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Kinetic Studies on the Control of the Bean Rust Fungus (*Uromyces phaseoli* L.) by an Inhibitor of Polyamine Biosynthesis[1]

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ABSTRACT

α-Difluoromethylornithine (DFMO), a specific and irreversible inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase, effectively inhibits mycelial growth of several phytopathogenic fungi on defined media *in vitro* and provides systemic protection of bean plants against infection by *Uromyces phaseoli* L. race 0 (MV Rajam, AW Galston 1985 Plant Cell Physiol 26: 683–692; MV Rajam *et al.* 1985 Proc Natl Acad Sci USA 82: 6874–6878). We now find that application of 0.5 millimolar DFMO to unifoliolate leaves of Pinto beans up to 3 days after inoculation with uredospores of *U. phaseoli* completely inhibits the growth of the pathogen, while application 4 or 5 days after inoculation results in partial protection against the pathogen. Spores do not germinate on the surface of unifoliolate leaves treated with DFMO 1 day before infection, but addition of spermidine to the DFMO treatments partially reverses the inhibitory effect. The titer of polyamines in bean plants did not decline after DFMO treatment; rather, putrescine and spermidine contents actually rose, probably due to the known but paradoxical stimulation of arginine decarboxylase activity by DFMO.

It has been established, through the use of genetic mutants and specific chemical inhibitors, that polyamines are essential for optimal growth and development in bacteria and fungi (9), higher plants (5, 8), and mammals (3). The formation of PA[2] in bacteria and higher plants may proceed through either of two enzymes, *e.g.* ADC or ODC, while only the ODC pathway operates in many fungi. DFMA and DFMO which specifically and irreversibly block ADC (1) and ODC (4), respectively, have been used to specify the initial route of Put biosynthesis in many organisms. Since Spd and Spm are formed from Put, effective blockage of Put formation can deprive the organism of all PA. The presence of but a single Put biosynthetic pathway in fungi (via ODC) suggested a possible approach to the use of DFMO as a protectant or possibly a chemotherapeutic agent in the prevention of certain types of fungal pathogenesis in higher plants.

In growth experiments on defined media with several phytopathogenic fungi (6), we observed that DFMO is an effective inhibitor of mycelial growth, and that such inhibitions are completely reversed by application of Put or Spd to the culture medium. In a recent paper (7), we reported on the remarkable efficacy of DFMO as a protectant of bean plants against infection caused by uredospores of *Uromyces phaseoli* race 0, the common bean rust fungus. Not only could we obtain complete protection by as little as 400 μl of 0.5 mM DFMO applied to a single unifoliolate leaf, but DFMO was found to confer protection as well on unsprayed parts of treated plants, indicating the translocation of some protective effect from the sprayed areas. The present work extends that study, reporting on the kinetics and reversibility of the DFMO effect.

Unifoliolate leaves of 10-d-old greenhouse-grown bean seedlings (*Phaseolus vulgaris* cv 'Pinto') were sprayed with DFMO or DFMA in 0.01% Tween-20 at pH 7.0 before or after inoculation with uredospores of *U. phaseoli* L. race 0. Control plants were sprayed similarly with Tween-20 without inhibitor. In experiments involving the reversal of DFMO effects, we utilized 0.05 mM DFMO (which yielded ~ 50% inhibition of uredial development) together with 0.01, 0.1, and 1.0 mM Put of Spd. After inoculation with rust uredospores (25 mg/100 ml of 0.01% Tween 20), all plants were placed in dew chambers (100% RH) for 16 h at 19°C in total darkness, as previously described (6). Plants were returned to the greenhouse and arranged randomly following exposures to inhibitor and uredospores. Disease severity was evaluated 7 d after inoculation by counting the lesions on the leaf.

PA analysis was performed on leaf samples collected from inhibitor-sprayed bean plants. Leaf samples were ground in prechilled mortars with 10% (w/v) $HClO_4$ at a ratio of 200 mg fresh weight leaf per ml $HClO_4$. Homogenates were centrifuged at 26,000*g* for 20 min at 4°C. The clear supernatant fractions were used for dansylation according to a procedure previously described (4). Briefly, 0.4 ml of freshly prepared dansyl (diaminonaphthylsulfonyl)-Cl (Sigma), 5 mg/ml in acetone, and 0.2 ml of saturated Na_2CO_3 were added to 0.2 ml of the supernatant fraction. The dansylated PA were extracted with 0.25 ml benzene and the clear benzene layer was used for PA determinations by TLC on LK6D high resolution silica gel plates (Whatman). After development in chloroform:triethylamine (25:2 v/v) for about 1 h, and location by fluoresence under a UV lamp, the dansylpolyamine bands were scraped off, eluted in 4 ml of ethyl acetate and quantified with an Aminco-Bowman fluorimeter.

We reported previously that DFMO at 0.5 mM or higher gives complete protection against the pathogen in both pre- and post-

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[2] Abbreviations: PA, polyamine; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; DFMA, α-difluoromethylarginine; DFMO, α-difluoromethylornithine; Put, putrescine; Spd, spermidine; Spm, spermine.

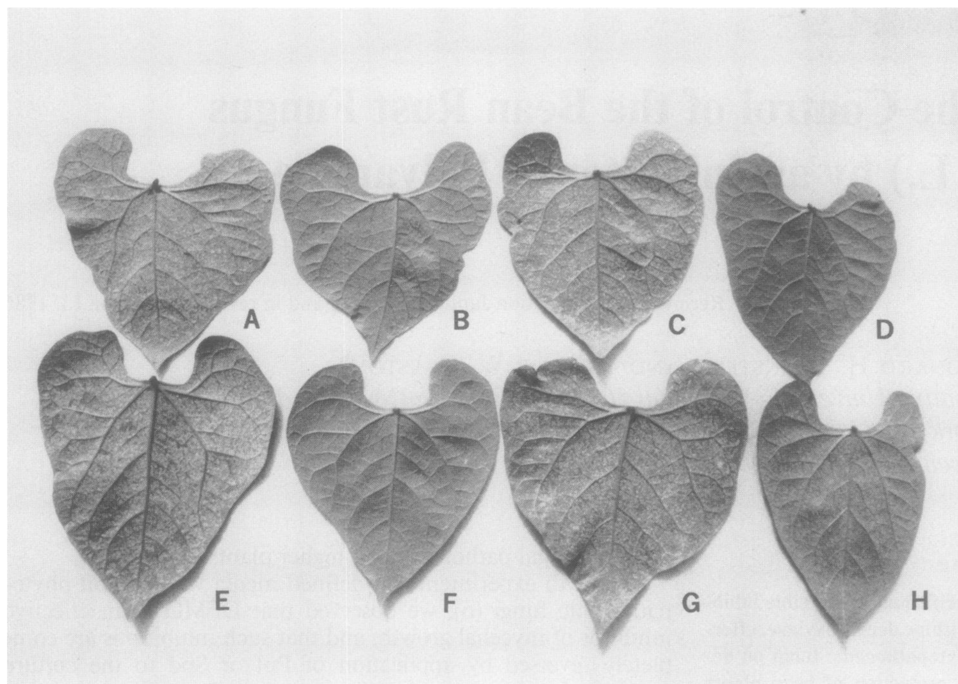

FIG. 1. Kinetics of pathogen inhibition following postinoculation exposures to 1.0 mM DFMO. Unifoliolate leaves of controls (A, C, E, and G) and DFMO treatments (B, D, F, and H) on 1, 2, 3, and 4 d after inoculation with uredospores of *U. phaseoli*, respectively.

Table I. *Kinetics of Inhibition of Pathogenesis Resulting from Postinoculation Application of DFMO to Unifoliolate Leaves of Bean Plants after Different Intervals of Time*

Unifoliolate leaves were inoculated with uredospores and then, at daily intervals, a different group of plants was sprayed with 0.01% Tween 20 lacking or containing 1.0 mM DFMO.

Time between Spore Inoculation and DFMO Spray	DFMO Concentration	Lesions	
d	*mM*	*per cm²*	*per leaf*[a]
1	0	74 ± 6	4673 ± 373
	1.0	0	0
2	0	69 ± 6	4568 ± 398
	1.0	0	0
3	0	64 ± 4	3172 ± 276
	1.0	0	0
4	0	65 ± 6	4419 ± 461
	1.0	41 ± 5[b]	2330 ± 211[b]
5	0	69 ± 3	3962 ± 217
	1.0	53 ± 4[b]	3278 ± 326

[a] Each value is Mean ± SE, based on six replicates (one leaf per plant). [b] Significant difference at 5% level.

Table II. *Effect of Postinoculation Application of DFMO, and of PAs, Alone or in Combination, on the Severity of Bean Rust Disease on Unifoliate Leaves*

Treatment	Lesions	
mM	*per cm²*	*per leaf*[a]
Control	64 ± 4	4104 ± 187 (100)[b]
Put, 0.01	57 ± 2	4013 ± 264 (98)
Put, 0.1	54 ± 5	3189 ± 478 (78)
Put, 1.0	44 ± 9	2698 ± 260 (66)
Spd, 0.01	56 ± 5	3209 ± 360 (78)
Spd, 0.1	58 ± 6	3416 ± 457 (83)
Spd, 1.0	50 ± 10	3926 ± 358 (96)
DFMO, 0.05	22 ± 4	1223 ± 268 (30)
DFMO, 0.05 + Put, 0.01	30 ± 5	1823 ± 289 (44)
DFMO, 0.05 + Put, 0.1	24 ± 4	1440 ± 337 (35)
DFMO, 0.05 + Put, 1.0	32 ± 2	2009 ± 129 (49)
DFMO, 0.05 + Spd, 0.01	29 ± 3	1678 ± 240 (41)
DFMO, 0.05 + Spd, 0.1	38 ± 7	2366 ± 488 (58)
DFMO, 0.05 + Spd, 1.0	39 ± 3	2334 ± 245 (57)

[a] Each value is mean ± SE, based on six replicates (one leaf per plant). [b] Percent of control values are in parentheses.

inoculation exposures when inoculation and treatment are separated by 24 h (7). In the present work, we determined the effect of varying delays in postinoculation application of DFMO on the extent and course of pathogenesis. Test plants were exposed to 1.0 mM DFMO either 1, 2, 3, 4, or 5 d after inoculation with uredospores of the pathogen. Complete protection against the pathogen was found when DFMO was first applied up to 3 d after inoculation (Fig, 1). Disease symptoms occurred when the interval between inoculation and DFMO application was 4 or 5 d, although the number of lesions was significantly reduced below control (unprotected) values (Table I).

In vitro, uredospore germination starts 30 min after incubation at 19°C in the light, and the germ tube reaches an effective size for leaf penetration within 3 h in the presence of 10 mM Mes (pH 7.0), 3 mM $CaCl_2$, 2 mM $MgSO_4$, and 1% purified agar. We attempted to visualize spore germination on the surface of the unifoliolate bean leaf with crystal violet, 1 d after spore inoculation. The plants had been sprayed with DFMO at 0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM 1 d before inoculation. Germination was normal on control leaves, completely inhibited on leaves treated with more than 0.5 mM DFMO, and partially inhibited at lower concentrations of DFMO.

To determine whether the effect of DFMO was related to inhibition of PA biosynthesis, we conducted experiments to determine the effects of PA application, alone or in combination with 0.05 mM DFMO (which yielded ~ 50% inhibition of uredial formation), on the severity of the disease. There were generally no significant differences between controls and PAs alone except at 1 mM Put, which reduced the number of lesions by about one-

third. This is probably due to the well known toxic effect of mM Put (8, 9). The number of lesions was reduced by 70% in plants exposed to 0.05 M DFMO alone; when Spd was supplied 1 h after DFMO, the inhibition conferred by DFMO was substantially reduced (Table II).

The effect of DFMO and DFMA sprays (0.01, 0.1, and 1.0 mM) on PA titers was also examined. Unifoliolate leaves were sprayed in the usual way and examined 1 and 3 d later, while the first trifoliolate leaves, unexpanded at the time of spray and themselves unsprayed, were examined 8 d later. There were no significant reductions of PA levels in any of the treated leaves; in fact, Put and Spd titers were increased by the highest concentration of DFMO (MV Rajam, LH Weinstein, AW Galston, unpublished data). This appears to be due to the paradoxical promotion of ADC activity by DFMO (8). There was no effect of DFMO or DFMA sprays on the growth of the plants.

Thus, in the absence of any depression in the PA titer of the host plant, we reason that the protective effect of DFMO against the fungus results in part from its persistence on the surface of the leaf, where fungal spores germinate and initiate growth. Since rust uredosporelings contain progressively higher Spd as their growth and differentiation progresses (2), it is reasonable to believe that PA deprivation caused by DFMO would inhibit both processes. The translocatability of the protective effect of DFMO (7) indicates that this substance may also initiate internal protective metabolic changes that are not reflected in the PA titer of the host plant.

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LITERATURE CITED

1. Kallio A, P McCann, P Bey 1981 DL-α-(Difluoromethyl)arginine: a potent enzyme-activated irreversible inhibitor of bacterial arginine decarboxylase. Biochemistry 20: 3163–3166
2. Kim WK 1971 Folate and polyamine content of undifferentiated and differentiated wheat stem rust uredosporelings. Can J Bot 49: 1119–1122
3. Mamont PS, M-C Duchesne, J Grove, P Bey 1978 Anti-proliferative properties of DL-α-difluoromethyl ornithine in cultured cells. A consequence of the irreversible inhibition of ornithine decarboxylase. Biochem Biophys Res Commun 81: 58–66
4. Metcalf BW, P Bey, C Danzin, MJ Jung, P Casara, JP Vevert 1978 Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C. 4.1.1.17) by substrate and product analogues. J Am Chem Soc 100: 2551–2553
5. Palavan N, AW Galston 1982 Polyamine biosynthesis and titer during various developmental stages of *Phaseolus vulgaris*. Physiol Plant 55: 438–444
6. Rajam MV, AW Galston 1985 The effects of some polyamine biosynthetic inhibitors on growth and morphology of phytopathogenic fungi. Plant Cell Physiol 26: 683–692
7. Rajam MV, LH Weinstein, AW Galston 1985 Prevention of a plant disease by specific inhibition of fungal polyamine biosynthesis. Proc Natl Acad Sci USA 82: 6874–6878
8. Slocum RD, R Kaur-Sawhney, AW Galston 1984 The physiology and biochemistry of polyamines in plants. Arch Biochem Biophys 235: 283–303
9. Tabor CW, H Tabor 1985 Polyamines in microorganisms. Microbiol Rev 49: 81–99