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Ice-Nucleation Activity of *Pseudomonas syringae* Isolated in Korea

Yong Hwan Kim*, Young Cheol Kim, Baik Ho Cho and Ki Chung Kim

College of Agriculture, Chonnam National University, Kwangju 505, Korea
**Institute of Agricultural Sciences, Suwon 170, Korea*

韓國에서 分離한 *Pseudomonas syringae*의 氷核活性

金龍煥* · 金永哲 · 趙白皓 · 金基清

全南大學校 農科大學
* 農村振興廳 農業技術研究所

ABSTRACT

Cell suspensions of two isolates of *Pseudomonas syringae*, PS8401 from sweet persimon and PS8402 from tea plant, were active in ice nucleation at -2.5 and -3.8°C, respectively. Ice nucleation at those temperature was, using micropipette method, detected in suspensions (10^8 colony forming unit/ml of distilled water) of cells that had been grown on nutrient agar supplemented with 2.5% glycerol. Using the same method, on the other hand, the freezing temperature of distilled water only was approx. -21.8°C, and those of various plant saps including corn were lower than -11.6°C. Corn seedlings sprayed with cell suspensions (10^8 cfu/ml of nutrient broth) of PS8401 began to be damaged at -2°C and were almost completely damaged at -4°C, whereas seedlings sprayed with nutrient broth only were not injured until the temperature down to -9°C. Amounts of frost damage measured 48 hr after application of PS8401 suspensions increased as applied bacterial cell densities were increased. Ice-nucleation activity of the cell suspensions *in vitro* increased with increasing the number of cells in suspension. The activity also affected by growth-medium composition or growth-temperature. Ice nucleation thus occured at -4.0, -4.4 and -7.2°C in suspensions (10^2 cfu/ml) of PS8401 that had been grown at 25°C on nutrient agar with 2.5% glycerol, nutrient agar with 2.5% glucose and nutrient agar only, respectively, and occured at -4.0 and -7.6°C in suspensions (10^2 cfu/ml) of PS8401 that had been grown on nutrient agar with 2.5% glycerol at 15-25°C and 30°C, respectively.

Key words: ice-nucleation active bacteria, *Pseudomonas syringae*, frost injury.

要 約

단감나무와 차나무의 表面에서 分離한 *Pseudomonas syringae* 2系統 PS8401, PS8402를 2.5% glycerol이 添加된 nutrient agar에서 培養한 다음, 증류수로 細胞懸濁液(10^8 colony forming unit/$m\ell$)을

調劑하여 마이크로피펫法으로 凍結溫度를 測定하여 본 結果, 各各 −2.5와 −3.8℃에서 凍結하여 氷核活性이 認定되었다. 한편 同一한 方法에 依한 증류수의 凍結溫度는 −21.8℃였고 옥수수를 비롯한 8가지 作物汁液의 凍結溫度는 −11.6℃ 以下였다. PS 8401의 nutrient broth 懸濁液(10^8 cfu/mℓ)을 撒布한 옥수수幼苗는 −2℃에서부터 얼기 시작하여 −4℃가 되면, 거의 全體 植物이 霜害를 입었으나 nutrient broth만 撒布한 對照區의 幼苗은 溫度가 −9℃로 내려가기 前까지는 被害를 입지 않았다. PS8401 懸濁液을 撒布한 後 48時間에 測定한 幼苗의 被害는 撒布한 細菌의 量이 增加함에 따라 심해지는 傾向이었다.

上記 PS 8401의 氷核活性은 懸濁液內 細菌의 數가 많아짐에 따라 增加하였으며 細菌을 培養한 培地의 組成이나 培養溫度에 따라서도 氷核活性이 變하였는데 2.5% glycerol 혹은 2.5% glucose를 添加한 nutrient agar와 탄소원을 별도로 添加하지 않은 nutrient agar에서 자란 細菌懸濁液(10^2 cfu/mℓ)의 凍結溫度는 各各 −4.0, −4.4, −7.2℃였고, 培地를 2.5% glycerol을 含有한 nutrient agar로 固定하고 溫度를 달리하여 生育시킨 細菌懸濁液(10^2 cfu/mℓ)의 凍結溫度는 −4.0℃(生育溫度가 15～25℃인 경우)와 −7.6℃(生育溫度가 30℃인 경우)였다.

INTRODUCTION

Frost injury has been described as one of the main limiting factors foi crop production in many locations in temperate zone. But, until recently, little attention has been paid to the mechanism of frost injury in frost-sensitive plants. Frost damage to frost-sensitive plants usually occurs between -2 and -5°C under natural conditions (2). At these temperature, ice formed in or on frost-sensitive plants spreads rapidly both intercellularly and intracellularly, causing mechanical disruption of cell membranes. In the absence of sites capable of ice nucleation, however, the water in plant tissues can supercool; freezing will not occur until the temperature becomes lower than -8°C to -10°C since plant materials are inefficient ice nuclei themselves (4). Because ice formation and subsequent frost damage occur at temperatures warmer than the determined temperature limits of plant supercooling, efficient heterogenous ice nuclei must limit supercooling under natural conditions. Ice nuclei have been presumed to be deposited on plant leaves from the atmosphere (8).

Recent research has focused on the search for biological sources of ice nuclei. Schnell and Vali (12) found a correlation between the content of ice nuclei and the content of organic matter in soils. Autoclaved soil lost all of its ice-nucleation activity at temperatures warmer than -10°C (13). Decaying leaf debris also contained many ice nuclei active at temperatures as warm as -5°C (12, 14). However, the most active ice nuclei that have been identified from terrestrial sources was the bacterium, *Pseudomonas syringae* van Hall (9). Certain strains of both *Erwinia herbicola* (Lönnis) Dye (6) and *Pseudomonas fluorescens* Migula (3) are also active in ice nucleation. The strains of *P. syringae* and *E. herbicola* catalyze ice formation at temperatures as warm as -1°C, and live in a commensal relationship with potential host and non host plants, and produce large epiphytic populations on the plant surface (5).

This study was conducted to identify the ice nucleation active bacteria isolated from some of the frost-sensitive plants distributed in Jeonnam province in Korea, and to demonstrate that they are active in ice nucleation *in vitro* and in promoting frost damage of corn plants.

MATERIALS AND METHODS

In 1984, the isolates of ice nucleation active (INA) becteria were obtained from the winter buds or sprouts of sweet persimon, tea tree and onion distributed in Jeonnam province in Korea.

Isolation of INA bacteria. Two grams (Fresh wt.) of winter buds or sprouts in 50ml of sterilized water were shaken at 24°C for 1hr, and the supernatants were diluted, and streaked on yeast-extract peptone agar (pH 6.8). INA bacteria were isolated,

using the method described below, on the basis of their ice nucleation activity. Unless otherwise specified, cultures were grown and maintained on nutrient agar (pH 7.2).

Identification of INA bacteria. Physiological and chemical characteristics of each isolate were determined to identify the isolates using Api 20-E kit (France Api International S. A.) in the help of Schaad's method (11), and the reactions referred to Bergey's manual, 1984.

Measurement of freezing temperature. Bacterial cells were removed from discrete colonies, suspended in sterile distilled water, and diluted to the desired cell densities (subsequently determined by dilution plating). The ice nucleation temperature of each suspension was determined by micropipette method (10). A micropipette (Type 10 LAMBDA Drummond Scientific Co. U. S. A.) was filled with 10μl of cell suspension and then the bottom of the pipette was sealed using gas burner. When being sealed, the surface of suspension was at a distance (20mm) from the sealing point for protection from being heated. A part of micropipette containing suspension was dipped in ethanol bath so that the upper surface of suspension in micropipette would be on the same line with the surface of ethanol. With circulation, ethanol in bath was cooled down at the rate of 0.3°C per minute by refrigeration. Freezing of the supension in the micropipette was easily detected with naked eyes since freezing forced to raise the surface of cell suspension (approx. 8.3% increase in volume). Plant saps were extracted in mortor, strained through 0.25μm milipore filter, and their freezing temperature were measured using the same method described above.

Measurement of frost damage to corn seedlings. Frost damage to three-leaf-stage corn seedlings at -4.5°C was measured by the similar method reported by Arny et al. (1). Plants were sprayed with suspensions of *P. syringae* in nutrient broth at various times before freezing. Plants were incubated in a mist chamber for 24hr and then incubated for another 24 hr in ambient air (wet treatment), or left in ambient air for the entire 48hr (dry treatment) at about 24°C in the dark. After incubation, plants were chilled for 5 min in a refrigerator previously adjusted at -4.5°C, and finally allowed to warm in ambient air until symptoms appeared. Frost injury was estimated by measuring the percent leaves damaged. Each of the three leaves of every corn seedling was rated for frost injury. Ten plants were included in each treatment. The leaf damaged was scored as one regardless of that the extent of injury in the leaf was severe or not. Frost injury was also estimated by measuring electrolyte leakage of the damaged leaves. Ten grams (fresh wt.) of frozen leaves were cut into segments (about 3mm in length) and immersed in 200ml of distilled water, and then shaken at 24°C for 1hr. The electrolyte of the supernatant was measured using a conductivity meter (Beckman Model RC-16C). The total conductivity of the leaves that had been boiled for 5 min in distilled water was about 1.7 m mhos.

RESULTS AND DISCUSSION

To isolate the bacteria active in ice nucleation (INA– bacteria) that were commonly present as epiphytes on frost sensitive plants in Korea, winter buds or sprouts of various plants were sampled and numbers of discrete colonies were isolated from them. Although most of the isolates had not shown ice nucleation activity, two isolates of *Pseudomonas syringae* were active in ice nucleation at -2.5 and -3.8°C, respectively (Table 1). The isolate PS8401 which was more active in ice nucleation has 1-2 polar flagella, and was selected for further study.

The presence of *P. syringae* on corn leaves resulted in badly damaged plants at -4°C but comparable leaves without *P. syringae* were not injured until the temperature down to -9°C (Fig. 1). Moreover, the freezing temperatures of various plant saps including corn plant were lower than -11.6°C (Table 2). It appears, therefore, that if the isolate of *P. syringae* is representative of the bacteria in ice nucleation on leaves of field-grown plants in Korea, there will be a large population of the isolate on plants under the natural conditions that frost-

Table 1. Freezing temperature of suspensions of the bacteria isolated from the surface of frost sensitive plants in Korea[a]

Bacterial suspension	Origin	Freezing temperature (°C)
Distilled water only	–	-21.8 ± 0.89[b]
Pseudomonas syringae 8401	Sweet persimon	-2.5 ± 0.44
Pseudomonas syringae 8402	Tea plant	-3.8 ± 0.98
Pseudomonas fluorescens 8401	Onion	-8.1 ± 0.46
Erwinia herbicola	Onion	-13.3 ± 0.52
Others	Sweet persimon, Tea plant and Onion	-14.7 ~ -21.7

[a] Bacterial concentration in a micropipette (10μl) was approx. 10^8 cfu/ml of distilled water. Cultures grown on nutrient agar with 2.5% glycerol for 48hr were used.
[b] Values are means of 15 replicates with standard error.

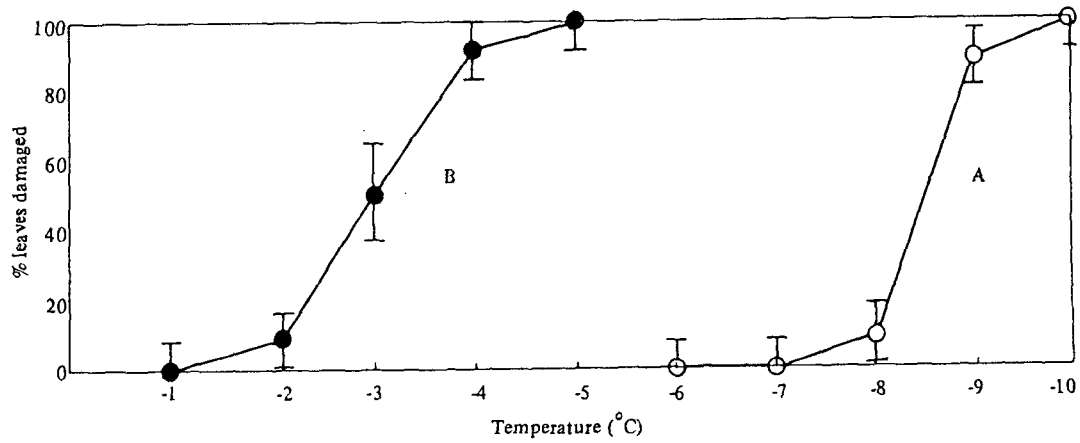

Fig. 1. Effect of *Pseudomonas syringae* 8401 on frost damage in corn. Corn seedlings were sprayed with nutrient broth only (A) or with bacterial suspension of PS8401 in nutrient broth (10^8 cfu/ml) (B), and held in a mist chamber of 48 hr before exposure at the temperatures shown on the abscissa. Vertical bars represent the standard deviations.

Table 2. Freezing temperature of different plant leaf saps held in micropieptte (10 μl)

Plant	Freezing temperature (°C)	Plant	Freezing temperature (°C)
Corn	-12.4 ± 0.72[a]	Tomato	-15.2 ± 0.39
Apricot	-11.9 ± 0.85	Jujube	-12.5 ± 0.45
Persimmon	-13.5 ± 0.64	Apple tree	-12.7 ± 0.73
Tea plant	-11.6 ± 0.56	Walnut	-14.4 ± 0.62

[a] Values are means of 15 replicates with standard error.

sensitive plants are apt to be chilled. We are, now, trying to examine relationships between PS8401 population and frost damage in field condition as to know whether they play a major role on frost damage of crops in Korea. The result that ice nucleation activity of the INA bacterial suspensions increased with increasing the number of cells in suspension (Table 3) and that frost damage increased as cell densities of PS8401 applied to the plants were increased (Fig. 2), indirectly ensure that the isolate may be a causal agent on frost damage of crops and also indicate that frost sensitivity may be determined by the size of the population of INA bacteria. But because the INA bacteria had been

Table 3. Increase in the ice nucleation temperature of bacterial suspensions with increasing bacterial concentration held in micropipette (10 μl)[a]

Isolates	Freezing temperature (°C)				
	10^{1} [b]	10^{2}	10^{4}	10^{6}	10^{8}
Pseudomonas syringae 8401	-4.8 ± 0.56[c]	-4.0 ± 0.69	-3.8 ± 0.86	-3.6 ± 0.39	-2.5 ± 0.73
Pseudomonas syringae 8402	-7.2 ± 0.85	-6.8 ± 0.64	-5.8 ± 0.62	-4.5 ± 0.73	-3.8 ± 0.45
Pseudomonas fluorescens 8401	-21.6 ± 0.72	-21.4 ± 0.85	-21.4 ± 0.76	-15.9 ± 0.76	-8.1 ± 0.35

[a] Cultures grown on nutrient agar with 2.5% glycerol for 48hr were used.
[b] Bacterial concentration (cfu/ml of distilled water).
[c] Values are means of 15 replicates with standard error.

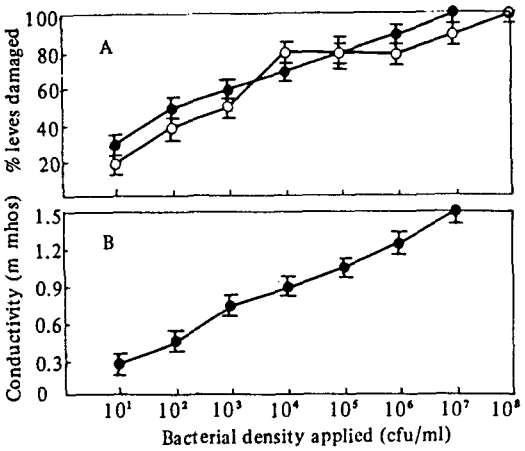

Fig. 2. Increase of frost damage of corn leaves with increasing cell density of *Pseudomonas syringae* 8401. Frost damage was rated by measuring the percent leaves damaged (A) or by measuring electrolyte leakage of the leaves damaged (B). Suspensions (in nutrient broth) of cell densities of *P. syringae* given on the abscissa were sprayed on corn seedlings 48 hr prior to freezing at -4.5°C. Plants represented by the curve (-●-) were placed in a mist chamber for 24hr and then in ambient air until freezing (wet treatment) Plants represented by the curve (-○-) were left in ambient air for the entire 48 hr (dry treatment). Vertical bars represent the standard deviations.

applied 48hr prior to freezing on the leaves of corn plants, they might have enough time to be multiplicated before freezing so that the treatment of INA bacteria as dilute concentration as 10^{4} cells/ml made the corn plants significantly damaged compare to the control plants (Fig. 2). Frost damage of corn leaves was increased with increasing the incubation time between application of PS8401 and exposure at -4.5°C (unpublished data). *P. syringae* sprayed on corn plants had an exponential increase from 6 to 36 hr (unpublished data).

Ice nucleation activity of PS8401 was affected by growth-medium composition (Table 4). This was shown by the comparison of ice nucleation activities of suspensions of PS8401 grown on nutrient agar of nutrient agar supplemented with 2.5% glycerol or glucose. Cultures supplemented with glycerol were more active in ice nucleation than those supplemented with glucose; but both were highly active in the case that the cell concentration in suspensions was 10^{8} cfu/ml. Even though the bacterium grew well on nutrient agar without an additional carbon source, its ice nucleation activity decreased more than those grown on enriched media with additional carbon sources. Thus, supplement of a suitable carbon source can affect the ice nucleation activity of bacterial suspensions. Growth-temperature also affected the the ice nucleation activity of bacterial suspensions. Growth-temperature also affected the the ice nucleation activity of the bacterium grown on nutrient agar supplemented with glycerol (Table 5). When the bacterium was grown at 15 to 25°C, no difference could be found in the ice nucleation activities of the cell suspensions. However, the activity decreased clearly in the case that the bacterium had been grown at a relatively high temperature (30°C) on the same medium. *In vitro* cultural conditions including medium composition, solid versus liquid growth medium, aeration, and growth temperature affected profoundly the ice nucleation efficiency

Table 4. Effect of growth-medium composition on the ice nucleation activity of *Pseudomonas syringae* 8401 at the various bacterial concentrations[a]

Growth medium	Freezing temperature (°C)					
	Aqueous medium[b]	10^{1}[c]	10^{2}	10^{4}	10^{6}	10^{8}
Nutrient agar (NA)	-14.2 ± 0.37[d]	-8.9 ± 0.96	-7.2 ± 0.65	-6.7 ± 0.46	-5.9 ± 0.58	-2.9 ± 0.53
NA + 2.5% glycerol	-13.2 ± 0.58	-4.8 ± 0.37	-4.0 ± 0.34	-3.7 ± 0.89	-3.6 ± 0.89	-2.5 ± 0.77
NA + 2.5% glucose	-13.6 ± 0.65	-5.3 ± 0.48	-4.4 ± 0.39	-4.0 ± 0.71	-3.7 ± 0.36	-2.5 ± 0.92

[a] Bacterial suspension (in distilled water) was held in a micropipette (10 μl). The cultures were grown for 48hr.
[b] The same consituents without agar.
[c] Bacterial concentration (cfu/ml of distilled water).
[d] Values are means of 15 replictaes with standard error.

Table 5. Effect of growth temperature on the ice nucleation activity of *Pseudomonas syringae* 8401 at the various bacterial concentrations[a]

Growth temperature (°C)	Freezing temperature (°C)				
	10^{1}[b]	10^{2}	10^{4}	10^{6}	10^{8}
15	-4.7 ± 0.58	-4.0 ± 0.87	-3.8 ± 0.54	-3.6 ± 0.35	-2.5 ± 0.65
20	-4.7 ± 0.32	-4.0 ± 0.60	-3.8 ± 0.50	-3.6 ± 0.59	-2.5 ± 0.99
25	-4.8 ± 0.76	-4.0 ± 0.65	-3.8 ± 0.87	-3.6 ± 0.90	-2.5 ± 0.20
30	-11.4 ± 0.78	-7.6 ± 0.45	-5.2 ± 0.45	-4.0 ± 0.34	-3.2 ± 0.65

[a] Bacterial suspension (in distilled water) was held in a micropipette (10μl). Cultures grown on nutrient agar with 2.5% glycerol for 48hr were used.
[b] Bacterial concentration (cfu/ml of distilled water).
[c] Values are means of 15 replicates with standard error.

of cells of many ice nucleation active strains of *P. syringae* and *E. herbicola*, as well as the temperature at which ice nucleation is expressed in these cells (6, 7, 15, 16). However, Maki et al (9) reported that the cell to ice nucleus ratio of their *P. syringae* isolate was constant under different growth conditions.

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