イネいもち病菌可溶性たんぱく質および酵素の比較電気泳動

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Comparative Gel Electrophoresis of Soluble Proteins and Enzymes of Rice Blast Fungus *Pyricularia oryzae* Cav.

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松山宣明*・高坂淖爾**：イネいもち病菌可溶性たんぱく質および酵素の比較電気泳動

Abstract

One hundred and thirty-two isolates of the rice blast fungus (*Pyricularia oryzae* Cav.) of various sources were tested. Soluble proteins extracted from mycelia and enzymes from culture filtrates were subjected to the polyacrylamide gel disc and thin layer electrophoresis. Two isolate groups were distinguished in the soluble protein patterns and peroxidase zymograms, but these have no significant correlations with the geographical distribution of the isolates or their pathogenicity.

In the non-specific esterase zymograms, 3 types were distinguished and the types showed significant correlations with the geographical distribution of the isolates. The taxonomic and phytopathological significance of these findings were discussed.

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Introduction

The identification and delimitation of the microorganisms based on physiological characteristics have been attempted and their significances have been discussed. In the phytopathological fields, many attempts to estimate the virulence and the pathogenicity of parasites *in vitro* have been done by many workers. Especially, serological, electrophoretic and immunoelectrophoretic techniques have been employed in recent researches because of sensitivity and good resolutional ability[2,3,5,7,8,11,13,14,15,16,18,19].

These procedures, however, are not so simple and easy to practice, because a parasite reveals its pathogenicity as a result of the interaction between virulent or avirulent genes of the pathogen and susceptible or resistant genes of the host[6].

In this paper, the electrophoretic patterns of the soluble proteins and the zymograms of peroxidase and of non-specific esterase of rice blast fungus (*Pyricularia oryzae* Cav.) collected from various countries were compared. It was also attempted to divide the many isolates into several groups by the similarities of the electrophoretic patterns and further to find the relationship between the patterns obtained and the geographical distribution of the isolates. The possibility to estimate the pathogenicity *in vitro* by using such procedures were also discussed. The abstracts were already reported[14,15] and in this report the details will be presented.

Materials and methods

Exp. 1 Twenty five isolates of *Pyricularia oryzae* Cav. were tested. Twelve were isolated from Japan and 13 from Southeast Asia and India. The isolates were shake-cultured in the

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synthetic medium of following constituent: 5.0 g yeast extract, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4$, trace of $CaCl_2$, 20.0 g glucose, and 1 *l* distil. water.

The mycelial mats were separated by filtration and washed with distil. water. Ten grams of the mycelial mats was homogenized by a French press with 50 m*l* of cold 1/15 M phosphate buffer (pH 7.4) containing 12.5% glucose at 0-5°C. The homogenate was centrifuged at 1,700 ×g for 10 min, and the supernatant was centrifuged at 105,000×g for 60 min. Equal volume of cold acetone was added to the supernatant and centrifuged at 1,700×g for 10 min. The resulting precipitate was dissolved in 2 m*l* of distil. water. Polyacrylamide gel disc electrophoresis[4] was performed and each protein band was detected by amidoblack 10 B staining.

Exp. 2 One hundred and thirty-two isolates of various sources (Japan, Korea, Taiwan, Hong Kong, Cambodia, Vietnam, Philippines, Indonesia, Ceylon, Thailand, West Pakistan, India, Guinea, Hungary, Mexico and Brazil) and the isolates collected from gramineous weeds were used. Each isolate was cultured in the synthetic medium as stated in Exp. 1. The mycelial mats were separated by filtration and discarded. The culture filtrates were used in this experiment. Two folds of cold acetone was added to 100 m*l* of the filtrate and kept at 0-5°C for 30 min and then centrifuged at 1,700×g for 10 min. The resulting precipitate was harvested and acetone was completely eliminated *in vacuo*. Then the precipitate was dissolved in 2 m*l* of 1/15 M phosphate buffer (pH 7.4) containing 12.5% of glucose.

Thin layer electrophoresis: Since the Ef value is sometimes uncertain in the disc electrophoresis, the horizontal, thin layer polyacrylamide gel electrophoresis was employed. This method is suitable for these experiments because of its sensitivity, excellent resolutional ability, and good reproduction, and is also convenient to run many samples at the same time on the same gel sheet under same condition. Nitto GE (acrylamide 92% and ethylene urea bisacrylamide 8%) was used as a supporting medium and DMAPN (dimethylaminopropionitrile) and ammonium persulfate as a catalyst. Four % gel was used and the discontinuous buffer system (gel buffer: 0.019 M tris (hydroxymethyl) aminomethane-0.0013 M citric acid pH 8.8, electrode buffer: 0.3 M boric acid-0.05 M NaOH pH 8.2) was employed. Ten μl of each sample was poured with microsyringe into narrow channel at the origin. The electrophoresis was carried out at 5-10°C for about 80 min with 1 mA/cm (width of gel) current.

Detection of the isozymes: Peroxidase isozymes were detected with 0.2% benzidine and 0.03-0.003% H_2O_2 solution (pH 4.7) at 30°C. The non-specific esterase isozymes were detected with 0.1% fast violet B salt and 0.05% α-naphthyl acetate solution (pH 6.8) at 37°C.

Results

Exp. 1 Fifteen to twenty bands of the soluble protein were detected. The isolates showed respective patterns, and 2 groups were noted as shown in Fig. 1 and 2. The first group shown in Fig. 1 has a characteristic pattern consisting of 3 bands around Ef 0.1. Seven isolates (Or-10, In-30, WP-06, Ph-01, I-22, I-42, Hi 63-20) belong to this group. The second group shown in Fig. 2 has a distinct pattern consisting of 2 bands near Ef 0.2. Five isolates (Hoku-1, Hoku-373, Ken 62-04, No-8, Au-03) belong to the second group. Members of the first group were collected from Southeast Asia and India except for Hi 63-20, which is a Japanese origin and pathogenic to some *indica* varieties. The second group members were isolated from Japan except for Au-03, which is an Australian isolate and has similar pathogenicity as Japanese isolates.

Thus, this procedure is available to divide, though roughly, the various isolates into several groups. It is, however, practically not so easy because of uncertainty of the Ef value when

many protein bands are crowded together.

Exp. 2 There were found two groups which showed distinctly different isozyme patterns in peroxidase zymograms as shown in Fig. 3. Almost all the isolates tested belonged to type B and a few isolates (Hoku-373, Hoku-373 (0), Hoku-373 (62), Au-01, 02, 03, 04, 05, Cooch Behar) to type A.

In the non-specific esterase zymograms 3 distinct types were distinguished as shown in Fig. 4 and Table 1. These zymograms showed a good agreement with the geographical distribution of isolates. Type I has Ef 0.56 band which is characteristic to this type, and it includes most of the Japanese, Korean, and Taiwan isolates. Type II has two characteristic bands at Ef 0.92, 0.95, and it includes a large part of Indian and a small part of Southeast Asian isolates. Type III lacks the characteristics of Type I and II. This type includes most of the Southeast Asian and Central American isolates.

Discussion

Attempts to identify or to delimit various microorganisms and even to estimate *in vitro* the virulence and pathogenicity of the plant parasitic bacteria and fungi such as *Erwinia* spp.,

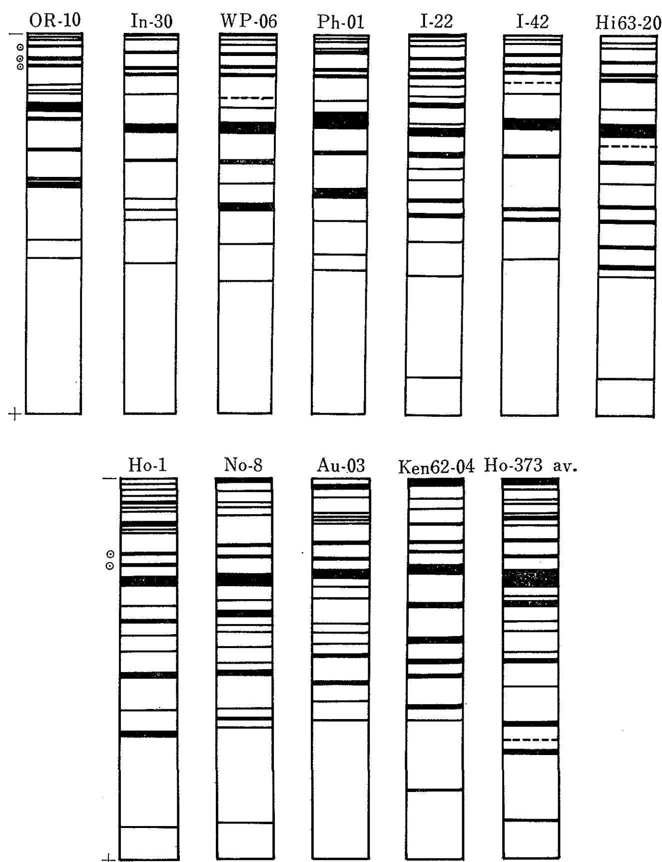

Fig. 1, 2. The disc electrophoretic patterns of soluble proteins detected with amidoblack 10 B staining.

Each figure shows the group which has partly significant similarities in protein patterns. ⊙ shows the characteristic bands of each group.

Table 1. The types of the non-specific esterase zymograms and the source of the isolates which belong to each type

	Type I	Type II	Type III
Japan	Ken 53-33, Hiro 63-20, Ken 62-89, Hoku 63-110, Ken 60-19, Hoku 373, Ken 69-04, Ken 63-23, Ken 62-34, O 65-114, Naga 87, Ai 69-226, Ken 69-11, Ken 64-38, Ken 61-14, Ken 69-08, Ken 54-04, Ken 62-04, Hoku 373 (0), O 63-69, Hoku 61-101, O 61-13		TH 65-103, TH 65-105
Korea	Wh 63-01, Wh 63-02, Wh 63-07, Wh 63-14, Wh 63-28, Wh 63-36, Wh 63-39		Wh 62-04
Taiwan	Tw-04, Tw-07, Tw-10		Tw-01, Tw-12
Hong Kong		HK-03, HK-04	HK-02, HK-06
Cambodia		Ca-04	Ca-06, Ca-12, Ca-16
Vietnam		Vn-07, Vn-11	Vn-01, Vn-04, Vn-14
Philippines		Ph-01, I-07	Ph-03, Ph-06, Ph-11, Ph-13, Ph-18, I-42
Indonesia		Is-56	Is-01, Is-04, Is-07, Is-08, Is-10, Is-12, Is-14, Is-28
Ceylon		Cy-05	Cy-01, Cy-02, Cy-03, Cy-04
Thailand		Th-04, Th-26, Th-42	Th-22
W. Pakistan		WP-07, WP-12	WP-04, WP-14, WP-19
India		In-15, In-28, In-30 Or-10, M-5, In-45, In-50, Deras, Pattambi, Srinagar, Lannan, Kalimpon, Tripwra,	In-14, Cooch Behar, M-4, In-07, In-35
Guinea		Gu-01, Gu-03, Gu-04, Gu-05	Gu-02
Hungary		Hu-07, Hu-10	
Mexico			Me-01, Me-02, Me-03, Me-04, Me-05, Me-06, Me-07, Me-08, Me-09, Me-10, Me-11, Me-12, Me-13, Me-14, Me-15, Me-16, Me-17, Me-18, Me-19, Me-20, Me-21, Me-22, Me-23, Me-24
Brazil			Br-126, Br-138

The source of each isolates had been detailed in another article[9].

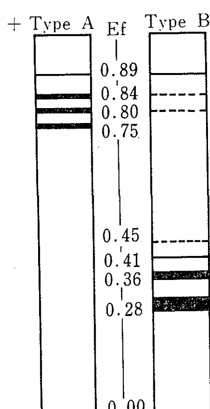

Fig. 3. Two types of the peroxidase zymograms obtained with thin layer electrophoresis.

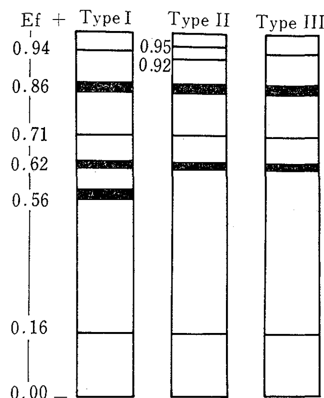

Fig. 4. Three types of the non-specific esterase zymograms obtained with thin layer electrophoresis.

One hundred and thirty-two isolates of various sources were divided into these 3 types and a significant correlation with the geographical distribution of the isolates was noted.

Agrobacterium spp., *Puccinia* spp., *Phytophthora* spp., *Pythium* spp., *Fusarium* spp., with their electrophoretic patterns of soluble proteins and enzymes have been made by many workers[2,3,5,7,8,11,13,14,15,16,18,19].

If the amount of cell wall-degrading enzymes, for example, excreted by pathogens decides the degree of virulence, it will be possible to estimate the virulence with such procedures. The estimation of the pathogenicity is not so simple and easy, because a pathogen reveals its pathogenicity as a result of the interaction between virulent or avirulent genes of parasite and susceptible or resistant genes of host[6]. Therefore, for this purpose, it is desirable to find some distinct physiological markers derived from genes which are closely linked with the virulent or avirulent genes, or to detect characteristic proteins or nucleic acids determined by allelic virulent or avirulent genes. Unfortunately, analysis of such genes have not been completed in rice plant[17], and the mechanism of resistance in molecular biological level is still vague. Therefore, at present, it seems more reasonable to ascertain whether it is possible to divide the isolates into groups with physiological similarities, and to find correlation between the physiological characteristics and the geographical distribution or the pathogenicity of the isolates. The present experiment showed that in some groups the isolates show characteristic similarities in the electrophoretic patterns of soluble proteins and that the zymographic patterns are closely correlated with the geographical distribution of the isolates. It was especially distinct in the case with non-specific esterase zymogram.

The world wide isolates of rice blast fungus were differentiated into many pathogenic races using international differential varieties[9], and it was proposed to divide these isolates into 3 groups, Japanese, Southeast Asian, and Indian groups, based on the pathogenicity against various rice varieties[12]. It is interesting that the 3 types of the non-specific esterase zymograms showed a fairly good agreement with such groups based on the pathogenicity. The mobility of the Ef 0.56 band varied slightly when the isolates Ken 54-04 lost its pathogenicity. This may indicate

a possibility that these isozymes somehow connected with the pathogenicity.

Although most of the Japanese isolates showed the type I zymogram, the Japanese isolates TH 65-103, TH 65-105 showed type III zymogram. Both these isolates were obtained from the Japanese variety "*Fukunishiki*" which has a resistant gene from U.S. variety "*Zenith*" (*intermediate* but shows a similar resistance as *indica*). If the genes of the host varieties affect the parasite genes and change the isozyme patterns, the correlation between the zymogram and the geographical distribution of isolates will gradually become vague in future, because foreign resistant genes have been transferred into Japanese varieties and *vice versa*. Although the sexual stage of *Pyricularia oryzae* is unknown and artificial mating is difficult now, the gene analysis of the parasite and host must be made to clarify these subjects. The fact that all isolates obtained from gramineous weeds belonged to type III is interesting from the evolutional view point.

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和文摘要

イネいもち病菌可溶性たんぱく質および酵素の比較電気泳動

松山宣明・高坂淖爾

日本産および外国産イネいもち病菌 *Pyricularia oryzae* Cav. の132菌株を供試した。菌体中の可溶性たんぱく質および培養沪液中から分離したパーオキシダーゼ，非特異的エステラーゼを，ポリアクリルアミ

ドゲルディスク，薄層電気泳動法によって泳動させ，アミドブラック 10B，ベンジジン—H_2O_2，ファストバイオレットB塩—α-ナフチル酢酸によりそれぞれ検出し，その電気泳動像を比較した。

可溶性たんぱく質およびパーオキシダーゼ泳動像にはそれぞれ二つの型が認められたが，菌の地理的分布，病原性等との間に明確な関係は認められなかった。一方，非特異的エステラーゼの泳動像には三つの型が検出された。これらの各型は菌の地理的分布とかなりよく一致した。すなわちI型：Ef 0.56 に特異的な染色帯をもち，日本，韓国，台湾産いもち病菌の大部分が含まれた。II型：Ef 0.92, 0.95 に特徴的な2本の染色帯をもち，主としてインド産菌および東南アジア産菌の一部が含まれた。III型：I，II型の特徴を欠く。主として東南アジア，中南米産菌が含まれた。泳動像型を用いた分類の病理学的意義についても論義を加えた。