

アジア各国から収集したXanthomonas campestris pv.oryzaeの血清型

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
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発行元	日本植物病理學會
巻/号	46巻2号
巻号補足	
掲載ページ	p. 209-215
発行年月	1980年4月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Serovars of *Xanthomonas campestris* pv. *oryzae* Collected from Asian Countries*

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崔 在乙**・松山宣明**・脇本 哲** : アジアの各国から
収集した *Xanthomonas campestris* pv. *oryzae* の血清型*

Abstract

By means of gel diffusion test with antisera produced against *Xanthomonas campestris* pv. *oryzae* isolates Q7472 (pathogenic group I) and Q7502 (pathogenic group IV), three serovars, A, B-I and B-II, were designated in *X. campestris* pv. *oryzae* isolates collected from Asian countries. Among 160 isolates tested, 132 produced precipitin bands with both antisera and were designated as serovar A. The other 28 isolates gave bands with anti-Q7502-serum but not with anti-Q7472-serum and were designated as serovar B. Serovar B was further separated into B-I and B-II, in terms of heat tolerance of antigen. Among 28 isolates belonging to serovar B, 20 fell into B-I because they did not show any changes in reaction pattern even if heat treatment (dip into boiling water for 1 hr) was given to the antigens. The remaining 8 isolates were designated as B-II as they lost their antigenicity by heating.

The degree of autoagglutination of isolates in 0.1-10% CaCl_2 or 1% acriflavine hydrochloride solution closely related with serovars. Serovar B-I and B-II showed marked autoagglutination while serovar A did not or much less in degree even if it occurred. The same phenomenon was also observed when heat treatment was given to the isolates suspended in 0.85% NaCl solution.

Serovar A is distributed all over Asian countries more widely with higher frequency as compared to serovar B.

(Received January 4, 1980)

Introduction

In regard to serological studies on *Xanthomonas campestris* pv. *oryzae*, some attempts were made to group isolates into strains by their serological characteristics. For this purpose, the agglutination test and gel diffusion test were commonly applied. In agglutination test, Fang *et al.*⁶⁾, Mukoo and Isaka¹⁶⁾ and Mahanta and Addy¹⁵⁾ reported that they could not find any distinct variations among *X. campestris* pv. *oryzae* isolates. By means of gel diffusion test, Addy and Dhal¹⁾ also reported similar results. Lin *et al.*¹¹⁾, however, showed an antigen correlated with virulence by gel diffusion test and the same result was obtained also by Addy and Dhal¹⁾.

At present, gel diffusion test has been applied most widely for analysing antigenic structures and serological grouping. By means of this technique, Grogan *et al.*⁸⁾ reported antigens species-specific for *Pseudomonas syringae* pv. *lachrymans* and *P. syringae* pv.

* Partially supported by Grant-in-Aid for Overseas Scientific Survey No. 404333 from the Ministry of Education, Science and Culture, Japan

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phaseolicola, and Lucas and Grogan¹³⁾ made clear at least three distinct serovars among *P. syringae* pv. *lachrymans* isolates. In *Xanthomonas campestris* pv. *vesicatoria*, two serovars based on the presence or absence of certain bands in gel diffusion test were designated by Charudattan *et al.*⁴⁾. De Boer *et al.* reported that the potato strains of *Erwinia carotovora* were able to be classified into 18 serogroups by diffusible somatic antigens detected by gel diffusion test⁵⁾. For the purpose of detection and identification of some kinds of plant pathogenic bacteria, Obata and others^{17,18)} tested serological specificity among species of phytopathogenic bacteria and suggested that serological method was useful for identification of bacterial species and for diagnosis of the diseases.

To make clear the serological characteristics of *X. campestris* pv. *oryzae*, gel diffusion test was carried out by using many isolates collected from Asian countries.

Materials and Methods

Isolates. One hundred and sixty isolates of *X. campestris* pv. *oryzae* were used in this study. Of these isolates, 63 originated from Japan, 8 from Indonesia, 5 from India, 29 from the Philippines, 48 from Thailand and 7 from Vietnam. The isolates have been preserved at -20°C after freeze-drying.

Preparation of antigens. Each bacterial culture grown on PSA slant medium (potato decoction 1 liter, $\text{Ca}(\text{NO}_3)_2$ 0.5 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 2.0 g, peptone 5 g, sucrose 15 g, agar 15 g, pH 7.0) was suspended in 10 ml of 0.85 % NaCl solution and washed two times by centrifugation at $8,000 \times g$ for 20 min. The precipitant was resuspended in the same solution and adjusted bacterial concentration at approximately 10^8 cells/ml. Each suspension was divided into three parts for giving treatments of heating in boiling water for 1 hr, sonication with Tominaga-150 ultrasonic vibrator for 20 min in ice box, and no treatment as a control. These antigen preparations were used immediately after the treatment or stored at -20°C until needed.

Preparation of antisera. *X. campestris* pv. *oryzae* isolates Q7472 (pathogenic group I), Q7502 (pathogenic group IV) and N5837 were used for preparing antisera. Each bacterial isolate suspended in saline solution and sonicated bacterial suspension of the isolate Q7472 were injected 6 times into the ear vein of adult New Zealand white rabbits (2.5–3.0 kg) with doses increasing from 0.5 to 2 ml at 4-day intervals. Seven days after the final injection, the rabbits were bled and antisera were prepared by ordinary procedures. The antisera thus prepared were stored at -20°C .

Serological agglutination test. Each bacterial suspension was mixed with different antisera serially diluted with saline solution in micro-precipitin tubes. The tubes were then incubated at 37°C for 1 hr and allowed to stand overnight at 4°C before observation.

Gel diffusion test. Gel diffusion test was carried out in Petri dishes (10 cm diam.) plated with 10 ml of 0.75 % agar (special agar noble, Difco) in 0.01 M potassium phosphate buffer (pH 7.4) containing 0.85 % NaCl and 0.04 % azide. Wells for antigen and antiserum were cut in sets of six peripheral wells surrounding a center well with an agar gel cutter (4 mm diam.) keeping a distance of 6 mm from each other. The wells were filled either with antisera or with antigens and Petri dishes thus prepared were incubated at room temperature for 48 hr until the precipitin bands appeared.

Autoagglutination test. The concentrated suspensions (ca. 10^8 cells/ml) of each

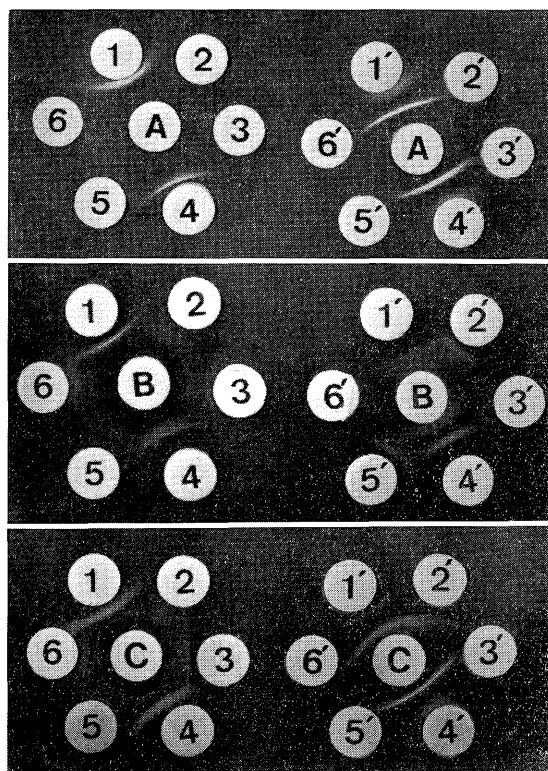


Fig. 1. Serological reactions of three different serovars of *X. campestris* pv. *oryzae*. Center wells A, B and C contain anti-Q7472-serum, anti-Q7502-serum and anti-Q7472 (sonic.)-serum, respectively. Outer wells were filled with whole cell suspensions in 0.85% NaCl solution, i. e., the wells 1 and 4 contain the suspension of the bacteria belonging to serovar A, 2, 5; serovar B-I, and 3, 6; serovar B-II. The wells 1', 2', 3', 4', 5' and 6' contain the cell suspensions of respective numbers heated at 100 C for 1 hr.

bacterial isolate in the solutions of NaCl (0.85%), CaCl₂ (0.1–10%) and acriflavine hydrochloride (1%) were incubated at room temperature for 6 hr. The degrees of agglutination were compared with each other. The effect of heat treatment (dipping into boiling water for 1 hr) on agglutination of the bacteria was also tested by using the same suspensions.

Results

One hundred and sixty isolates of *X. campestris* pv. *oryzae* showed different reactions against two kinds of antisera suggesting three serovars as shown in Table 1. Of these isolates, 132 isolates produced precipitin bands with both anti-Q7472-serum and anti-Q7502-serum, were designated as serovar A. The other 28 isolates, produced bands with anti-Q7502-serum but did not with anti-Q7472-serum, were designated as serovar B. Twenty isolates belonging to serovar B were designated as B-I because they did not show any changes in reaction pattern even if heat treated antigens were used. The remaining eight isolates belonging to serovar B were designated as B-II because they lost their antigenicity by heating. All isolates produced precipitin bands with anti-N5837-serum but spur

Table 1. Serovars of *X. campestris* pv. *oryzae* in gel diffusion test

Serotype	Treatment	Reaction to antiserum of			
		Q7472	Q7502	N5837	Q7472 (sonic.)
A	WC	+	+	+	+
	H	+	+	+	+
B	I	WC	—	+	+
		H	—	+	+
	II	WC	—	+	+
		H	—	+	+

WC: Whole cell antigen, H: Antigen heated at 100 C for 1 hr, +: Band formed, —: No band formed.

Table 2. Homologous and heterologous agglutination reaction of three serovars of *X. campestris* pv. *oryzae*

Antiserum	Isolate	Antiserum dilution									
		20	40	80	160	320	640	1280	2560	5120	10240
Anti-Q7472-serum	Q7472*	++	++	++	++	++	+	-	-	-	-
	Q7502	++	++	++	+	+	+	+	-	-	-
	N5837	++	++	++	+	+	+	+	+	-	-
Anti-Q7502-serum	Q7472	++	++	++	+	+	-	-	-	-	-
	Q7502*	++	++	++	++	++	+	+	+	-	-
	N5837	++	++	++	+	+	+	+	-	-	-
Anti-N5837-serum	Q7472	++	++	+	+	+	-	-	-	-	-
	Q7502	++	++	+	+	-	-	-	-	-	-
	N5837*	++	++	+	+	+	+	+	+	+	-
Anti-Q7472 (sonic.)-serum	Q7472*	++	++	++	++	++	+	+	+	-	-
	Q7502	++	++	++	++	++	+	+	+	-	-
	N5837	++	++	++	++	+	+	+	-	-	-

++: Complete agglutination, +: Partial agglutination, -: No agglutination, *: Homologous antiserum-antigen reaction.

was observed between the bands produced by serovar A and serovar B.

Sonication of bacterial cells changed their serological specificity. The antiserum against sonicated cells of the isolate Q7472 produced bands with intact cells of all the isolates tested (Fig. 1-C). When sonicated antigens were tested with anti-Q7472-serum, some isolates belonging to serovar B-I and B-II changed their serological properties.

Serological agglutination test was carried out with unheated antigens of each serovar. Although the titers of each antiserum somewhat varied depending upon the bacterial isolates used for titration, all the isolates belonging to each serovar showed agglutination with four antisera without showing any specificity (Table 2).

Autoagglutination of the bacteria in the solutions of 0.1–10 % CaCl_2 , 1% acriflavine hydrochloride and 0.85 % NaCl was observed. The effect of heating on the agglutination of each isolate was also tested in 0.85 % NaCl solution. The isolates belonging to serovar B could be distinguished clearly from those belonging to serovar A, because the former showed marked autoagglutination. In the case of serovar A, autoagglutination did not occur or much less in degree even if it occurred (Table 3).

The isolates belonging to serovar A are distributed more widely in Asian countries as compared to those belonging to serovar B. The isolates belonging to serovar B were not detected so far among the isolates from India and Vietnam.

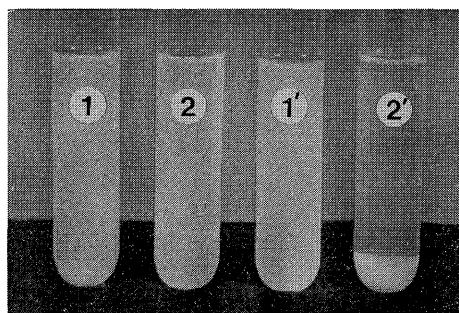


Fig. 2. Autoagglutination of *X. campestris* pv. *oryzae* in 0.85% NaCl solution. 1; serovar A unheated, 2; serovar B unheated, 1'; serovar A heated at 100 C for 1 hr, 2'; serovar B heated at 100 C for 1 hr.

Table 3. Autoagglutination in the solutions of various reagents

Reagent	Serovar A	Serovar B-I	Serovar B-II
Distilled water	—	—	—
" (H)	+	+	+
0.85 % NaCl	+	+	+
" (H)	+	‡‡	‡‡
0.1 % CaCl ₂	+	‡‡	‡‡
1 % "	+	‡‡	‡‡
10 % "	+	‡‡	‡‡
1 % Acriflavine hydrochloride	+	‡‡	‡‡

‡‡: Complete autoagglutination, +: Partial autoagglutination, —: No autoagglutination, H: Heated at 100 C for 1 hr.

Table 4. Serovars of *X. campestris* pv. *oryzae* isolates

Serovar	Origin	Isolate
A	Japan	Q7418, Q7424, Q7428, Q7447, Q7467, Q7472, Q7464, Q7484, Q7512, Q75114, Q7644, Q7649, Q7653, Q7647, Q7657, Q7660, Q7667, Q7670, Q7673, Q7781, N5701, N5710, N5801, N5805, N5818, N5824, N5831, N5834, N5846, N5850, N5854, N5861, N5863, N5879, N5881, N5843, N5802, Tokai, Ibaraki, Yoshioka, H5809, H5809-SR, T7147, T7133, T7174, H75373, H75304
	Indonesia	N6901-1, N6803-1, 2, 1-2, Ku7811, Ku7812, Ku7813
	India	India5, India25, N6914, N6910, N6916
	Philippines	PXO12, PXO15, PXO19, PXO25, PXO26, PXO29, PXO32, PXO43, PXO44, PXO54, PXO60, PXO63, PXO64, PXO65, PXO66B, PXO73, PXO81B, Ku7820, Ku7821, Ku7822, Ku7823, Ku7824, Ku7825, Ku7826, B-69
	Thailand	TB7226, TB7234, TB7245, TB7246, TB7309, TB7310, TB7312, TB7409, TB7412, TB7422, TB7423, TB7507, TB7509, TB7517, TB7523, TB7525, TB7538, TB7613, TB7615, TB7622, TB7633, TB7639, TB7702, TB7706, TB7712, TB7713, TB7715, TB7719, TB7802, TB7803, TB7804, TB7809, TB7810, TB7815, TB7816, Ku7801, Ku7802, Ku7804, Ku7805, NO1, NO2
	Vietnam	B21B1, B2A1, B2B6, B2B10, B2C3, B2C4, Ku7505
B	Japan	Q7502, Q7512, N5806, N5829, N5842, N5816, N5847, N5859, N5875, H5809 (Ku), Kagawa
	Indonesia	Ku7813
	Philippines	S-2, S-24, 4
	Thailand	TB7236, TB7302, TB7540, TB7525, TB7806
	Japan	N5833, N5845, N5868, N5837, Shiga2
II	Philippines	B-70
	Thailand	TB7205, TB7721

Discussion

Although marked differences were not observed among the isolates of *X. campestris* pv. *oryzae* in serological agglutination test, three serovars were detected in gel diffusion test.

Since the intact bacteria cannot immigrate inside gel, the precipitates observed in gel diffusion test suggest that bacterial cells exude some diffusible antigens which easily migrate inside gel. By using sonicated cells, many bands appeared in gel diffusion test, which suggests the liberation of some kinds of antigens tightly combined within cell structures by the treatment. Sonicated bacteria, however, are considered to be uncommendable for serotyping *X. campestris* pv. *oryzae*, because they did not show clear-cut specificity in gel diffusion test. The reliable method for serotyping *X. campestris* pv. *oryzae* was considered to analyze diffusible somatic antigens exuded from the cells as already reported on *Rhizobium* strains by Gibbins⁷⁾ and on *Erwinia carotovora* by De Bore *et al.*⁵⁾

The bacterial cells belonging to serovar B caused autoagglutination in 0.85 % NaCl, 0.1–10 % CaCl_2 and 1 % acriflavine hydrochloride but those belonging to serovar A did not or much less in degree even if it occurred. According to the reports by Kropinski *et al.*^{9,10)} autoagglutination patterns in 4 % NaCl and 1 % acriflavine proved to be useful for distinguish rough mutant of *Pseudomonas aeruginosa* from its wild-type strain. This technique was also used by Lindberg *et al.* in order to differentiate smooth strain from rough strain of *Salmonella*.¹²⁾ Some kinds of cations are considered to play important roles in bacterial autoagglutination.^{2,3)} The elements necessary to autoagglutination of *X. campestris* pv. *oryzae* are not known, but supposingly they might be polysaccharides and/or proteins. The effect of cations on these elements may be different depending upon serovars, that is, very strong activation may be seen on the elements of serovar B but not on those of serovar A.

With regard to geographical distribution of the serovars, serovar A is distributed more widely with higher frequency in Asian countries as compared to serovar B. Serovar B was not detected from India and Vietnam in this experiment, however, further experiments using more number of isolates will be needed to make conclusion.

Since *X. campestris* pv. *oryzae* shows at least three serovars in gel diffusion test, one has to be cautious in identification of this bacterium by serological techniques.

Acknowledgement

We wish to express our hearty thanks to the staff members of Department of Plant Pathology and Entomology, National Institute of Agricultural Sciences, Japan, Division of Environment, Kyushu National Agricultural Experiment Station, Japan, Department of Plant Pathology, the International Rice Research Institute, the Philippines, Division of Plant Pathology and Microbiology, Department of Agriculture, Thailand, and Department of Pests and Diseases, Central Research Institute for Agriculture, Indonesia, for their kind supply of the bacterial isolates and collaboration in collecting isolates in those countries.

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和 文 摘 要

アジアの各国から収集した *Xanthomonas campestris* pv. *oryzae* の血清型

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イネ白葉枯病菌菌株のうち、病原型 I 群に属する Q7472 菌と N 群に属する Q7502 菌株の抗血清を使用して、アジア各国から収集したイネ白葉枯病菌の血清学的性質を寒天ゲル内拡散法によって検討した結果、3 つの血清型に大別することができた。供試 160 菌株のうち、132 菌株は抗-Q7472-血清および抗-Q7502-血清の両者と反応し、これを血清型 A と命名した。他の 28 菌株は後者に対してのみ反応し、これを B 型とした。B 型に含まれる 28 菌株のうち、20 菌株はそれらの抗原を熱処理 (100 C, 1 時間) しても反応性を失わなかったが、8 菌株は熱処理することにより抗原性を失った。したがって前者を B-I とし、後者を B-II とした。

血清型 B-I および B-II に属する菌株の懸濁液は 0.1-10% CaCl₂, 1% acriflavine hydrochloride 液中で顕著な自己凝集反応を示したのに対し、A に属する菌株はほとんどその反応を示さなかった。また同様の現象は 0.85% NaCl 液に懸濁した細菌液を 100 C, 1 時間熱処理した場合にも認められ、抗原因子と自己凝集因子との関連性が示唆された。