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Effect of α-DL-Diflouromethylornithine on *Aecidium mori* Barclay and Its Efficacy for Controlling Mulberry Red Rust

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ABSTRACT

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The effects of α-DL-diflouromethylornithine (DFMO) on the aeciospore germination of *Aecidium mori* and its infection on mulberry (*Morus alba* var. *atropurpurea* cv. Taishan 3) plants were investigated. The presence of DFMO was found to be greatly inhibitory to both spore germination and germ tube development of the test fungus at a millimolar level. Complete inhibition to the spore germination was observed with the addition of DFMO at 5 mM; which was comparable to that by the addition of the officially registered fungicide Plantvax at 4000 X dilution. The amendment of 1 mM putrescine significantly alleviated the inhibitory effect of 2 to 5 mM DFMO, indicating the observed inhibitory effect was due to the specific inhibition of ornithine decarboxylase and the importance of this polyamine compound in the aeciospore germination. In greenhouse and field trials performed, a weekly application of 1 to 5 mM DFMO was found to be of greatly inhibitory to the red rust infection. The disease control efficacy of 5 mM DFMO application was comparable to that by the two commercially available fungicides Plantvax and Saprol. The DFMO treatment didnot show any observable deleterious effect on the mulberry plants; and the treated plant parts were found to be safe to feed the silkworms even right after treatment. The potential value of this target specific, low toxicity chemical in the control of mulberry red rust disease deserves great attention.

Key words: *Aecidium mori*, aeciospore germination, DFMO, disease control, mulberry red rust, polyamine biosynthesis

INTRODUCTION

In Taiwan, Mulberry red rust caused by *Aecidium mori* Barclay is one of the most important diseases for mulberry cultivation and silkworm rearing. The disease is generally prevailing during the cool season while mulberry leaves are in great demand for silkworm rearing (6,7,18). The pathogen affects mulberry plant mainly on new buds, young leaves, new shoots, petioles, catkins or even fruits (6,8). During the past decade, the disease caused quite substantial yield losses in mulberry tender shoot production every year; it thus appeared to be a major limiting factor for silkworm rearing industry (7). In Taiwan, for the disease control in the field, Plantvax (5,6-Dihydro-2-methyl-1, 4-oxathiin-3-carboxanilide-4, 4-dioxide; 75% wettable powder) is the main officially registered fungicide (1). However, in many cases, its efficacy of disease control was not satisfactory; and the pesticide residue used to be a concerned problem for silkworm rearing.

Polyamines are known to be ubiquitous small molecular weight organic cations among most living cells and are essential for their growth and development (5,11,16,17). It has been well recognized that living cells produce polyamines mainly via the arginine decarboxylase (ADC)-and/or the ornithine decarboxylase (ODC)-mediated pathway. It was also noted that higher plants and bacteria had both ADC and ODC activities; whereas mammalian and lower eukaryotes including many fungi had only the later ones (2,3,5,10,17). α-DL-diflouromethylornithine (DFMO) is a specific and irreversible inhibitor of ODC. In medical science, DFMO has been successfully used in clinical application as a curative agent for controlling protozol infections (8,15). For the control of plant fungal pathogens, it was already noted by several workers that presence of DFMO was of greatly inhibitory to the growth and development of quite a few phytopathogenic fungi including *Botrytis spp., Rhizoctonia solani, Monilinia fructicola,* and *Biopolaris maydis,*

indicating a possibility of application of this enzyme-specific inhibitor in the related disease control (4,9,12). Rajam *et al.* (13,14) recently demonstrated the potential value of using DFMO as an agent for controlling bean plants from uredospore infection by the rust fungus *Uromyces phaseoli*. Since DFMO appeared to be remarkably non-toxic and well tolerated by animal cells, its application would not likely endanger our living environments including silkworm. In this study, the effects of DFMO on aeciospore infection on mulberry were investigated; the potential of using it as a control agent for mulberry red rust disease is herein discussed.

MATERIALS AND METHODS

Biological materials

Aeciospores of *Aecidium mori* were originally collected from infected leaves of Taishan 3 cultivar (*Morus alba* var. *atropurpurea*) in a trial field located at Miaoli County in November 1986. They were adjusted to approximately 10^5 spores/ml in concentration with sterile distilled water and used as inoculum to paint-inoculate (18) Taishan 3 mulberry plants which were grown on sandy loam in a greenhouse in Taiwan Apicultural and Sericultural Experiment Station (TASES) at Kung-Kuan. Approximately 2 weeks after inoculation, aeciospores were collected from a single pustule developed on the inoculated leaves. These single pustule-derived fungal propagules were then maintained and propagated on Taishan 3 cultivar plants by repeated mass inoculation in the greenhouse for the remaining of the investigation. For all the experiments performed, aeciospores produced on the mulberry leaves 2 to 4 weeks after the artificial inoculation were used as test inocula.

Chemicals

DFMO was kindly provided by Dr. Ekkehard H. W. Bohme of Merrell Dow Research Institute (Cincinnati, Ohio, U.S.A.). Plantvax, and Saprol (N, N-[1,4-Piperazinediyl-bis-(2,2,2-trichloroethylidene)-bis] foramide, 18.6% E. C.) were purchased from local pesticide distributor. Putrescine was obtained from Sigma Chemical Co. (St. Louis, MO U.S.A.); and Bacto Agar was from Difco Laboratory (Detroit, MI, U.S.A.). All other chemicals used were obtained from Merck Chemical Co. (Germany). Unless specified, glass double distilled water was freshly prepared and used for the preparation of test chemicals and spore suspensions.

Effect of DFMO on aeciospore germination

DFMO at 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, and 5.0 mM in concentration were incorporated into 3% Bacto water agar (Difco) right before pouring plates. Plantvax at 4000 X dilution and Saprol at 750 X dilution as that suggested by the Technical Bulletin of mulberry protection (1) were included as the control chemicals; and the untreated controls contained only sterile distilled water. Approximately 100 μl of aeciospore suspension at 2×5^5 spores/ml in concentration (with 100 ppm Tween 80 as the dispersing agent) were smeared evenly onto each 9 cm agar plate containing test chemicals and incubated at 24 C. The rate of spore germination was then examined by an Olympus BH-2 phase contrast light microscope.

Effect of putrescine on inhibitory effect of DFMO to spore germination

On thoroughly cleaned and sterilized glass slides, approximately 10 μl aeciospore suspension at 2×10^5 spores/ml were mixed with equal volume of 0.2, 0.4, 1.0, 2.0, 4.0 and 10.0 mM DFMO solution respectively. To one set of these test slides, 1 mM of putrescine was included in test spore suspension. The test slides were then set in Petri plate moist chambers and incubated in a 14–18 C incubation room. The spore germination and germ tube development were then examined as above described.

Effect of DFMO on red rust infection

New shoots of pot grown Taishan 3 mulberry plants in greenhouse were spray treated with 0.5, 1.0, 2.0, and 5.0 mM DFMO solutions, respectively, until run-off. Control plants were treated likewise with only water. About 3 days after the spray application, the test plants were artificially paint-inoculated with freshly collected aeciospore suspension as above described. Percent leaf colonization of the top 5 fully expanded leaves were scored 2 to 3 weeks after inoculation. And disease severity was calculated by the following equation:

$$\text{Disease Severity} = \frac{n_1 \times 1 + n_1 \times 2 + n_3 \times 3 + n_4 \times 4 + n_5 \times 5}{5 \times \text{total leaf numbers.}} \times 100\%$$

wherein n_1 = numbers of leaves with 1 to 10% rust colonization,

n_2 = numbers of leaves with 11 to 20% rust colonization,

n_3 = numbers of leaves with 21 to 30% rust colonization,

n_4 = numbers of leaves with 31 to 50% rust colonization,

and n_5 = numbers of leaves with more than 50% rust colonization.

In addition to greenhouse trials, the efficacy of DFMO application was also tested in a field plot at TASES. During April to May of 1988, about the onset of the seasonal disease epidemic, new shoots of Taishan 3 mulberry plants were spray-treated with 0.5, 1.0, 2.0, and 5.0 mM DFMO solution twice at 10-day intervals.

The control plants were sprayed with only water. About 8 days after second application, disease severities of test plants due to natural infection were scored as that in greenhouse trials.

Effect of DFMO application on silkworm rearing

One set of field grown mulberry plants which were spray treated with DFMO solution as above described were harvested and utilized for daily silkworm rearing from the second day after chemical application. For each treatment concentration, a total of 200 heads of Kou-Fu × Nom-Fon Hybrid silkworms (50 per replicate) at their first larval stages were used. Upon cocoon forming stage, the cocoon forming uniformity, percentages of healthy pupae, total cocoon weight, average weight of unit cocoon shell and percentages of cocoon shells were scored.

RESULTS

Effect of DFMO on aeciospore germination

The inhibitory effect of DFMO to aeciospore germination of the test fungus was evidently dependent on the concentration applied. As shown in Figure 1, the rate of spore germination of control treatment on water agar was approximately 47%, whereas that of compared 0.5 mM DFMO treated ones was only about 17%. The application of Saprol at 750 X in dilution completely inhibited the aeciospore germination. DFMO at 5 mM in concentration, although didnot show 100% inhibition, reduced the rate of germination to approximately 8% which was about the same level as that by Plantvax 4000 X treatment. The rate of germination of test aeciospores in distilled water appeared to be somewhat less than that on water agar (Figs. 1 and 2A); and similary to that on water agar, the inhibitory effect of DFMO was evidently a function of applied concentration (Fig. 2A). At 5 mM in concentration, DFMO nearly completely inhibited the aeciospore germination. Whereas with the addition of 1 mM putrescine, the inhibitory effect of DFMO appeared to be slightly alleviated (Fig. 2B). The presence of DFMO also showed a concentration dependent inhibitory effect on germ tube development of the test fungus (Figs. 3A and 3B). At 32 hours after incubation, the average length of germ tubes of 0.5 mM DFMO treated aeciospores has reached only about 50% of the compared control treatment. The increase of DFMO concentration to 2 mM then completely inhibited the germ tube growth and development (Fig. 3A). This inhibitory effect, however, was obviously alleviated with the addition of 1 mM of putrescine (Fig. 3B).

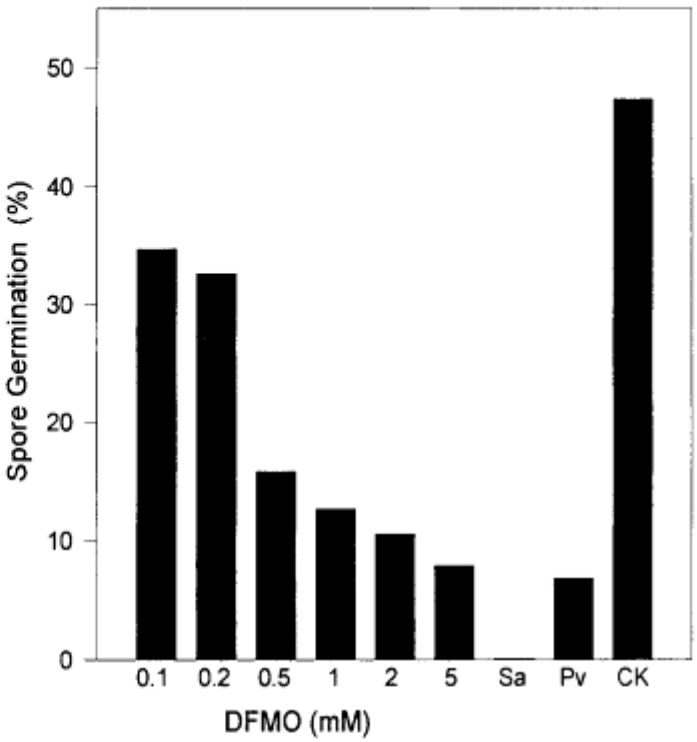

Fig. 1. Effect of α-DL-diflouromethylornithine (DFMO), Plantvax (Pv, 4000 X in dilution), and Saprol (Sa, 700 X in dilution) treatment on the aceiospore germination of *Aecidium mori* Barclay. The test chemicals at indicated concentrations were added to the agar medium aseptically before pouring plates. Data shown were average spore germination of 4 replicates determined 32 hours after incubation.

Effect of DFMO on red rust infection

In greenhouse trial, spray application of DFMO at the test range of concentration 3 days before artificial inoculation all greatly reduced the infection of test fungus (Fig. 4). The disease severity index of 0.5 mM DFMO treated plants about 2 weeks after inoculation was approximately 20%, which was only about one third of that of the compared control plants. The inhibitory effect of DFMO on the fungal infection then became more and more prominent with the increased treatment dosage. The application at 5 mM in concentration completely inhibited the red rust infection as that by a compared Plantvax application (at 4000 X in dilution, data not shown).

The efficacy of DFMO in red rust control in the field was similary to that of greenhouse trial. The survey of disease severity 8 days after second DFMO application showed that the application at 1 to 2 mM range led to a reduction of disease severity to approximately 30% of that of the compared control plants (Fig. 5). While applied at 5 mM in concentration,

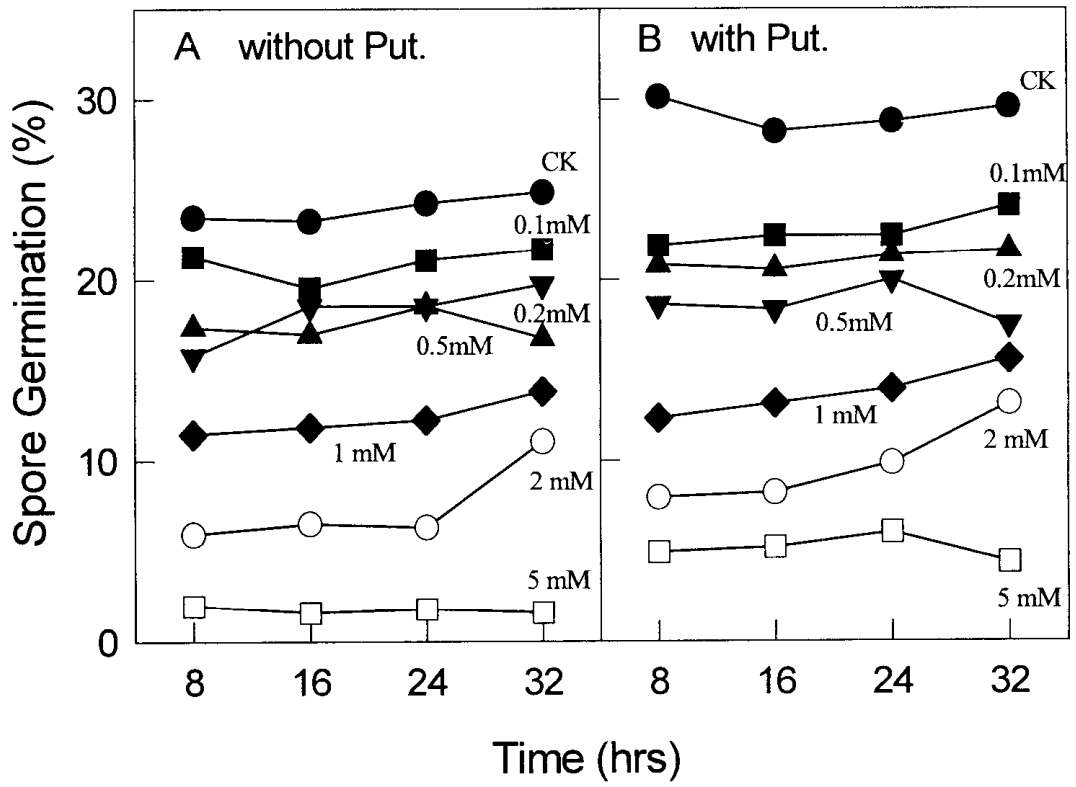

Fig. 2. Effect of putrescine (1 mM) amendment on the inhibitory effect of α-DL-diflouromethylornithine (DFMO) on aeciospore germination of *Aecidium mori* Barclay. DFMO at indicated concentrations were added to the agar medium, with (B) or without (A) putrescine (Put.) amendment, aseptically before pouring plates. Data shown were average germination rate of 4 replicates, determined at the time indicated.

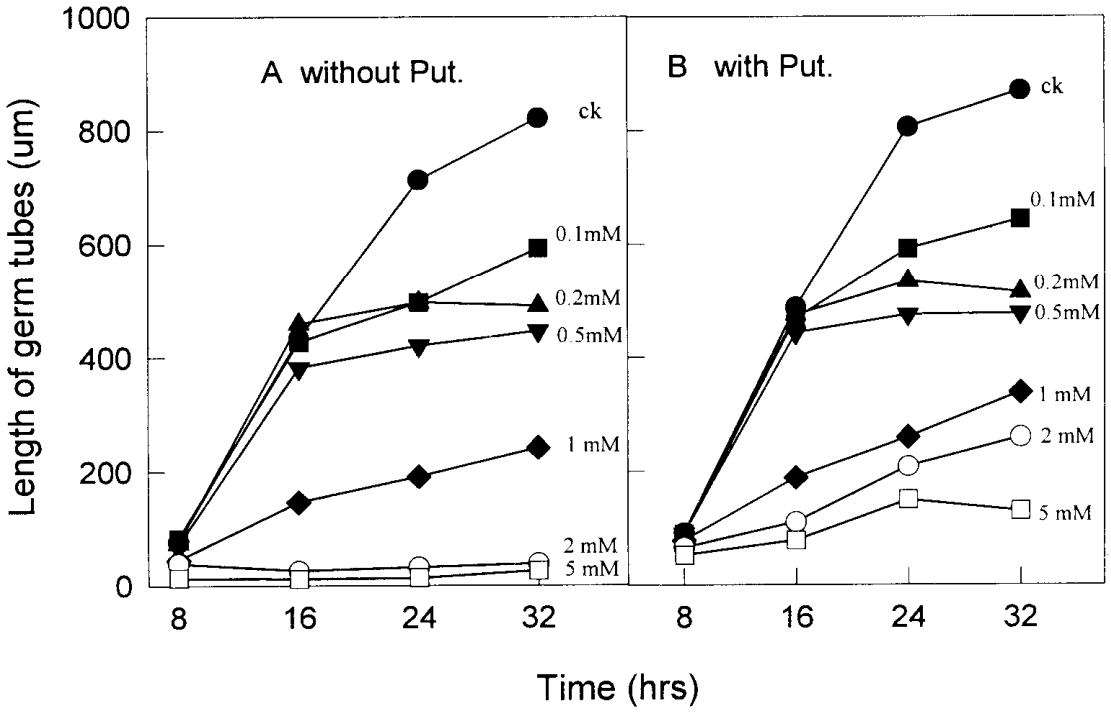

Fig. 3. Effect of putrescine (1 mM) amendment on the inhibitory effect of α-DL-diflouromethylornithine (DFMO) on aeciospore germ tube development of *Aecidium mori* Barclay. DFMO at indicated concentrations were added to the agar medium, with (B) or without (A) putrescine (Put.) amendment, aseptically before pouring plates. Data shown were average germ tube length of 4 replicates, determined at the time indicated.

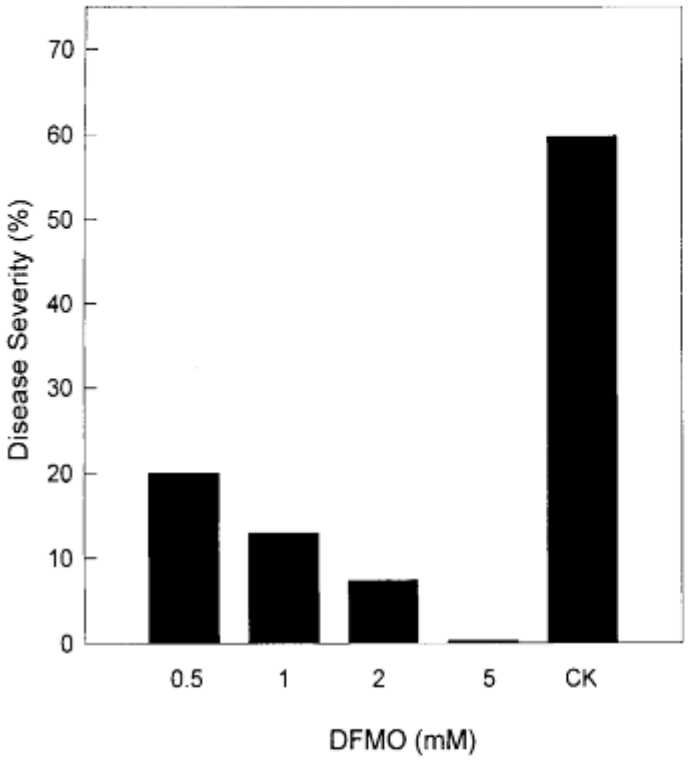

Fig. 4. Effect of spray application of α-DL-diflouromethylornithine (DFMO) on the infection of *Aecidium mori* Barclay on mulberry (cv. Taishan 3) plants grown in a greenhouse. The test plants were sprayed with the test chemicals 3 days before artificial paint-inoculation with aeciospore suspension as that described in the text. The control plants were treated with only water. Data shown were average disease severity indexes determined 2 weeks after inoculation.

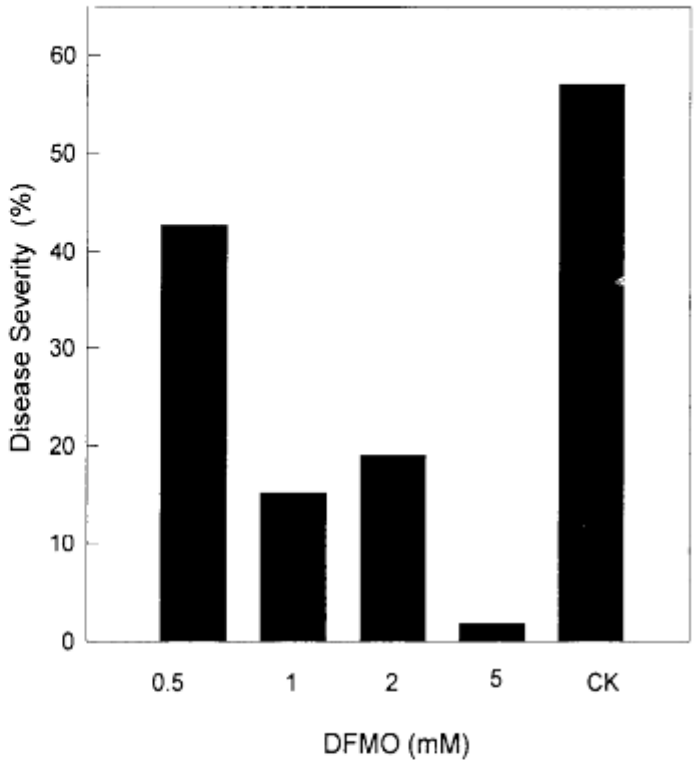

Fig. 5. Effect of α-DL-diflouromethylornithine (DFMO) spray treatment on the infection of *Aecidium mori* Barclay on mulberry plants (cv. Taishan 3) grown in a trial field. The test plants were spray treated twice with DFMO solution (at 10 days interval) at the indicated concentrations. The control plants (CK) were treated with only water. Data shown were average disease severity index of test plants determined 8 days after the second spray.

DFMO appeared to inhibit the fungal infection nearly completely. The disease control efficacy by 5 mM DFMO application was not significantly different to that by the compared Plantvax application (data not shown).

Effect of DFMO application on silkworm rearing

Throughout the experimental period, no significant changes of feeding habits were ever observed with the DFMO treated leaf samples. Upon the end of silk worm rearing stage, the cocoons were harvested and the quality and yield were determined. The appearance of the cocoons and the pupae harvested from DFMO-treatments were essentially not different as compared to that harvested from the control treatment (data not shown). And as that shown in Table 1, the total cocoon weight, the average weight of whole cocoon, and the average weight of unit cocoon shell of silkworms fed with DFMO treated mulberry leaves were not significantly different from that of the compared control treatment.

DISCUSSION

Periodical chemical treatment for the control of red rust disease is an essential cultural practice for mulberry cultivation in Taiwan because of the prevailence of the disease and the lack of suitable resistant cultivars (6,7,18). Without chemical application, the yield loss due to severe red rust infection was generally quite substantial as regards to the supply of tender mulberry shoots for silkworm rearing. A major problem associated with this chemical control activity was the deleterious effect on silkworm rearing due to chemical residues. To the fungicides currently available for this purpose, a 5 to 10 days safety period was generally recommended after the chemical application (1). For certain chemicals, e.g. Bordeaux mixture, an even longer safety period (about 20 days) was required. Since young shoots of mulberry plant were needed in great amounts for the daily consumption by silkworm rearing, the need of long safety period was always a puzzled problem for the growers. A most commonly

TABLE 1. Effect of α-DL-diflouromethylornithine (DFMO) application on silkworm rearing as examplified by the yield and quality of cocoon production

Treeatment concentration	Total cocoon weight(g)	Average cocoon weight(g)	Average weight of unit cocoon shell
5 mM	114 ± 10.78	2.32 ± 0.05	49 ± 0.96
2 mM	104 ± 3.37	2.26 ± 0.04	47 ± 0.5
1 mM	94 ± 20.7	2.32 ± 0.01	48 ± 0.96
0.5 mM	108 ± 7.62	2.32 ± 0.04	49 ± 0.96
CK	111 ± 3.77	2.34 ± 0.04	50 ± 0.96

observed deleterious effect on silkworms due to the residual chemical toxicity was the reduced feeding activities. The affected silkworm might stop moving, shrinkage in size, and unable to molting (7). In some cases, they might develop deadly diarrhea. A highly target-specific, silkworm-safe fungicide is urgently in need.

The inhibitory effectivity of DFMO in spore germination and infection of the red rust fungus shown in this study has clearly demonstrated its potential value as a pesticide for the disease control. The effectiveness of DFMO application for the disease control was apparently comparable to that by Plantvax or Saprol-the commercially available fungicide officially recommended. However, unlike the two traditional fungicides, DFMO is known to be rather biologically safe and well tolerated by animals. In medical science, DFMO is well known in its application as an curative agent for the remedy of African trypanomiasis (or sleeping sickness)(8,15). Enzymologically it is well recognized as a target-specific, suicidal inhibitor for ornithine decarboxylase (ODC), one of the two key enzymes known for putrescine biosynthesis (5,11,17). Another key enzyme involved in putrescine biosynthesis is arginine decarboxylase (ADC). The inhibitory effect of DFMO on aeciospore germination and the germ tube development indicated the essentialty of ODC in these biological activities. The alleviating effect of putrescine supplementation further implicated the need of putrescine and the dependence of the test fungus on ODC for the polyamine biosynthesis. The dependence on ODC and the lack of ADC seemed to be common among phytopathogenic fungi (4,10,12,13). In contrast to the test fungus, for mulberry plant and silkworm, the application of DFMO appeared to be well tolerated. In both greenhouse as well as field experiment proceeded, no observable deleterious effects of test plants were ever detected due to the DFMO application. For higher plants, both ODC-and ADC-mediated putrescine biosynthetic pathways were known to be functioning (3). In fact, in certain plant species, an ADC dominated pathway has been reported. The lack of deleterious effect of DFMO application on silkworm growth and cocoon production (Table 1) further demonstrated the superiority of its practical value. During the course of this experiment, mulberry leaves sprayed with DFMO was used to feed some first stage silkworm larvae even right after the chemical treatment, the test silkworms all appeared to be normal (data not shown). The use of DFMO as an environmentally safe fungicide for the control of mulberry red rust is greatly recommended.

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摘　　要

林洋三[1]、曾德賜[2]．1994．二氟甲基鳥胺酸(α-DL-diflouromethylornithine)對 *Aecidium mori*之影響及其於桑赤銹病之防治效果．植病會刊3:168-174．(1. 苗栗縣公館　蠶蜂業改良場，2. 臺中市　國立中興大學植物病理學系)

本研究旨在探討二氟甲基鳥胺酸(α-DL-diflouromethylornithine, DFMO)對 *Aecidium mori* Barclay 春孢子發芽之影響及其於桑樹(臺桑三號栽培品種，*Morus alba var. atropurpurea*)赤銹病防治之應用效果；DFMO的存在，只要有毫莫耳(mM)濃度程度，與 *A. mori*感染有關的春孢子發芽以及發芽管之發育作用均即很明顯受到抑制；5 mM DFMO之添加可完全抑制春孢子之發芽作用，其抑制效果與市售桑赤銹病防治用殺菌劑 Plantvax 稀釋4000倍濃度之效果相當。處理DFMO時同時添加1 mM Putrescine可以明顯減輕DFMO之發芽抑制作用，顯示其效果確係由於鳥胺酸脫羧酵素(Ornithine decarboxylase)之抑制作用，且多胺類化合物之生合成與測試菌之發芽作用顯有極密切之關係；於溫室及田間之病害防治試驗中發現，每週一次噴灑1-5 mM之DFMO可以顯著降低赤銹病菌之感染作用，其中5 mM DFMO之噴灑處理，其對病害之防治效果較之市售Plantvax與Saprol之防治效果並不遜色，DFMO之噴灑處理，其對桑樹之生長發育均未見有明顯影響，且處理植株即使在噴藥後立即剪採桑條作爲飼育之用，對家蠶之取食、發育、與結繭等正常生理功能，均未見有不利之影響，此一作用機制專一之低毒、安全性藥劑，其在桑赤銹病防治上之應用價值甚值得有關從業人員之注意。

關鍵詞：赤銹病菌(*Aecidium mori*)、春孢子發芽作用、二氟甲基鳥胺酸(DFMO)、病害防治、桑樹赤銹病。