ペフラゾエートに対するイネばか苗病菌の感受性

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Sensitivity of *Fusarium moniliforme* Isolates to Pefurazoate

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Abstract

From the infected rice seeds with "Bakanae" disease collected in various districts in Japan, 518 isolates of *Fusarium moniliforme* were isolated and examined for their sensitivity to pefurazoate, an inhibitor of an ergosterol biosynthesis. The minimum inhibitory concentration (MIC) values of the compound were in a range from 0.78 ppm to 12.5 ppm, and the less sensitive isolates, which were reported for triflumizole known as an ergosterol biosynthesis inhibitor (EBI), to pefurazoate were not observed. However, both the fungicides had a similar trend in the sensitivity pattern of *F. moniliforme*, so that the isolates less sensitive to triflumizole were also low sensitive to pefurazoate (MIC: 6.25 ppm to 12.5 ppm). On the base of the different sensitivity to those EBIs and benomyl, the isolates could be classified into four types; isolates sensitive to both of the EBIs and benomyl (I), isolates sensitive to EBIs and resistant against benomyl (II), isolates moderately sensitive to the EBIs and sensitive to benomyl (III) and isolates moderately sensitive to the EBIs and resistant against benomyl (IV). The ratios of the isolates I, II, III and IV were 8.5%, 83.0%, 8.1% and 0.4%, respectively. The ability of these isolates to produce gibberellins and fusaric acid was estimated. The isolates I, II and IV produced gibberellins, but the isolate III did not produce them. Fusaric acid was produced by all the types of isolates, and there was no clear difference in the sensitivity to the fungicides. Rice seeds artificially inoculated with the isolate IV were disinfected with pefurazoate which showed similarly high effect as in the seeds inoculated with the isolate I, indicating that the difference in sensitivity *in vitro* was irrelevant to the actual effect of seed treatment.

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Key words: *Fusarium moniliforme*, pefurazoate, sensitivity, gibberellins, fusaric acid.

INTRODUCTION

Pefurazoate (code name: UHF 8615, trade name: Healthied), pent-4-enyl *N*-furfuryl-*N*-imidazole-1-ylcarbonyl-DL-homoalaninate, is a new seed disinfectant[15] and shows an excellent effect against major seed borne diseases such as "Bakanae" disease, Helminthosporium leaf spot and blast. The mixtures of benomyl or thiophanate-methyl and thiram have been widely used as conventional rice seed disinfectants, but in recent years, the appearance of resistant strains against benzimidazole fungicides has been reported[7,12,18] in some districts. On the other hand, triflumizole[5] was registered as a seed disinfectant effective to resistant strains to benzimidazole

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fungicide, and prochloraz[2] and many other disinfectants are now being developed. Most of these fungicides have a *N*-substituted imidazole ring in the structural formula, and it has been reported[6,13] that their modes of action were involved in the inhibition of ergosterol biosynthesis in the cell membranes of fungi. Amano *et al.*[1] reported that some EBIs had very widely distributed MIC values for *F. moniliforme*, and they were highly effective to resistant isolates against benzimidazole fungicides but rather low to sensitive isolates. Furthermore, Hamamura *et al.*[3] examined the sensitivity of *F. moniliforme* to triflumizole, and clarified the existence of less sensitive isolates valued at 1,000 ppm or more of MIC. These less sensitive isolates were poor in the ability to produce gibberellins and very weak in pathogenicity. Pefurazoate[15,16] is also an ergosterol biosynthesis inhibitor like triflumizole and prochloraz. The present report describes the sensitivity of *F. moniliforme* to pefurazoate in relation to the ability of gibberellin production, and the effect of pefurazoate treatment on the rice seeds inoculated with moderately sensitive isolates.

MATERIALS AND METHODS

Isolation. *F. moniliforme* was isolated from infected rice seeds collected from 9 places in 8 prefectures of Japan. The rice seeds were surface-sterilized by 20-fold diluted sodium hypochlorite solution for one min, washed with sterilized water and placed on a 1% sucrose agar medium. The colonies formed around the seeds were observed using a microscope to confirm the existence of microconidia of single cell in typical chains. They were identified[9] as *F. moniliforme* and the monospores were isolated. Sampled isolates were listed in Table 1.

Determination of MIC value. Pefurazoate and benomyl were used as technical products, and triflumizole as 30% wettable powder. Each fungicide dissolved in acetone was added to a potato dextrose agar (PDA) medium (50 C) to obtain the predetermined concentration. Mycelial disks (5 mm in diam.) of *F. moniliforme* were placed on the PDA plate amended with the fungicide and incubated at 24 C for 3 days, to measure the MIC values.

Extraction of gibberellins. Isolates of *F. moniliforme* cultured in Richard's medium (KNO_3 10 g, KH_2PO_4 5 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, $FeCl_3$ 0.02 g, sucrose 30 g, distilled water 1,000 ml, pH 5.4) at 28 C for 5 days on a reciprocating shaker at 135 rpm. The cultured liquid was centrifuged for separation into a mycelial pellet and filtrate, and the mycelial pellet was freeze-dried. The pH of the filtrate was adjusted to 3.0 with 1 N HCl, and 10 ml of ethyl acetate was added to 10 ml of the filtrate. The mixture was shaken and subjected to centrifugal extraction at 1,000 rpm for 5 min. The ethyl acetate layer was evaporated to dryness and the residue was redissolved in a small amount of 50% acetone to make a test sample. On the other hand, 5 ml of *n*-butanol was added to 5 ml of the lowest water phase layer and centrifuged at 1,000 rpm for 5 min, and the *n*-butanol layer was evaporated to dryness under reduced pressure and dis-

Table 1. Source of *Fusarium moniliforme* isolates used in this experiment

Isolate	No. of isolates	Cultivar	Locality	Year isolated
G 50–G119	70	Akihikari	Iwate	1985
G120–G188	69	Ohozora	Fukushima	1986
G200–G243	44	Sasanishiki	Toyama	1987
G260–G299	40	Akihikari	Aomori	1988
G330–G390	61	Nipponbare	Shimane	1988
G500–G555	56	Nipponbare	Wakayama	1988
G560–G600	41	Hidasakaemochi	Gifu	1989
G601–G663	63	Koganebare	Ohita	1989
G664–G737	74	Toyosachi	Ohita	1989
	(Total 518)			

solved as an aqueous solution. In the ethyl acetate extracts, free gibberellins are fractionated, and in the *n*-butanol extracts, polar gibberellins and bound gibberellins are fractionated[8].

Bioassay for gibberellins. Dwarfed rice seeds (cultivar: tanginbozu) were used for bioassay according to Murakami's dripping method[10] and dipping method[11]. Dripping method was the same as described previously[17]. In dipping method, five germinated rice seeds were placed side by side in a 22 mm diam. × 60 mm high tubular vial, and 0.5 ml of test solution was put into it. With a glass cover for prevention of transpiration, they were incubated for 5 days at 30 C under continuous irradiation. Five days later, the length of second sheath was measured. The quantity of gibberellins produced by each isolate was determined on the base of a standard curve expressing the relation between the concentration of GA_3 and the length of second sheath of rice. Though each isolate was estimated to produce several kinds of gibberellins, the total quantity produced was obtained in terms of GA_3.

Extraction of fusaric acid. As done for the extraction of gibberellins, 6 ml of *n*-butanol was added to 3 ml of *F. moniliforme* culture filtrate obtained by culturing in Richard's medium for 5 days. The *n*-butanol extract was evaporated under reduced pressure. The residue was dissolved in 50% acetone to estimate the amounts of fusaric acid.

Estimation of fusaric acid. The estimation was carried out according to thin layer chromatography (TLC) using a two-wavelength chromatoscanner. Five μl of a sample was spotted on a silica-gel plate for development, and detection and measurement were carried out at UV 254 nm. The developing solvent used was *n*-butanol–acetic acid–water (3:1:1). Using the calibration curve of standard fusaric acid, the amount of fusaric acid produced by each isolate was calculated.

Thin layer chromatography. Each ethyl acetate extract which confirmed to contain gibberellin-like substances by bioassay was analyzed by TLC. An ethyl acetate extract was chromatographed on silica-gel thin layers (No. 5715, Merck), using ethyl acetate–chloroform–acetic acid (20:8:1) as a developing solvent. After development, TLC plate was sprayed with 20% H_2SO_4, and heated at 120 C for 2 min. Detection was carried out at UV 366 nm so that the respective gibberellins emit fluorescence under ultraviolet rays[14].

Pathogenicity test. Rice seeds (cultivar: nipponbare) soaked in water at 24 C for 4 days were dipped for 24 hr in a conidial suspension (2×10^6 spores/ml) of *F. moniliforme* cultured in Richard's medium at 28 C for 5 days, to prepare artificially inoculated seeds. The seeds were sown on a nursery box, and cultured in a glasshouse. At the 4-leaf-stage, 30 days after sowing, the number of elongated rice seedlings was counted.

Seed treatment. The conidial suspension obtained by culturing in Richard's medium at 28 C for 3 days with shaking was centrifugally washed at 1,000 rpm for 5 min, to attain 2×10^6 spores/ml. Fifty grams of rice seeds (cultivar: nipponbare) were put into a 300-ml Erlenmeyer flask, and 9 ml of the conidial suspension was added. The mixture was thoroughly stirred and incubated at 24 C for 2 days. The seeds were air-dried at a room temperature, and used as infected seeds for a seed treatment test. The seed treatment was carried out according to the method described previously[17].

RESULTS AND DISCUSSION

Sensitivity of F. moniliforme isolates to pefurazoate and benomyl

Five hundred and eighteen isolates of *F. moniliforme* were isolated and the MIC values of pefurazoate and benomyl against those isolates were measured. As shown in Fig. 1, the MIC values of pefurazoate were in a range from 0.78 ppm to 12.5 ppm, showing a distribution with almost one peak at MIC 1.56 ppm. Such less sensitive isolate (MIC>1,000 ppm) reported[3,4] for triflumizole, known as an ergosterol biosynthesis inhibitor (EBI) like pefurazoate, was not observed. These 518 isolates could be classified in reference to the sensitivity to benomyl into 432 resistant isolates (MIC≧100 ppm) and 86 sensitive isolates (MIC≦12.5 ppm). The

sensitivity to pefurazoate showed a normal distribution with one peak at MIC 1.56 ppm against benomyl resistant isolates, but showed an irregular distribution with two peaks at MIC 1.56 and 6.25 ppm against benomyl sensitive isolates. That is, benomyl sensitive isolates contained many

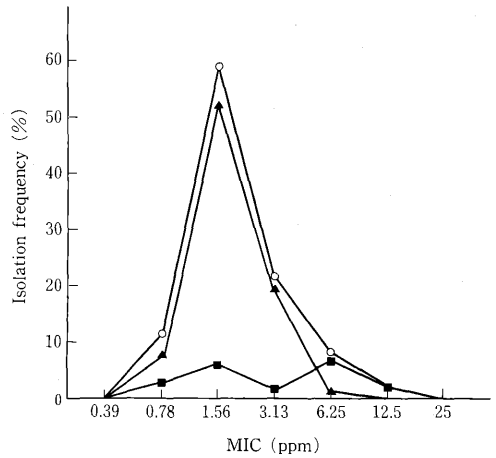

Fig. 1. Distribution of pefurazoate sensitivity in *Fusarium moniliforme* isolates.
○: total isolates (518 isolates), ▲: benomyl-resistant strains (432 isolates), ■: benomyl-sensitive strains (86 isolates).

Table 2. Sensitivity of *Fusarium moniliforme* isolates to pefurazoate, triflumizole and benomyl

Isolate	MIC (ppm)			Sensitivity to		Sensitivity type
	Pefurazoate	Triflumizole	Benomyl	EBIs[a]	Benomyl	
G 63	1.56	6.25	1.56	S[b]	S	I
G 86	1.56	0.39	3.13	S	S	
G547	3.13	0.39	1.56	S	S	
G 73	1.56	6.25	>1,600	S	R	II
G270	1.56	3.13	>1,600	S	R	
G294	1.56	6.25	>1,600	S	R	
G347	1.56	0.20	800	S	R	
G548	1.56	0.39	200	S	R	
G588	1.56	0.20	200	S	R	
G607	1.56	6.25	400	S	R	
G 76	12.5	400	3.13	MS	S	III
G 77	12.5	100	3.13	MS	S	
G166	6.25	100	3.13	MS	S	
G168	6.25	400	6.25	MS	S	
G385	12.5	100	1.56	MS	S	
G543	12.5	50	3.13	MS	S	
G552	6.25	50	3.13	MS	S	
G560	6.25	100	1.56	MS	S	
G180	6.25	25	>1,600	MS	R	IV
G236	6.25	100	400	MS	R	

a) EBIs: ergosterol-biosythesis inhibitors, here indicated pefurazoate and triflumizole.
b) S: sensitive to pefurazoate (MIC≦3.13 ppm), triflumizole (MIC≦12.5 ppm) and benomyl (MIC≦12.5 ppm).
MS: moderately sensitive to pefurazoate (MIC≧6.25 ppm) and triflumizole (MIC≧25 ppm).
R: resistant to benomyl (MIC≧100 ppm).

isolates moderately sensitive to pefurazoate (MIC 6.25 to 12.5 ppm). Therefore, from 518 isolates, 10 isolates each of moderately sensitive isolates being relatively high in MIC (MS isolates: MIC 6.25 to 12.5 ppm) and sensitive isolates (S isolates) were selected, and the MIC values of triflumizole against these isolates were measured. As shown in Table 2, the MS isolates to pefurazoate were also less sensitive to triflumizole, and the S isolates were also sensitive. Thus it was found that both the fungicides had a very similar trend in the sensitivity of *F. moniliforme*. As indicated by Amano *et al.*[1], the MIC values of triflumizole against *F. moniliforme* were widely distributed in a range from 0.2 ppm to 400 ppm, and in this regard, it was different from pefurazoate. These 20 isolates could be classified into 4 types, I to IV, as shown in Table 2 in reference to the sensitivity to pefurazoate and triflumizole (hereinafter both were expressed as the EBIs) and benomyl. The isolates in the type I showed sensitivity to both the EBIs and benomyl, accounting for 8.5% of 518 isolates. Those in the type II were sensitive to the EBIs and resistant against benomyl, accounting for the largest rate of 83.0%. Those in the type III were moderately sensitive to the EBIs (MS isolates) and sensitive to benomyl, accounting for 8.1% and those in the type IV were moderately sensitive to the EBIs and resistant against benomyl, being only 2 isolates (0.4%) among the sample isolates. Thus, *in vitro* sensitivity tests showed that the wild isolates which have not been given pressure to the EBIs contained as much as 8.5 % of isolates moderately sensitive to the EBIs (isolates of III and IV types).

Production of gibberellins and fusaric acid by moderately sensitive isolates

Hamamura *et al.*[3,4] had reported that the isolates less sensitive to triflumizole were poor in the ability to produce gibberellins and very weak in pathogenicity. Therefore, with regard to 20 isolates in Table 2, the relation between the sensitivity to the fungicides and the ability to produce gibberellins were tested according to Murakami's bioassay using Tanginbozu rice seeds. The ethyl acetate extracts and *n*-butanol extracts of the culture filtrate from the liquid culture incubated in Richard's medium for 5 days at 28 C were bioassayed. It was reported[8] that the ethyl acetate extract fraction had free gibberellins, and the *n*-butanol extract fraction had polar gibberellins and bound gibberellins. However, in the present bioassay, the gibberellin activity in the *n*-butanol extract could not be observed, and in the ethyl acetate extract remarkable gibberellin activity was observed. In view of the relation between the sensitivity of each type of

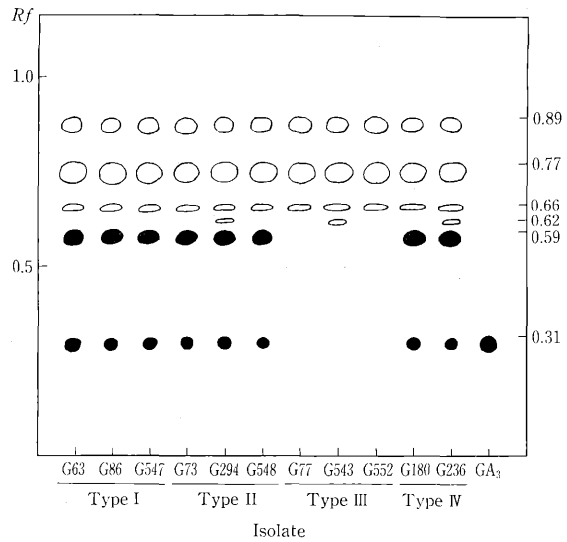

Fig. 2. Thin layer chromatographic separation of the ethyl acetate extracts from the culture filtrate of *F. moniliforme* [Ethyl acetate–chloroform–acetic acid (20:8:1)].
●: gibberellins, ○: other metabolites.

isolates to the fungicides and the ability to produce gibberellins, the isolates of the type III moderately sensitive to the EBIs and sensitive to benomyl did not show any gibberellin activity to cause the elongation of rice second sheaths. On the other hand, the isolates belonging to the I, II and IV types showed remarkable gibberellin activity. Furthermore, when the ethyl acetate extract was analyzed by silica-gel TLC, the sample from the sensitive isolates belonging to the I, II and IV types showed the existence of gibberellins at *Rf* value 0.31 position corresponding to GA_3 and furthermore at *Rf* value 0.59 position (Fig. 2). However, the isolates belonging to the type III did not show any gibberellin activity as in the case of bioassay. These results endorsed the result indicated by Hamamura *et al.*[4], that the isolates less sensitive to triflumizole were poor in the ability to produce gibberellins, but the present tests furthermore proved that the isolates less sensitive to the EBIs and sensitive to benomyl were low in the ability to produce gibberellins and that the isolates less sensitive to the EBIs and resistant against benomyl could produce gibberellins. Table 3 showed the relation between gibberellin production and fusaric

Table 3. Productivity of gibberellin-like substances by *Fusarium moniliforme* isolates and their pathogenicity

Type[a]	Isolate	Production of (mg/g of mycelial dry wt.)		Percent of elongated seedling[d] (%)
		Gibberellins[b]	Fusaric acid[c]	
I	G 63	3.8	98	43
	G 86	3.4	26	—[e]
	G547	1.6	4	71
II	G 73	2.6	90	73
	G270	1.3	42	—
	G294	1.6	137	68
	G347	4.0	nt[f]	—
	G548	1.2	10	—
	G588	7.0	nt	—
	G607	3.5	47	—
III	G 76	nd[g]	nt	—
	G 77	nd	33	0
	G166	nd	nt	—
	G168	nd	nt	—
	G385	nd	nt	—
	G543	nd	9	0
	G552	nd	10	—
	G560	nd	nt	—
IV	G180	2.9	158	37
	G236	0.6	54	56

a) See table 2.
b) Bioassay: rice seedlings (cultivar, Tanginbozu) were put on agar medium when the second leaf grew 1–2 mm in length. The ethyl acetate extracts containing gibberellin-like substances were applied to basal region between the first and the second leaf. The second leaf sheath was measured 5 days after incubation. The quantity of gibberellins produced by each isolate was determined on the base of a standard curve expressing the relation between the concentrations GA_3 and the second sheath length of rice.
c) The estimation was carried out according to TLC method using a two-wavelength chromatoscanner. Using the calibration curve of standard fusaric acid, the amount of fusaric acid produced by each isolate was calculated.
d) Pathogenicity test: rice seeds were inoculated by dipping in the conidial suspension.
e) —: no experiments.
f) nt: not tested.
g) nd: not detected.

acid production, and no clear trend could be observed between fusaric acid production and sensitivity to disinfectants.

Pathogenicity of isolates moderately sensitive to EBIs

The isolates of the type III, which did not produce gibberellins in the above bioassay, were artificially inoculated to germinated seeds and were sown on nursery culture soil, to observe whether elongation symptom appeared in seedling stage.

As shown in Table 3, the isolates of the type III did not show any elongation symptom at all, even though pink colonies peculiar to *F. moniliforme* were formed around the seeds on the culture soil, to endorse the results of *in vitro* bioassay. On the other hand, the seeds inoculated with the isolates of the type IV, less sensitive to the EBIs and resistant against benomyl, showed apparent elongation symptom. Thus it was found that many of the isolates less sensitive to the EBIs were not able to produce gibberellins and might be suitable for control by the EBIs.

However, it was confirmed that isolates of the type IV less sensitive to the EBIs and capable of producing gibberellins also exist, even though their distribution frequency is very low.

Effect of seed treatment with pefurazoate

Rice seeds were artificially inoculated with isolates less sensitive to the EBIs and capable of producing gibberellins, and the effect of seed treatment with pefurazoate was compared with that of the seeds inoculated with sensitive isolates (Table 4). Pefurazoate showed an equal effect on the seeds inoculated with either type of isolates, and the difference in MIC value were not related the effect of seed treatment at all.

As described above, there is a specific phenomenon that many of the isolates of *F. moniliforme* less sensitive to the EBIs are remarkably poor in the ability to produce gibberellins, and in addition, it was found that the phenomenon has close relation with the sensitivity to benomyl. It is a very interesting problem whether the origin of the phenomenon is concerned with the resistance mechanism of benomyl. On the other hand, the existence of the isolates less sensitive to the EBIs and capable of producing gibberellins was also observed though the rate was a very low. It is an important problem whether the effect of pefurazoate against the seeds infected with these isolates is equal to or not to that of the seeds infected with sensitive

Table 4. Effect of seed treatment with pefurazoate on control of "Bakanae" disease

Fungicide	Treatment[a]	% Control			
		MS isolates[b]		S isolates[c]	
		G180	G236	G 86	G547
Pefurazoate 20% wp	Soaking for 10 min				
	10,000 ppm	98	96	96	100
	5,000 ppm	98	95	96	100
	2,500 ppm	98	96	96	95
	Soaking for 24 hr				
	1,000 ppm	96	96	96	99
	500 ppm	95	91	93	95
	250 ppm	83	92	74	68
	Wetted dressing				
	0.5% of dry seed wt.	98	97	97	97
Untreated	Soaking in water	(48.1)[d]	(70.6)	(23.9)	(100)

a) Used seeds: inoculation was made by dipping rice seeds in the conidial suspension of *F. moniliforme* isolates.
b) MS isolates: moderately sensitive isolates to pefurazoate and triflumizole.
c) S isolates: sensitive isolates to pefurazoate and triflumizole.
d) Figures in parentheses are % diseased seedling.

isolates, and it was confirmed that the effect of seed treatment with pefurazoate against these isolates was as high as that of the seeds inoculated with sensitive isolates. The difference in MIC value were not related to the actual effect of seed treatment at all.

It is presumed that seed disinfectants with an ergosterol biosynthesis inhibitor will increase in near future, and an advent of resistant strains may occur. It is an interesting problem as a coming studies whether there are any different nature between the less sensitive strains already existing before the use of the EBIs and the resistant strains may be expected to appear after start of use.

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和 文 摘 要

和田拓雄・久津間誠一・竹中允章：ペフラゾエートに対するイネばか苗病菌の感受性

全国各地のイネばか苗病罹病種子からばか苗病菌518株を分離し，ペフラゾエートに対する感受性を検定したところ，MIC 値は0.78～12.5 ppm の範囲にあった。ペフラゾエートと同様にエルゴステロール生合成阻害剤 (EBI) であるトリフルミゾールの場合に報告されている低感受性菌 (MIC>1,000 ppm) の存在は認められなかった。しかし，両剤に対する本菌の感受性傾向は類似しており，トリフルミゾールに低感受性の菌株はペフラゾエートでも感受性が低かった (MIC 6.25～12.5 ppm)。一方，ばか苗病菌の薬剤感受性を EBI およびペノミルとの関係でみると4タイプに分類された。すなわち，(I): EBI に感受性でペノミルにも感受性の菌株，(II): EBI に感受性でペノミルに耐性の菌株，(III): EBI にやや感受性が低くペノミルには感受性の菌株，(IV): EBI にやや感受性が低くペノミルにも耐性の菌株であり，おのおのの分布比率は，8.5, 83.0, 8.1 および 0.4% であった。これら菌株のジベレリン産生およびフザリン酸産生について検定したところ，(I), (II) および (IV) の菌株はジベレリンを産生したが，(III) の菌株はいずれも非産生株であった。フザリン酸の産生は全菌株で認められ，薬剤感受性との間に明らかな相関関係はなかった。(IV) の菌株を人為接種した種籾をペフラゾエートで種子消毒したが，(I) タイプの菌株を接種した種籾の場合とまったく同様の高い効果を示し，*in vitro* での MIC 検定による感受性差は実際の種子消毒効果とは相関していなかった。