Prevention of Infection of Soybean Seeds by *Colletotrichum truncatum* by Polyamine Biosynthesis Inhibitors

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ABSTRACT

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The mechanism-based inhibitors of polyamine biosynthesis α-difluoromethylornithine (DFMO) and α-difluoromethylarginine (DFMA) were used to prevent soybean seed infection by the pathogenic fungus *Colletotrichum truncatum*. DFMA (2 mM) and DFMO (4 mM) protected soybean seeds against infection by *C. truncatum*. The effect of these inhibitors on the growth of the fungus was examined. Similar sensitivity to both DFMO and DFMA was observed; however, growth inhibition was reversed by the addition of putrescine or spermidine. This fungus was found to contain both ornithine and arginine decarboxylases. The intracellular polyamine levels of putrescine, spermidine, and spermine were lowered by the presence of either DFMA or DFMO. Spermidine was the predominant polyamine found in this fungus. Because DFMA, contrary to DFMO, did not affect seed germination, DFMA treatment could be used to prevent infection of soybean seeds by *C. truncatum*.

Additional keyword: *Glycine max*.

The polyamines putrescine, spermidine, and spermine participate in controlling the growth and development of both prokaryotic and eukaryotic cells (3,6,16,18). Putrescine is the precursor of the other two polyamines and is synthesized in vivo through two different pathways. Decarboxylation of ornithine by ornithine decarboxylase constitutes the only pathway present in mammalian tissues (6). In higher plants and bacteria, putrescine also can arise from the decarboxylation of arginine by arginine decarboxylase (3,16). Inhibition of either ornithine or arginine decarboxylases is accomplished by the mechanism-based irreversible inhibitors α-difluoromethylornithine (DFMO) or α-difluoromethylarginine (DFMA), respectively (7,10), which deplete intracellular polyamine content. Early work suggested that putrescine synthesis in fungi is governed by the rate-limiting enzyme ornithine decarboxylase (1,11–14,17). A recent report, however, indicates the presence of arginine decarboxylase in a variety of fungi (8). Moreover, both DFMO and DFMA were found to inhibit the growth of a number of pathogenic fungi, suggesting that in these organisms, as in higher plants, both pathways may be functional (8).

Soybean (*Glycine max* (L.) Merr.) is one of the most important crops in the United States, Brazil, and Argentina. Soybean seeds can be attacked by the fungus *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore, which causes a pod disease known as anthracnose. This endemic disease results in infected seeds that either do not germinate or produce crops of reduced quality.

Attempts have been made to protect crop plants from attack by pathogenic fungi through the use of specific inhibitors of polyamine biosynthesis (11,13,19–21). To apply this approach to infected soybean seeds, it is necessary to discriminate between the polyamine metabolism of the host and of the attacking fungus. We have observed that cadaverine (1,5-diaminopentane) is essential for normal germination of soybean seeds, and inhibition of its synthesis by DFMO produced an abnormal rooting of the germinating seeds (5). In contrast, when putrescine biosynthesis was inhibited in soybean seeds by DFMA, no abnormalities were observed up to 2 wk after the onset of germination. Thus, DFMA could have an inhibitory effect on growth of *C. truncatum* without affecting seed germination. DFMA treatment might represent a potential control measure for seeds infected by this pathogen. The present study examines the effect of DFMO and DFMA on polyamine biosynthesis of this pathogenic fungus, and analyzes the changes in its infectivity on soybean seeds.

MATERIALS AND METHODS

Fungal growth. Stock cultures of *C. truncatum* were mantained on potato-dextrose agar. Experimental cultures were started with mycelial plugs taken from the advancing edge of 5- to 8-day-old cultures with a cork borer (4 mm in diameter). One mycelial plug was placed in the center of each petri dish on Czapek-Dox synthetic medium containing 1.5% (w/v) purified agar. Inhibitors, polyamines, and polyamine plus inhibitor solutions were filter-sterilized and added to autoclaved medium cooled to 48 ±2 C. Final concentration of inhibitors ranged from 1 to 6 mM, depending on the inhibitor used. Polyamine treatments consisted of 1 mM putrescine, cadaverine, spermidine, and spermine. Inhibitor plus polyamine treatments consisted of 1–6 mM DFMO or DFMA plus 1 mM of the aforementioned polyamines. All cultures were maintained at 25±1 C in the dark. Growth measurements were taken at 1, 2, 4, and 6 days of incubation by measuring the distance between the new growth and the edge of the original mycelial plug in four directions. For enzyme and polyamine analysis, the fungus was grown in liquid Czapek-Dox synthetic medium. All growth experiments involved six replicates and were repeated at least five times. Student's *t* test was used for means comparison.

Enzyme assay. Activities of ornithine and arginine decarboxylases were assayed as follows. Mycelia grown in liquid culture for 3 days were homogenized in the extraction buffer (0.5 ml/g fresh weight) with glass beads in a prechilled mortar and pestle. The extraction buffer contained 50 mM potassium phosphate buffer (pH 8.0), 0.5 mM pyridoxal phosphate, 5 mM dithioerythritol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1%

polyethylene-glycol (PEG), 0.5 mM EDTA, and 0.2% Brij 35. The homogenate was centrifuged at 20,000 g for 30 min at 4 C, the supernatant (soluble fraction) was separated, and the precipitate was resuspended in the extraction buffer to the original volume (particulate fraction). Alternatively, the homogenates were sonicated before centrifugation. Enzymatic ornithine and arginine decarboxylase activities were assayed in both fractions by measuring the $^{14}CO_2$ released from their respective substrates. The reaction mixture in a final volume of 200 μl contained 100 μl of the supernatant or the resuspended fraction (50 μg of protein) and 50 mM phosphate buffer (pH 8.0), 5 mM dithioerythritol, 0.5 mM pyridoxal phosphate, 1 mM PMSF, 0.5 mM EDTA, 1% PEG, and either 1 mM L-[1-^{14}C]-ornithine (200,000 dpm, 60 mCi/mmol) or 1 mM L-[1-^{14}C]-arginine (250,000 dpm, 55 mCi/mmol). Controls using boiled extract were performed simultaneously. Incubations were run at 37 C and the $^{14}CO_2$ released from the reaction was measured as described elsewhere (5).

Polyamine analysis. Polyamines were extracted following the modified procedure of Dion and Herbst (2). In brief, mycelia were homogenized in ice-cold 5% perchloric acid (5 ml/g fresh weight) with glass beads in a prechilled mortar and pestle. The homogenate was centrifuged at 20,000 g for 20 min at 4 C and the supernatant was used for polyamine analysis using the dansylation method with 2-methylputrescine as an internal standard. The dansylated polyamines were separated on silicagel chromatofoil (Merck) using a two-solvent system, n-hexane/ethyl acetate (2:1)

and chloroform/triethylamine (9:1). The dansylated derivatives were visualized by fluorescence and quantified after elution from the foil using ethyl acetate. Their fluorescence was measured at 365 nm excitation and 510 nm emission. Standard concentration curves of the dansylated putrescine, spermidine, and spermine were simultaneously run for each polyamine analysis.

Infection of the seeds by *C. truncatum*. Soybean seeds were surface sterilized with dilute hypochlorite solution (50 mg of active Cl/ml) for 30 min, washed thoroughly, and dried under sterile conditions in a laminar flow hood. Seeds were separated into six batches and soaked in 20 ml of sterile water, 20 ml of inhibitor solution (1 mM DFMO or DFMA), 20 ml of 1×10^4 spores per milliliter of *C. truncatum* or 20 ml of inhibitor solution plus *C. truncatum* spores. All treatments were made under intermittent vacuum for 15 min. After inoculation, the seeds were placed in petri dishes and incubated for 3 days at 25 C in the dark. Each experiment consisted of six plates containing eight seeds each and was repeated at least three times. Inoculation under this condition yielded a high percentage of infected seeds.

RESULTS

Effect of DFMO and DFMA on growth of *C. truncatum*. To analyze the effect of DFMO and DFMA on *C. truncatum* growth, the fungus was cultivated in the presence of inhibitors in solid medium. After 2 days of treatment, both compounds drastically reduced mycelial growth (Fig. 1A). Culture of the fungus with increasing concentrations of the inhibitors indicated that both chemicals produced similar dose-dependent inhibitory responses (Fig. 1B).

If the inhibition of growth induced by both inhibitors is due to their interference with normal polyamine biosynthesis, it would be possible to compensate for their detrimental effect by the addition of exogenous polyamines. As anticipated, the effect of the inhibitors on mycelial growth was completely reversed by the addition of 1 mM putrescine or spermidine but not by the addition
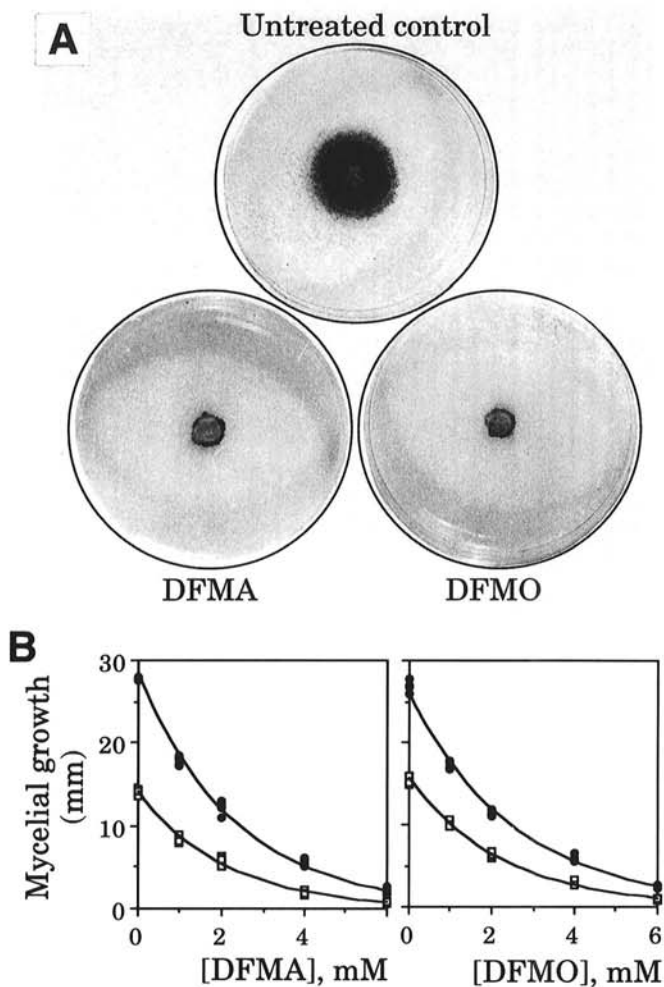
Fig. 1. Effect of α-difluoromethylornithine (DFMO) or α-difluoromethylarginine (DFMA) on radial growth of *Colletotrichum truncatum*. **A,** Fungus was grown for 6 days on solid medium. Inhibitors were added at 4 mM (final concentration). **B,** Effect of increasing concentrations of DFMO and DFMA. Fungal radial growth was determined at 4 days (□) and 6 days (●). Data were adjusted using a nonlinear regression program ($R^2 = 0.98–0.99$).
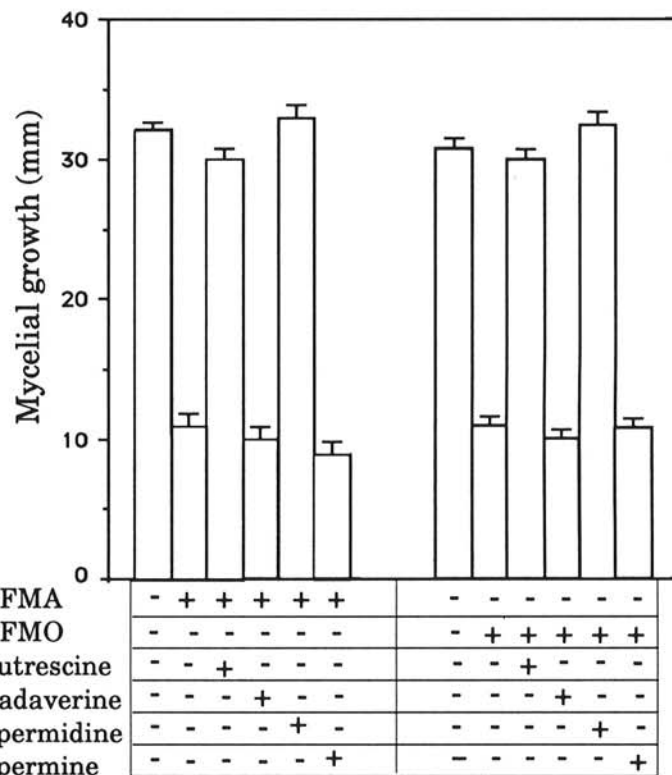
Fig. 2. Effect of polyamines on inhibition of mycelial growth of *Colletotrichum truncatum* by α-difluoromethylornithine (DFMO) and α-difluoromethylarginine (DFMA). Polyamines were used at 1 mM; DFMO and DFMA were used at 2 mM. Fungal radial growth was measured at 6 days. Bars indicate standard deviation of the mean.

of either 1 mM cadaverine or spermine (Fig. 2). None of these diamines and polyamines by themselves affected the growth of the fungus (data not shown). The reversal of inhibition by putrescine and spermidine was already visible within the first 2 days after their addition.

The above results suggest that the negative effect on growth produced by DFMO and DFMA is a direct consequence of the depletion of endogenous polyamine pools. To confirm this hypothesis, we measured the concentration of the polyamines putrescine, spermidine, and spermine in a culture extract of *C. truncatum* grown for 8 days in liquid medium, in the absence or presence of the inhibitors. As shown in Table 1, spermidine was the most abundant polyamine, with a concentration approximately 10 times higher than that of putrescine. Treatment of *C. truncatum* with DFMO and DFMA, at a concentration that inhibited fungal growth, reduced the polyamine levels to 20–50% of the untreated control (Table 1). Because putrescine reversed the inhibition of mycelial growth (Fig. 2), pool concentrations of polyamines were also examined in culture extracts of the fungus grown in the presence of putrescine. This diamine appears to be taken up very efficiently as putrescine levels increased markedly. Added putrescine was presumably converted into spermidine, since spermidine levels also increased. In contrast, the spermine pool was not affected by putrescine addition (Table 1). Addition of putrescine to either DFMO- or DFMA-treated fungi cultures similarly produced a marked increase in the intracellular putrescine and spermidine pools. In this case, polyamine concentrations reached levels similar to those observed in controls incubated with no inhibitor (Table 1). Thus, reversal of the inhibitory effect by the addition of putrescine to the fungal culture correlates with its efficacy in restoring intracellular pools of polyamines.

Ornithine and arginine decarboxylases activities. As mentioned before, the intracellular levels of putrescine, spermidine, and spermine are determined by the activity of either ornithine or arginine decarboxylases, or both. Because DFMO and DFMA have such a profound effect on fungal polyamine content, we assayed the effect of these compounds directly on the enzymatic activity of the fungal decarboxylases.

Decarboxylase activities were examined in a soluble and a particulate fraction extracted from 3-day-old mycelial culture. A DFMO-sensitive ornithine decarboxylase activity was detected only in the soluble fraction, whereas arginine decarboxylase was found in both the soluble and the particulate fractions (Table 2). Arginine decarboxylase is sensitive to DFMA but the particulate fraction activity appears to be inhibited more strongly than the soluble fraction. Because the activity was measured using $[1-^{14}C]$-arginine, this decarboxylation could not be attributed to urease activity contained in the extracts. The fact that *C. truncatum* contained arginine decarboxylase activity in addition to ornithine decarboxylase activity may explain the similar inhibitory efficacy observed for DFMO and DFMA treatment.

Effect of DFMA and DFMO on seed infection. We have shown that inhibitors of polyamine biosynthesis can prevent fungal

growth in liquid and agar media. We were interested, however, in the effect that such inhibitors might have on fungi growing on soybean seeds, their natural host. Soybean seeds were inoculated with *C. truncatum* in the absence or presence of DFMA or DFMO. Three days after inoculation, fungal growth (acervuli borne on small developed stromata) was observed as gray spots on seeds cultivated without inhibitor (Fig. 3A). As expected, DFMA and DFMO treatment drastically reduced fungal growth (Fig. 3A and B). The DFMA treatment induced no changes in

TABLE 2. Activities of ornithine and arginine decarboxylases and their in-vitro inhibition by DFMO and DFMA

Enzyme[a]	Activity[b] nmol $^{14}CO_2$ h^{-1} (mg prot)$^{-1}$					
	Soluble			Particulate		
	Control	DFMO[c]	DFMA[c]	Control	DFMO	DFMA
ODC	10.0 ± 0.2	2.0 ± 0.1	10.5 ± 0.3	ND[d]	ND	ND
ADC	4.0 ± 0.2	4.0 ± 0.2	3.2 ± 0.2	3.6 ± 0.3	3.6 ± 0.3	1.8 ± 0.2

[a]ODC = ornithine decarboxylase, ADC = arginine decarboxylase.
[b]Enzyme activities were determined as described in Materials and Methods. The inhibitors were used at 1 mM. Data are the mean ± SD of four experiments each with three replications.
[c]DFMO = α-difluoromethylornithine; DFMA = α-difluoromethyl-arginine.
[d]Not detectable.

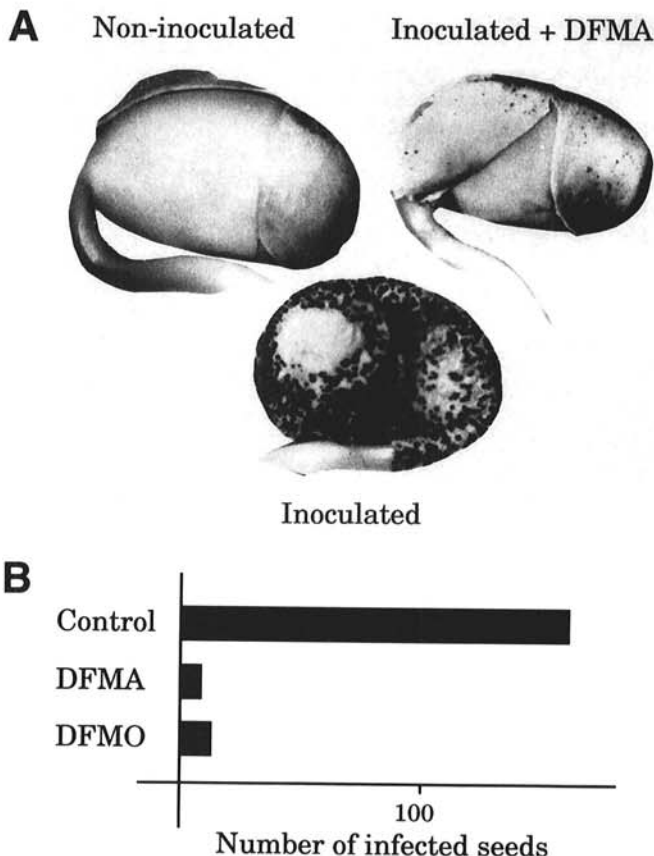**A** Non-inoculated Inoculated + DFMA

Inoculated

B

Number of infected seeds

Fig. 3. Protection by α-difluoromethylornithine (DFMO) and α-difluoromethylarginine (DFMA) of soybean seeds inoculated with *Colletotrichum truncatum*. **A,** Seeds were noninoculated, inoculated, or inoculated in the presence of DFMA (2 mM) with fungal spores and incubated for 3 days. **B,** Quantification of infected seeds under different treatments. All seeds were inoculated as described in Materials and Methods in the absence (Control) or presence of DFMO or DFMA. Seeds were considered infected if fungal growth covered more than 50% of the seed as shown in **A** ("Inoculated").

TABLE 1. Effect of DFMO and DFMA on the cellular concentrations of polyamines in 8-day-old cultures of *Colletotrichum truncatum*

Treatment[a]	Putrescine	Spermidine	Spermine
Control	6.0 ± 0.3[b]	58.0 ± 2.0[b]	3.5 ± 0.2[b]
DFMO	1.8 ± 0.2	16.0 ± 1.0	1.8 ± 0.2
DFMA	1.6 ± 0.2	18.0 ± 1.3	1.9 ± 0.2
Putrescine	85.0 ± 5.0	175.0 ± 8.0	3.6 ± 0.2
DFMA + Putrescine	79.0 ± 5.0	162.0 ± 8.0	3.4 ± 0.2
Control*	11.5 ± 0.5	56.0 ± 2.0	3.5 ± 0.2

[a]DFMO = α-difluoromethylornithine; DFMA = α-difluoromethyl-arginine. DFMO and DFMA were used at 2 mM and putrescine at 1mM. Control* = *C. truncatum* grown under control conditions with 1 mM putrescine added just before polyamine extraction.
[b]Data are the mean ± SD of three different experiments each with three replications. Values are nmol/g fresh weight.

the germination process of the seeds compared with noninoculated, nontreated controls, whereas DFMO treatment produced an abnormal elongation of the roots (data not shown).

DISCUSSION

Polyamines are essential for cell growth and proliferation (6); thus, a number of compounds that selectively inhibit polyamine biosynthesis have been tested to suppress pathogenic infection in plants (11,13,19–21). In fact, previous studies on fungal plant pathogens suggest that specific inhibition of polyamine biosynthesis could be an effective method for disease control. In previous studies, DFMO was shown to reduce the growth of a number of plant pathogenic fungi in vitro (1,11–14,17) while exerting no apparent effect on the host's physiology. This was attributed to the fact that plants have an alternative pathway for putrescine biosynthesis, via arginine decarboxylase, which is not inhibited by DFMO (3,16). Inhibition of the mycelial growth of some pathogenic fungi by DFMA has also been observed. This effect could be due to either the conversion of DFMA into DFMO by arginase activity present in the fungal cells (15), or a direct inhibition of arginine decarboxylase by DFMA (8).

Interestingly, *C. truncatum* has a soluble (cytosolic) and a particulate arginine decarboxylase, but no particulate ornithine decarboxylase could be detected. Cytosolic and nuclear ornithine decarboxylases have been reported in the phytopathogenic fungus *Pyrenophora avenae* Ito & Kuribayashi (4) and in germinating barley seeds (9), but to our knowledge there have been no previous literature reports as to the existence of a particulate arginine decarboxylase. It is conceivable that these two enzymes play distinct, noninterchangeable roles during fungal growth.

Because DFMO affects germination of soybean seeds, its applicability is of limited value in disease suppression (5). In contrast, DFMA does not present such a detrimental effect on seed germination, suggesting that this compound has broader potential. Thus, understanding the polyamines biosynthesis in *C. truncatum* and DFMA action acquires an additional importance. Growth of *C. truncatum* in vitro was found to be inhibited by both DFMO and DFMA. This effect was reversed by the addition of putrescine or spermidine, suggesting a direct action of these inhibitors on the polyamine biosynthetic enzymes.

Previous studies suggest that growth sensitivity of phytopathogenic fungi to DFMO and DFMA varies widely (8,11,17) and cannot always be taken as a reliable indicator of their efficacy for disease control (11,13). The results reported here show that the sensitivity to polyamine inhibitors has to be evaluated for each host and its fungal pathogen before deciding which could be the most efficacious method of disease control. It is evident, however, that interference with polyamine biosynthesis in the phytopathogenic fungi is a useful method for controlling their growth.

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