A Comparison of the Esterase and Catalase Zymograms of *Fusarium* Species with Special Reference to the Classification of a Causal Fungus of *Fusarium* Leaf Spot of Rice

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松山宣明*・脇本 哲* : *Fusarium* 属菌とくにイネ褐色葉枯病菌分類の為の
エステラーゼ, カタラーゼザイモグラムの比較

Abstract

The causal agent of *Fusarium* leaf spot of rice (Kasshokuhagare-byo) was identified as *Fusarium nivale* (pink snow mold) in 1970, however, there are still some objections. To clarify this problem zymograms of non-specific esterase and catalase excreted by the causal fungi were compared. A preliminary comparison of various formae speciales of *F. oxysporum* and *F. solani* showed that the zymograms are species specific and useful for identification and taxonomy of *Fusarium* species. The zymograms of the leaf spot of rice were strikingly different from those of *F. nivale* causing typical pink snow mold. Differences were also observed between *F. nivale* from pink snow mold and that from various graminaceous plants in summer. The significance of zymogram diversity in the taxonomy of *Fusarium* species and the classification of the leaf spot fungus of rice were discussed.

(Received July 1, 1977)

Introduction

There are many etiological studies on *Fusarium* leaf spot disease of rice^{10-13, 22}). In 1970 the causal fungus was identified as *Fusarium nivale* (*Micronectriella nivalis*) and in 1975 *Fusarium nivale* f. sp. *graminicola* and *Micronectriella nivalis* were proposed as new names²¹). However, Naito and Koshimizu²²) objected to this nomenclature by morphological and physiological diversity.

Recently, the increasing usefulness of protein spectra and zymograms from gel electrophoresis have been emphasized^{2, 4-9, 15-20, 24}). Although the taxonomy of plant pathogenic fungi depends on morphological and pathological criteria, physiological markers are useful when these markers are correlated with the major criteria. Matsuyama and Kozaka¹⁵) and Matsuyama, Kato and Yamaguchi¹⁶) studied the electrophoretic zymograms and reported their usefulness in differentiating *Pyricularia* species. In this experiment, esterase and catalase zymograms of various formae speciales of *Fusarium oxysporum* and *F. solani* were compared to find out whether they could be correlated with morphological criteria. Then zymograms of *Fusarium* leaf spot fungus of rice and *F. nivale* were compared. A preliminary report has already appeared¹⁸).

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Materials and Methods

Isolates. Fourteen isolates of *Fusarium oxysporum*, ten of *F. solani*, three of *F. nivale* isolated in the spring from typical pink snow mold of barley, nine of *F. nivale* isolated in the summer from various graminaceous plants and nine of *Fusarium* leaf spot fungus of rice (Kasshokuhagare-byo) were used in this experiment. The details of each fungus are presented in Table 1.

Culturing. Each isolate was cultured on a shaker in 100 ml of liquid synthetic medium supplemented 0.5% yeast extract for 9 days at 23 C and 25 C (*F. oxysporum*, *F. solani*).

Enzymes. Two volumes of cold acetone were added to chilled culture filtrates (100 ml). The precipitates were collected by centrifugation at $3,200 \times g$ for 10 min. The supernatant was discarded and remained acetone was eliminated *in vacuo*. The pellets were stored in deep freezer until needed. For electrophoresis the precipitates were dissolved in 1 ml of 1/60 M phosphate buffer (pH 7.4) containing 12.5% glucose.

Thin layer electrophoresis. Nitto GE (acrylamide 92% and ethyleneurea bis-acrylamide 8%) was used as a supporting medium with DMAPN (dimethylamino propionitrile) and ammonium persulfate as catalysts. A 4% gel was used with a discontinuous buffer system (Gel buffer : 0.038M Trishydroxymethylaminomethane-0.0026M citric acid pH 8.8, Electrode buffer : 0.3M boric acid - 0.05M NaOH pH 8.2). Ten μ l of each sample was poured into the gel slots. Electrophoresis was carried out at ca. 5 C for about 100 min at constant 130 V.

Detection. Non-specific esterase isozymes were detected on the gel with 0.1% Fast violet B salt and 0.05% 1- or 2-naphthylacetate in 1/60M phosphate buffer (pH 6.8). Catalase isozymes were detected with 1% H_2O_2 in acetate buffer (pH 4.7). Each isozyme was clearly detected as zone with small bubbles.

Results

Esterase zymograms of *F. oxysporum* and *F. solani*

All zymograms of various formae of *F. oxysporum* resemble each other. The esterase zymograms were very simple and the active isozyme was detected around Ef 0.35 (Fig 1, 2). Small differences in Ef value were observed between f. sp.

Fig. 1. Diagrammatic representation of esterase zymograms of *Fusarium oxysporum*. Common isozyme around Ef 0.35 was observed.

Table 1. The list of the isolates used in this experiment

<i>Fusarium</i> spp.	Isolate	Host	Place	Date	Source (Collectors)
<i>Fusarium</i> sp. (<i>Fusarium</i> leaf spot of rice)	F-11	Rice	Okayama	1974	Yamaguchi and Ito (NIAS)
	F-21	"	"	"	"
	F-41	"	Aichi	"	"
	F-51	"	Fukuoka	"	"
	F-75	"	Saga	"	"
	F-92	"	Shizuoka	"	"
	F-121	"	Iwate	"	"
	F-183	"	Miyazaki	"	"
	F-221	"	Hokkaido	"	"
<i>F. nivale</i> (From various plants in summer)	IFO-7435	Barley	Chiba	1957,5	Yokoyama (IF), Nishihara
	IFO-7436	Wheat	"	1957,6	"
	IFO-7442	Rescue grass	"	1959,5	"
	FNW	Wheat	Tochigi	1976,6	Yamaguchi (NIAS)
	FNA	Agropyron	"	"	"
	FNB	Barley	"	"	"
	FNAB	Agropyron	"	"	"
	FNR	Rye	"	"	"
	Fn-5065	Barley	"	1975,6	Nishihara (NGRI)
<i>F. nivale</i> (Pink snow mold)	Fn-1	Barley	Hokkaido	1975,4	Tsuboki, Yamaguchi (NIAS)
	Fn-2	"	(Shari)	"	" "
	Fn-3	"	(Bihoro)	"	" "
<i>F. oxysporum</i> f. sp. <i>lycopersici</i> <i>fabae</i> <i>luffae</i> <i>phaseoli</i> <i>lagenariae</i> <i>niveum</i> <i>melonis</i> <i>cucumerinum</i> <i>fragariae</i> <i>lini</i>	F-211	Tomato			Saito (Kochi Agr. Exp. stn.)
	SUF-803	"			Matsuo (Shinshu Univ.)
	SUF-960	"			via Kiso (Kurume Br. VORS)
	SUF-888	Broad Bean			"
	SUF-363	Dishcloth gourd			"
	SUF-672	Kidney Bean			"
	SUF-797	White flowered gourd			"
	SUF-366	Watermelon			"
	KF-320	melon			Kiso (Kurume Br. VORS)
	F 1-8-1	"			NIAS, Matsuo (Shinshu Univ.)
	KF-326	Cucumber			Kiso (Kurume Br. VORS)
	F 1-4-1	"			NIAS, Matsuo (Shinshu Univ.)
	KF-328	Strawberry			Fujii, Kiso (Kurume Br. VORS)
	F 1-6-1	Hemp			NIAS
<i>F. solani</i> f. sp. <i>mori</i> (MF+) <i>radicicola</i> (MF-) <i>robiniae</i> (MF-) (MF+) <i>xanthoxyli</i> (MF-) (MF+) <i>phaseoli</i> <i>pisi</i> " "	SUF-232	Mulberry			Matsuo (Shinshu Univ.)
	SUF-208	Potato			"
	SUF-578	Blacklocust			"
	SUF-577	"			"
	SUF-XV-1	<i>Xanthoxylum</i> sp.			"
	SUF-XV-23	"			"
	SUF-386	Kidney bean			"
	SUF-II-7	Pea			Matsuo (Shinshu Univ.)
	SUF-654	"			via Kiso (Kurume Br. VORS)
	F-1-10-1	"			NIAS, MATSUO (Shinshu Univ.)

note : NIAS : National Institute of Agricultural Sciences

VORS : Vegetables and Ornamental Crops Research Station

NGRI : National Grassland Research Institute

IF : Institute for Fermentation

Fig. 2 Diagrammatic representation of esterase zymograms of *Fusarium oxysporum*. J-1, 2, 3 represent races of f. sp. *lycopersici*.

lycopersici and other formae but no differences were observed among races (J-1, J-2, J-3).

The zymograms of *F. solani* were also simple. Though slight differences were observed for each forma, most isozymes were found around Ef 0.90 (Fig 3). These zymograms were distinctly different from those of *F. oxysporum*. The results show that the zymograms reflect the morphological classification. They also indicate that fungi which show the same zymogram could belong to the same species.

The esterase zymograms of *Fusarium* sp. isolated from the leaf spot of rice and *F. nivale*.

The zymograms of *Fusarium* sp. from rice were slightly more complex than those of *F. oxysporum* or *F. solani*. Isolates from various places in Japan showed almost same the zymograms (Fig 4). The isozyme at Ef 0.98 was a distinct marker for this fungus. This isozyme was undetectable without substrate and lost its activity on heating. Its electrophoretic mobility was great and it was resistant to eserine inhibition like arylesterase.

Fusarium nivale isolated in summer from various plants and in spring from a typical pink snow mold, also showed complex zymograms (Fig. 5,6). However, they were obviously different from those of the leaf spot fungus of rice. Differences were also observed between *F. nivale* isolated in summer and *F. nivale* from pink snow mold. The zymograms of both fungi were roughly similar, however, the Ef values were different.

Fig. 3 Diagrammatic representation of esterase zymograms of *Fusarium solani*. Main isozymes were always detected around Ef 0.90.

Fig. 4 Sites of esterase activity for nine isolates of *Fusarium* leaf spot fungus of rice. Ef 0.98 isozyme was commonly observed in this group.

Catalase zymograms

Catalase zymograms were very simple and one or two isozymes were detected (Fig. 7). The Ef 0.55 isozyme has high activity and was only detected in the *Fusarium* leaf spot fungus of rice. Most isolates of this fungus also had the Ef 0.84 isozyme. While *F. nivale* isolated in summer also had the Ef 0.84 isozyme and *F. nivale* from pink snow mold had an obviously different isozyme at Ef 0.77. No catalase isozymes were detected in *F. oxysporum* and *F. solani* under these experimental conditions.

Zymograms at various culture stages of the fungi

A comparison of esterase and catalase zymograms at different culture stages was carried out. The zymograms varied according to the stage of growth and gave different patterns at early stages. However, almost same patterns were obtained from 6th to 11th day (Fig. 8). The isozyme at Ef 0.98 which was specific in the zymograms of *Fusarium* leaf spot fungus of rice appeared by 6th day and was observed even 11 days after inoculation. The zymogram changes in catalase were also observed and steady zymogram was obtained from 6th to 11th day.

Fig. 5. Sites of esterase activity for three isolates of *F. nivale* isolated from pink snow mold of barley.

Discussion

Zymogram comparison, in spite of its limitations, could be a handy and reliable method for identification and taxonomy of fungi^{2,4-9,15-20,24}). Matsuyama *et al*¹⁵⁻¹⁸) stressed the usefulness of thin layer polyacrylamide gel electrophoresis for this purpose and its convenience in comparing many samples on same gel sheet.

Esterase zymograms were species specific in *Pyricularia* spp.¹⁶). Araki and Suzuki²) reported that the esterase zymograms of *F. oxysporum* f. sp. *lycopersici* and *cucumerinum* are different from that of *niveum*. Meyer and Renard²⁰) compared *F. oxysporum* f. sp. *melonis* with f. sp. *elaedis* and failed to distinguish between

Fig. 6 Sites of esterase activity for nine isolates of *F. nivale* isolated from various graminaceous plants in summer. Common isozyme around Ef 0.18 was observed in every zymograms.

Fig. 7 Diagrammatic representation of catalase zymograms of *Fusarium* leaf spot fungus of rice (left), *F. nivale* from pink snow mold (middle) and *F. nivale* isolated in summer (right).

them. Our data on 10 formae of *F. oxysporum* showed a strong similarity among them. However, slight differences in Ef values of the marker isozyme were observed between *F. oxysporum* f. sp. *lycopersici* and the other formae. Though *Fusarium oxysporum* is a huge species and shows considerable variation in morphology and pathogenicity, the zymograms of each forma were quite similar.

The zymograms of each forma of *F. solani* were almost identical and were distinctly different from those of *F. oxysporum*. This result agrees with morphological and immunological observations³⁾. Thus, the usefulness and reliability of esterase zymograms for the taxonomy were confirmed by these results.

The causal fungus of the leaf spot of rice (Kasshoku-hagare-byo) has long been studied by many workers^{10 ~13, 22)}. But the identification of the fungus has been suspended because of its difficulties of inoculation.

Around 1967 this disease was epidemic in Japan. Kitani and Ohata¹²⁾ reported that the causal fungus was morphologically same with the leaf blotch fungus of rice formerly identified as *Rynchosporium oryzae* Hashioka et Yokogi (Kumogata-byo)¹⁰⁾. However, they were uncertain if this fungus was really *Rynchosporium* sp.. In 1970 Koshimizu *et al.*¹³⁾ summarized earlier reports and concluded that the causal fungi of both diseases were the same and are Ascomycetes. In the same year this fungus

was identified as *Fusarium nivale* (*Micronectriella nivalis*)²⁶⁾. This name was listed formally as *Fusarium nivale* (Fries) Cesati f. sp. *graminicola* (Berkeley et Broome) Snyder et Hansen, *Micronectriella nivalis* (Schaffnit) Booth²¹⁾. Naito and Koshimizu²²⁾ recognized that this fungus was one of the *Fusarium* spp.. However, they have suspended if this fungus is *F. nivale* and *Micronectriella* sp. because of morphological and physiological diversity. They reported that the leaf spot fungus

Fig. 8 Esterase profiles of *Fusarium* leaf spot fungus of rice (F-51) and *F. nivale* isolated from pink snow mold of barley (Fn-3) at various culture stages.

of rice seemed to have double walled asci and chlamyospore-like structures and that the optimal temperature for mycelial growth was higher than that of *Fusarium nivale*.

All isolates from diseased rice leaves from various parts of Japan showed almost identical zymograms and had the characteristic isozyme at Ef 0.98. They form a distinct group that is quite different from *F. nivale*. Differences were also observed in catalase zymograms although one isozyme was common to the leaf spot fungus of rice and *F. nivale* isolated in the summer. This may mean that although these fungi are not the same, they may have an evolutionary relationship. The differences between zymograms support partly Naito and Koshimizu's observation²²⁾.

In 1940, it was reported that the causal fungus of pink snow mold (*Fusarium nivale* f. sp. *graminicola*) caused dark fleck on wheat leaves even in summer¹⁾. Similar symptoms were also reported on various other plants in early and mid summer and isolates were identified as *F. nivale*²³⁾. Some of these isolates were used in our studies. However, their esterase and catalase zymograms were different from those of *F. nivale* isolated from typical pink snow mold in early spring.

In earlier experiments with *Pyricularia* spp¹⁶⁾, the zymogram differences were correlated with classification at the species level based on morphology and in this studies the same phenomenon was observed. The zymogram differences between the leaf spot fungus of rice and the two kinds of *F. nivale* seem to indicate diversity at the species level. However, the Snyder-Hansen system for *Fusarium* classification²⁵⁾ is reduced for convenience to only 8 species and 34 formae speciales instead of Wollenweber's 16 sections, 66 species, 57 varieties and 22 forms²⁷⁾. Therefore, a single species sometimes involves several physiologically, and even morphologically, different isolates. Thus, different zymograms may not always mean different species in *Fusarium*. However, we can say that the leaf spot fungus of rice could not be same with *F. nivale* because of large diversity of zymograms. *Fusarium nivale* isolated in the summer and *F. nivale* isolated from typical snow mold in the spring might be in same species so far as the Snyder-Hansen system is concerned, but these two fungi are clearly not identical. *Fusarium* leaf blotch of various plants in summer is clearly different from the pink snow mold in winter. Further detailed mor-

phological comparisons are needed.

Authors are grateful to Dr. T. Yamaguchi and Dr. M. Ito of Natl. Inst. Agric. Sci., Dr. T. Araki of Hokkaido Natl. Agric. Exp. Station., Dr. N. Nishihara of Natl. Grassland Res. Institute, Dr. A. Kiso of Kurume Branch of Vegetable and Ornamental crops Res. Station, Dr. T. Yokoyama of Institute for Fermentation and Prof. Dr. T. Matsuo of Shinshu University for their supplying of cultures and many advices. We also appreciate to Dr. P. R. Day of Conn. Agric. Exp. Station, USA for his critically reviewing the manuscript.

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和 文 摘 要

Fusarium 属菌とくにイネ褐色葉枯病菌分類の為の
エステラーゼ, カタラーゼザイモグラム比較

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イネ褐色葉枯病菌は1970年 *F. nivale* (ムギ類紅色雪腐病菌) と同定されたが形態の部分的相違, 生理的性質の違いから異論もみられる。この問題を明らかにする為, 前報においてその実用性が認められた非特異的エステラーゼ, カタラーゼのザイモグラム比較を行なった。まず予備試験として *f. sp.* を異にする *F. oxysporum*, *F. solani* の比較を行なった結果, 種のレベルでザイモグラムが明らかに異っており, この手法が *Fusarium* 属菌の分類同定にも利用出来ることが確認された。イネ褐色葉枯病菌, ムギ類紅色雪腐病菌 (*F. nivale*), 夏期に各種植物から分離され *F. nivale* と同定されている菌株のザイモグラムは互に明確な違いを示した。これらの結果に基づき, *Fusarium* 属菌の分類に於てザイモグラムの差違がもつ意義, イネ褐色葉枯病菌の所属について論議を加えた。