Amino Acids (1997) 13: 347–354

Amino
Acids

Growth and polyamine metabolism in *Pyrenophora avenae* exposed to cyclohexylamine and norspermidine

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Accepted June 20, 1997

Summary. The effectiveness of inhibitors of polyamine biosynthesis in controlling plant pathogenic fungi is well established. The spermidine synthase inhibitor cyclohexylamine (CHA) and the spermidine analogue norspermidine were evaluated against *in vitro* growth of the oat stripe pathogen *Pyrenophora avenae*. Mycelial growth was reduced by 55% upon exposure to 2.0 mM CHA while the same concentration of norspermidine reduced growth by 63%. Neither inhibitor had any effect on ODC or AdoMetDC activities, nor the flux of label from ornithine through to the polyamines. Levels of free polyamines in fungal tissue exposed to 0.01 mM norspermidine were unaltered, although 1.0 mM CHA did produce a 75% increase in fungal putrescine content. These data suggest that CHA and norspermidine do not reduce fungal growth as a result of a perturbation in polyamine biosynthesis.

Keywords: Amino acids – Polyamines – Spermidine – Cyclohexylamine – Norspermidine – Spermidine synthase – Plant pathogenic fungi

Abbreviations: ODC: ornithine decarboxylase; ADC: arginine decarboxylase; AdoMetDC: S-adenosylmethionine decarboxylase; DFMO: α-difluoromethylornithine; CHA: cyclohexylamine

Introduction

Polyamines are essential for the normal growth and development of all cells (Tabor and Tabor, 1981). The initial step in polyamine biosynthesis in plants is the decarboxylation of either ornithine or arginine, catalysed by the enzymes ornithine decarboxylase (ODC; EC 4.1.1.17) and arginine decarboxylase (ADC; EC 4.1.1.19) respectively. The decarboxylation of S-adenosylmethionine via the enzyme S-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50) results in the formation of an aminopropyl group. Donation of such a group to putrescine, catalysed by the enzyme spermidine synthase (EC 2.5.1.16), results in the formation of spermidine, with a subse-

quent addition of the moiety via spermine synthase (EC 2.5.1.22) yielding spermine (Walters, 1995). Since most fungi only possess the ODC route to putrescine, inhibition of this enzyme has long been recognised as a possibility for specific control of fungal diseases on plants (Rajam and Galston, 1985). Indeed, α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, provides good control of a number of plant pathogens (Walters, 1986; Havis and Walters, 1992). Polyamine metabolism can also be perturbed by polyamine analogues (Porter and Sufrin, 1986). Thus, a number of putrescine analogues have been shown to control a variety of plant fungal diseases and to alter biosynthetic enzyme activities and intracellular polyamine concentrations in fungal cells *in vitro* (Foster and Walters, 1993; Havis et al., 1994a,b). However, spermidine levels were never totally depleted through the use of such analogues (Foster and Walters, 1993; Havis et al., 1994a,b). Since spermidine appears to be the most important polyamine in fungi (Stevens and Winther, 1979), inhibition of spermidine synthase may be more effective in reducing fungal growth. Thus, a study was undertaken to examine the effects of two commercially available spermidine analogues, cyclohexylamine (CHA) and norspermidine on fungal growth, enzyme activities and polyamine concentrations in the oat stripe pathogen, *Pyrenophora avenae*. CHA is known to inhibit spermidine synthase in various systems (Walters, 1995), while norspermidine has been shown to possess potent antiproliferative activity (Prakash et al., 1988). This paper presents the results of the study.

Materials and methods

Fungal growth on solid medium

Pyrenophora avenae Ito and Kuribay was maintained on potato dextrose agar (PDA). Filter-sterilized aqueous solutions (10 ml) containing CHA and norspermidine were added to 70 ml of sterile PDA at 45–47°C to obtain final concentrations between 0 and 2 mM. Control plates contained culture medium only. Sterile medium (20 ml) was added aseptically to a 90-mm single-vent sterile plastic Petri dish and a sterile 8-mm diameter cork borer used to remove plugs of mycelium from the edges of stock cultures, which were inverted and placed in the centre of each Petri dish. Inoculated plates were incubated in the dark at 24°C. Radial mycelial growth, excluding the mycelial plug, was measured 3, 6 and 8 days after inoculation.

Fungal growth in liquid medium

Filter-sterilized solutions (10 ml) containing the compounds were added to 140 ml of sterile potato dextrose broth (PDB) in 250 ml conical flasks to obtain the desired final inhibitor concentration. Control flasks contained medium only. Flasks were inoculated with a 10-mm disc of mycelium and placed in a Gallenkamp orbital shaker (140 rpm) at 24°C. After 2, 3 and 4 days, the fungus was washed with distilled water through a fine mesh sieve and centrifuged at 16,000 g at 0°C for 10 min. The pellet obtained was weighed and used for enzyme and polyamine analysis.

In order to study fungus which had been exposed to the analogues for one day, fungus was grown in unamended medium for 2 days before being transferred to amended medium for one further day. This procedure was necessary to ensure that enough fungal material was obtained for analysis.

Enzyme, polyamine assays and the formation of polyamines from [U-^{14}C]ornithine

ODC and AdoMetDC assays, and the determination of polyamine levels were performed as described by Zarb and Walters (1993). The formation of polyamines from radio-labelled ornithine was studied by taking a 100μl aliquot from the ODC reaction mixture, in which [U-^{14}C]ornithine was used, and treating as described for polyamines. Following chromatographic separation, the spots were scraped into 10ml of scintillant (Emulsifier Safe, Packard, UK) for radioactive counting in a 1900 TR liquid scintillation analyzer (Packard, UK).

For mycelial growth on solid media, results were calculated as the means of 12 replicates. All other results were calculated as the means of 4 replicates. All experiments were repeated with similar results and statistical significance was assessed using Student's *t*-test. In all studies, apart from the growth experiments, fungus was exposed to either 1.0mM CHA or 0.01 mM norspermidine although selected analyses were carried out for fungus exposed to higher concentrations of the analogues. The lower concentrations were used most extensively to ensure that enough fungal material was obtained to carry out the various procedures. Where enzyme activities, polyamine concentrations and the formation of polyamines from radio-labelled ornithine were determined in fungal tissue exposed to the inhibitors for 2, 3 and 4 days, all results were similar, thus only those for 4 day old fungus are presented.

Results and discussion

Mycelial growth of *P. avenae* on solid media was reduced in the presence of 2.0mM CHA by 55.2%. Lower concentrations of CHA had little effect on fungal growth (Table 1). Norspermidine produced a slightly greater effect on fungal growth, with 1.0mM reducing growth by 40.3% and 2.0mM reducing growth by 63.3% (Table 1). Growth of the fungus in liquid culture was completely inhibited by 0.5mM norspermidine, while a concentration of 3.0mM CHA reduced growth by 92.9% (Table 2).

The spermidine synthase inhibitor CHA and the spermidine analogue norspermidine are known to inhibit the growth of tumours in rodents (Ito et

Table 1. Effect of CHA and norspermidine on mycelial growth of *Pyrenophora avenae* 8 days after inoculation

Concentration (mM)	Mycelial growth, measured as mean colony radius (mm)	
	CHA	norspermidine
0	36.2 ± 0.79	36.2 ± 0.79
0.05	37.5 ± 0.31	37.6 ± 0.36
0.1	37.9 ± 0.26	38.0 ± 0.36
0.5	37.0 ± 0.47	33.8 ± 0.68*
1.0	35.4 ± 1.00	21.6 ± 2.39***
2.0	16.2 ± 2.04***	13.3 ± 0.70***

Note. Significant differences are shown as *$p \leq 0.05$ and ***$p \leq 0.001$.

Table 2. Effect of CHA and norspermidine on growth of *Pyrenophora avenae* in liquid culture 4 days after inoculation

Growth in liquid culture, measured as mean fresh weight (g)			
Concentration (mM)	CHA	Concentration (mM)	norspermidine
0	4.2 ± 0.37	0	5.2 ± 0.30
2.00	4.3 ± 0.16	0.1	1.8 ± 0.11**
2.25	2.6 ± 0.06*	0.2	1.3 ± 0.11**
2.50	1.7 ± 0.19**	0.3	0.7 ± 0.08***
2.75	0.7 ± 0.37***	0.4	0.3 ± 0.02***
3.00	0.3 ± 0.02**	0.5	NG

Note. Significant differences are shown as $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. *NG* no growth.

Table 3. ODC and AdoMetDC activities in *Pyrenophora avenae* exposed to CHA and norspermidine for 4 days

Treatment	Enzyme activity [pmol CO_2 (mg protein)$^{-1}$ hr^{-1}]	
	ODC	AdoMetDC
Control	48.2 ± 7.61	92.5 ± 11.01
1.0 mM CHA	50.5 ± 7.40	99.4 ± 10.32
Control	54.2 ± 8.13	83.0 ± 2.06
0.01 mM norspermidine	61.2 ± 6.69	75.2 ± 6.66

al., 1982; Prakash et al., 1988) as well as cultured animal cells (Mitchell et al., 1985; Sunkara et al., 1988). Indeed, such antiproliferative properties have already been demonstrated for CHA in fungi with inhibition of mycelial growth of *Gaeumannomyces graminis* (West and Walters, 1989) and reductions in powdery mildew infection on barley in the glasshouse (West and Walters, 1988). The results of the present study agree, therefore, with this previous work, and show that both CHA and norspermidine possess antifungal properties, with the inhibitory effect on *P. avenae in vitro* being more pronounced with norspermidine.

Neither 1.0 mM CHA nor 0.01 mM norspermidine had any effect on ODC or AdoMetDC activities in *P. avenae* grown *in vitro* (Table 3). Treatment with the inhibitors also produced no effect on the formation of polyamines from radio-labelled ornithine. All radioactivity was observed in fungal spermidine and spermine, with none found in putrescine in any of the treatments examined (Table 5). Similar results were observed for fungus exposed to the higher concentrations of 2.75 mM CHA and 0.2 mM norspermidine for 1 or 4 days (Table 6).

Table 4. Polyamine concentrations in *Pyrenophora avenae* grown in the presence of CHA and norspermidine for 4 days

Treatment	Polyamine concentration (μmol g^{-1} FW)			
	Putrescine	Cadaverine	Spermidine	Spermine
Control	369 ± 22.4	103 ± 8.3	270 ± 9.7	86 ± 4.1
1.0 mM CHA	645 ± 29.1**	126 ± 12.2	266 ± 10.7	97 ± 13.8
Control	523 ± 35.2	222 ± 15.1	419 ± 33.4	150 ± 18.6
0.01 mM norspermidine	587 ± 42.8	201 ± 16.6	457 ± 19.9	167 ± 14.6

Note. Significant differences are shown as **$p \leq 0.01$.

Table 5. Formation of polyamines from [U-^{14}C]ornithine in *Pyrenophora avenae* after exposure to CHA and norspermidine for 4 days

Treatment	Radioactivity in polyamine (dpm g^{-1} FW)	
	Spermine	Spermidine
Control	12.0 ± 2.88	78.0 ± 9.54
1.0 mM CHA	15.4 ± 2.19	56.6 ± 8.39
0.01 mM norspermidine	16.9 ± 4.21	60.3 ± 9.26

Table 6. Formation of polyamines from [U-^{14}C]ornithine in *Pyrenophora avenae* after exposure to increased concentrations of CHA and norspermidine for 1 or 4 days

Treatment (1 day)	Radioactivity in polyamine (dpm g^{-1} FW)	
	Spermine	Spermidine
Control	32.0 ± 1.77	184 ± 18.2
2.75 mM CHA	36.1 ± 1.90	177 ± 10.4
0.2 mM norspermidine	45.0 ± 5.07	197 ± 12.1
Treatment (4 days)	Spermine	Spermidine
Control	22.1 ± 3.6	83.8 ± 9.73
2.75 mM CHA	22.9 ± 1.55	89.1 ± 9.28
0.2 mM norspermidine	25.5 ± 5.53	98.4 ± 10.92

These findings for CHA are in contrast to many which indicate that CHA inhibits spermidine synthase from a variety of sources. Examples include spermidine synthase from mammalian cells, trypanosomes, certain bacteria (Pegg and Williams-Ashman, 1987) and from Chinese cabbage leaves (Sindhu

and Cohen, 1984). However, work on other polyamine biosynthetic enzymes has shown that enzymes will react differently upon exposure to an inhibitor *in vitro* and *in vivo* (Walters et al., 1995). Such a phenomenon could, therefore, explain the lack of any effect of CHA on spermidine synthase in fungi. Nevertheless, CHA is known to inhibit spermidine synthase in protoplasts extracted from Chinese cabbage leaves (Greenberg and Cohen, 1985). Other authors, however, have observed CHA to cause an inhibitory growth effect on potatoes (Masse et al., 1988) and in tumours (Ito et al., 1982), but have been unable to fully account for this effect by the perturbation of polyamine biosynthesis. Little information exists on the effects of norspermidine on spermidine synthase, although, Masse and co-workers (1988) did indicate that a norspermidine-induced growth effect in potatoes was in fact due to perturbation of spermidine biosynthesis. Previous work with the inhibitors in other organisms aside, the findings of the present study with fungi were not entirely unexpected. Indeed, work using putrescine analogues had concluded that observed effects on polyamine biosynthesis in fungi grown *in vitro* were unlikely to have been wholly responsible for reductions in fungal growth (Havis et al., 1994b).

Norspermidine had little effect on the levels of free polyamines in *P. avenae* (Table 4). This is in keeping with the data presented in Tables 3, 5 and 6 showing that the analogue had no effect on spermidine biosynthesis. CHA, however, produced a 74.8% increase in the level of putrescine found in the fungus, although spermidine levels were not altered (Table 4). Increased putrescine and decreased spermidine levels are the widely reported results of spermidine synthase inhibition. Such CHA-induced changes in polyamine levels have been observed in *Helianthus tuberosus* extracts (Torrigiani et al., 1987), pine cotyledons (Biondi et al., 1986) and cultured animal cells (Mitchell et al., 1985). In the present work, since CHA had no effect on spermidine levels, did not affect the flux of radio-label through to spermidine and had no effect on ODC, an explanation for the observed increase in putrescine requires further work. It is known that putrescine can be formed by a "back-conversion" pathway, involving the action of polyamine oxidase on acetylspermidine (Large, 1992) and it is possible that the CHA-induced increase in putrescine was the result of such a mechanism. It is worth noting that an accumulation of putrescine was suggested by Davis and Ristow (1991) to be responsible for the reduction in growth of a mutant of *Neurospora crassa*. Excess putrescine has been shown to be toxic in the cyanobacterium *Anabaena* (Davis, 1990), while the action of diamine oxidase upon putrescine to yield free radicals and hydrogen peroxide and consequently, cause membrane damage may also have led to the reduction in growth which resulted from exposure of the fungus to CHA (DiTomaso et al., 1989). It seems unlikely however that the observed increase in putrescine concentration, from 369 to 645 μmol g^{-1} FW, was large enough to be responsible for the antifungal effect of CHA.

Irrespective of the mechanism responsible for the increase in putrescine in fungal tissue exposed to CHA, it would appear that the fungicidal effects of CHA and norspermidine are not a result of altered polyamine biosynthesis.

Polyamine analogues were initially hypothesised by Porter and Sufrin (1986) to be of use in chemotherapeutic strategies since they might be able to utilise the polyamine uptake system of a cell, and once in that cell, affect growth by a number of mechanisms, only one of which was inhibition of polyamine biosynthetic enzymes. The absence of an effect on polyamine biosynthesis by the spermidine analogues suggests that the compounds act in some other way to exert their antiproliferative effects. Clearly, further work to determine the mode of action of the spermidine analogues is warranted.

Acknowledgments

Caroline Mackintosh is grateful to SAC and the University of Glasgow for the award of a Dr. William Stewart Postgraduate Scholarship. SAC receives funding from SOAEFD.

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Received August 28, 1996