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Prevention of a plant disease by specific inhibition of fungal polyamine biosynthesis

(ornithine decarboxylase/putrescine/α-difluoromethylornithine/polyamines/bean rust)

M. VENKAT RAJAM*†, LEONARD H. WEINSTEIN‡, AND ARTHUR W. GALSTON*§

*Department of Biology, Kline Biology Tower, Yale University, New Haven, CT 06511; and ‡Boyce Thompson Institute, Ithaca, NY 14853

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ABSTRACT **DL-α-Difluoromethylornithine (DFMO), an inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase (EC 4.1.1.17), strongly retards the growth of several species of phytopathogenic fungi *in vitro*. Such inhibition can be completely reversed by putrescine or spermidine, confirming the essentiality of polyamines for growth of fungal hyphae. We now show that DFMO can protect bean plants (*Phaseolus vulgaris* Linnaeus cv. Pinto) against infection by uredospores of the bean rust fungus, *Uromyces phaseoli* Linnaeus, race O. Unifoliolate leaves of 10-day-old greenhouse-grown seedlings were sprayed with 400 μl per leaf of DFMO at various concentrations in 0.01% Tween 20 at pH 7.0 before or after inoculation with uredospores of *Uromyces*. After 16 hr in darkness in dew chambers to facilitate spore germination, plants were transferred to the greenhouse, arranged randomly, and examined for local lesions 7 days later. All concentrations of DFMO 0.50 mM or higher gave complete protection against the pathogen; at lower concentrations, postinoculation treatments with DFMO were generally more effective than preinoculation. The appearance of lesions on plants treated with lower concentrations of DFMO was retarded 2–6 days. DFMO also confers protection on unsprayed parts of treated plants, indicating the translocation of some protective effect from sprayed areas. DL-α-Difluoromethylarginine, an analogous inhibitor of arginine decarboxylase (EC 4.1.1.19), which is the rate-limiting enzyme in an alternative pathway for polyamine biosynthesis in higher plants, confers no protection even at 5 mM. This emphasizes ornithine decarboxylase as the biochemical locus of choice for the prevention of plant diseases by inhibiting polyamine metabolism.**

Fungi attack a wide variety of economically important crop plants, substantially reducing their quality and yield. The major weapon used against such phytopathogens has been synthetic fungicides. We report here on the fungicidal and plant protective efficacy of DL-α-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase (ODCase; EC 4.1.1.17), the enzyme that provides fungi with the polyamines needed for normal growth and development (1). The differential toxicity of DFMO for fungi and higher green plants depends on the fact that the latter have an alternative pathway, based on arginine decarboxylase (ADCase; EC 4.1.1.19), for the synthesis of essential polyamines. In previous *in vitro* experiments with fungi grown on synthetic media (2), we showed that DFMO is an effective inhibitor of mycelial growth and that such inhibitions are readily reversed by applied putrescine or spermidine. For the important rust, smut, and mildew diseases, however, the obligately parasitic organisms cannot be grown on synthetic media. We accordingly undertook these experiments, which now show that DFMO can similarly inhibit germination, growth, and pathogenic effects of fungi inoculated onto leaves of susceptible plants.

MATERIALS AND METHODS

Pinto bean (*Phaseolus vulgaris* Linnaeus cv. Pinto) seeds were sown in a peat/vermiculite mix in 10-cm fiber pots. Four seeds were planted per pot and each pot was thinned to two uniform seedlings after emergence. The plants were then grown in a greenhouse supplied with air filtered through charcoal and Purafil II (Purafil, Atlanta) at 23–25°C ambient temperature, 70% relative humidity, and a 16-hr photoperiod.

Unifoliolate leaves of 10-day-old bean seedlings were sprayed (400 μl per leaf) with DFMO or α-difluoromethylarginine at concentrations of 0.01–5 mM, before or after inoculation with uredospores of the bean rust, *Uromyces phaseoli*, race O. The inhibitor solutions were prepared in 0.01% Tween 20, with the pH adjusted to 7.0. Control plants without inhibitor were sprayed similarly and were allowed to dry before inoculation with the pathogen. After inoculation with rust uredospores (25 mg/100 ml of 0.01% Tween 20) all plants were placed in dew chambers (100% relative humidity) for 16 hr at 19°C in total darkness. After exposures to inhibitors and uredospores, all plants were returned to the greenhouse and arranged randomly. Disease severity was evaluated 7 days after inoculation by counting local foliar lesions. In control plants and those treated with low concentrations of DFMO, uredospores were collected randomly from each treatment to determine percent germination and pathogenicity. For determination of germination, spores were dusted onto Petri plates containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) at pH 7.0, 3 mM $CaCl_2$, 2 mM $MgSO_4$, and 1% purified agar, and incubated for 3 hr at 19°C in the light.

Each exposure consisted of 12 plants in six replicate pots. All experiments were repeated at least once, with similar results.

RESULTS

Foliar lesions characteristic of infection by *Uromyces phaseoli* uredospores were apparent on control plants without inhibitor within 4–5 days after inoculation and attained their maximal size after 10–12 days. Abundant lesions occurred in the control plants, while plants with pre- or postinoculation exposures to increasing concentrations of DFMO developed progressively fewer lesions (Fig. 1). No disease symptoms could be detected on plants treated with DFMO at 0.5 mM or higher. At the lower concentrations, inhibition of uredial development was more pronounced on

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Abbreviations: ADCase, arginine decarboxylase; ODCase, ornithine decarboxylase; DFMO, α-difluoromethylornithine.

†Permanent address: Department of Botany, Kakatiya University, Warangal 506009, India.
§To whom reprint requests should be addressed.

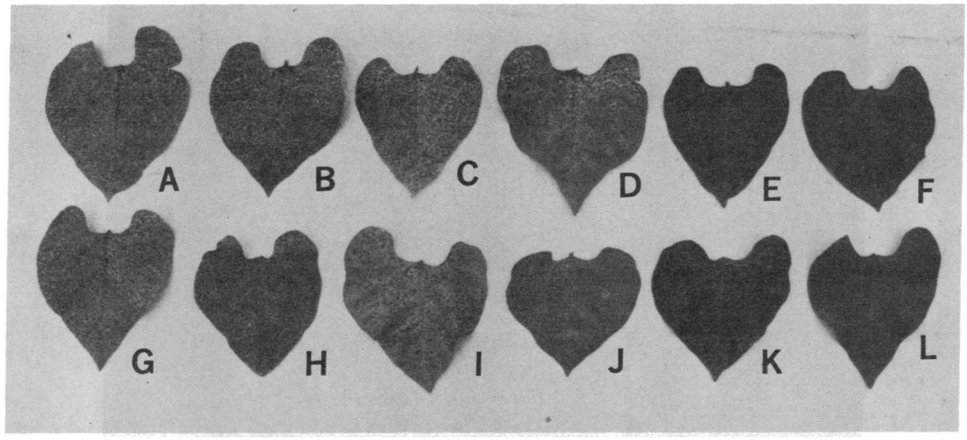

FIG. 1. Severity of symptoms of rust disease induced by inoculation of unifoliolate leaves of pinto beans with uredospores of *Uromyces phaseoli* preceding (*A–F*) and following (*G–L*) DFMO treatments. DFMO treatments were at 0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM, in *A–F* and *G–L*, respectively.

plants given postinoculation exposures to the inhibitors. In such cases, uredial appearance was delayed for 2–6 days in DFMO-treated plants, and the extent of delay was dose-dependent. Increasing the concentration of DFMO from 0.01 to 0.25 mM resulted in a gradual decrease in lesion number and disease severity. The ED_{50} was found to be at 0.05 mM and 0.025 mM for pre- and postinoculation exposure to DFMO, respectively. In contrast, α-difluoromethylarginine was ineffective in reducing infection, even at 5.0 mM. The average numbers of lesions per square centimeter and per leaf produced by pre- and postinoculation exposures to different concentrations of both the inhibitors are summarized in Table 1. Uredospores collected from plants treated with low concentrations of DFMO and α-difluoromethylarginine show no decrease in germination or pathogenicity.

The protective effects of DFMO are not limited to the region of application, indicating translocation of either DFMO or some substance formed as a result of its application. Thus, when the petiolar or apical half of a unifoliolate leaf was treated with 1 mM DFMO and inoculated with uredospores, the other half was also protected against infection. Transfer of the protective effect was better from leaf base to leaf apex than in the reverse direction. In the half-leaf treatment experiments, postinoculation treatment was once

Table 1. Mean number of lesions induced by uredospores of *Uromyces phaseoli* on unifoliolate leaves of bean plants exposed to DFMO or α-difluoromethylarginine (DFMA) before or after inoculation

Treatment		Preinoculation exposure		Postinoculation exposure	
Inhibitor	Conc., mM	Lesions per cm^2	Lesions per leaf	Lesions per cm^2	Lesions per leaf
None		59 ± 4	3708 ± 375	61 ± 2	3091 ± 71
DFMO	0.01	47 ± 6	2677 ± 397	40 ± 5*	1888 ± 249**
	0.025	34 ± 1**	2071 ± 103**	28 ± 2**	1526 ± 160**
	0.05	29 ± 4**	1712 ± 375*	17 ± 2**	1026 ± 131**
	0.075	19 ± 2**	1177 ± 159**	12 ± 1**	669 ± 96**
	0.10	14 ± 2**	659 ± 104**	5 ± 1**	221 ± 39**
	0.25	2 ± 1**	85 ± 20**	2 ± 1**	73 ± 4**
	0.50	0**	0**	0**	0**
	1.0	0**	0**	0**	0**
	5.0	0**	0**	0**	0**
DFMA	1.0	51 ± 4	3101 ± 217	56 ± 3	2829 ± 242
	5.0	46 ± 4	2928 ± 317	50 ± 2*	2195 ± 169**

Each value is the mean ± SEM, based on six replicates (one leaf per plant). ED_{50} values were approximately 0.05 and 0.025 mM DFMO for pre- and postinoculation exposures, respectively. * and ** denote significant differences from controls at the 5% and 1% level, respectively.

Table 2. Evidence for translocation of a protective effect against bean rust disease from unifoliolate leaves of pinto bean partially treated with 1 mM DFMO

Treatment		Preinoculation exposure				Postinoculation exposure			
		Lesions per cm^2		Lesions per half leaf		Lesions per cm^2		Lesions per half leaf	
Inhibitor	Conc., mM	Treated side	Untreated side	Treated side	Untreated side	Treated side	Untreated side	Treated side	Untreated side
None		80 ± 12	75 ± 13	2379 ± 358	2259 ± 399	71 ± 7	68 ± 8	1955 ± 192	1869 ± 263
DFMO	1.0	0**	22 ± 4*	0**	617 ± 112*	0**	0**	0**	0**

DFMO in 0.01% Tween 20 was applied with an artist's brush to the adaxial surface of one longitudinal half (divided by the midvein) of unifoliolate bean leaves, before or after inoculation with uredospores of *Uromyces*. Controls were treated similarly with Tween 20 alone. Each value is mean ± SEM, based on six replicates (one leaf per plant). * and ** denote significant differences from control at 5% and 1% level, respectively.

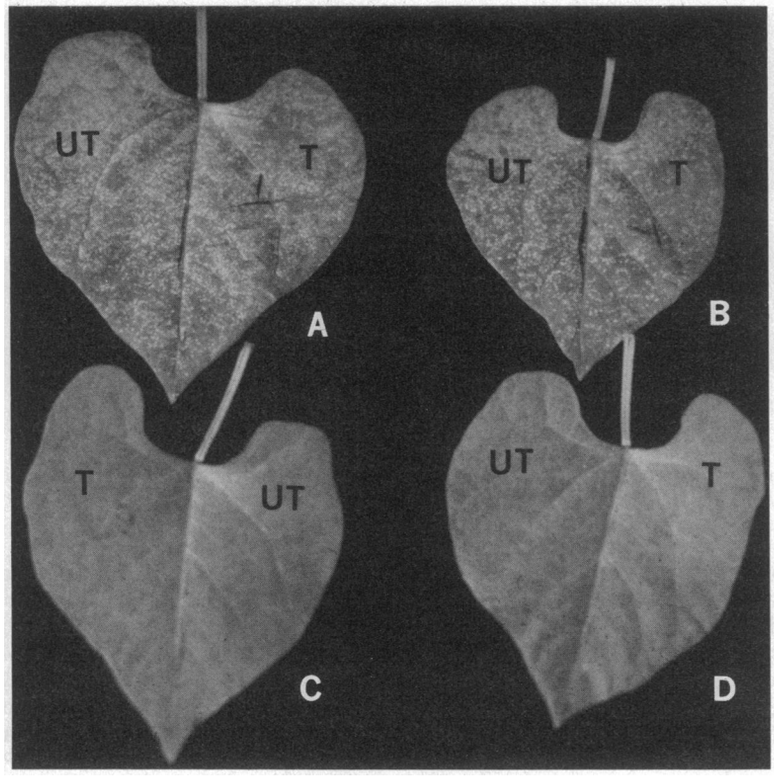

FIG. 2. Transfer of the protective effect from sprayed to unsprayed regions. Pre- and postinoculation treatments in control (*A* and *B*) and 1.0 mM DFMO (*C* and *D*), respectively. T, treated side; UT, untreated side.

again somewhat more effective than preinoculation exposures to DFMO. Similar translocated protective effects were noted when a longitudinal half or the unifoliolate leaf up to the midvein was treated with DFMO (Table 2; Fig. 2).

In further experiments, plants that had been treated with DFMO and pathogen were reinoculated with pathogen after 1 week, at a time when disease symptoms were apparent on the unifoliolate leaves. The number of lesions on the newly emerged trifoliolate leaves was considerably reduced in plants previously treated at 0.05, 0.1, 0.5, and 1.0 mM DFMO, while 5.0 mM afforded complete protection (Table 3; Fig. 3).

Plants sprayed with DFMO at all concentrations looked healthy, exhibiting no malformation or reduction in growth rate. By contrast, unprotected, infected plants showed a marked reduction in height (Fig. 4).

Table 3. Mean number of lesions induced by uredospores of *Uromyces phaseoli* in reinoculated trifoliolate leaves of bean plants

DFMO, mM	Preinoculation exposure		Postinoculation exposure	
	Lesions per cm^2	Lesions per leaflet	Lesions per cm^2	Lesions per leaflet
0	69 ± 6	1525 ± 99	71 ± 3	1402 ± 88
0.05	64 ± 5	1458 ± 92	59 ± 1*	1246 ± 73
0.1	55 ± 3	1274 ± 120	58 ± 4*	1289 ± 131
0.5	39 ± 7*	1172 ± 158	41 ± 4**	982 ± 84*
1.0	16 ± 2**	320 ± 22*	22 ± 2**	422 ± 13**
5.0	0**	0**	0**	0**

Trifoliolate leaves were reinoculated with pathogen after 1 week, before or after exposure to DFMO, to detect transfer of a protective effect to other parts of the plant. Each value is the mean ± SEM, based on six replicates (one leaf per plant). * and ** denote significant differences from controls at the 5% and 1% level, respectively.

DISCUSSION

Polyamines are now regarded as essential for normal growth and development in bacteria (1), fungi (1, 3), higher plants (4–6), and mammals (7). The diamine putrescine is produced from arginine and ornithine via the rate-limiting enzymes ADC and ODC, respectively (1). Bacteria and higher plants have both the ADC and ODC pathways (1, 6), while fungi are largely limited to the ODC pathway (3, 8, 9), with occasional indications of a biodegradative form of ADC (10, 11). The recent availability of DFMO (12) and α-difluoromethylarginine (13) as specific enzyme-activated "suicide inhibitors" of ODC and ADC, respectively, has made it possible to pinpoint which of these pathways operates in a variety of physiological responses attributable to polyamines (6). *S*-Adenosyl methionine decarboxylase, an enzyme important for furnishing aminopropyl groups for transfer to putrescine to make spermidine and spermine (1), has been detected in many fungal extracts. It has been purified from *Saccharomyces cerevisiae* (14) and shown to be activated by putrescine (10). Thus, fungal pathways for polyamine biosynthesis beyond putrescine seem to resemble those in mammals and higher plants.

DFMO was introduced as an anticancer drug (12). Studies on its relation to polyamine depletion (6, 15) have confirmed ODC as the biochemical locus of its effect. In fungi (2) and in higher plants (16) it has been found to cause some enlargement of cell diameter, although its major effect is clearly inhibition of cell division (4). In fungi, use of both specific mutations and enzyme inhibitors has shown the importance of polyamines not only for growth (1, 6, 8, 17) but also for meiosis and sporulation (1, 18).

Our experiments show that DFMO protects bean plants against infection by uredospores of *Uromyces phaseoli*. Previous data (2) and current experiments (unpublished) indicate effective inhibition of other phytopathogens *in vitro*. Since 400 μl of 0.25 mM DFMO applied to a single leaf gives

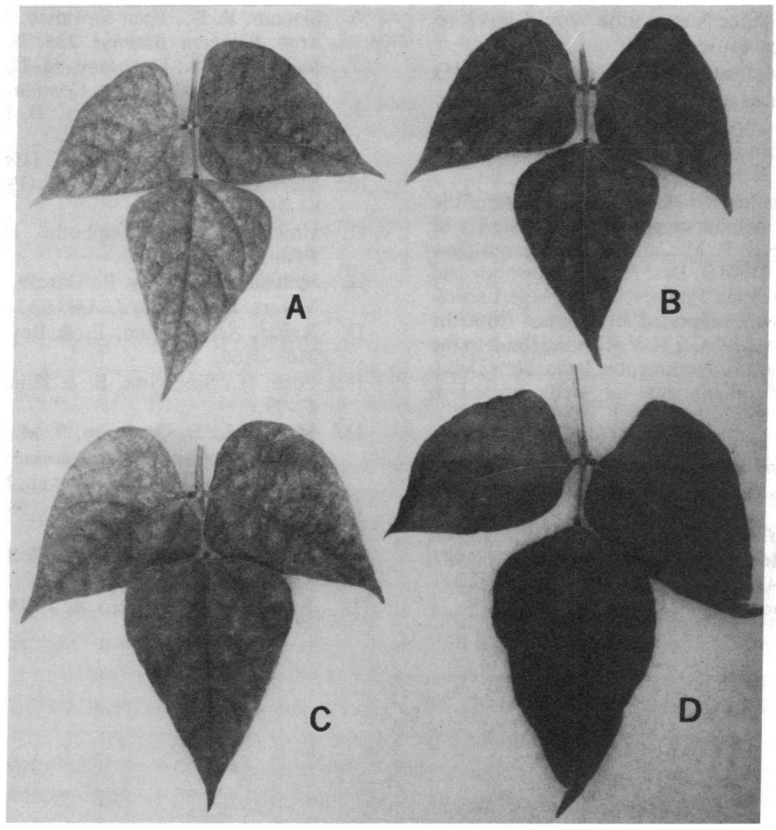

FIG. 3. Trifoliolate leaves of bean plants reinoculated with uredospores 1 week after unifoliolate leaves were exposed to DFMO. Pre- and postinoculation effect in controls (*A* and *C*) and 5.0 mM DFMO (*B* and *D*), respectively.

complete protection against infection, we estimate that at a spray rate of 100 gallons to the acre (940 liters/hectare), about 25 g per acre would be adequate for protection of a bean crop in the field. In view of the low toxicity of DFMO (1), this level of application should pose minimal problems for animals and humans. Furthermore, the lack of effectiveness of α-difluoromethylarginine underscores the specificity of DFMO action and explains the lack of toxicity of DFMO to green plants.

The translocatability of the protective effect of DFMO could be due to the movement of DFMO, a DFMO metabolite, or an induced antifungal compound such as a phytoalexin. The fact that postinoculation application of DFMO was more effective than preinoculation speaks, however,

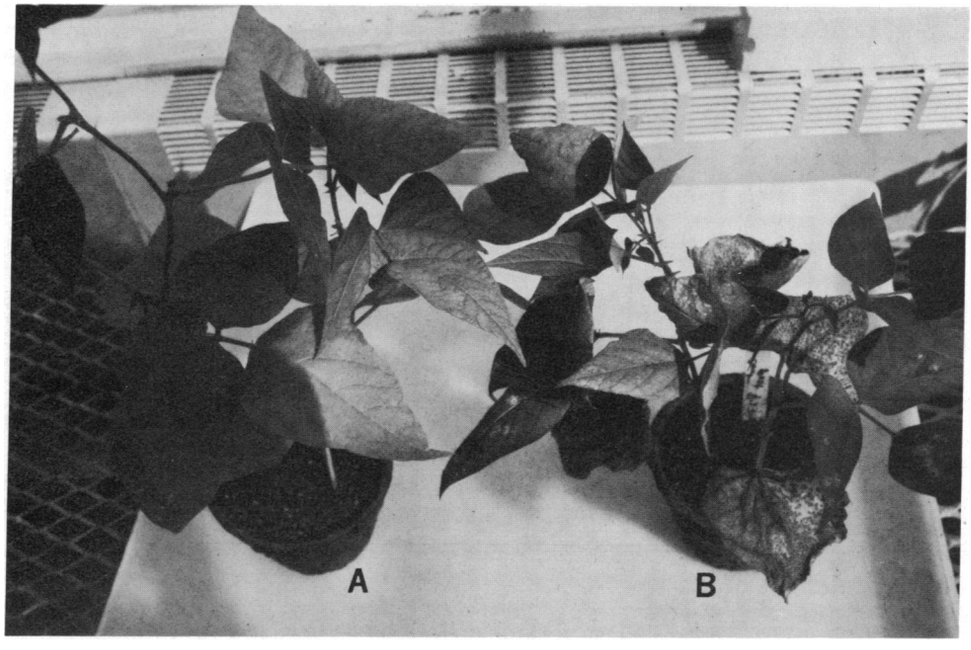

FIG. 4. Appearance of 2-week-old bean plants after postinoculation exposures to control (*B*) and 5.0 mM DFMO (*A*) sprays.

against any indirect effect, since some time would have to elapse for metabolic changes to occur.

On the basis of these experiments we believe that DFMO and other inhibitors of enzymes in the polyamine biosynthetic pathway may prove to be applicable to the alleviation or prevention of crop losses due to phytopathogenic fungi.

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