Original Article

Lowered Virulence to Rice Plants and Decreased Biosynthesis of Gibberellins in Mutants of *Gibberella fujikuroi* Selected with Pefurazoate

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Mutants of *Gibberella fujikuroi* were obtained by selection from a large number of conidia of a wild type field isolate of the fungus on agar plates containing lethal concentration of a sterol demethylation inhibitor, pefurazoate. The mutants isolated from the colonies grown on the plates had higher minimal growth-inhibitory concentrations of pefurazoate and the EC_{95} values were higher than that for the mother isolate but only a small increase was observed in the EC_{50} values. Though the slope of the dosage-response curve was changed in the mutants, the mechanism of action of the fungicide in a mutant proved to be its inhibition of sterol 14α -demethylation as shown by an experiment on inhibition of ergosterol biosynthesis using ^{14}C -acetate. Gibberellin production in the mutants in liquid culture was tested by bioassay of gibberellins and assay of gibberellin A_3 using HPLC. Mutants were also tested for their virulence to rice plants. Decreased virulence due to decreased production of gibberellins was observed in the mutants. A mutational change in a site related to 14α -demethylation in ergosterol biosynthesis and to kaurene oxidation in gibberellin biosynthesis in common is discussed.

INTRODUCTION

Sterol demethylation inhibitors (DMIs) such as triflumizole and pefurazoate have been developed and used as seed fungicides for the last several years in Japan to control Bakanae disease of rice caused by *Gibberella fujikuroi* (*Fusarium moniliforme*). Since fungal resistance to modern selective fungicides is sometimes a problem in the field, monitoring of sensitivity to the above-mentioned fungicides has already been conducted. Hamamura *et al.*¹⁾ observed a wide range of sensitivity to triflumizole among field isolates of the fungus. However less sensitive isolates were less virulent to host plants presumably due to low production of gibberellins. Wada and his coworkers²⁾ also surveyed sensitivities of field isolates of the fungus to triflumizole and to

pefurazoate, fungal gibberellin production and their virulence to rice plants. They observed that the range of distribution of sensitivity to the fungicides was seemingly narrower and the relationship between sensitivity to the fungicides and virulence to rice plants was roughly similar but a little more variable as compared with the observation by Hamamura *et al.*

The relation between 14α -demethylation in fungal sterol biosynthesis and kaurene oxidation in gibberellin biosynthesis in higher plants has already been reported and discussed by Sisler & Ragsdale.³⁾ They pointed out the similarity of enzymes involved in the two processes and their sensitivity to similar inhibitors.

The relation between inhibition of fungal biosynthesis of gibberellins and fungal growth inhibition was studied by Coolbaugh *et al.*,⁴⁾

using three DMI fungicides and two plant growth regulators which inhibit kaurene oxidation. The order of effectiveness of the five test compounds as inhibitors of fungal biosynthesis of gibberellins was similar to that as inhibitors of fungal growth. Since the order of effectiveness as inhibitor of gibberellin biosynthesis in higher plants was different from that in the fungus, they suggested that the kaurene oxidation systems from the two sources have somewhat different molecular characteristics.

The present experiments were conducted to elucidate how the mutational change in sensitivity to a DMI fungicide affect the fungal gibberellin production and virulence to rice plants, using laboratory-derived mutants of *G. fujikuroi* selected with pefurazoate.

MATERIALS AND METHODS

1. Test Fungus and Fungicide

A wild type (W) field isolate of *G. fujikuroi* coded as 167-1 was kindly supplied in 1987 by Shimane Prefectural Agricultural Experiment Station. It was originally obtained by single spore isolation from a rice field where no DMI fungicides had been used in rice cultivation. The isolate was subcultured on potato dextrose agar (PDA) medium and stored in the laboratory. Technical grade pefurazoate (Healthied®), pent-4-enyl *N*-furfuryl-*N*-imidazol-1-ylcarbonyl-DL-homocysteinate, having 95% purity was used for most tests. Enantiomers of the fungicide⁵⁾ were also used in some of the present experiments.

2. Selection of the Conidia with the Fungicide

Pefurazoate was dissolved in acetone and added to molten PDA medium which was then dispensed into Petri dishes of 9 cm diameter and allowed to solidify. One hundred microliters of a suspension of the conidia collected from fungal mats on PDA medium was added and spread uniformly on the surface of the medium in each plate. The medium contained 10^6 µg/ml of the fungicide. Several colonies per 10^6 conidia grew and each of the colonies was transferred to fungicide-free PDA 10 days after inoculation. Single spore isolation was then made from the subcultures of the in-

sensitive colonies. Nine insensitive mutants thus obtained from the field isolate were used for the present experiments.

3. Sensitivity Evaluation

Measurements of minimal inhibitory concentration (MIC) of the fungicide to fungal growth at 28°C and of growth rates in the presence of the fungicide were made three days after inoculation of fungicide-amended PDA medium with 1 mm square segments from mycelial mats. This method is similar to that in the relevant report⁵⁾ except for the modification of incubation period from four days to three days.

4. Effect of Enantiomers of Pefurazoate on Ergosterol Biosynthesis in a Mutant

Effect of the enantiomers of pefurazoate on biosynthesis of ergosterol and related sterols from $1\text{-}^{14}\text{C}$ -labeled acetate in one of the mutants, M7, was tested by the method reported elsewhere.⁵⁾

5. Evaluation of Virulence

The mutants obtained were tested for virulence to a dwarf rice cultivar, Wai-Tou C. Seeds of the test cultivar were inoculated with the mother field isolate and its mutants by soaking the seeds in suspension of the fungal conidia (2×10^6 spores/ml) for 24 hr at 25°C followed by sowing on soil in plastic containers. After 20 days under greenhouse conditions, 60 plants per container at the 3- to 4-leaf stage were evaluated for leaf elongation as compared with the uninoculated control plants. Rate of disease incidence was also calculated from the number of diseased plants which were clearly elongated and appeared abnormal as compared with the control plants. Three replications were used for these tests for virulence.

6. Examination of Fungal Production of Gibberellins

To examine the fungal production of gibberellins, 2×10^6 conidia of the mother field isolate and of its mutants were cultured in 100 ml of Richard's medium with shaking in the dark for 5 days at 28°C. This liquid medium is said to be favorable for production of gibberellin A₃ (GA₃) without causing con-

siderable production of fusaric acid and is often used in this kind of experiments.^{1,2)} Bioassay of gibberellins in the culture liquid of the mother isolate and its mutants was made using the dwarf rice cultivar Wai-Tou C as the test plant.⁶⁾ The culture filtrate was adjusted to pH 3.0 with HCl and extracted with ethyl acetate. Solvent was evaporated and the extract was dissolved again in acetone-water (1:1), adjusted to pH 7.0 with NaOH, and assayed for gibberellins. Amount of gibberellins was estimated as GA₃ equivalent from a calibration curve obtained from the elongation effect on the rice seedlings produced by authentic GA₃ purchased from Ishizu Pharmaceutical. The value by this bioassay includes various gibberellins and may be affected by stunting effect of fusaric acid in the culture extracts and will be discussed later. The above extract was also used for evaluation of GA₃ and fusaric acid by HPLC. It was conducted with Shimadzu LC-6A apparatus equipped with a loop type sample injector (Rheodyne, USA), TSK-gel ODS-80TM column (4.6 mm ϕ $\times$ 250 mm, Tosoh Co.), Shimadzu SPD-6A spectrometer for detection and Chromatopak C-R4AX integrator (Shimadzu) for data process. Elution was made with a mixture of 0.1 M phosphate buffer (pH 2.1) and acetonitrile (4:1) containing 0.8 mM sodium 1-octanesulfonate as a mobile phase at a flow rate of 1.0 ml/min at 40°C. For quantification of fusaric acid and GA₃, salicylic acid was used as an internal standard and they were monitored by UV absorption at 210 nm. Authentic standard of fusaric acid was purchased from Sigma.

RESULTS

1. Result of the Selection of the Mutants Less Sensitive to Pefurazoate

In the present experiment, frequency of emergence of pefurazoate-insensitive mutants was between 10^{-8} and 10^{-7} . No difference was observed between the mutants obtained and the wild type mother field isolate with respect to appearance and growth rate in culture in the laboratory except pefurazoate sensitivity.

The sensitivity of the mutants to pefurazoate was shown by the effective concentration required for 50% response (EC₅₀), for 95%

Table 1 Sensitivity to pefurazoate of a wild-type field isolate of *Gibberella fujikuroi* (W) and of its mutants (M1 to M7) selected with the fungicide.

Test isolate and mutants	Sensitivity ($\mu\text{g/ml}$)		
	EC ₅₀	EC ₉₅	MIC
(Field isolate)			
W	0.19	2.1	2.5
(Mutants)			
M1	0.25	7.6	>20
M2	0.22	5.7	20
M3	0.36	7.8	20
M4	0.22	3.2	10
M5	0.22	6.7	20
M6	0.27	5.4	>20
M7	0.25	7.3	>20
M8	0.32	7.6	>20
M9	0.26	8.9	20

Fig. 1 Dosage-response (DR) curves to pefurazoate of a wild-type field isolate (W) of *Gibberella fujikuroi* and one of its mutants, M7, selected with the fungicide.

response (EC₉₅) and minimal inhibitory concentration (MIC) in comparison with those required to produce a similar response of the mother isolate (Table 1). Considerable difference was found in MIC and EC₉₅ between the mother isolate and its mutants but the difference in EC₅₀ was less remarkable. It means the slope of dosage-response (DR) curve

is gentle in the mutants as compared with that for the mother isolate. A DR curve for one of the mutants M7 and that of the mother isolate are shown in Fig. 1. All the other mutants showed DR curves close to that of M7 though that of a mutant M4 laid between M7 and the mother field isolate.

2. Antifungal Mechanism of Pefurazoate in a Mutant

Fungicidal mechanism of enantiomers of pefurazoate to one of the mutants was studied. The effects of the enantiomers on ergosterol biosynthesis are shown in Table 2.

Incorporation of radioactivity into ergosterol fraction from ^{14}C -acetate was strongly inhibited by the (S)-(-)-isomer. Concomitant accumulation of 14-methylsterols, such as 24-methylenedihydrolanosterol and obtusifolliol, was also observed showing the inhibition of 14 α -demethylation in ergosterol biosynthesis. The (R)-(+)-isomer also inhibited the ergosterol biosynthesis and caused accumulation of 14-methylsterols, but the effect was smaller than with the (S)-(-)-isomer.

3. Evaluation of Fungal Gibberellin Production

Four mutants, M1, M4, M6 and M7, were tested for gibberellin production. The results of evaluation of gibberellins and fusaric acid are shown as amount per g dry weight of mycelia in Table 3. The results of bioassays of gibberellins were thought to be affected by stunting effects of fusaric acid produced along with gibberellins as stated before. The stunting effect of fusaric acid on elongation by GA_3 was, therefore, checked on the test dwarf rice plants. Higher concentration of fusaric acid really cancelled the elongation effect of GA_3 especially at higher concentration of GA_3 . However, lower concentration of fusaric acid did not show the stunting effect in the presence of GA_3 but showed a slight amplifying effect on the elongation by GA_3 at lower concentration of GA_3 . At the concentrations of GA_3 (around 20 $\mu\text{g}/\text{ml}$ or lower in test solution for application to test plants) and of fusaric acid (around 25 $\mu\text{g}/\text{ml}$ or lower) used, the effect of fusaric acid on elongation of the test plants seemed negligible under the present test conditions. Thus, amounts of gibberellins evaluat-

Table 2 Percentages of incorporation of 1- ^{14}C -acetate into lipid fractions of the mutant M7 of *Gibberella fujikuroi* in the presence of isomers of pefurazoate.

Isomers added	Sterols			Other lipids
	24-MDL	OF	ES	
Untreated control	0.7	1.0	11.2	87.1
(S)-(-)-isomer	8.2	8.7	1.4	83.1
(R)-(+)-isomer	5.0	4.2	4.6	86.2

Concentration of isomers added: 0.24 $\mu\text{g}/\text{ml}$.

Abbreviations of sterol names: 24-MDL, 24-methylenedihydrolanosterol; OF, obtusifolliol; ES, ergosterol.

Table 3 Amounts of gibberellins evaluated by bioassay, and gibberellin A_3 and fusaric acid evaluated by HPLC in extract of culture filtrate of *Gibberella fujikuroi*.

Test isolate and mutants	Bioassay	Assay by HPLC	
	Gibberellins (mg GA_3 eq./g dry wt.)	Gibberellin A_3 (mg/g dry wt.)	Fusaric acid (mg/g dry wt.)
Field isolate W	0.91	1.40	0.72
Mutant M1	0.03	0.51	1.67
M4	0.43	0.82	1.61
M6	0.14	0.10	0.71
M7	0.34	0.12	1.13

ed by bioassay and those of GA_3 assayed by HPLC produced by the mutants were lower than those produced by the wild type mother field isolate.

GA_3 production *versus* pefurazoate sensitivity as expressed in EC_{50} is shown in Fig. 2. GA_3 production decreased with decrease in sensitivity to pefurazoate.

4. Virulence of the Mutants on Rice Plants

The above four mutants of the fungus were also tested for virulence to the host plants. Disease incidence and development of the symptom, *i.e.* elongation of the host plants, are shown *versus* sensitivity of the mutants to pefurazoate (Fig. 2) and *versus* GA_3 production (Fig. 3). Lowered virulence in the mutants in comparison with their mother

Fig. 2 Relationships to EC₉₅ value of production of gibberellin A₃ (GA₃) in liquid culture and of virulence as expressed by disease incidence and by elongation of rice plants among a field isolate of *G. fujikuroi* and its mutants selected with pefurazoate.

Fig. 3 Relationships of disease incidence and of elongation of rice to GA₃ production among a field isolate of *G. fujikuroi* and its mutants selected with pefurazoate.

Fig. 4 Rice plants infected by a field isolate of *G. fujikuroi* (left) and by its mutant, M6, (middle) and uninoculated rice plants (right).

isolate seemed to be connected with decreased sensitivity to pefurazoate and with decreased production of GA₃. A part of the experiment is also shown by a photograph (Fig. 4).

DISCUSSION

For the present investigation, mutants of the fungus produced by a single gene mutation are most desirable. Chemical or physical treatment followed by the selection or training of the fungus with increasing concentration of fungicides in each of several transfers are often used to obtain mutants insensitive to fungicides. By these methods multiple mutations were more likely than by the present method. The mutants obtained were believed to have resulted from naturally occurring spontaneous mutations and multiple mutation seemed unlikely in the present selection.

The mutants obtained by the selection had DR curves with different slopes in comparison with that of the mother isolate. Slope of the curve has often been discussed as an indication of mechanism of action of a group of fungicides.⁷⁾ Since a difference in the slope of the DR curve was shown between the mother isolate and its mutants, the fungicidal mechanism in mutants was examined. The results showed that the mechanism of action of pefurazoate was inhibition of 14 α -demethylation in ergosterol biosynthesis just as in the wild type fungus.⁵⁾ Since slope of DR curve shows the range of distribution of sensitivity

among cells of the test fungus, a gentle slope of a DR curve and an increased EC₉₅ value in mutants suggest that the site of action of pefurazoate or some factor(s) related to the site is modified in such a way that the range of distribution of the sensitivity among cells is widened in a less sensitive direction.

With respect to gibberellin production in the mutants obtained, the results presented in Fig. 2 shows that mutation to a lower sensitivity to the fungicide is associated with a decrease in gibberellin production. The point to be discussed here is the connection of the gene related to sensitivity to the DMI and to fungal production of gibberellins. As Sisler & Ragsdale suggested,³⁾ the site of action of DMI fungicides, which is the 14 α -demethylation step mediated by a mixed function oxygenase (mfo) in fungal ergosterol biosynthesis, may be similar to the site of kaurene oxidation, which is a step of gibberellin biosynthesis involving an mfo. Thus, DMI fungicides, such as azole fungicides, often cause stunting effect on growth of higher plants. But there might be some difference between the DMI site in fungal cells and the site of kaurene oxidation in higher plants as elucidated by Takano and his coworkers⁸⁾ who reported the biological actions of optical isomers of another DMI fungicide, diniconazole. The (*R*)-(-)-isomer of this compound is the stronger fungicide, while the (*S*)-(+)-enantiomer shows stronger stunting effect on growth

Fig. 5 Pathways of fungal biosyntheses of ergosterol and gibberellins in *G. fujikuroi* and the proposed sites where a mutational change affects the gibberellin biosynthesis and sensitivity to pefurazoate in ergosterol biosynthesis.

of cucumber plants. However, in studies of the effect of the same fungicide on gibberellin biosynthesis in *G. fujikuroi*, it was found that the fungicidal (*R*)-(-)-isomer is more potent also as an inhibitor of fungal gibberellin biosynthesis.⁹⁾ In the case of pefurazoate, the (*S*)-(-)-isomer is the fungicidal principle for the wild type fungus as well as for the mutants mentioned above, but its inhibition of fungal gibberellin biosynthesis was not tested in the present experiments and remains to be studied in the future.

The results on virulence and gibberellin production and on antifungal characteristics of pefurazoate shown by DR curves for the mother isolate and its fungicide insensitive mutants obtained in the present experiments may be plausibly explained as follows:

The mixed function oxygenase involved in 14 α -demethylation in ergosterol biosynthesis or

a factor or an enzyme linked to this mfo is common to or at least closely related to that involved in kaurene oxidation in gibberellin biosynthesis in the fungal cells (Fig. 5). A single mutation of the gene coding for the enzyme or the factor caused a decrease in sensitivity to the fungicide and a concomitant reduction in kaurene oxidation followed by decreased production of gibberellins in the fungal cells.

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要 約

**Pefurazoate で選抜したイネばか苗病菌変異株
におけるイネへの病原力低下とジベレリン生合
成の減少**

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野生型イネばか苗病菌の分生孢子多数を pefurazoate 含有寒天培地上に接種して、生育する同剤低感受性変異菌株を分離した。変異菌株は野生型母株に比し、最低生育阻止濃度 (MIC) や 95% 有効濃度 (EC_{95}) はかなり高いが、50% 有効濃度 (EC_{50}) では大きな差はなかった。変異菌株では葉量-反応曲線の勾配が緩やかとなっていたが、 ^{14}C -標識酢酸からの菌体脂質生合成への同剤の影響を調べた結果では、作用機構はステロール 14 α -位脱メチル阻害であった。変異菌株の液体培養でのジベレリン生産を生物定量および HPLC による定量を行ない、一方、変異菌株のイネに対する病原力試験を行なった結果、変異菌株においてはジベレリン生産の減少と病原力の低下とが観察され、同菌のステロール生合成系の 14 α -位脱メチル過程とジベレリン生合成の一過程であるカウレン酸化に共通に関与する酵素または因子に変異を生じたものと推察された。