Effects of isoprothiolane on *Pyricularia oryzae* Cavara. (II) Effects on hyphal mass*

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Summary

Effects of isoprothiolane (IPT: diisopropyl 1,3-dithiolane-2-ylidenemalonate) on hyphal mass of *Pyricularia oryzae* Cav. were investigated. Preliminary to the exploration of the IPT effects, the IPT-tolerant isolate, designated as PI13, was obtained through selection of a tolerant colony which had originated from a single protoplast derived from the IPT-sensitive isolate P2. Growth of hyphal mass of P2 was perfectly inhibited on PDA and in Vogel's liquid medium, respectively, at 50 ppm and 25 ppm, whereas that of PI13 was suppressed but not perfectly even at 50 ppm. Any concetrations of IPT up to 50 ppm did not show prominent effects on conidium germination and appressorium formation of P2 but 25 and 50 ppm IPT reduced the occurrence of pigmented appressorium markedly, while IPT did not interfere with these events of PI13 at all at any concentrations up to 50 ppm. It was also found that young hyphae obtained from the colony margin of P2 were more sensitive to IPT than those from the intervening and central regions of the colony, suggesting that the IPT effects on *P. oryzae* might be closely associated with ages of hyphal cells: apical cells of hyphae being highly sensitive to IPT.

Introduction

Isoprothiolane (IPT: diisopropyl 1,3-dithiolane-2-ylidenemalonate) is known as a fungicide to specifically control the rice blast disease caused by *Pyricularia oryzae* Cav[10]. Araki and Miyagi[1] reported that IPT perfectly inhibited hyphal growth of this fungus in a liquid medium and penetration of cellophane film and rice leaf sheath at a concentration of 10 ppm. Hirooka *et al.*[3] studied the effect of IPT on penetration process of *P. oryzae* appressoria by micromanipulation in a scanning electron microscope and found that penetration of this fungus was inhibited by 10 ppm IPT through interference with the emergence of infection pegs from appressoria on rice leaves. On the other hand, Kakiki and Misato reported that IPT markedly interfered with fatty acid[8] and cell wall synthesis[7] of *P. oryzae* at a concentration of 50 ppm.

Primarily we wondered why the effective concentration of IPT to *P. oryzae* obtained by biochemical means[7,8] was relatively higher than that by physiological and cytological means[1,3]. Thus, we dsigned a series of comparative experiments to elucidate the IPT effects on *P. oryzae* at the levels of hyphal mass, single hypha and protoplast isolated from hyphae by cytological and physiological means in more detail. This paper describes the IPT effects on hyphal mass as the first step.

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Materials and methods

Test chemical, test fungus and preparation of hypha and conidium suspension

IPT (99.9% technical grade, Nihon Noyaku Co. Ltd) was used in this study. *Pyricularia oryzae* Cav. isolate P2 (sensitive to IPT) and isolate PI13 (tolerant to IPT, as described below) were used throughout the experiments. The respective test fungi were grown on PDA (potato-dextrose-agar) slants at 25°C for 7–10 days. The hypha suspension was prepared by adding 5 ml of sterilized deionized water to the slant, followed by stirring the surface of mycelial mat with a sterilized platinum loop. To allow the fungus to form conidia, 0.5 ml of the above hypha suspension was added to V–8 juice agar medium (V–8 juice, 250 ml; $CaCO_3$, 3 g; deionized water, 750 ml; agar, 15 g) in Petri dishes and expanded evenly on the medium surface, followed by incubation at 25°C for 5 days. Ten ml of sterilized water were added to the medium and the mycelial mat was stirred with a brush to release conidia. The conidium suspension was prepared by filtration of the above suspension of conidia and hyphae with 3 sheets of cheese cloth to eliminate fragmented hyphae from the suspension. The density of conidia in the suspension was adjusted at approximately 4×10^6 conidia/ml with sterilized water.

Selection of the IPT-tolerant isolate

To attempt to obtain the IPT-tolerant isolate, protoplasts were prepared from hyphae of P2 by employing the method previously described[6,9]. The protoplast suspension was adjusted to include approximately 10^6 protoplasts/ml, and 0.1 ml of the suspension was added to PDA contianing 25 ppm IPT and 0.6 M KCl in Petri dishes, then expanded on the surface with a sterilized glass lod. After a 6-day incubation at 25°C, mycelial colonies which had originated from isolated single protoplasts were transferred onto PDA containing no IPT. The IPT tolerance of the respective isolates was tested in terms of colony diameter, dry weight of mycelia and conidium germination in the presence of various concentrations of IPT, as described below.

Evaluation of the IPT sensitivity of test isolates

i) Colony diameter. The isolates of P2 and PI13 were grown on PDA at 25°C for 8 days. Mycelial discs of 6 mm diameter were obtained from the marginal region of colony of the respective isolates, and placed each on the center of PDA medium containing 0, 5, 10, 25 or 50 ppm IPT. The colony diameter was measured every 2 days after the start of incubation at 25°C.

ii) Dry weight of mycelia. The hypha suspension of the respective isolates prepared as above was added to 100 ml Vogel's liquid medium in 200 ml flasks and incubated on a rotary shaker at 25°C for 48 hr. Small colonies of less than 1 mm diameter were collected with a micropipette and resuspended in Vogel's medium. One half ml of the colony suspension was added to 10 ml of Vogel's medium in L-shaped glass tubes and IPT was added to the incubation mixture at a final concentration of 0, 5, 10, or 25 ppm at the start of incubation. After a 1–5 day incubation on a reciprocal shaker at 25°C, mycelia in the respective glass tubes were collected with glass filters, and then dried at 65°C for 24 hr. Subsequently, the dry weight of mycelia was determined.

iii) Conidium germination and appressorium formation. Pieces of filter paper, 1×1 cm^2, were immersed in 0, 5, 10, 25 or 50 ppm IPT in 0.01 M Na-phosphate buffer (pH 5.6). Each of them was placed on a watch glass in a moist Petri dish. A cellophane sheet, 1×1 cm^2, which had been boiled previously in deionized water, then dried, was placed on the respective pieces of filter paper. The conidium suspension was sprayed over the cellophane sheets and incubated in moist Petri dishes at 25°C for 24 hr. The rates of germination, appressorium formation and appressorium pigmentation were determined by light microscopy using the following equations.

$$\text{Conidium germination rate} = \frac{\text{No. of germinated conidia}}{\text{Total No. of conidia observed}} \times 100\ (\%)$$

Appressorium formation rate =

No. of appressoria/No. of germinated conidia ×100 (%)

Appressorium pigmentation rate =

No. of pigmented appressoria/Total No. of appressoria observed ×100 (%)

Sensitivity of mycelia of various ages to IPT

In order to find differences in sensitivity of mycelia of various ages to IPT, mycelial discs of 3 mm diameter were obtained from the center to the margin of P2 and PI13 colony, respectively (Fig. 1). The respective discs were placed on PDA containing 0, 5, 10 or 25 ppm IPT and incubated at 25°C for 6 days and then diameter of the respective colonies which had developed from each disc was measured.

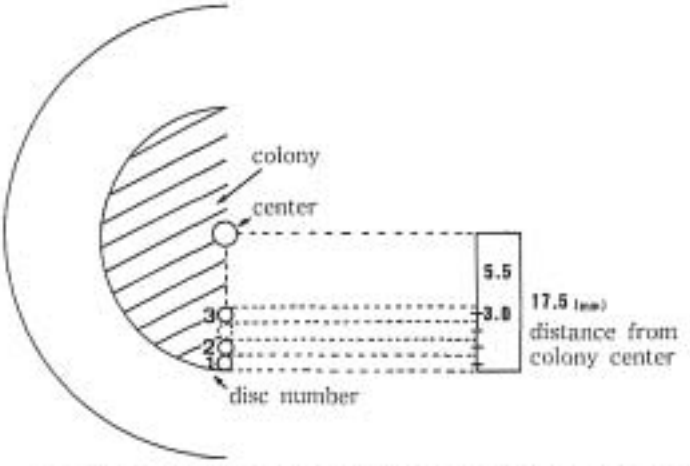

Fig. 1. A schematic figure of sampling mycelial discs of various ages from a colony of the IPT-sensitive and -tolerant isolates of *P. oryzae* growing on potato-dextrose-agar medium.

Results

Selection of IPT-tolerant isolate

Several colonies which had originated from single protoplasts grew on PDA containing 25 ppm IPT. However, only one isolate formed abundant conidia on V-8 juice agar medium as similarly as P2. Various visible characters of its colony such as color and growth rate were all similar to those of P2. Moreover, this isolate showed a similar pathogenicity to a rice cultivar, Jukkoku, as P2. While mycelia of this isolate were transferred onto fresh PDA repeatedly, twenty times, the above characters of this isolate were not changed at all. As far as we tested in the laboratory, this isolate was different from P2 only in the IPT-tolerance. Some of the data showing such differences between two isolates are shown below in detail. Thus, this isolate was designated as PI13 and used in the present study.

Effect of IPT on mycelial growth

Increase of colony diameter of P2 was markedly affected by 5 and 10 ppm IPT and was almost perfectly suppressed by the concentrations higher than 25 ppm (Fig. 2A). On the other hand, mycelial growth of PI13 in terms of colony diameter was not prominently affected by 5–25 ppm IPT but suppressed markedly by 50 ppm (Fig. 2B).

The dry weight of P2 mycelia in control prominently increased from the 1st to the 3rd day after the onset of incubation and reached a maximum at the 4th day (Fig. 3A). When P2 mycelia were treated

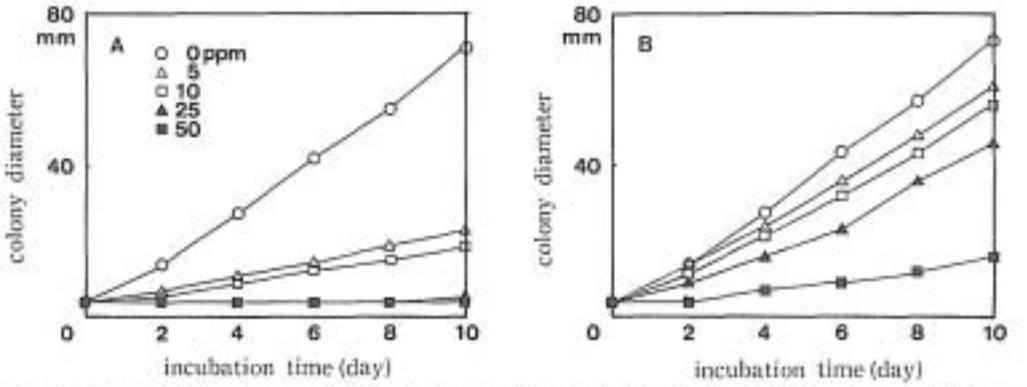

Fig. 2. Effect of various concentrations of IPT on mycelial growth of the IPT-sensitive isolate P2 (A) and the IPT-tolerant isolate PI13 (B) of *P. oryzae* on potato-dextrose-agar medium.

with 5 ppm IPT, their dry weight was suppressed to increase for initial 2 days and then increased markedly thereafter. In the presence of 10 ppm IPT, P2 mycelia increased in dry weight slowly until the 5th day after the onset of incubation, but their growth was completely suppessed by 25 ppm IPT (Fig. 3A). When P2 mycelia were grown in Vogel's medium containing no IPT, their colony colour appeared white at first, turned light grey at 2.5 day after the start of incubation and finally dark grey at 3.5 day. However, mycelial colour appeared unaffected during a 5-day incubation, when IPT was added to the medium at any concentrations (Fig. 3A).

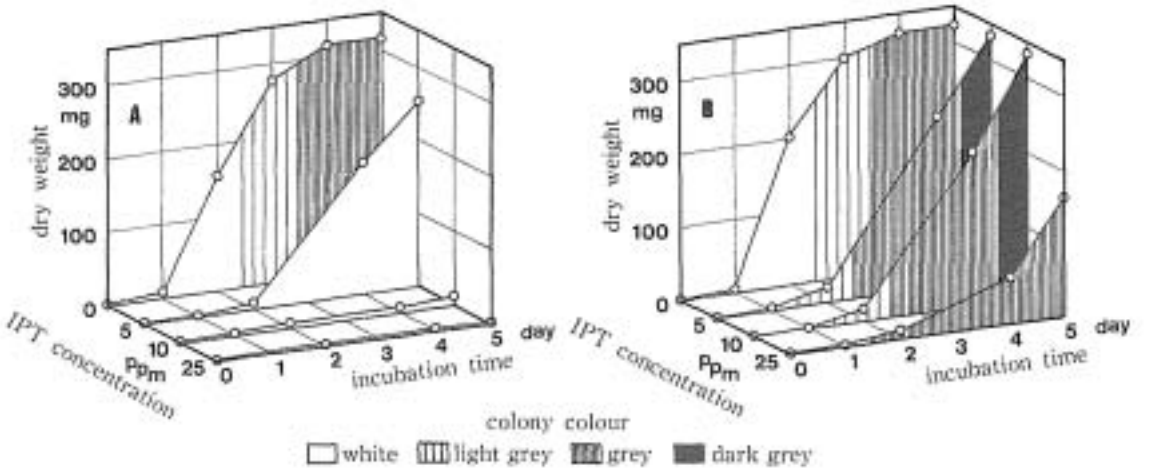

Fig. 3. Effect of various concentrations of IPT on dry weight and pigmentation of the IPT-sensitive isolate P2 (A) and the IPT-tolerant isolate PI13 (B) of *P. oryzae* in Vogel's liquid medium.

In control, mycelial growth of PI13 was similar to that of P2 (Fig. 3B). In the presence of 5 or 10 ppm IPT, mycelial growth of PI13 was markedly suppressed for initial 2 days but increased rapidly thereafter to reach the same level as that of control at the 5th day. When PI13 was treated with 25 ppm IPT, their growth was suppressed for initial 2 days but recoverd gradually thereafter (Fig. 3B). In contrast with P2, colony colour of PI13 turned light grey to dark grey even in the presence of any concentrations of IPT (Fig. 3B).

Effect of IPT on conidium germination and appressorium formation

When P2 conidia were treated with 5–50 ppm IPT, their germination was unaffected but appressorium formation was considerably affected with increased concentration of IPT (Fig. 4A). The most conspicuous effect appeared on appressorium pigmentation: pigmented appressoria occurred at the rate of only ca. 15% in the presence of 25 and 50 ppm IPT (Fig. 4A). By contrast, conidium germination and appressorium formation and pigmentation of PI13 were not affected by any concentrations of IPT up to 50 ppm, as shown in Fig. 4B.

Sensitivity of mycelia of various ages to IPT.

As already indicated in Fig. 2, growth of P2 mycelia was markedly suppressed by 5–25 ppm IPT

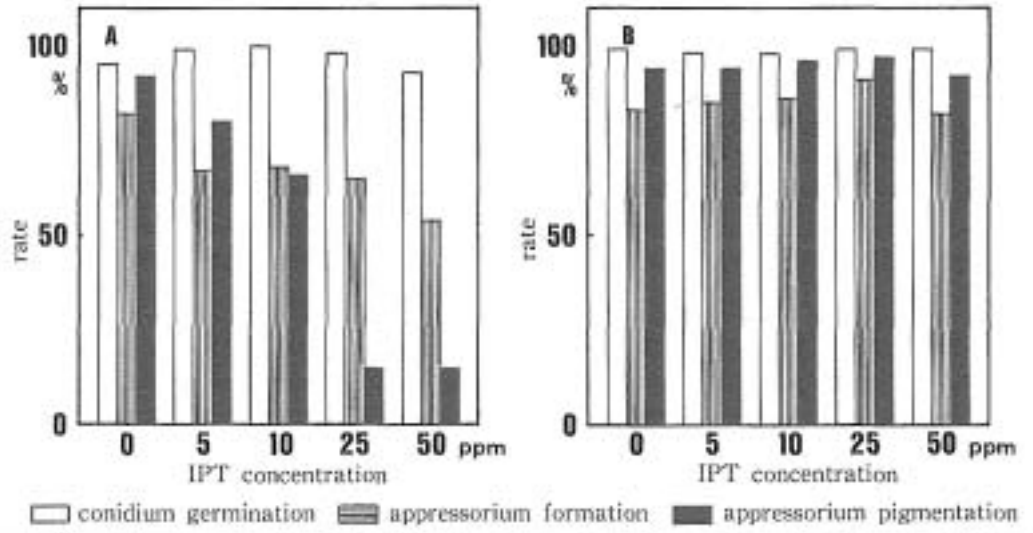

Fig. 4. Effect of various concentrations of IPT on conidium germination, appressorium formation and appressorium pigmentation of the IPT-sensitive isolate (A) and the IPT-tolerant isolate (B) of *P. oryzae* on cellophane films.

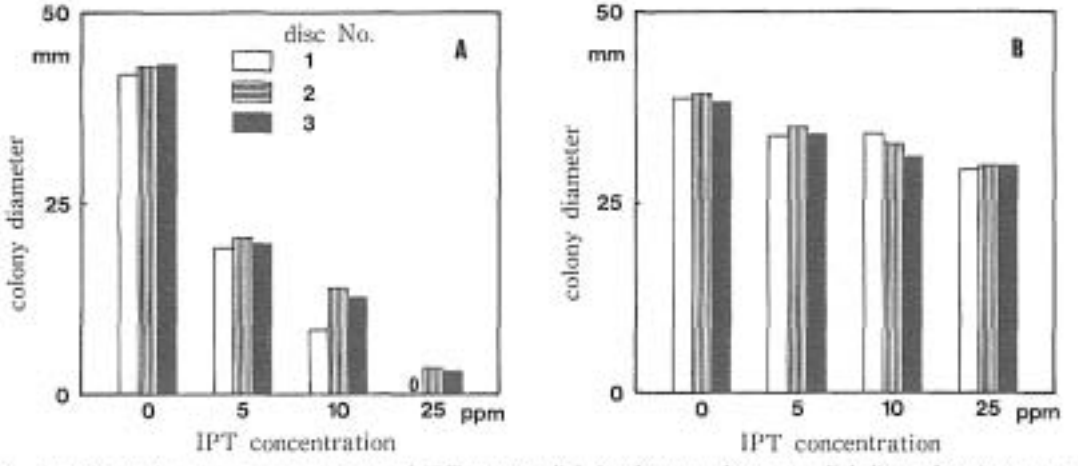

Fig. 5. Effect of various concentrations of IPT on mycelial development from mycelial discs of various ages obtained from the colony of the IPT-sensitive isolate P2 (A) and IPT-tolerant isolate PI13 (B) of *P. oryzae*. (For disc No., see Fig. 1).

(Fig. 5A). However, the sensitivity of mycelia to IPT was closely associated with their ages: in the presence of 5 ppm mycelia developed from all discs which had been obtained from various regions of the original mycelial colony (Figs. 1, 5A) but in the presence of 10 ppm mycelia developed less from the disc obtained from the marginal region of original colony than from other discs obtained from the intervening and the central region; moreover, in the presence of 25 ppm mycelia never developed only from the disc obtained from the marginal region (Fig. 5A). By contrast, PI13 mycelia developed similarly from all discs, obtained from various regions of original mycelial colony, at any concentrations of IPT, although their growth tended to be suppressed slightly with increased concentration of IPT (Fig. 5B).

Discussion

When P2 mycelia were grown on PDA containing 25 ppm IPT (Fig. 2A) and in Vogel's liquid medium containing 10 ppm (Fig. 3A), their growth was markedly suppressed but these concentrations allowed the mycelia to grow slowly, respectively, during 10- and 5-day incubation. Such a slow growth indicates that P2 mycelia were not killed by these concentrations: at least some of hyphae or hyphal cells remaining alive, and suggests the possibility that P2 mycelia include hyphae or hyphal cells of varied sensitivities to IPT. The data of Fig. 5A indicate that young hyphae growing at the marginal region of P2 colony were more sensitive to IPT than older hyphae at the intervening and central regions of colony. Similarly, Ishizaki *et al.*[4] obtained the result that younger hyphae of the present fungus were more sensitive to IBP than older ones. They[5] furthermore studied the IBP effect on single hyphae and found that they stopped elongation approximately 10 min after the onset of treatment with 2 ppm IBP and their apical cells vacuolated at 50 min. Considering the similarity of IPT and IBP in their effects on hyphal mass, it is plausible that apical cells of P2 hyphae might be more sensitive to IPT than other older cells.

When the conidium germination was tested in the presence of IPT, its most prominent effect was seen in suppression of appressorium pigmentation which was associated with appressorium maturation[2]. This observation is consistent with Araki and Miyagi's results[1]. These results are probably associated with the fact that when P2 conidia were treated with IPT, their appressoria failed to produce penetration pegs because of their immaturity[3].

As far as mycelial growth and conidium germination are concerned, IPT was much less effective to PI13 than to P2. Especially at the stages from conidium germination through appressorium pigmentation, PI13 appeared unaffected even by 50 ppm IPT. In the presence of 50 ppm and 25 ppm, respectively, in PDA and Vogel's liquid media, PI13 mycelia grew to some extent, although their growth was considerably suppressed. Thus, it can be considered that PI13 is much more tolerant to IPT than P2, although it is uncertain at present whether PI13 is a type of IPT-tolerant strain which causes a serious damage on rice through its tolerance to IPT at field level.

As indicated in Figs 2 and 3, the effective concentration of IPT to P2 was less in Vogel's medium than PDA. A similar tendency was obtained in our previous work[4] concerning IBP effects. As Yamamoto and Hosoi[11] reported, effects of some fungicides are often influenced by the type and/or component of the medium. In a separate experiment, we confirmed that the effective concentration of IPT on P2 was almost same in both Vogel's and potato-dextrose liquid media. Thus, the above difference of the effective concentration of IPT in Vogel's liquid medium and PDA is probably due to the type of medium. However, why this difference happens between liquid and solid media is uncertain at present.

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摘　　要

イネいもち病菌 *Pyricularia oryzae* Cav. に及ぼす IPT の影響
（Ⅱ）菌叢に及ぼす影響

大熊妙子・石崎　寛・久能　均

Pyricularia oryzae Cav. の菌叢に及ぼすイソプロチオラン (IPT) の影響を調べた。供試菌として IPT 感受性の分離株 P2 及び耐性の P113 を用いた。前者の菌糸から調製したプロトプラストを 25 ppm IPT 含有の PDA 培地に接種し，その上で伸長してきたコロニーを選抜して後者の株を得た。P2 の菌叢の伸長は PDA 上及び Vogel 液体培地中でそれぞれ 50, 25 ppm IPT によってほぼ完全に阻害された。一方いずれの培地を用いても P113 の菌叢の伸長は 50 ppm 存在下でも完全に阻害されることはなかった。セロファン膜上の P2 分生胞子の発芽は阻害されなかったが，付着器形成は IPT 濃度の上昇とともに阻害される傾向を示した。また付着器着色は 25 ppm で強く抑制された。しかし，P113 の胞子発芽，付着器形成は 50ppm IPT でもほとんど影響をうけなかった。次に PDA 培地上で生育する P2 および P113 の菌叢周縁部から中央部にかけて直径 3 mm の菌叢小円板をコルクボーラーで打ち抜き，それぞれ齢の異なる菌叢小円板を得た。これらを 0–25 ppm IPT 含有の PDA 培地に移植して培養したところ，P2 菌叢周縁部から得た小円板のみに 25 ppm 存在下で菌叢の発達が認められなかった。以上の結果は，*P. oryzae* に及ぼす IPT の影響は菌叢を構成する菌の齢に関係しており，若い菌糸は IPT 感受性が著しく高いことを示唆している。