Notes

Development of Resistance to Organophosphorus Fungicides in *Pyricularia oryzae* in the Field

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Development of resistance to organophosphorus thiolate (PTL) fungicides in *Pyricularia oryzae* in the field was monitored. In 1977, development of moderate resistance was demonstrated by the frequency distribution of sensitivity to IBP evaluated by minimal growth-inhibitory concentration (MIC) among field-isolates from Toyama prefecture, as compared with stock strains isolated before PTL fungicides were practically used. Discrimination of the moderately resistant (MR) strains from normal strains, however, was difficult with the evaluation of MIC. The discrimination was achieved by evaluation of radial growth of mycelia on PSA amended with IBP or by testing sensitivity to synergistic fungicidal action of IBP and a *N*-phenylphosphoramidate (PA) by the crossed paper technique. Both PTL-MR isolates, which were frequently found, and a PTL-resistant (PTL-R) isolate, which was rarely found, were resistant to the synergistic action. PTL-R and PTL-MR mutants obtained by *in vitro* selections and the PTL-R isolate were more sensitive to PA than normal strains, while PTL-MR isolates were not. The PTL-R isolate therefore seemed similar to PTL-R mutants, but the PTL-MR isolates differed from the PTL-MR mutants. No lesion was found from which both PTL-sensitive and PTL-MR strains were isolated.

INTRODUCTION

Organophosphorus thiolate (PTL) fungicides have been widely used for 14 years in Japan to control rice blast caused by *Pyricularia oryzae*. Mutants of the fungus resistant to PTL fungicides have already been reported to be easily obtained under laboratory conditions by the selection from the conidia of the fungus on agar plates containing the fungicides.^{1,2)} However, development of resistance to the fungicides in the paddy field has never been recognized until recently. In 1977, Yaoita *et al.* reported that frequency distribution of sensitivity to a PTL fungicide, IBP (*S*-benzyl diisopropyl phosphorothiolate, Kitazin P®), among isolates of the fungus collected in Niigata Prefecture in 1976, had been widened to a lower sensitivity region.³⁾ They also reported later that the sensitivity distribution differing in localities in the same

prefecture may be due to the different rates of PTL fungicides applied.⁴⁾ These results suggest the development of the resistance to the PTL fungicide, but no definite evidence has yet been obtained.

We have also been monitoring resistance to the PTL fungicide in the field since 1974, and recently found that resistance to PTL fungicides in field-isolates of the fungus had developed.

MATERIALS AND METHODS

1. Isolation of the Fungus

Infected leaves and panicles were sampled from rice fields of various locations and stored in a refrigerator at about 5°C. Surface of the samples was treated with 80% ethanol followed by 0.1% mercuric chloride before isolation. Each lesion to be tested was cut out of the sample and placed on an agar plate in a petri dish and irradiated with near-ultraviolet light

through the cover of the plate 40 cm under five tubes of FL 20 BL at 27°C for two to seven days, depending on the appearance of the sporulation on the lesion by microscopic observation. The agar plate around the sporulated lesion and the lesion were cut off, removed carefully from the dish and placed on the inside of a cover of another petri dish. The cover, on which the piece of the agar plate with the sporulated lesion was put, was then placed on a dish containing another plain agar plate. The dish was kept at 27°C to let the conidia fall on the agar plate for an appropriate time period and then the petri dish was turned upside-down so that no more conidia could fall on the plate. Conidia on the agar plate were isolated under a microscope using an ophthalmological knife and transferred onto potato sucrose agar (PSA, decoction of 200 g potato, 20 g sucrose and 15 g agar in 1 liter). Generally, an isolate was picked from one lesion unless otherwise stated.

2. Evaluation of Sensitivity of Isolates to Fungicides

Minimal growth-inhibitory concentration (MIC) was determined by observation of the fungal growth on PSA amended with the test fungicide seven days after inoculation with conidial suspension of the test isolate.

Inhibition of the mycelial growth on PSA by IBP at 100 μM was observed seven days after inoculation of a disc of a mycelial mat of 5 mm diameter, and expressed by inhibition of radial increase of the mycelial mat as compared with that on an untreated PSA plate.

Sensitivity to synergistic action of IBP and dibutyl *N*-methyl-*N*-phenylphosphoramidate (BPA) was determined by the crossed paper technique as previously reported,⁵⁾ and expressed by the expanded zone of growth-inhibition around the paper strip impregnated with IBP when crossed with BPA.

3. Fungicides

IBP and edifenphos (*S,S*-diphenyl ethyl phosphorodithiolate, EDDP, Hinosan®) which were kindly supplied by the Kumiai Chemical Co. Ltd. and Nihon Tokushu Noyaku Co. Ltd. had a purity of 100% and 85.1% respectively. BPA and dihexyl *N*-methyl-*N*-phenylphos-

phoraidate (HPA) were prepared as previously reported.⁵⁾

4. Fungal Strains

In addition to field-isolates obtained by the above method, the following strains were tested in comparison. Stock strains of the fungus, isolated from infected rice plants which had been collected from different locations in Japan before 1964 when no PTL fungicides were practically used, were kindly given by Dr. T. Yamaguchi of the National Institute of Agricultural Sciences. A mutant resistant to PTL fungicides selected from conidia of a normal wild-type strain, Hoku 373, on PSA medium which had been amended with IBP at 200 μM ,²⁾ was also tested.

Another mutant moderately resistant to PTL fungicides was also selected from Hoku 373 with IBP at 100 μM . In this selection with IBP at 100 μM , 21 colonies emerged from 6.4×10^6 conidia. Nine strains, isolated from nine out of the 21 colonies were tested for resistance. Among the nine strains, seven were resistant to IBP at 200 μM and seemed similar to the resistant mutants previously reported, but two strains were resistant at 100 μM and yet sensitive at 200 μM and seemed to be another type of mutant moderately resistant to PTL fungicides. The frequency of emergence of resistant (R) mutants was thus, $(21/(6.4 \times 10^6)) \times (7/9) = 26 \times 10^{-7}$ showing good accord with the previous result,²⁾ while that of the moderately resistant (MR) mutants was $(21/(6.4 \times 10^6)) \times (2/9) = 7.3 \times 10^{-7}$. The two PTL-MR mutants obtained seemed similar in their sensitivities to PTL and related fungicides.

RESULTS AND DISCUSSION

1. Results of Monitoring of Resistance to IBP

Since the MIC of IBP for the laboratory-derived PTL-R mutants and that for the normal wild-type strains were 400 μM and 100–50 μM respectively, 100 μM was chosen as the discriminating concentration for the monitoring of the resistant strains among field isolates in 1974 and 1976. Among 165 isolates from Ibaraki and Yamagata Prefectures in 1974, no isolate was found which was resistant to IBP at 100 μM (MIC : $\geq 200 \mu\text{M}$), while

among 173 isolates from 17 locations in Toyama Prefecture in 1976, eight isolates were resistant (Table 1). In 1977, 78 isolates from 14 locations in Toyama Prefecture were tested for sensitivity to IBP at 100 μM and 50 μM in comparison with 30 stock strains isolated before 1964. The results are shown in Table 1. Before 1964, 90% of the isolates were sensitive to IBP at 50 μM ($\text{MIC} : \leq 50 \mu\text{M}$), while 59% were at the same sensitivity level in 1977. Isolates resistant at 50 μM but sensitive at 100 μM ($\text{MIC} : 100 \mu\text{M}$) were 10%

Table 1 Result of monitoring of resistance to a PTL fungicide in field-isolates of *Pyricularia oryzae*.

Minimal growth-inhibitory concentration by IBP (μM)	Number of isolates ^{a)}			
	Before 1964	1974	1976	1977
≥ 200	0	0	8	4
100	3	165	165	28
≤ 50	27			46

^{a)} Source of isolates: Stock strains isolated before 1964, isolates from Ibaraki and Yamagata Prefectures in 1974, and isolates from Toyama Prefecture in 1976 and 1977.

before 1964 but 36% in 1974. No isolates were resistant at 100 μM ($\text{MIC} : \geq 200 \mu\text{M}$), before 1964 but 5% isolates were so in 1977. The result clearly showed a moderate development of resistance to IBP in the field, but discrimination of the moderately resistant strains from normal strains were impossible for the isolates resistant at 50 μM but sensitive at 100 μM ($\text{MIC} : 100 \mu\text{M}$). The discrimination of the moderately resistant strains might be better judged if the sensitivity was tested at closer concentration steps, but it may cause another difficulty in the judgement of MIC in the experiment, because fungal growth on PSA amended with a fungicide is hardly differentiated by the different but close concentrations.

2. Sensitivity of Isolates to Other Fungicides Related with IBP

Four out of the eight strains resistant to IBP at 100 μM which were isolated in 1976 were further tested for sensitivity to PTL and related fungicides. One of them isolated from Tonami, Toyama Pref. coded as Tn 14-1, seemed similar to the PTL-R mutant in its higher resistance to PTL fungicides, while

Table 2 Sensitivity of field-isolates and mutants of *Pyricularia oryzae* to PTL and related fungicides.

Fungicide and concentration (μM)		Isolates			Mutants ^{a)}	
		Tn 14-1	Fm 3-1	Hoku 373	PTL-MR	PTL-R
Fungal growth ^{b)}						
IBP	100	++	+	—	+	++
	200	++	—	—	—	+
Edifenphos	20	++	+	—	++	++
	40	+	—	—	—	+
HPA	100	—	++	++	++	—
	200	—	++	++	—	—
BPA	200	—	++	++	+	—
Synergistic fungitoxicity ^{c)}						
IBP and BPA		—	±	++	±	—

^{a)} Mutants were selected from conidia of a normal wild-type strain, Hoku 373. PTL-MR is moderately resistant and PTL-R is resistant to PTL fungicides.

^{b)} Fungal growth on potato sucrose agar medium containing fungicides was observed 4–7 days after inoculation with suspension of the conidia in comparison with that on untreated medium. ++: normal growth, +: growth but slightly inhibited, ±: faint growth, —: entirely inhibited.

^{c)} Synergism was observed by the crossed paper technique. ++: remarkably synergistic, +: synergistic, ±: slightly synergistic, —: not synergistic.

other three isolates from Fukumitsu, Toyama Pref., had only moderate resistance to IBP as in the case of the PTL-MR mutants. Since the three IBP-MR isolates had similar sensitivities not only to IBP but also to other PTL and related fungicides, they are represented in the present paper by just one of them, coded as Fm 3-1.

N-methylphosphoramidate (PA) compounds were reported to be specifically fungitoxic to PTL-R mutants.⁵⁾ This negative correlation between PTL and PA was also found in PTL-MR mutants obtained *in vitro*, though the levels of both the resistance to PTL and the increased sensitivity to PA were moderate. Field-isolates were tested for this negative correlation and it was found in the PTL-R isolate, Tn 14-1, but not in the PTL-MR isolate, Fm 3-1. The results are listed in Table 2.

This means that the PTL-R isolate is similar to the PTL-R mutants, but that the PTL-MR isolate is different from the PTL-MR mutant. Later work on the fungal metabolism of IBP and BPA by these strains, which will be published elsewhere, also supported this result.

Synergism of fungicidal action between PTL and PA on normal wild-type strains has also been reported.⁵⁾ It was tested on field-isolates and mutants with varied sensitivity to PTL and PA using IBP and BPA (Table 2). The synergism was observed only on the normal wild-type strain but hardly at all on the PTL-R isolate, the PTL-MR isolate, the PTL-R mutant and the PTL-MR mutant (Table 2).

3. Discrimination of PTL-MR Isolates

As discussed above, among isolates in 1977 showing a resistance level of MIC at 100 μM against IBP, there may be at least two classes of strains, the normal wild-type and PTL-MR ones.

Synergistic fungicidal action of IBP plus BPA was tested with all the IBP field-isolates in 1977 and the 30 stock strains isolated before 1964. Distribution in synergism represented by the expansion of the growth-inhibitory zone of IBP by BPA showed two peaks for the isolates in 1977 (Fig. 1). The peak of higher synergism, the right peak on Fig. 1,

Fig. 1 Sensitivity distribution of field-isolates of *P. oryzae* to synergistic fungicidal action of IBP plus BPA as evaluated by the crossed paper technique.

Fig. 2 Relationship of fungal sensitivities to inhibition of radial growth of mycelia by IBP, to synergistic fungicidal action of IBP plus BPA as tested by the crossed paper technique, and to toxicity of IBP as tested by the agar dilution method using conidial suspensions as inocula and expressed by MIC.

MIC of isolates are, $\leq 50 \mu\text{M}$ (●), $100 \mu\text{M}$ (◐) and $\geq 200 \mu\text{M}$ (○).

consisted of all the isolates sensitive to IBP at 50 μM and a small part of those resistant at 50 μM but sensitive at 100 μM , and the peak of lower synergism, the left peak on Fig. 1, consisted of all the isolates resistant to IBP at 100 μM and a large part of the isolates sensitive to 100 μM but resistant at 50 μM . Strains isolated before 1964 were distributed in the range of the higher synergism peak. Therefore, isolates belonging to the lower synergism, the left peak on Fig. 1, were assumed to be newly developed strains having varied sensitivity to PTL and related fungicides.

The radial growth of mycelia of 21 isolates

on PSA amended with IBP at 100 μ M were tested (Fig. 2). A close correlation between the synergistic activity of IBP and BPA and the inhibition of radial growth by IBP was found, and a grouping of isolates was quite clear with the inhibition of radial growth as well as with the synergistic activity. The grouping was not necessarily identical with the MIC classification, however.

The results shown in Figs. 1 and 2 indicate that the discrimination of PTL-MR isolates from normal strains will be better achieved by testing the radial growth of mycelia on IBP-containing medium or by testing for synergistic fungicidal action of BPA plus IBP rather than by determination of MIC.

By these methods, two or more isolates from each lesion were sampled and compared, but there was no case in which normal strains and PTL-R or PTL-MR strains were found in the same lesion in tests on ten IBP-sensitive isolates from two lesions and 23 IBP-MR isolates from five lesions. This result is similar to the result with the kasugamycin-resistant strains of *P. oryzae* in the field.⁶⁾

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REFERENCES

- 1) Y. Uesugi, M. Katagiri & K. Fukunaga: *Bull. Natl. Inst. Agric. Sci.* **C33**, 93 (1969)
- 2) M. Katagiri & Y. Uesugi: *Ann. Phytopathol. Soc. Jpn.* **44**, 218 (1978)
- 3) T. Yaoita, N. Go, K. Aoyagi & H. Sakurai: *Ann. Phytopathol. Soc. Jpn.* **43**, 357 (Abstract)

(1977)

- 4) T. Yaoita, N. Go, K. Aoyagi & H. Sakurai: *Ann. Phytopathol. Soc. Jpn.* **44**, 401 (Abstract) (1978)
- 5) Y. Uesugi, M. Katagiri & O. Noda: *Agric. Biol. Chem.* **38**, 907 (1974)
- 6) H. Miura, M. Katagiri, T. Yamaguchi, Y. Uesugi & H. Ito: *Ann. Phytopathol. Soc. Jpn.* **42**, 117 (1976)

要 約

圃場におけるイネいもち病菌の有機リン殺菌剤耐性の発達

片桐政子, 上杉康彦, 梅原吉広
チオール型有機リン殺菌剤 (PTL 剤) である IBP を用いてイネいもち病菌圃場分離株に対する最低生育阻止濃度 MIC を求め, PTL 剤感受性頻度分布を調査した結果, PTL 剤が実用される以前に分離された菌株に比較して, 1977 年に富山県下で分離された菌株では耐性が発達していることが明らかであったが, その程度は多くの場合大きくなく, 中程度耐性 (MR) の菌株が多く出現していると思われ, またまれには耐性度のそれより高い耐性 (R) 菌株も見いだされた。しかし, 通常の菌株と MR 分離株とを区別することは MIC 測定だけでは不十分であった。寒天培地上の菌叢生育の IBP による阻害により,あるいは, IBP と *N*-phenylphosphoramidate (PA) 間に見られる殺菌協力作用を濾紙交さ法により測定することにより通常菌と MR 分離菌株を識別することが可能と思われた。この殺菌協力作用は通常菌に対してとくに顕著であり, R 分離菌株や, MR 分離菌株にはほとんど見られないからである。実験室内選択で得た PTL-R 変異菌株や PTL-MR 変異菌株は PA 剤に特異的に感受性が高く, PA と PTL の間で負相関交差耐性の関係が成り立ち, 圃場分離菌株でも, PTL-R 株においてはこの関係が成り立つが, PTL-MR 株では成り立たず, PTL-MR 分離菌は実験室的に得た変異菌株とは異なると思われた。なお, 同一病斑から通常菌株と PTL-MR 菌株とが分離された例は本試験では見られなかった。