한국식물병리학회지 2(3) : 150-157(1986)
Korean J. Plant Pathol. 2(3) : 150-157(1986)

Preservation Methods of *Xanthomonas campestris* pv. *oryzae* in Relation to Virulence and Colony-Type Variation.

In Gyu Hwang and Yong Sup Cho

Department of Agricultural Biology, College of Agriculture, Seoul National University, Suweon 170, Korea

벼 흰빛잎마름병균의 菌株保管에 따른 病原性 및 集落變異現象

黃 仁 奎 · 趙 鏞 涉

서울大學校 農科大學 農生物學科

ABSTRACT

Effects of presservation methods on viability and virulence of *Xanthosmonas compestris* pv. *oryzae* were investigated. The incidence of colony-type variants from freeze-dried and deep-frozen cultures was also determined. The suspending medium for freeze-dried cultures containing 10% sucrose and 1% gelatin showed the highest viability, and the virulence was well maintained in the suspending medium containing 2% dextrin, 0.5% ascorbic acid, 0.5% ammonium chloride, 0.5% thiourea, and 0.85% NaCl. Serially transferred cultures became attenuated. Rough colonies which had wrinkled surface occurred at a frequency of 1.9% to 15.8% during freeze-drying and freezing. The rough colonies consisted of nonseptated filamentous cells and rod-shaped cells. The virulence of rough colonies was weak as compared with the normal colony type.

Key words: Xanthomonas campestris pv. *oryzae*, maintenance.

要 約

벼흰빛잎마름병균의 균주를 효과적으로 보관하기 위하여 동결건조, 동결, 액체파라핀, 살균수, 계대배양 등의 방법을 사용하여 생존율과 병원성유지 정도를 4개월에 걸쳐 조사하였다. 실험결과 10% sucrose 와 1% gelatin 을 함유한 현탁액에서 가장 높은 생존율을 나타냈으며 병원성은 2% dextrin, 0.5% ascorbic acid, 0.5% ammonium chloride, 0.5% thiourea, 0.85% NaCl 등을 함유한 현탁액에서 가장 잘 유지되었다. 계대배양한 균주는 병원성이 약화되었다. 거친집락형은 동결건조 또는 동결과정에서 모두 나타났으며 이는 정상집락에 반해 주름진 집락모습과 사상형이었다. 거친집락형은 병원성이 매우 약하였으며, 탄소원 요구도가 다양하였으므로 동결건조 과정에서 대사작용의 저해가 일어난 것으로 추측되었다.

INTRODUCTION

The regular subculturing of bacterial cultures by transfering at certain time intervals is often used for a short-term storage of bacterial cultures. However, this method is not proper to preserve phytopathogenic bacteria because the bacterial

cultures may become attenuated or mutation may occur after serial transfers on culture media (7). Other preservation methods such as lyophilization, deep-freezing, drying on a suitable menstruum, suspending in sterile distilled water, and immersing in liquid paraffin or mineral oil are often preferable to subculturing for short- or long-term storage of bacterial cultures (1, 5, 6, 8, 10, 12, 13, 15, 16, 17).

Although freeze-drying or lyophilization is one of the most economical and effective methods for a long-term preservation of bacteria and other microorganisms, mutations and loss of virulence and viability may occasionally occur during freeze-drying and thawing procedures (5, 8, 9, 16). Various suspending media were developed as cryoprotective agents to increase viability of bacterial cells after lyophilization (5, 16). In the case of *Xanthomonas campestris* pv. *oryzae* which is the causal organism of bacterial leaf blight of rice (*Oryza sativa* L.), attenuation of virulence over the periods of subculturing on culture media was reported by lee at al(7).

The objective of this study is to find appropriate preservation methods for *X. campestris* pv. *oryzae* to maintain its original physiological and pathological properties. Preservation of the bacterium without changes in viability, virulence, and other characteristics is a critical procedure for various researches like resistance breeding projects which often take a long period of time.

MATERIALS AND METHODS

Preservation of bacterial isolates. The isolate, JB8206, of *X. campestris* pv. *oryzae* which belongs to race K-1 was obtained from, Institute of Agricultural Science, Rural Development Administration, Korea (22). Preservation methods and formula for each suspending medium used in this study are listed in Table 1. The cultures of *X. campestris* pv. *oryzae* were grown for 48 hours at 28 C on potato semi-synthetic agar (PSA) medium (20). For freeze-drying or deep freezing methods, bacterial cell suspensions in each of the suspending media were turbidometrically prepared to make a concentration of approximately 10^9 cells/ml. Bacterial suspensions were freeze-dried on a freeze-dryer (Thermovac FDC-8). During 26 hours of the total operation time for freeze-drying, the time required for cooling was 19 hours. The freeze-dired cultures were stored at -20 C. For freezing without drying, bacterial cell suspensions in each susupending medium were placed in a freezer at -20 C. For the liquid paraffin method, sterile liquid paraffin was added to completely cover a PSA slant culture medium on which JB8206 had grown for 48 hours at 28 C. The paraffin-covered cultures were stored

Table 1. Preservation methods and the formulae of each suspending medium in freeze-drying and freezing

Treatments	Suspending media[a]
Freeze-drying 1	10% Skim milk, 0.5% Yeast extract, 0.85% NaCl
Freeze-drying 2	0.5% Soluble starch, 1% Sodium glutamate, 1% Gelatin
Freeze-drying 3	10% Sucrose, 1% Gelatin
Freeze-drying 4	0.5% Cystein HCl, 0.1% Tryptophan, 0.5% Lactose, 0.85% NaCl
Freeze-drying 5	0.5% Ascorbic acid, 0.5% Ammonium chloride, 0.5% Thiourea, 2% Dextrin, 0.85% NaCl
Freezing 1	1% CMC[b], 0.1M $MgSO_4$
Freezing 2	10% Glycerol, PSA broth[c]
Liquid paraffin	–
Distilled water	–
Serial transfer	–

[a] pH = 7.2 – 7.4.

[b] CMC = Carboxymethylcellulose sodium.

[c] PSA broth : Potato 300g, $Ca(NO_3)\cdot 4H_2O$ 0.5g, $NaHPO_4\cdot 12H_2O$ 2.0g, Peptone 5.0g, Sucrose 15.0g, Distilled water 1l.

at 5 C. For the sterile distilled water method, the 48-hour-old culture of the bacterium at 28 C was suspended in sterilized distilled water to a concentration of approximately 10^9 cells/ml. The cell suspension was stored at 5 C.

Viability and virulence of stored cultures. Viability of the cultures preserved by various method was determined for four months. The stored cultures were resuspended and diluted to appropriate concentrations. One tenth ml of diluted culture was pipetted directly on solidified nutrient agar plates, and then spread with a glass rod. Colonies appeared on the plates after 7-day incubation at 28 C were counted.

Virelence of the stored cultures was evaluated by inoculating the cultures to rice plants of cultivar Milyang 23 grown for 50-60 days in pots in a greenhouse. Inoculum was prepared from 48-hour-old cultures which were originated from a single colony from the stored culture of each of the preservation methods. The inoculum for each treatment was turbidometrically adjusted to the final concentration of approximately 10^8 cells/ml. Inoculation was done by clipping the tips of rice plant leaves using scissors with the inoculum on them (3,4). Virulence of the stored cultures was evaluated by measuring the lesion length 15 days after inoculation. Pots of inoculated plants for all treatments were arranged in a completely randomized design with three replications.

Morphological and physiological properties o colony-type variants. The surface of single colonie recovered from the stored cultures was examined a X40 magnification under a light microscope. Th shape of bacterial cells from rough colonies whic occurred during the storage period was examined a X1,000 magnification under a light microscop with oil-immersion after the simple staining wit methylene blue. The methods described by Harriga and McCance (2) were used for the tests of gelati liquefaction, hydrolysis of casein, catalase reduc tion, oxidase test, hydrolysis of starch, productio of hydrogen sulfide, litmus milk reaction, methy red test, production of ammonia, reduction o nitrate, and acid production from various carbohy drates.

RESULTS

Viability and virulence of stored cultures. N detectable loss of viability of freeze-dried culture occurred for four months. However, the number o viable cells in cultures which were deep-frozen o stored in distilled water decreased during th

Table 2. Effects of bacterial culture storage methods on the survival and virulence of *Xanthomonas campestris* pv. *oryzae*

Storage method[a]	No. survival (X10^7 cells/ml) after:				Virulence (cm) after:			
	1 Mon	2 Mon	3 Mon	4 Mon	1 Mon	2 Mon	3 Mon	4 Mon
Freeze-drying 1	4.4[d]	3.8	4.2	4.2	3.7dcd	2.abc	2.5cd	2.6de
Freeze-drying 2	4.7	4.9	3.5	3.8	5.5a	6.3a	4.6b	3.5bc
Freeze-drying 3	5.5	5.4	5.4	5.3	3.1bc	5.9a	4.9b	3.8b
Freeze-drying 4	3.8	1.0	1.0	1.0	5.6a	2.7bc	2.0cd	2.5de
Freeze-drying 5	3.2	2.9	2.8	2.9	5.5a	5.9a	6.7a	6.5a
Freezing 1	1.9	1.9	1.9	2.0	3.0bc	3.0bc	2.5cd	1.9de
Freezing 2	1.9	1.9	2.1	1.9	3.8d	3.2c	2.8cd	2.5de
Liquid paraffin					2.5b	2.9bc	2.9d	2.7ce
Distilled water	1.3	1.2	1.3	1.0	2.8bc	2.8bc	2.9d	2.6de
Serial transfer					3.2cd	1.9b	1.7c	1.7d

[a] Each of storage methods is presented in Table 1.

[b] Means of 5 observations.

[c] Means of 3 observations.

[d] Means followed by a common letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

period. The highest viability was observed when 10% sucrose and 1% gelatin were used as the suspending medium and the lowest when the suspending medium consisted of 0.5% lactose, 0.5% cystein HCl, 0.1% tryptophan, and 0.85% NaCl (Table 2). There was no difference in viability between cultures which were frozen and stored in distilled water at 5 C. The virulence of cultures was maintained better when freeze-dried than when stored by other methods. Serially transferred cultures on PSA slants became attenuated during the period of four months (Table 2).

Morphological characteristics and incidence of colony type variants. Five different types of colony variants were obtained in this study (Table 3). Growth rates of the colony type variants did not

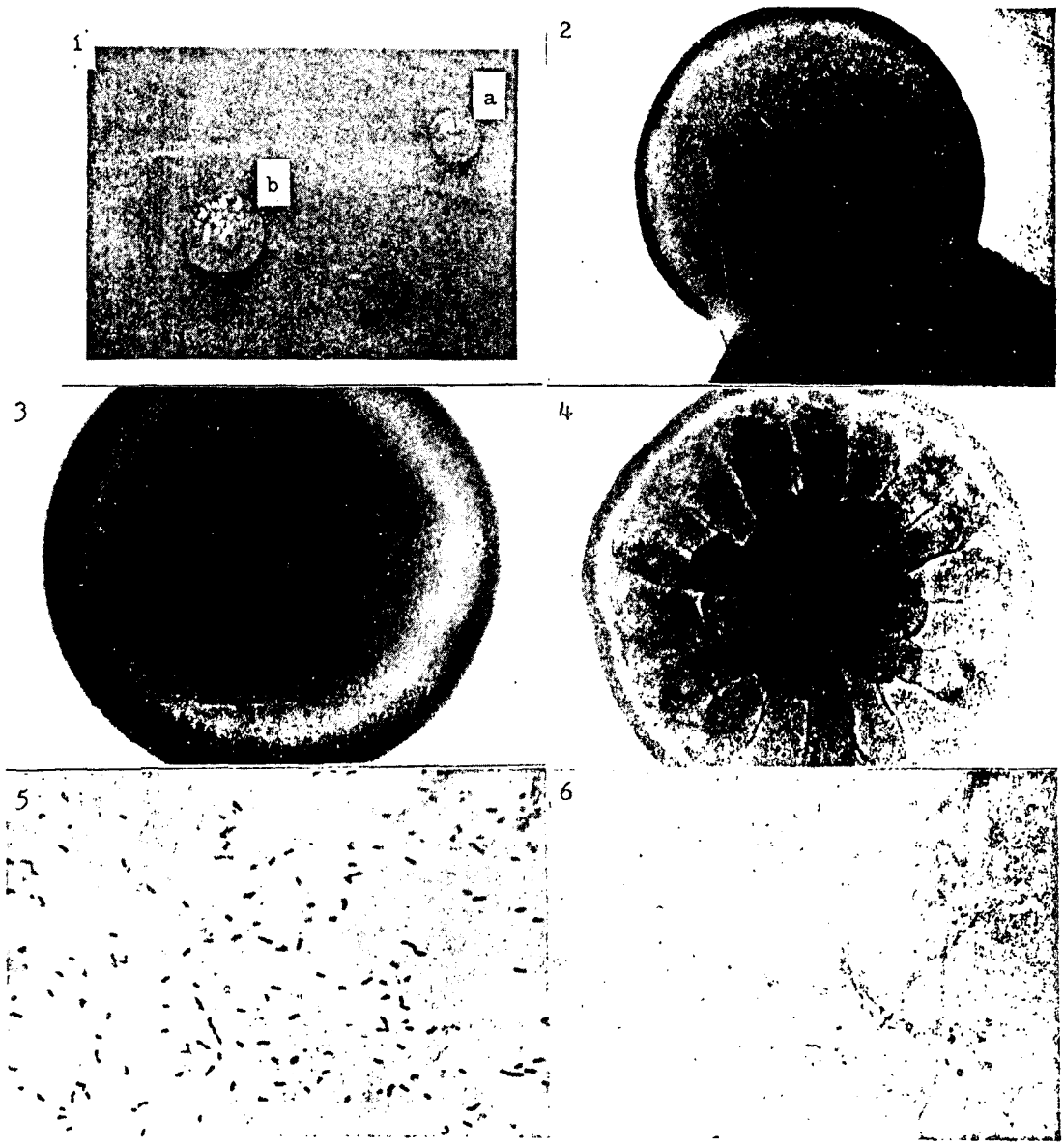

Fig. 1. Morphological characteristics of colony-type variants.
1. a) and b) show normal and rough colony types, respectively.
2. Normal colony type surface (x40).
3,4. Rough colony type surface (x40).
5,6. Normal rod and filamentous shape stained with methylene blue (x1000).

Table 3. The colony types in relation to the shape of colony surface of *Xanthomonas campestris* pv. *oryzae* due to storage condition

Types	Colony size[a] (diam. in mm)	Features of colony
Mdy	1.5-2.0	Mucoid, dark yellow, contents homogeneous
Mswy	1.5-2.0	Mucoid, semiwrinkled, yellow, contents heterogeneous
Rwy	2.0-2.5	Rough, wrinkled, yellow, contents heterogeneous
Mpy	3.0-3.5	Mucoid, flat, pale yellow, contents heterogeneous
Mcy	2.5-3.0	Mucoid, convexed white core, yellow, contents heterogeneous

a Cultures on nutrient agar for 7 days at 28°C.

Table 4. Incidence of colony-type variants after 4 months(%)[a]

Origin	Colony type				
	Mdy	Mswy	Rwy	Mpy	Mcy
Freeze-drying 1 ($4.2x10^7$/ml)	ND[b]	2.4	4.8	ND	ND
Freeze-drying 2 ($3.8x10^7$/ml)	2.6	ND	5.2	ND	ND
Frreze-drying 3 ($5.3x10^7$/ml)	ND	ND	5.7	ND	1.9
Freeze-drying 4 ($1.9x10^7$/ml)	ND	ND	15.8	10.5	ND

a Incidence was determined by mean of 3 observations per treatment.
b Not detected.

differ significantly from that of the normal colony type isolates. The colony colors of variants were yellow, dark yellow, or pale yellow. The contents of colonies were heterogeneous except the Mdy colony type. Cells of rough colonies were filamentous or rod-shaped (Fig. 1). The incidence of colony type variants ranged from 1.9% to 15.8% (Table 4). The Rwy colonies occurred in all freeze-drying and freezing methods except freeze-drying 5 (Table 4).

Physiological properties of colony type variants. Five colony type variants derived from the parent strain JB 8206 of *X. campestris* pv. *oryzae* showed similar results in the most of bacteriological tests employed in this study (Table 5). All colony type variants were Gram negative, and showed negative reactions in the oxidase, methyl red, and nitrate reduction tests, and variable reactions in the production of hydrogen sulfide. Hydrogen sulfide was slightly produced from cystein by the normal, Mswy, Rwy, and Mcy colony types. The Mdy and Mpy colony types did not produce hydrogen sulfide. Ammonia was produced from the pepton water only by the normal colony type. There were some differences between the normal colony type and colony type variants in acid production from various carbohydrates. Reactions of all isolates were negative for lactose, mannitol, and raffinose, and variable for arabinose, dextrin, glucose, maltose, sorbitol, starch, and sucrose (Table 5).

Virulence of colony type variants. The leaves inoculated with the Nswy, Mpy, Mcy, and the normal colony types were curled, but those with the Mdy and Rwy colony types were not. Virulence of the Mswy and Mpy colony types were similar to that of normal colony type. However, the Mdy and Rwy colony types showed weak virulence compared with the normal colony type (Fig. 2).

Table 5. Physiological properties of colony-type variants

Tests[a]	Normal	Variants					Wakimoto et al. (1960)
		Mdy	Mswy	Rwy	Mpy	Mcy	
Gelatin liquefaction	–	–	–	–	–	–	–
Hydrolysis of casein	–	–	–	–	–	–	–
Catalase reduction	+	+	+	+	+	+	+
Oxidase test	–	–	–	–	–	–	–
Production of H_2S	±	–	±	±	–	±	±
Hydrolysis of starch	–	–	–	–	–	–	–
Production of NH_3	+	–	–	–	–	–	+
Litmus milk reaction	+	+	+	+	+	+	+
Methyl red test	–	–	–	–	–	–	–
Reduction of nitrate	–	–	–	–	–	–	–
Acid production from							
Arabinose	±	+	–	–	–	–	+
Dextrin	±	±	–	+	–	±	–
Glucose	+	–	+	+	–	+	+
Lactose	–	–	–	–	–	–	–
Maltose	+	–	–	+	±	–	–
Mannitol	–	–	–	–	–	–	±
Raffinose	–	–	–	–	–	–	–
Sorbitol	±	+	–	–	–	±	±
Starch	–	–	–	–	±	–	–
Sucrose	+	+	±	±	+	+	+

[a] Test results : + =positive, ± = weakly positive, – = negative.

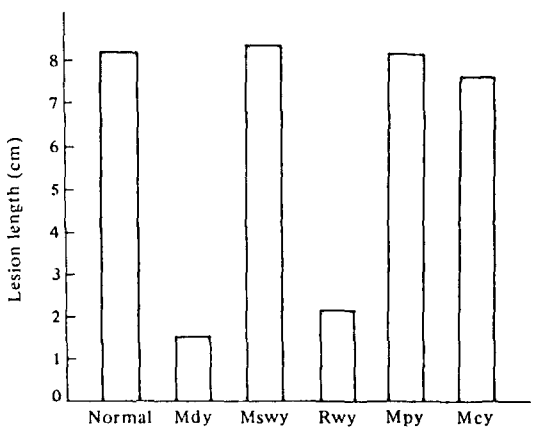

Fig. 2. Virulence of colony-type variants of *Xanthomonas campestris* pv. *oryzae* on rice cultivar Milyang 23.

DISCUSSION

The efficiency of freeze-drying depends on suspending medium, cooling and warming rates, and growth stage of bacteria (5). The results in this study indicated that the suspending media for bacterial culture storage influenced viability of cells. The protective effects of additives against freezing and drying were not clear, because there were no experimental evidences about the protective mechanisms. In general, it is known that the rapid cooling and dehydration are efficient for the preservation of bacterial cultures (16). The rehydrating materials were not considered as a factor affecting viability and permeability. However, bacterial survival and morphological change according to various cooling and warming rates and rehydrating materials must be considered to enhance the effectiveness during freeze-drying in furthur studies. Freeze-drying combines a number of stresses that may cause injuries to microorganisms. The possibility of metabolic injury to bacteria due to coldness is frequently mentioned (14, 18). Variations in production of hydrogen sulfide and ammonia, and acid production from various carbohydrates suggested that metabolic changes might occur during freeze-drying and deep-freezing.

In the case of *E. coli*, the *ruv* and the *lon* genes are involved in the septum formation after DNA synthesis inhibition by exposure to low doses of UV. The UV radiation inhibits the septum formation as a result of alterations in the DAN metabolism (11, 14, 19, 21). The formation of nonseptated filamentous cells of *X. campestris* pv. *oryzae* by freeze-drying was not previously reported. This study indicates that the formation of nonseptated filaments may occur by freeze-drying or deep-freezing.

Considering the incidence of morphological mutants and metabolic changes, we conclude that cultures of *X. campestris* pv. *oryzae* should be preserved by the freeze-drying method. It is also necessary to select the normal colonies from freeze-dried cultures prior to using the cultures for other purposes. Bacterial isolates recovered from freeze-dried cultures without selecting the normal colonies may not provide consistant results in the virulence tests. The nonseptated filamentous cells obtained in this study could be used as an useful tool for future studies on the normal regulation of cell division.

REFERENCES

1. CLEMENT, M. T. (1961). Effect of freeze-drying, and storage in the freeze-dried and frozen state on biability of *Escherichia coli* cell. *Can. J. Microbiol.* 7: 99-106.
2. HARRIGAN, W. F. & McCANCE, M. E. (1966) Laboratory methods in microbiology. Academic Press, London.
3. KAUFFMAN, H. E., REDDY, A. P. K., HSIEH, S. P. T. & MERCA, S. D. (1973). An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. *Plant Dis. Reptr.* 57: 537-541.
4. IRRI. (1966). Inoculation technique for bacterial leaf blight of rice. *IRRI Annual Report* 92-94, 1966.
5. LAPAGE, S. P., SHELTON, J. E. & MITCHELL, T. G. (1970). Culture collections and the preservation of bacteria. Pagas 136-228 *in Methods in Microbiology*. Vol. 3A, ed. by J. R. Norris & D. W, Ribbons. Academic Press Inc., London.
6. LEBEN, C. (1982). Preservation of plant-pathogenic bacteria on silica gel. *Plant Disease 66: 327.*
7. LEE, S. G., LEE, T. H., CHOI, Y. C. & CHO, Y. C. (1979–. Effect of serial transfer on the virulence of *Xanthomonas oryzae*. *Korean J. Plant Prot.* 18: 73-76.
8. MARTIN, S. M. (1963). Conservation of microorganisms. *Ann. Rev. Microbiol.* 18: 1-16.
9. MAZUR, P. (1970). Cryobiology: The freezing of biological system. *Science* 168: 939-949.
10. MOORE, L. W. & CARLSON, R. V. (1975). Liquid nitrogen storage of phytopathogenic bacteria. *Phytopathology* 65: 246-250.
11. OTSUJI, N., IYEHARA, H. & HIDESHIMA, Y, (1974). Isolation and characterization of an *Escherichia coli* ruv mutant which forms nonseptate filaments after low doses of ultraviolet light. *J. Bacteriol.* 117: 337-344.
12. PERSON, L. H. (1969). Preservation and virulence of *Xanthomonas vesicatoria* isolates maintsined in sterilized tap or distilled water. *Plant Dis. Reptr.* 53: 927-929.
13. QUADLING, C. (1960). Preservation of *Xanthomonas* by freezing in glycerol broth. *Can. J. Microbiol.* 6: 475-477.
14. SINSKEY, T. J. & SILVERMAN, G. J. (1970). Characterization of injury incurred by *Escherichia coli* upon freeze-drying. *J. Bacteriol.* 66: 327 & 7.1 7. 1 7. LEE 101: 429-437.
15. SLEESMAN, J. P. & LEBEN, C. (1978). Preserving phytopathogenic bacteria at -70 C or with silica gel. *Plant Dis. Reptr.* 62: 910-913.
16. SOUREX J. (1974). Long-term preservation by freeze-drying of pathogenic bacteria of the Czechoslovak National Collection of Type cultures. *Int. J. Syst. Bacteriol.* 24: 358-365.
17. SUSLOW, T. V. & SCHROTH, M. N. (1981). Bacterial culture preservation in frozen and

dry-film methylcellulose. *Appl. Environ. Microbiol.* 42: 872-877.

8. STRAKA, R. P. & STOKES, J. L. (1959). Metabolic injury to bacteria at low temperatures. *J. Bacteriol.* 78: 181-185.
9. TANAKA, Y., YOH, M., TAKEDA, Y. & MIWATANI, T. (1979). Induction of mutation in *Escherichia coli* by freeze-drying. *Appl. Environ. Microbiol.* 37: 369-372.
0. WAKIMOTO, S. (1960). Classification of strains of *Xanthomonas campestris* pv. *oryzae* in the basis of their susceptibility to bacteriophages. *Ann. Phytopathol. Soc. Japan* 25(4): 193-198.
21. WALKER, J. R. & PARDEE, A. B. (1967). Conditional mutations involving septum formation in *Escherichia coli*. *J. Bacteriol.* 93: 107-114.
22. YUN, M. S., LEE, E. J. & CHO, Y. S. (1985). Pathogenic specialization of the rice bacterial leaf blight pathogen, *Xanthomonas campestris* pv. *oryzae:* Race classification based on reactions of Korean differential varieties. *Korean J. Plant Prot.* 24(2): 97-101.