 gauge needles. A piece of filter paper (10 mm diam.) impregnated with 10 μl 2 M-KOH was fitted to each needle to trap ¹⁴C0₂ released during the reaction. The test-tubes were placed in a water-bath at 37 °C for 30 min after which 0-2 ml 6% (v/v) perchloric acid was injected into each tube; the tubes were then incubated for a further 30 min. The filter paper was removed and placed in a scintillation vial containing 12 ml Emulsifier Safe scintillant (Packard). The samples were counted for radioactivity using a LKB 1215 Rackbeta liquid-scintillation counter. Activity was expressed as pmol ¹⁴C0₂ (mg protein)⁻¹ h⁻¹. Protein was assayed by the Lowry method using BSA as a standard. All results are the means of five replicates. Significance was assessed using Student’s t-test.

Polyamine analysis. Fungus (600 mg) was macerated with 1 ml 4% (v/v) perchloric acid. The samples were sonicated as described for enzyme assays, then centrifuged at 12000 r.p.m. for 25 min at 0 °C. To each 200 μl volume of supernatant, 17 mg Na₂CO₃ and 400 μl dansyl chloride (30 mg ml⁻¹) in acetone were added. This mixture was incubated in darkness overnight at 22 °C. Excess dansyl chloride was converted to dansylproline by incubating for 30 min with 0-1 ml L-proline (100 mg ml⁻¹). The dansylated polyamines were extracted in 250 μl toluene. Portions (25 μl) of this toluene extract were spotted onto activated LK6D silica-gel plates (Whatman) and left to develop in tanks containing chloroform/triethylamine (5:1, v/v). The spots were traced using a UV-lamp and cut-out; the dansylated derivatives were then extracted in 5 ml ethyl acetate. Standards between 0-1-10 μg were measured. Fluorescence was measured in a Perkin-Elmer LS-5 luminescence spectrometer at excitation 365 nm/emission 506 nm. Polyamine content is expressed as nmol (g fresh wt)⁻¹. All results are the means of four replicates. Significance was assessed using Student’s t-test.

Respiration rate measurements. Fungus (500 mg) in 5 ml of fresh, liquid PDA was placed in the compartment of a Rack oxygen electrode at 24 °C. Each sample was aerated to 100% before measuring oxygen uptake. Measurements are expressed as percentage change in oxygen as compared to the control. This experiment was done twice with similar results.

Results

Mycelial growth on solid media

The addition of the inhibitors MGBG, DFMO, EMGBG and MGBG/DFMO combined reduced mycelial growth of P. avenae (Figs 1–4). Although DFMO was less effective than MGBG, it reduced growth of the fungus by 64% when used at 2-0 mM (Fig. 1). In contrast, MGBG almost completely inhibited fungal growth at 2-0 mM (Fig. 2).

Significant reduction of mycelial growth was obtained using EMGBG, which at 0-1 mM reduced growth by
Inhibitors of fungal polyamine biosynthesis

Fig. 1. Effect of DFMO on growth of *P. avenae*. ■, 1st day after inoculation; □, 3rd day after inoculation; ◼, 6th day after inoculation.

Fig. 2. Effect of MGBG on growth of *P. avenae*. ■, □, ◼ see Fig. 1.

about 50% (Fig. 4). MGBG/DFMO combined were very effective in controlling mycelial growth, although no more so than MGBG alone. Nevertheless, a 2.0 mM concentration of this mixture resulted in complete inhibition of growth on day 1 of measurement, with only a small tuft of mycelium appearing on the 3rd and 6th days (Fig. 3).

The addition of putrescine at 0-1 mM had no effect on growth (Fig. 5). Similarly, 0-1 mM-spermidine increased growth only by day 6 (Fig. 6). Inhibitory effects of 0-1 mM-EMGBC, 0-5 mM-MGBG and 0-5 mM-MGBG/DFMO combined were not reversed by the addition of either 0-1 mM-putrescine or 0-1 mM-spermidine. However, both putrescine and spermidine reversed inhibition by 1-0 mM-DFMO (Figs 5 and 6).

For all treatments, effects of inhibitor were more pronounced by the 6th day in comparison with measurements made on previous days.

There were no significant differences in cell length of mycelia grown on media containing inhibitor or inhibitor + polyamine, with the exception of media containing the 1-0 mM-MGBG/DFMO mixture, which significantly reduced cell length (data not shown). The diameter of all the cells was about 3.8 μm.
Table 1. Effects of inhibitors on the activity of ODC in P. avenae

Values are means of five replicates ± SE. Significant differences are shown as follows: (a) *P = 0.001; (b) *P = 0.01; (c) *P = 0.1. ns, Not significant.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>ODC activity [pmol CO₂ (mg protein)]⁻¹ h⁻¹</th>
<th>Cytosolic</th>
<th>Nuclear</th>
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<tr>
<td>Control</td>
<td>6.14 ± 1.45</td>
<td>9.53 ± 1.67</td>
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<td>MGBG</td>
<td>2.72 ± 0.17</td>
<td>4.54 ± 0.26</td>
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<td>MGBG + Spd</td>
<td>1.93 ± 0.20</td>
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<td>MGBG + Put</td>
<td>1.98 ± 0.08</td>
<td>3.43 ± 0.53</td>
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<tr>
<td>MGBG + DFMO</td>
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<td>5.98 ± 0.54</td>
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<tr>
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</table>

*The concentration of inhibitors was 0.1 mM, except DFMO, 1.0 mM. Put, putrescine (0.1 mM); Spd, spermidine, 0.1 mM.

Enzyme activities

The activity of ODC in P. avenae grown in media containing MGBG, DFMO and MGBG/DFMO combined was significantly reduced in both nuclear and cytosolic fractions (Table 1). EMGBG had no effect on ODC activity in the cytosolic fraction, although a slight reduction was observed in nuclear ODC activity (Table 1).

As shown in Table 2, SAMDC activity was greatly reduced when P. avenae was grown in the presence of EMGBG. A mixture of MGBG/DFMO had no significant effect on SAMDC activity and MGBG reduced activity only slightly. DFMO increased SAMDC activity (Table 2).

With only a few exceptions, the addition of 0.1 mM putrescine or spermidine did not reverse inhibition of ODC or SAMDC activities and in most cases reduced activity further (Tables 1 and 2).

Polyamine concentrations

As well as putrescine, spermidine and spermine, cadaverine was also detected in P. avenae. Although this is the first report of cadaverine from a plant pathogenic fungus, this diamine has been reported from the fungi Aspergillus oryzae and Coprinus atramentarius (Stevens & Winther, 1979) and from Neurospora crassa (Paulus et al., 1982). Putrescine, spermidine and cadaverine concentrations were significantly reduced when P. avenae was grown in media containing DFMO or MGBG/DFMO combined (Table 3). EMGBG and MGBG reduced the concentrations of spermidine and cadaverine. Spermine levels were lower in the presence of MGBG.

Respiration rate

The respiration rate of P. avenae was reduced when grown in media containing MGBG, a mixture of MGBG/DFMO or a mixture of MGBG/carnitine. An
increase in respiration occurred in the presence of DFMO and EMGBG (data not shown).

**Discussion**

The results in this paper show that interference with polyamine biosynthesis using specific enzyme inhibitors reduced mycelial growth of *P. avenae*. This agrees with work using DFMO to inhibit fungal growth *in vitro* (Rajam & Galston, 1985; Birecka *et al.*, 1986) and *in vivo* (Rajam *et al.*, 1985; Walters, 1986). Treatment with DFMO strongly reduced ODC activities in *P. avenae*. Furthermore, the intracellular concentrations of putrescine, spermidine and cadaverine were substantially reduced but spermine levels remained unchanged in *P. avenae* grown in the presence of DFMO. Since DFMO did not reduce respiration rate in *P. avenae*, it is probable that the decrease in growth of this fungus caused by DFMO was due to a reduction in polyamine concentration. Indeed, inhibition by DFMO of mycelial growth and, to a lesser extent, ODC activity was almost completely reversed by the addition of exogenous putrescine and spermidine, supporting suggestions that polyamines are essential for fungal growth (Stevens & Winther, 1979). The large increase in SAMDC activity which occurred in the presence of DFMO may be due to stabilization of the active protein as found in animal systems by Shirahata & Pegg (1985). Another possible explanation is that the accumulated, highly basic, decarboxylated SAM could substitute for a polyamine (though it is not a polyamine analogue) ensuring that the low concentrations of putrescine made in the presence of DFMO are converted into spermine (Pegg, 1984). This would explain why DFMO does not reduce spermine content in most cells (Pegg & McCann, 1982; Table 5). Some workers have reported that in animal cells, uptake of DFMO is slow (Erwin & Pegg, 1982), and that various compensatory mechanisms occur in an attempt to prevent total loss of polyamines (Janne *et al.*, 1983). However, despite the increase in SAMDC activity, only spermine levels were maintained at control values.

The substantial reduction in cadaverine concentration in fungal cells grown in the presence of DFMO, MGBG and EMGBG is difficult to explain, since we can provide no data on the activity of its biosynthetic enzyme, lysine decarboxylase, or of diamine oxidase, which could break down cadaverine. Moreover, other research has shown that cadaverine concentration increased considerably in *Neurospora* and in mycoplasma-infected tumour cells depleted in intracellular polyamines (Paulus *et al.*, 1982; Alhonen-Honings *et al.*, 1982).

MGBG, singly and in combination with DFMO, was more effective at reducing mycelial growth than DFMO alone. Since DFMO inhibits putrescine and spermidine synthesis, but has no effect on spermine levels, the addition of MGBG, thus inhibiting SAMDC, should prevent spermine accumulation. Combinations of DFMO and MGBG have been tested as anti-cancer treatments (Porter & Sufrin, 1986), and recent work has shown mixtures of DFMO and MGBG to be powerful inhibitors of powdery mildew infection of barley (West & Walters, 1988).

Pretreatment of cells with DFMO depletes spermidine pools and induces an increased uptake of MGBG via a stimulated polyamine transport mechanism (Porter & Sufrin, 1986). The results here indicate that MGBG was not very efficient at inhibiting SAMDC activity although a decrease in spermine concentration was observed. Other workers have found large increases in SAMDC activity when looking at animal cells treated with MGBG and suggest that the increase is due to enzyme stabilization, by the bound inhibitor, against proteolytic degradation (Pegg *et al.*, 1973). MGBG did, however, cause a significant reduction in ODC activity in *P. avenae*, and although putrescine levels remained unchanged, this may have been the result of reduced diamine oxidase activity, as has been reported by Kallio & Janne (1983). This MGBG-induced (and EMGBG-induced) reduction in ODC activity is surprising, since other workers have reported substantial increases in ODC activity in cultured tumour cells induced by treatment with MGBG and its analogue (see Porter & Sufrin, 1986; Janne *et al.*, 1985). The latter authors suggest that this may be due to stabilization of ODC protein in MGBG-treated cells.

As well as inhibiting SAMDC, MGBG also inhibits cellular respiration resulting in severe mitochondrial damage (for references see Janne *et al.*, 1985). This effect of MGBG may account for the lack of response when
exogenous putrescine and spermidine were added to the media. Nikula et al. (1984) found that MGBG inhibited mitochondrial fatty acid oxidation and that the addition of carnitine prevented this. They suggest that impaired fatty acid oxidation due to MGBG may be one of the 'mechanisms' which leads to mitochondrial damage. Our data show that use of MGBG and DFM0/MGBG combined resulted in decreased respiration rates. This was reversed, in most cases, in the presence of carnitine. However, addition of carnitine to MGBG-treated fungus resulted in a further, unexplained reduction in respiration rate. It would appear, therefore, that the effect of MGBG on growth of *P. avenae* may not be due solely to inhibition of polyamine biosynthesis.

EMGBG, an analogue of MGBG, is a very potent inhibitor of SAMDC (Elo et al., 1986; Table 2). However, unlike MGBG, it has no anti-proliferative activity against mouse leukaemia cells *in vitro* (Elo et al., 1986). It was surprising, therefore, to find that EMGBG was a very effective inhibitor of mycelial growth in *P. avenae*. The reason for this difference between animal and fungal cells in their response to EMGBG is not known. It would appear from the data here that, unlike MGBG, EMGBG does not have a toxic effect on the mitochondria. However, the addition of exogenous putrescine and spermidine did not reverse the inhibitory effects of EMGBG on mycelial growth.

In all cases where spermidine was added to incubation media, in an attempt to reverse the effects of inhibitor, SAMDC activity was reduced substantially below that found in cells treated with inhibitor only. Similarly, ODC activity was reduced well below that found for inhibitor-treated cells when spermidine or putrescine were added to the media. This apparent regulation of ODC and SAMDC activities by polyamines has also been found in animal cells (Pegg & McCann, 1982; Pegg, 1984), and may be useful in attempts to find novel ways of inhibiting fungal polyamine biosynthesis.

Cell length and diameter remained largely unaffected by the addition of inhibitors and/or polyamines. This does not agree with the work of Rajam & Galston (1985), who showed reduced cell length and increased cell diameter when various fungi were exposed to DFMO. Increases in cell length and diameter were also reported in the presence of exogenous polyamines. As suggested by Birecka et al. (1986) these differences may be accounted for by differing sensitivities between genera and perhaps also in the uptake and distribution of the inhibitor within the cell. The mechanisms underlying the differing fungal responses to inhibitors of polyamine biosynthesis are currently under investigation in this laboratory.

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References


