Effect of Thiophanate-methyl on the Infection Process of Some Plant Pathogenic Fungi

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安田 康*・中田 昭*・野口照久*: 数種の植物病原菌の感染
過程におよぼすチオフアネートメチルの作用

Abstract

Effect of a new fungicide, thiophanate-methyl (Th-M) on the infection process of three fungi, *Pyricularia oryzae*, *Colletotrichum lagenarium* and *Sphaerotheca fuliginea* was investigated microscopically.

Th-M inhibited almost completely the development of hyphae of *P. oryzae* and *C. lagenarium* in host cells, whereas spore germination and appressorium formation were scarcely inhibited on the host treated with 500 ppm Th-M. The growth of germ tube of *S. fuliginea* spore was also inhibited on 50 ppm Th-M-treated cucumber leaves. Moreover, the swelling or breakage of spore and the distortion of germ tube of *P. oryzae* spore when directly contacted with 500 ppm Th-M. On the other hand, the rice leaf sheath cells treated with Th-M revealed normal convex plasmolysis and the epidermal cell wall of treated cucumber leaves never showed morphological abnormality.

Methyl 2-benzimidazolecarbamate (MBC), a metabolite of Th-M also inhibited the development of invaded hyphae, but no difference between the effects of Th-M and MBC was observed in the infection process. It could be concluded that these acted characteristically rather in the process of penetration than in spore germination and appressorium formation on host cells.

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Introduction

A new systemic fungicide, thiophanate-methyl (Th-M), dimethyl 4, 4'-*o*-phenylenebis (3-thioalophanate) has been recently found to be effective for the control of numerous fungal diseases of plants and its efficacy was curative as well as protective^{3,4,5}. Th-M was reported to be converted into methyl 2-benzimidazolecarbamate (MBC) in plants^{6,8}, animals⁶ and fungi¹⁰. The mechanism of antifungal action of Th-M has been partially studied in connection with research of its metabolism^{10,12}, and it was found that MBC has more effective antifungal activity than that of Th-M. This paper reports the result of experiments on the effect of Th-M and MBC on the infection process of some phytopathogenic fungi, causing the rice blast disease, the anthracnose and the powdery mildew of cucumber.

Materials and methods

Hosts, inocula and inoculation *Pyricularia oryzae* Cav. was cultured on an oatmeal agar medium at 25 C for 3 weeks. Five days after washing away old spores, newly formed ones on the medium were used as inocula. Inner epidermal tissues of rice leaf sheath (*Oryza sativa* L. cv.

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Kinmaze), about 7 cm in length were inoculated with spore suspension ($10^6-2 \times 10^6$ spores/ml) by Sakamoto's sheath inoculation method⁷⁾, and incubated in Petri dish for given hours at 23-25°C. *Colletotrichum lagenarium* (Pass.) Ellis et Halsted was cultured on a potato sucrose agar at 24°C for 6 days. The spore suspension (10^6-10^7 spores/ml) was dropped on the upper or lower epidermis of cucumber leaves (*Cucumis sativus* L. cv. Sagamihanjiro) treated with Th-M or MBC. The spores of *Sphaerotheca fuliginea* (Schlechtendahl) Pollacci which were obtained from heavily diseased cucumber leaves were dusted by using a hair brush on the lower surface of Th-M-injected leaves. The cucumber plants were incubated for given hours at 25°C.

Application of fungicide Rice seedlings, five leaf stage were treated with 500 ppm fungicides for 2 days by infiltration through their roots. Intact leaves of cucumber seedlings, one leaf stage were treated with 50 ppm Th-M by injection using an injector to tissues beneath the lower epidermal cell layer. In another experiments, 500 ppm Th-M or MBC was sprayed on leaves of seedlings or poured into soil in pot.

Observation The inner epidermis with 2-3 cell layers of rice leaf sheath or the lower epidermis of cucumber leaves was peeled away by using a razor blade and observed under a microscope. For the observation of invaded hyphae of *C. lagenarium*, the pieces of cucumber leaves were fixed in formalin-acetic acid-ethanol solution and dehydrated through ethanol-butanol series solution. They were embedded in paraffin, then sectioned in 10 μ m width. Sections were stained in hematoxyline and eosine, gentian violet and acid fuchsine solution. For germination test, collodion solution was dropped on the inoculated leaves and the dried collodion membrane was peeled away from the surface and stained with cotton blue. Plasmolysis in rice leaf sheath cells was observed after staining with 0.2% neutral red solution and dipping in 1 M KNO₃ solution.

Results

The germinated spore of *P. oryzae* formed appressorium on the surface of inner leaf sheath not treated with fungicide and also vegetative hypha developed in the invaded cells. Thereafter, the leaf sheath cells were occupied with hypha which further invaded into neighboring cells about 54 hours after inoculation (Plate I, 1). In treated sheath, it was observed that Th-M and MBC suppressed the formation of infection hypha and almost completely inhibited the growth of invading hypha (Plate I, 2), although spore germination was scarcely inhibited and appressorium formation was occasionally suppressed in some degree at the same hours after inoculation. When *P. oryzae* spores contacted with 500 ppm Th-M on rice leaf sheath, the swollen or breaked spores and the distorted germ tubes were found (Plate I, 4-7) as observed *in vitro*⁹⁾. On the other hand, the sheath cells treated with Th-M revealed convex plasmolysis (Plate I, 3), whereas the cells invaded with mycelia 50 hours after inoculation did not show any plasmolysis phenomenon.

Most of spores of *C. lagenarium* germinated and formed appressorium on the cucumber leaves treated with 500 ppm Th-M or MBC, as well as on untreated leaves or glass slide 24 hours after inoculation as shown in Table 1. In this case, any morphological abnormality of germ tube or appressorium on treated leaves was not observed. The rate of penetration through appressorium of *C. lagenarium* into untreated host cells was 65% 96 hours after inoculation (Table 2). In this infection stage, penetration peg mostly might grow to hyphae which extended and branched profusely in the palisade and spongy parenchyma cells¹¹⁾ (Plate II, 1). In treated leaves, infection hyphae which derived from appressorium was never detected (Table 2 and Plate II, 2). The epidermal cell wall beneath the appressorium formed on treated leaves did not exhibited any abnormality, such as swelling, thickening or callosity formation^{1,2)}.

Table 1. Effects of 500 ppm Th-M and MBC on spore germination and appressorium formation of *C. lagenarium* on cucumber leaves treated 4 days before inoculation

Fungicide	Treatment	Number of spores counted	% of spore germination	% of appressorium formation
Th-M	sprayed on leaves	492	82	100
	poured into soil in pot	449	82	100
MBC	sprayed on leaves	505	72	100
	poured into soil in pot	433	85	100
Untreated leaves		533	94	99
Glass slide		459	97	97

Table 2. Effects of 500 ppm Th-M and MBC on the invasion of *C. lagenarium* to the epidermal cells of cucumber leaves sprayed 4 days before inoculation

Fungicide	Number of appressoria counted	% of the invasion to host epidermal cells
Th-M	1,444	0
MBC	1,026	0
Untreated	1,309	65

A network of superficial mycelia of *S. fuliginea* was produced on the lower surface of untreated leaves 4 days after inoculation (Plate II, 3). The growth of germ tubes of the germinated spores was inhibited by the treatment of the host plants with 50 ppm Th-M (Plate II, 4), suggesting that the haustorium formation in host cell was suppressed and thereby aerial mycelia on leaf surface could not grow as in the dissimilar way as in untreated leaves.

Discussion

Appressorium formation of *P. oryzae* was not easily affected by the treatment of the host plants with Th-M or MBC. The germinated spore of *C. lagenarium* formed appressorium on treated leaves even at the concentration that could completely prevent the occurrence of symptom and exhibit strong antifungal activities against many fungi including *C. lagenarium* by agar dilution method⁴⁾. On the other hand, these fungicides inhibited completely the hyphal growth of *P. oryzae* and *C. lagenarium* in treated respective host cells. Th-M also suppressed the development of superficial mycelia of *S. fuliginea*. Here inhibition is thought to attribute to preventing the formation of a globose haustorium by which the fungus obtains its nutrients. Thus, it may be concluded that the limitation of infection of pathogenic fungi by Th-M or MBC ascribes rather to suppressed the formation of infection peg or haustorium than to inhibition of spore germination and appressorium formation. In the present experiments, no difference between the effect of Th-M and MBC was observed in the infection process of *P. oryzae* and *C. lagenarium*, although MBC has more effective fungitoxicity than that of Th-M *in vitro*^{10,12)}.

In the case of contact with *P. oryzae* spores on rice leaf sheath, Th-M and MBC occasionally caused the swelling or breakage of spore and the distortion of germ tubes. Yasuda *et al.*⁹⁾ reported that ¹⁴C-Th-M was incorporated concurrently with spore germination and development of germ tubes

and mainly accumulated at the abnormal regions in organ of *P. oryzae*. Th-M was converted in plants⁶⁾ and fungi¹⁰⁾ into MBC responsible for the fungitoxic effect¹²⁾. Whether or not this characteristic phenomenon was attributed to fungitoxicity of MBC could not be confirmed in the present experiments. On the other hand, Th-M-treated rice leaf sheath cells revealed normal convex plasmolysis, and the epidermal cell wall of treated cucumber leaves beneath the appressoria did not exhibit any morphological abnormalities^{1,2)}. These may indicate that Th-M never cause phytotoxicity to rice leaf sheath and cucumber leaves at the concentration that suppressed the formation of hyphae³⁾.

From the present observations, it was suggested that Th-M has the possible fungitoxic actions as follows; (1) This fungicide cause the swelling or breakage of spore itself when it was directly contact with fungi at sufficient doses. (2) It also causes abnormal phenomenon on germ tubes or acts on the growing tip of germ tube and then affects appressorium formation or aerial mycelium development. (3) Even when appressoria were mostly formed, this fungicide inhibits characteristically the formation of penetration peg or hypha, thus preventing spread of invaded hypha in epidermal cells. It can be presumably concluded that Th-M acts mainly on process of penetration and not on pre-penetration phase in some fungal infections.

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和文摘要

数種の植物病原菌の感染過程におよぼす チオファネート-メチルの作用

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新殺菌剤、チオファネート-メチル (Th-M) のイネいもち病菌 (*Pyricularia oryzae*), キュウリ炭疽病菌 (*Colletotrichum lagenarium*) およびキュウリうどんこ病菌 (*Sphaerotheca fuliginea*) の感染過程におよぼす作用を光学顕微鏡観察により追究した。

Th-M (500 ppm) 処理葉上での *P. oryzae* や *C. lagenarium* の孢子発芽および附着器形成は阻止されな
いが、同葉内への侵入糸の形成あるいはその生育が完全に抑えられた。また Th-M は 50 ppm でも *S. fuliginea*

のキュウリ葉上での菌糸の伸長を強く抑え、吸器の形成を阻止するようであった。一方、500 ppm の Th-M をイネ葉鞘上で *P. oryzae* の孢子と接触させた場合、Th-M は孢子自身の細胞を膨張させたりあるいは破かいし、また発芽管の先端を彎曲した。他方、処理イネ葉鞘細胞は正常な原形質分離能を有し、また処理キュウリ葉の表皮細胞の細胞壁には形態的な異常は見られなかった。

Th-M の主な代謝産物である MBC は侵入糸の生育を阻止したが、感染過程におよぼす Th-M と MBC の作用の差異は認められなかった。これらの殺菌剤の特徴は寄主葉上の孢子発芽や附着器形成に対するよりもむしろ、病原菌の侵入過程に主に作用する点にあることが結論された。

Explanation of plates

Plate I.

1. Appressorium (A) of *Pyricularia oryzae* and hyphae (H) developed in inner epidermal cell of rice leaf sheath 54 hours after inoculation.
2. Appressorium (A) of *P. oryzae* spore (S) formed on inner epidermal cells of 500 ppm Th-M-treated sheath. Inner hyphal growth was suppressed.
3. Plasmolysis shown in 500 ppm Th-M-treated sheath cells.
4. and 5. Distorted germ tubes (arrows) of *P. oryzae* spores on 500 ppm Th-M-treated sheath cells.
6. and 7. Swollen (Sw) and broken (B) spores of *P. oryzae* on 500 ppm Th-M-treated sheath cells.

Plate II.

1. Appressorium (A) of *Colletotrichum lagenarium* and infection hyphae (H) grown in cucumber leaves 3 days after inoculation.
2. Appressorium (A) of *C. lagenarium* on 500 ppm Th-M-treated leaves of cucumber. No infection hyphae was observed.
3. A network of aerial mycelium (M) produced on the lower surface of epidermis of cucumber leaves 4 days after inoculation with *Sphaerotheca fuliginea* spore (S).
4. The suppressed growth of germ tubes (G) of *S. fuliginea* spore (S) on the surface of 50 ppm Th-M-treated cucumber leaves 5 days after inoculation.