Original Article

Effects of Isoprothiolane on Protoplasts Isolated from Hyphae of *Pyricularia oryzae**

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Cytological effects of isoprothiolane (diisopropyl 1,3-dithiolan-2-ylidenemalonate: IPT) on *Pyricularia oryzae* CAV. were investigated by applying it to protoplasts isolated from hyphae of IPT-sensitive (P2) and -tolerant (PI13) isolates. When protoplasts of the respective isolates were incubated in the presence of IPT at various concentrations, the number of P2 protoplasts decreased with time after the onset of incubation, while that of PI13 protoplasts was not affected, suggesting that the difference between P2 and PI13 in terms of tolerance to IPT might be associated with the difference between their symplasts. IPT concentration had no effect on the frequency of cell wall regeneration of both isolates, although it might interfere with the quality of the cell wall formed in the presence or absence of IPT. The most prominent effects of IPT on P2 were the suppression of reversion of hyphae at 10-25 ppm and the complete inhibition of colony development at 5 ppm. PI13 was not prominently influenced at either concentration. The results indicating the suppression of reversion are consistent with an earlier finding that IPT retards or inhibits mycelial growth. The present study demonstrated that the protoplast system could be used for cytological tests of fungicide effects.

INTRODUCTION

Isoprothiolane (abbreviated as IPT herein-after) has been known as a highly protective and curative systemic fungicide which can prevent the rice blast disease caused by *Pyricularia oryzae* CAV.¹⁾ As Araki and Miyagi²⁾ reported, IPT inhibits mycelial growth of the fungus at 40 ppm but not spore germination at up to 200 ppm. However, it markedly suppresses the emergence of infection hyphae from appressoria at about 10 ppm³⁾ and the

development of the disease at 1-5 ppm.¹⁾ Although biochemical experiments suggested that IPT might interfere with sterol metabolism^{4,5)} and cell wall synthesis,⁶⁾ it is hard to explain chemically why the inhibitory concentration of IPT varies at growth stages of the fungus.

In this paper, protoplasts isolated from hyphae of IPT-sensitive and -tolerant isolates of *P. oryzae* were examined at various IPT concentrations to elucidate its cytological effects on the fungus. As is well known, protoplasts of *P. oryzae*, when incubated in an appropriate medium soon after isolation, regenerate cell walls around them, and subsequently the regenerated cells protrude hyphae within 12 hr after the onset of incubation.^{7,8)} Thus, we assumed that the cytological changes by IPT treatment, which might appear at stages from protoplasts to hyphal elongation,

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would help to find out the action site of IPT. In addition, we attempted to examine a possibility whether the protoplast system can be used for a test to evaluate the fungicide effects on fungi.

In the present study, the formation of a cell wall around a protoplast is defined as cell wall regeneration, the cell surrounded by a regenerated cell wall as a regenerated cell, and the subsequent process of protrusion of a hypha as reversion, according to De Vries and Wesels⁹⁾ and Tanaka *et al.*¹⁰⁾

MATERIALS AND METHODS

1. Test Fungus

Pyricularia oryzae CAV. isolates P2 (sensitive to IPT) and PI13 (tolerant)¹¹⁾ were used in this study. The test fungi were grown on potato-glucose-agar (PGA) slants at 25°C for 7–10 days.

2. Test Fungicide

Isoprothiolane (diisopropyl 1,3-dithiolan-2-ylidenemalonate, 99.9% technical grade) was supplied by Nihon Nohyaku Co., Ltd. and used throughout the experiments. Preparation of IPT solution followed the method previously described.¹¹⁾

3. Preparation of Protoplasts and Treatment with IPT

Protoplasts of the respective isolates were prepared by the method described previously.⁷⁾ Protoplasts were suspended in 0.02 M Naphosphate buffer (pH 7.0) with 0.6 M KCl and 2 mM MgCl₂ at a density of 10⁸/ml (determined with a hemocytometer). The respective suspensions (200 μ l) were mixed with 4 ml of melting PGA supplemented with 0.6 M KCl containing 0, 5, 10 or 25 ppm IPT at 40°C by stirring gently on a thermo-controlled stirrer. Immediately after mixing, thin layer agar plates (TLA's) were prepared by using the above respective protoplast suspensions as previously described.⁷⁾ The TLA's were incubated in a moist petri dish at 25°C.

4. Observation of the IPT Effects

Following the incubation for various lengths of time, each TLA was cut into small pieces, *ca.* 1 $\times$ 1 cm² and subjected to one of the fol-

lowing experiments.

1) FITC-labeled wheat germ agglutinin (FITC-WGA), a kind of lectin which has been used to determine the molecular architecture of cell walls of various fungi¹²⁾ was applied to detect the cell wall regenerated around the protoplast. The FITC-WGA solution was flooded over the small pieces of TLA and a cover slip was placed on it, followed by observation with a Zeiss inverted interference contrast microscope equipped with an epifluorescence attachment. A filter set with BP 450-490 excitation filter, an FT 510 dichroic mirror, and an LP 515-565 barrier filter was used. The frequency of cell wall regeneration and reversion, and the incidence of granulated protoplasts were determined using the following equations.

Frequency of cell wall regeneration = (No. of regenerated cells per unit volume of TLA)/(No. of protoplasts originally embedded per unit volume (NP)) $\times$ 100 (%)

Frequency of reversion = (No. of hyphae emerged from regenerated cells)/(No. of regenerated cells per unit volume) $\times$ 100 (%)

Incidence of granulated protoplasts = (No. of granulated protoplasts per unit volume)/(NP) $\times$ 100 (%)

Since the thickness of TLA was kept constant (0.15 mm), it was possible to determine the number of protoplasts distributing in a unit volume of 3.14 (π) $\times$ 0.24² (radius of the field of view) $\times$ 0.15 (thickness of medium) (mm³).⁷⁾

2) Another small piece of TLA was stained with 0.25% trypan blue¹⁴⁾ and observed with a Zeiss IM35 microscope. The incidence was expressed as a percent of the number of positively stained protoplasts and regenerated cells per unit volume to NP.

3) Remaining small pieces of TLA were incubated in the above moist petri dishes for another 16 hr and the number of colonies which had developed from isolated, single protoplasts was determined with a Zeiss IM35 microscope. The frequency of colony development was expressed as a percent of the number of colonies per unit volume to the total number of hyphae having emerged from regenerated cells per unit volume.

RESULTS

1. Morphological Effects of IPT on the Growth Patterns of Protoplasts

Control protoplasts of both isolates (IPT-sensitive and -tolerant) appeared spherical with either a dense cytoplasm or a large vacuole, when observed immediately after embedded in the medium (Fig. 1-A). When incubated with IPT, some of protoplasts became irregular in shape with granulated cytoplasm as illustrated in Figs. 1-B and C, the incidence of such abnormal protoplasts depending on the IPT concentration applied. Most of these granulated protoplasts stained bluish violet, when treated with trypan blue (Fig. 1-D).

As reported in a previous paper,⁷⁾ cell wall regeneration in controls occurred within 1 hr after the onset of incubation. The regenerated cell wall, when treated with FITC-WGA, gave a greenish fluorescence under blue light of a fluorescence microscope whether or not IPT treatment was given. When treated with trypan blue, some of the regenerated cells stained bluish violet (Fig. 1-E).

The reversion to hyphae usually occurred 8–9 hr after the onset of incubation in controls. The reversion occurred only from regenerated cells but not from protoplasts. In controls of both isolates, hyphae which had emerged from regenerated cells showed normal, slender feature and repeated branching as illustrated in Fig. 1-G. However, when either 5 or 10 ppm IPT was present in the medium, short hyphae of slightly malformed cells developed from an original, regenerated cell (Figs. 1-F, H). Most of such hyphae did not develop further, although in a few cases thin hyphae of normal feature grew and kept elongating to a limited extent. In the medium containing 25 ppm IPT, no hypha-like structures developed from regenerated cells but only short protrusions emerged from some cells in many cases. Such cells were stained positively with trypan blue (Fig. 1-I). Only in a few cases, several malformed hyphae resembling those in Figs. 1-F and H developed from regenerated cells treated with 25 ppm IPT. In contrast, in PI13, colonies with an appearance similar to those in Fig. 1-G developed from most of the regenerated cells of irrespective of the presence

of IPT at concentration, although several cells near the original, regenerated cells were sometimes, slightly malformed.

2. Numerical Evaluation of the IPT Effects on Protoplast Persistence, Regeneration and Reversion

During a 24 hr incubation, about 55% of P2 control protoplasts which had been originally embedded became invisible, probably because of degeneration and/or bursting. Such disappearance increased as IPT concentration went up: 39.0, 34.5 and 18.0% of originally embedded protoplasts persisted until 24 hr after the onset of incubation, when the incubation medium contained, 5, 10 and 25 ppm IPT respectively (Table 1). Protoplasts of PI13 control also degenerated and/or burst almost as frequently as those of P2 control. The decline of their persistence was not markedly affected by IPT treatment in contrast with P2 (Table 1).

The frequency of cell wall regeneration of both P2 and PI13 was not markedly affected by IPT concentrations: 63.0–73.9% protoplasts regenerated their cell walls regardless of the presence of IPT (Table 1). On the other hand, reversion of P2 was prominently influenced by IPT concentrations: especially IPT higher than 10 ppm greatly suppressed reversion. Reversion of PI13, however, was not remarkably affected, although the frequency gradually declined with elevation of IPT concentration (Table 1).

Colonies developed from 93.5% of reversed hyphae of control P2, but the presence of IPT at any concentration suppressed colony development. On the contrary, colony development of PI13 was not markedly affected by IPT, although its incidence gradually decreased as IPT concentration elevated (Table 1).

As mentioned above, IPT higher than 10 ppm interfered with the reversion of P2. In order to clarify the cause, incidences of trypan blue-stained and granulated protoplasts and regenerated cells were examined. In P2 control, only 2–3% of protoplasts and regenerated cells were positively stained with trypan blue and granulated. However, their incidences increased with IPT concentration and finally 97.2% of protoplasts and regenerated cells were

Table 1 Effects of IPT concentration on incidence of changes during protoplast growth of *P. oryzae* isolates P2 and PI13 24 hr after the onset of incubation.

Isolate	Event ^{a)}	IPT concentration (ppm)			
		0	5	10	25
P2	A	45.0±3.2% ^{b)}	39.0±6.5	34.5±8.1	18.0±3.2
	B	73.6±5.0	63.0±2.9	63.8±6.1	72.2±8.2
	C	67.6±2.8	58.7±3.9	31.8±4.1	7.7±5.3
	D	93.5±4.3	0	0	0
	E	3.3±2.1	12.2±3.8	42.6±5.3	91.7±8.6
	F	2.2±1.9	16.7±4.6	51.5±6.2	97.2±3.0
PI13	A	42.5±2.8	38.5±6.3	40.5±8.2	40.0±3.6
	B	72.9±5.8	73.9±4.8	65.2±5.4	70.9±2.9
	C	82.3±4.2	82.8±7.1	72.1±6.5	68.9±9.1
	D	92.2±3.5	92.9±3.5	81.1±6.3	71.1±8.2
	E	5.3±1.1	5.2±2.1	7.4±2.8	10.0±3.1
	F	2.4±1.2	4.3±2.5	4.9±2.3	15.0±4.1

^{a)} A: persistence of protoplasts, B: cell wall regeneration, C: reversion of hypha, D: colony development, E: granulation of protoplasts and regenerated cells, F: trypan-blue-stained protoplasts and regenerated cells.

^{b)} Mean value ± standard deviation of 3–5 experiments.

stained positively and 91.7% of them were granulated in the presence of 25 ppm IPT (Table 1). On the other hand, granulation and positive stain of PI13 protoplasts and regenerated cells did not markedly increase: 25 ppm IPT granulated only 10% and positively stained 15% of protoplasts and cells.

DISCUSSION

The most prominent effect of IPT on the protoplast system of an IPT-sensitive isolate was the suppression of reversion at 10–25 ppm and complete inhibition of colony development

at concentrations higher than 5 ppm. As indicated by Hirooka *et al.*,³⁾ IPT, at approximately 10 ppm in the ambient water of inoculated conidia, reduced the emergence of infection pegs from appressoria of *P. oryzae* on rice leaves and cellophane film by 96 and 100%, respectively. Although the physiological similarity of regenerated cells and appressoria remains unknown, IPT seems to inhibit the emergence of a hypha or a similar structure whose formation probably depends on the metabolism activated in regenerated cells and appressoria. Another marked effect of IPT on

Fig. 1-A A spherical protoplast with a dense cytoplasm immediately after isolation.

B A protoplast with a granulated cytoplasm after treated with 25 ppm IPT for 24 hr.

C A granulated protoplast with an irregular shape after treated with 25 ppm IPT for 24 hr.

D A 25 ppm IPT-treated protoplast which stained bluish violet with trypan blue.

E A regenerated cell which stained bluish violet with trypan blue 10 hr after the onset of 10 ppm IPT treatment.

F A slightly malformed, thick hypha emerging from a regenerated cell (arrow) 24 hr after the onset of 10 ppm IPT treatment.

G Hyphae elongating from an original, single regenerated cell (arrow) in control 48 hr after the onset of incubation.

H Malformed hyphae emerging from an original regenerated cell (arrow) 24 hr after the onset of 25 ppm IPT treatment. Such hyphae usually elongate only to a limited extent.

I A regenerated cell with a short protrusion (arrow) which stained bluish violet 24 hr after the onset of 25 ppm IPT treatment.

Bar: 10 μm.

newly formed hyphae was also produced by malformed hyphae which appeared in the presence of IPT and elongated to a limited extent thereafter. These observations are probably associated with the previous results¹¹⁾ that young hyphae obtained from the colony margin of P2 were more sensitive to IPT than older ones from the intervening and central regions of the colony.

As indicated in Table 1, more protoplasts were granulated and stained with trypan blue, and more cells were regenerated as IPT concentration elevated. According to Yamada and Ohyama,¹⁵⁾ living cells cannot be stained as long as their plasmalemma functions normally, because they block infiltration of the dye into the cytoplasm, while dead cells whose plasmalemma has lost its normalcy stain bluish violet. Thus, according to their explanation, the positive stain of protoplasts and regenerated cells indicates the outcome of fungicidal action of IPT to these structures. However, it is also plausible that cells might be positively stained when IPT solely interferes with the permeability of plasmalemma without killing the cells. The reverse correlation between the frequency of reversion and the incidence of trypan blue-stained structures suggests that suppression of reversion at higher IPT concentrations might be due to the fungicidal action to these structures.

There are several possible explanations of IPT effects on the protoplast system and hyphae. First, the effects may appear when the incorporated IPT quantitatively reaches a certain inhibitory level in the cytoplasm. Second, the degradation products of IPT incorporated in the cytoplasm may interfere with the metabolism of regenerated cells and hyphae. Several hours may be required for the cytological effect to appear. Third, although IPT has a certain effect on the metabolic pathway and/or its product(s), it is not effective until the stock materials which protoplasts originally possess are used and supplemented by activation of a synthetic pathway for the materials.

Comparing the rate of persistence of P2 protoplasts with that of PI13 protoplasts in the presence of IPT, the former was affected by IPT concentration, while the latter was not.

The result may suggest that the tolerance of PI13 to IPT might be associated with its symplast or plasmalemma at least in part. Physical and/or chemical comparison between plasmalemma of both isolates might help to elucidate the occurrence of tolerant isolates.

Based on Table 1, it is most likely that IPT does not interfere with regeneration of cell walls, but there remains a possibility that cell walls formed in the presence or absence of IPT are not qualitatively identical with each other, as Ishizaki *et al.*¹³⁾ revealed by electron microscopy. Therefore, some more studies are needed to reach a final conclusion about the interference of IPT with cell wall regeneration.

As the present and previous works¹¹⁾ showed that the sensitivity of both isolates to IPT was expressed parallelly in the protoplast system and mycelia, although the former system was a little more sensitive to IPT than the latter. In addition, a possible effect of the interference with plasmalemma and/or symplast, which cannot be detected at the hyphal level,¹¹⁾ was revealed at the protoplast level. Another advantage to use protoplasts for a fungicide test is that changes associated with the fungicide effect can be observed within a relatively short time of 24 hr when IPT is used. Thus, the use of protoplasts will probably provide an advantageous system to test the fungicide effects on cell wall, hyphal formation and the effect of surfactants on plasmalemmae.

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要 約

イネいもち病菌菌糸から分離されたプロトプラストに及ぼすイソプロチオランの影響*

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イネいもち病菌 (*Pyricularia oryzae* CAV.) 菌糸から分離したプロトプラストに及ぼすイソプロチオラン (diisopropyl 1,3-dithiolan-2-ylidenemalonate: IPT) の影響を IPT 感性株 P2 および耐性株 PI13 を用いて細胞学

的に検討した。各種濃度の IPT 存在下で培養された P2 プロトプラストは時間とともに数が減少したが, PI13 のそれは減少しなかった。この結果は本菌の IPT 耐性は細胞膜を含む細胞質に関連していることを示唆している。各種濃度の IPT は両株プロトプラストの細胞壁再生率には影響を及ぼさなかったが, IPT 存在下で形成される両株の細胞壁に質的差異があるか否かは不明である。P2 の再生細胞からの菌糸復帰は 10~25 ppm IPT で抑制され, コロニーの発達は 5 ppm で完全に抑制された。この結果は, 5 ppm IPT 存在下で復帰した菌糸もその後の伸長過程で抑制をうけ, コロニーにまで発達しえないことを示唆している。なお, PI13 の菌糸復帰は 50 ppm に至るまで顕著な影響をうけなかった。本剤の効果は菌糸生長遅延および抑制に現われるとされていたが, 本研究のプロトプラスト系においても同様の結果が得られたことになり, プロトプラスト系が薬剤検定に利用されうることを示している。

* イネいもち病菌に及ぼすイソプロチオランの影響 (第3報)