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Effect of Isoprothiolane on the Infection Process of *Pyricularia oryzae*

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荒木不二夫*・宮城幸男*：イネいもち病菌の感染過程に及ぼすイソプロチオランの影響

Abstract

Isoprothiolane (IPT), diisopropyl 1,3-dithiolane-2-ylidenemalonate was tested for its effect on vegetative growth and infection process of *Pyricularia oryzae* Cav. Mycelial growth in a glucose-yeast extract liquid medium was inhibited completely at 20 ppm, and partially at 10 and 5 ppm. Abnormal hyphae like chlamydospore cells were frequently formed in the presence of IPT. IPT at 2 ppm did not affect conidial germination and appressorium formation, but almost completely inhibited penetration when treated at the pre-germination stage. Treatment of the fungus with IPT at different stages of the fungal infection process revealed that IPT inhibits penetration and growth of infection hyphae rather than conidial germination and appressorium formation.

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Introduction

Isoprothiolane (IPT), diisopropyl 1,3-dithiolane-2-ylidenemalonate has been known to be a highly protective and curative systemic fungicide for controlling the rice blast disease by foliar and root system application[7]. Although precise mode of action of IPT has not been elucidated, IPT seems to inhibit penetration of *Pyricularia oryzae* Cav. into host epidermal cells when the conidia were treated before germination[1]. This paper deals with the effects of IPT both on the growth of vegetative hyphae and on the infection process of rice blast fungus.

Materials and Methods

Fungus. Rice blast fungus, *P. oryzae* (P-2 isolate from the National Institute of Agricultural Science), was grown on rice straw medium in 300 ml flask at 25C. Conidia from 7-day culture were washed by centrifugation in deionized water, and suspended in water to give an appropriate conidial density.

Fungicide. IPT (Fuji-One, EC 40%) was used. Pentachlorobenzyl alcohol (PCBA, Blastin, WP 50%) was also used as a reference in the experiment on host because its characteristic mode of action has been elucidated[10, 13].

Experiment in vitro. The inhibitory effect on the growth of vegetative hyphae was examined in a liquid medium. Fifty ml of glucose-yeast liquid medium containing a known concentration of IPT in 300 ml flask was inoculated with an agar disk with mycelium (5 mm in diameter), prepared from a preliminary culture on

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PSA medium at 25C for 7 days, and cultured at 28C for 10 days in a reciprocating shaker. The growth of the fungus was estimated by determining dry weight and morphological change was examined under a light microscope. Effect of IPT in the hyphal penetration was examined by using the cellophane film as a model system, and the results were compared with those on host plants. Cellophane film of 1.5 cm square, prepared from Visking Cellulose Tube, was placed on the filter paper which was impregnated with the mixture of IPT and fresh rice leaf homogenate, in a watchglass (3 cm in diameter), and then spore suspension was dropped on the film. After incubation at 25C for 45 hr, the cellophane film was stained with a few drops of dilute zinc-chlor-iodide reagent ($ZnCl_2$ 50g, KI 20g, I 0.5g, deionized water 100 ml), and the frequency of the hyphal penetration into the film was counted under a microscope. The points of penetration were not stained due to the denaturation of cellulose, probably by the cellulase secreted by penetrating hyphae.

Experiment on Hosts. Primary leaves of barley (*Hordeum vulgare* L. cv. Kanto No. 6) was used, because pathogenesis of rice blast fungus on the leaves was similar to that on rice plant, and they could be used for the relative evaluation of fungicides, as was reported previously[1]. Moreover, barley leaves were easily cleared from the pigments by treatment with lactophenol solution, and were suitable for examining the hyphal growth within host tissue. Rice leaf sheath (*Oryza sativa* L. cv. Kimmaze) was also used to compare with barley leaves and cellophane film.

Barley seedlings were sprayed with conidial suspension, and kept in a moist chamber at 25C, followed by fungicide applications at intervals. Leaf samples were fixed in lactophenol 48 hr after inoculation and observed microscopically by the procedure reported in previous paper[1].

Inner epidermal tissue of rice leaf sheath of about 5 cm long was inoculated, by Sakamoto's sheath inoculation method[11], with the mixture of conidia and fungicide, and then incubated in a moist chamber at 25C for 48 hr.

Results

Effect of IPT on the growth of vegetative hyphae

Vegetative growth of the fungus was completely inhibited at 20 ppm, while the initiation of growth was delayed about a few days at 10 ppm, resulting in 90% inhibition (Fig. 1). In another experiment with agar medium, minimal inhibitory concentration (M. I. C.) was 50 ppm. As inhibitory effects of fungicides occasionally vary with conditions of culture, M. I. C. of IPT may be ranged between 20 and 50 ppm.

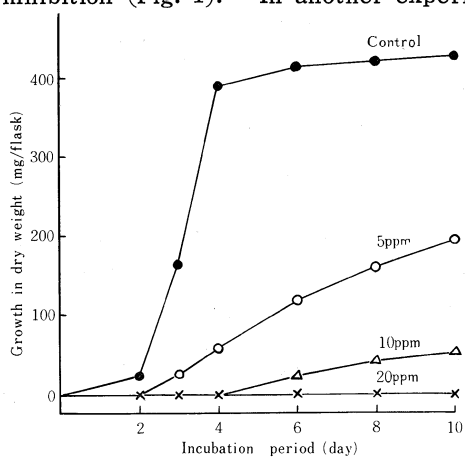

Fig. 1. Effect of IPT on the hyphal growth of *P. oryzae* in a liquid medium.

An abnormal form of hyphae was formed during the incubation period as shown in Fig. 2. Cell wall of hyphae treated with IPT became thickened, especially at the septum wall, and cells were shortened in length, giving rise to harshed and rugged hyphae. Such chlamydospore-like cells have also been observed in *Helminthosporium oryzae* treated with several D-amino acids[15].

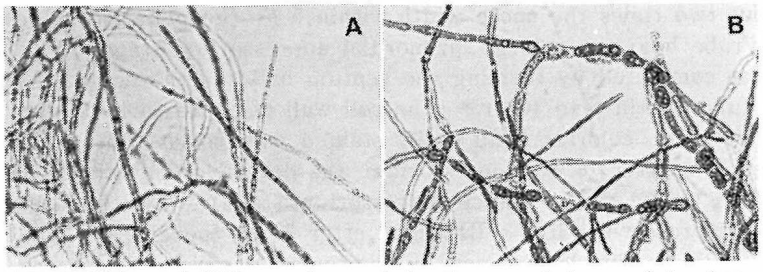

Fig. 2. Effect of IPT as observed in the morphology of hyphae. A: Normal hyphae; B: Abnormal hyphae produced in 5 ppm IPT

Effect of IPT on the hyphal penetration into cellophane film

The inhibitory effect of IPT on hyphal penetration into cellophane film was comparable to those on barley leaves and rice leaf sheath as shown in Table 1. IPT at 10 ppm completely inhibited the penetration and at 2 ppm over 90%, but conidial germination and appressorium formation on hosts were not affected at around these

Table 1. Inhibitory effect of IPT on hyphal penetration of *Pyricularia oryzae* into cellophane film and host cells

Concentration of IPT (ppm)	Inhibition of penetration (%)		
	Cellophane film	Barley leaf	Rice leaf sheath
10	100	100	100
2	94	98	92
1	68	80	89
0.5	51	57	62
0	0	0	0

concentrations. As Oku *et al.* reported, the penetration into cellophane occurs mainly at slightly enlarged tips of germ tubes[10], but partly from the appressoria as ordinarily seen on host plants.

Negative reaction to cellulose staining reagent was observed only around the penetration site (Fig. 3), and not around the elongating germ tube. Thus the frequency of unstained spots coincided with that of penetration sites.

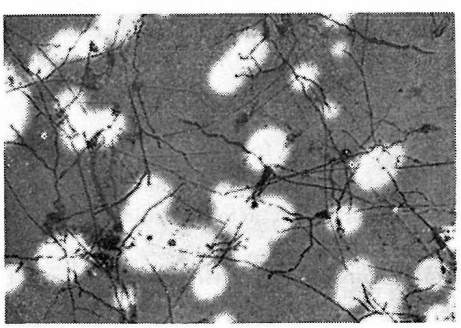

Fig. 3. Negative reaction of cellophane (halo zone) to cellulose staining reagent appearing around the penetration sites of *P. oryzae*.

Oku *et al.* also reported that the hyphae penetrated through the cellophane film[10]. Our observations, however, indicated that hyphal penetrations were limited within the film, because the infection hyphae were left in good morphology even after both surfaces of the film were wiped off, and their morphology was similar to that of the subcuticular penetrating hyphae shown by Knox-Davies[5].

Effect of IPT on each stage of infection process on barley

Most of conidia inoculated on barley leaves initiated their germination within 2 hr-incubation and the length of germ tube

reached about two times the spore width within 5 hr (germination stage). The tip of the germ tube began to enlarge (primordial appressorium stage) and appressorium formation was completed by forming the septum between the enlarged part and the basal germ tube within 8 to 10 hr. The cell wall of appressorium, after 14 hr-incubation was yet thin, colorless, and easily stained with cotton blue (immature appressorium stage). After 18 hr, appressorium was dark-colored with thicker cell wall and impervious to the stain (mature appressorium stage). Development of infection hyphae from appressorium into cells began after 22 hr (penetrating stage).

Effect of IPT on the development in the fungal infection process was summarized in Fig. 4. IPT treatment at the pregermination stage resulted in complete inhibition of conidial germination, appressorium formation, and penetration at 200, 100, and 10 ppm, respectively. IPT applied at the germinating stage inhibited the appressorium formation approximately 90% at 200 ppm and 38% at 100 ppm. Treatment during the primordial and mature appressorium stages brought about the decrease of penetration frequency, and furthermore suppressed the growth of infection hyphae. This

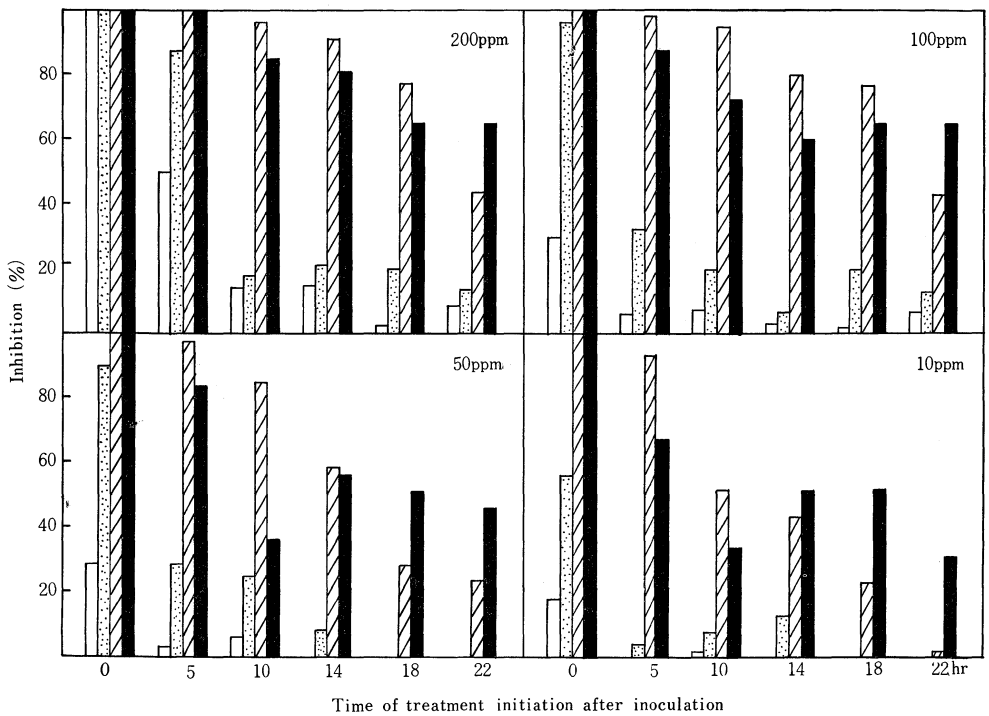

Fig. 4. Effect of IPT on the infection process of *P. oryzae*.
Conidial germination Appressorium formation
Penetration Growth of infection hyphae
The height of each column indicates inhibition percent 48 hr after inoculation.

activity on the growth of infection hyphae was also confirmed by the IPT treatment at 10 ppm at the penetrating stage. Thus, IPT exerted inhibitory effects on every phase of the fungal development, but most efficiently on penetration and growth of infection hyphae. Most of appressoria affected by IPT were unable to penetrate walls and they were not stained with cotton blue, although they resembled the immature appressoria in morphology (Fig. 5). On the other hand, PCBA, used as a reference,

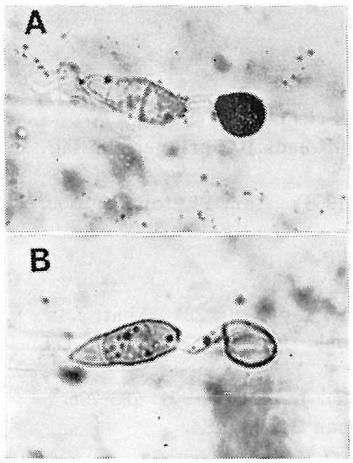

Fig. 5. Effect of IPT as observed in the morphology of appressoria. A: Normal appressorium; B: Abnormal appressorium produced in 50 ppm IPT.

inhibited the penetration only when it was applied at the germinating stage, and exerted little effect on the growth of infection hyphae when treated after the primordial appressorium stage. These results coincided with previous findings that IPT is a curative as well as a protective fungicide[7] and PCBA is only a protective one[13].

Discussion

Chlamydospore-like cells were formed of the blast fungus mycelium incubated in a liquid medium containing IPT, and abnormal appressoria were also produced on cellophane film and barley leaves when treated with IPT. Certain substances cause abnormal forms of fungal mycelium, e. g., griseofulvin and cerlenin produce 'curling' and polyoxin 'swelling'. Their modes of action, however, are different from one another, i. e., griseofulvin acts on the replicatory system of fungal cells[3], cerlenin disturbs lipid metabolism[9], and polyoxin inhibits the biosynthesis of chitin of fungal cell wall[12]. The mechanism of fomation of abnormal hyphae in the presence of IPT should be elucidated in future.

Cellophane film was in most cases penetrated directly by tips of germ tubes. The fungal penetration into host cells was commonly achieved by forming penetration peg from appressorium, although in some exceptional cases, direct hyphal penetration into epidermal cell was observed on a certain host plant[14]. Nevertheless cellophane film was almost as efficient as plant cell wall in evaluating cessation of hyphal penetration by fungicides such as PCBA[10], amino acid derivatives[4], and probenazole[16].

Staining with zinc-chlor-iodide reagent facilitated the detection of penetration sites on the cellophane film because of the halo zone formed around the sites. These halo zones were very similar to those on the epidermal cell wall of barley leaf attacked by *Erysiphe graminis hordei*[6]. These halos most likely occur as the result of denaturation of cellulose by cellulase which would probably be secreted during penetration peg development.

IPT affected every stage of infection process of *P. oryzae,* while the inhibitory activity on the formation of appressorium and the penetration peg was most remarkable when treated at the stages of germination and formation of primordial appressoria. Moreover, even after the growth of infection hyphae was initiated, IPT inhibited their extention into host cells.

Spore germination and appressorium formation in several fungi are accompanied with syntheses of RNA and proteins[2, 8]. Although little is known about these synthetic processes of rice blast fungus, the differential effects of IPT and PCBA observed in this experiment suggest that a morphogenetic pathways similar to these fungi may exist in the infection process of the blast fungus. The effect of IPT on the metabolisms of nucleic acids and proteins in rice blast fungus will be reported elsewhere.

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和文摘要

イネいもち病菌の感染過程に及ぼすイソプロチオランの影響

荒木不二夫・宮城幸男

イネいもち病の防除にすぐれた効果をもつ浸透性殺菌剤イソプロチオラン（ＩＰＴ）を用いて，栄養菌糸の生育および寄主体への侵入行動に及ぼす影響を各発育過程を通じて調べた。

ＩＰＴを含んだブドウ糖一酵母エキス培地でいもち病菌を培養すると，20ppmではまったく生育せず，10および5ppmでは生育の遅延が認められた。菌糸は形態的に異常となり，厚膜胞子様の細胞が多数観察された。未発芽胞子にＩＰＴを作用させると，2ppmでは付着器は形成されるが，菌糸の侵入はほぼ完全に阻止された。接種後経時的に菌の各発育段階，すなわち，未発芽期，発芽管伸長期，付着器形成前期，付着器成熟期および侵入初期のそれぞれの時期にＩＰＴを作用させると，侵入および侵入菌糸の生育が強く阻害され，胞子発芽および付着器形成はあまり阻害されなかった。