Ice Nucleation-Active Bacteria on Chinese Cabbage in Northern China: Population Dynamics and Characteristics and Their Possible Role in Storage Decay

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ABSTRACT

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Ice nucleation-active (INA) bacteria active at −5 C were detected on leaves of Chinese cabbage in the field and in storage facilities in the Beijing Municipality. Populations of INA bacteria in the field and those on plants in storage were as high as 2.45×10^6 and 3.12×10^6 cfu/cm^2 of leaf, respectively. About 93% of the INA bacteria isolated were identified as *Pseudomonas viridiflava*. Other species of INA bacteria isolated from Chinese cabbage included *P. fluorescens* and nonfluorescent pseudomonads. Chinese cabbage is a half-hardy species that risks exposure to several periods of subzero temperatures during its cultivation and storage in northern China and that may suffer serious postharvest soft rot. Under experimental conditions, no significant differences in frost damage were detected between chamber-grown plants inoculated with or free of INA bacteria. Frost sensitivity of Chinese cabbage was reduced significantly by hardening at 8 C for about 1 mo, although outer leaves of hardened plants remained somewhat frost sensitive. Frost-damaged Chinese cabbage leaf tissue was colonized and decayed more rapidly at 5 C by *P. viridiflava* than was non-frost-damaged tissue. The progress of soft rot could be inhibited by spraying leaves with a nonpectolytic strain of *P. viridiflava* 48 h before introduction of the pectolytic strain.

Additional keywords: bacterial competition, *Brassica campestris* ssp. *pekinensis*, postharvest pathology.

Ice nucleation-active (INA) bacteria are ubiquitous on plants. They have been found on many different species of herbaceous and woody plants, and they most probably occur worldwide in temperate and subtropical regions (4,6,8,15,16,22,25,26,33,37). However, at the time this study began in 1986, there were no reports of INA bacteria from China. Results of our initial attempts to isolate INA bacteria from vegetable crops in the Beijing Municipality indicated that total epiphytic bacterial populations in this region on beans, tomatoes, and cucumbers, for example, were very low, and INA bacteria were undetectable throughout the spring and summer until September.

Chinese cabbage is the principal vegetable crop grown in fields in northern China from September to November. The timing of harvest of Chinese cabbage and the conditions for storage put the cabbage heads at risk of exposure to several periods of subzero temperatures. The heads are usually harvested after the first frosts of late October (19). After harvest, the cabbages are left in the field 2 or 3 days to permit wound-healing. Cabbages sold to state-operated markets remain outdoors for several more days, either unprotected or in piles covered with straw mats, until they are distributed. After distribution, the cabbages are kept outdoors for up to two more weeks so that the cabbage tissue desiccates slightly before being put in storage. Conventional storage facilities in northern China consist of pits covered with straw mats or cement block cellars without any means of strict temperature regulation.

Results of experiments with INA bacteria suggest that their presence on plant surfaces is a necessary and sufficient condition for frost damage to frost-sensitive plant tissues at subzero temperatures above −5 C (23). However, INA bacteria may protect hardy or frost-tolerant tissue from excessive frost damage by inducing, at temperatures near 0 C, extracellular freezing. Such freezing may be tolerated by hardy plants and, hence, the probability of cataclysmic intracellular freezing that would occur at colder temperatures in the absence of INA bacteria is reduced (9). Chinese cabbage is considered to be half-hardy (14) and it has not been determined previously what role INA bacteria play in frost damage to this species.

During the 4–6 mo of storage of Chinese cabbage, temperatures in the storage facilities may drop below zero. The leading cause of loss of Chinese cabbage during this period is soft rot. It has been demonstrated that freezing of plant tissues and subsequent frost damage are predisposing factors for the development of certain plant diseases (1,28,31,32), in part because the freeze-thaw cycle facilitates the ingress of bacteria into plant tissues (31). To understand the epidemiology of soft rot, it is important to know if frost damage increases the susceptibility of Chinese cabbage to soft rot and what role INA bacteria play in this susceptibility.

The objectives of the work reported here were to determine if INA bacteria are present on Chinese cabbage in the field and in storage in the Beijing area, to identify the species present, and to assess their role in frost damage and subsequent soft rot of Chinese cabbage. Brief reports of the characteristics of the INA bacteria we have isolated from Chinese cabbage have been published elsewhere (36).

MATERIALS AND METHODS

Plant material. For quantification of epiphytic INA bacterial populations in the field and during storage, seedlings of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis* (Lour.) Olsson) cv. 106 were transplanted on 4 Aug 1986 in three 0.67-ha commercial production fields (referred to as Tree Village, Xiao Jia

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He Brigade 3, and Xiao Jia He Brigade 5) in Haidian district, Beijing Municipality. One hundred heads were harvested from each field on 10 Nov 1986. After being kept outdoors for about 2 wk, the harvested cabbages were moved to an underground cement block storage facility until March 1987 in the same manner as they are stored for institutional use.

To determine the effect of INA bacteria on frost sensitivity, plants of Chinese cabbage 106 were grown in a chamber with a 16-h photoperiod at 23 C and 8 h of darkness at 18 C and were watered daily from below. After 55–60 days, half of the plants were transferred to a chamber at 8 C with a 12 h photoperiod and were watered weekly for 28–29 days. The dry matter and water content of hardened and nonhardened plants were determined by drying (at 80 C for 48 h) disks cut from the lamina of an outer, a middle, and an inner leaf of each plant. Plants used in tests of soft rot development were grown in a growth chamber but were not hardened.

Inoculation and incubation of plants. To determine the effect of INA bacteria on frost sensitivity, hardened and nonhardened chamber-grown plants were sprayed to runoff with sterile buffer or a suspension (10^8 cfu/ml) of INA *Pseudomonas viridiflava* strain FMu-107 in 0.1 M phosphate buffer. As chamber-grown plants did not form heads, uniform inoculation could be achieved. After inoculation, plants were covered with plastic bags and incubated at 8 C for 36 h. To determine the effect of INA bacteria and frost damage on soft rot, chamber-grown plants were sprayed with sterile buffer or with a suspension of *P. viridiflava* strain FMa-50 (10^8 cfu/ml), streptomycin-resistant strain S107X (10^4 or 10^8 cfu/ml), or rifamycin-resistant strain R50X (10^6 cfu/ml). After inoculation, plants were covered with a plastic bag and incubated in the growth chamber for 48 h.

Strains FMu-107 and FMa-50 were isolated from field-grown Chinese cabbage cultivar 106. FMu-107 did not express pectolytic activity on Hildebrand's pectate medium (pH 7) (10) nor on frost-damaged leaf tissue, and it had a frequency of 7×10^{-8} INA cells at −4.5 C. FMa-50 expressed pectolytic activity on Hildebrand's pectate medium and on frost-damaged leaf tissue, and it had a frequency of fewer than 10^{-8} INA cells at −4.5 C. INA frequency was determined based on the freezing of twenty 0.1-ml aliquots of suspensions of 48-h-old cultures grown at 25 C on nutrient agar containing 2.5% glycerol (v/v) (NAG). Calculation of the cumulative number of nuclei was according to the method of Vali (34). Strain S107X originated from strain FMu-107 and R50X from FMa-50 by selecting for antibiotic resistance on King's medium B (17) supplemented with cycloheximide (50 mg/L) and with 100 mg/L of streptomycin or rifamycin, respectively. Cross-resistance of S107X and R50X were determined: rifamycin-resistant cells of S107X and streptomycin-resistant cells of R50X occurred at frequencies of 10^{-7} and less than 3×10^{-11}, respectively. Pectolytic and ice nucleation activities of antibiotic-resistant strains were similar to those of the parent isolates.

Quantification of bacterial populations. Outer cabbage leaves were sampled from 15 cabbages in each field on 11 October and 10 November 1986 and in storage from heads corresponding to each field on 17 December 1986, and 2 January and 20 February 1987. Each selected leaf was one of the four outermost leaves of the heads in the field or the outermost nondecayed leaf of heads in storage. After a leaf was taken from a cabbage in storage, the cabbage was discarded.

A 47-cm^2 hexagonal piece (about 10% of the total area of a leaf) was cut from a randomly selected position on each leaf and washed individually in 30 ml phosphate washing buffer (0.1 M phosphate buffer plus 1% [w/v] Proteose peptone [Difco], pH 7) on a rotary shaker (125 rpm) for 1.5 h at 5 C. Washings were dilution-plated on King's medium B supplemented with cycloheximide (100 mg/L) and cephalexin (20 mg/L). Plates were incubated for 2–5 days at 22–24 C. Colonies were counted and 5–10% of all colonies from one dilution having 30–300 cfu/plate for each leaf were randomly selected and purified. Purified isolates were stored on slants of NAG at 4 C.

After incubation of chamber-grown plants, bacterial populations were quantified from randomly sampled 5.3-cm^2 leaf disks by individually grinding each disk in 25 ml 0.1 M phosphate buffer for 30 s at 24,000 rpm with a surface-sterilized ultra-turax (Janke and Kunkel GMBH and Co. KG, D7813 Straufen, Germany). In tests of frost sensitivity of whole plants, disks were sampled from random positions on an inner, a middle, and an outer leaf of each plant before the plant was subjected to frost. The grinding was dilution-plated on King's medium B supplemented with 50 mg/L cycloheximide (Sigma). In treatments employing antibiotic-resistant strains of bacteria, the medium was additionally supplemented with 100 mg/L rifamycin to detect R50X or with 100 mg/L streptomycin to detect S107X.

Bacterial characterization and determination of ice nucleation activity. Ice nucleation activity of isolates obtained from field-grown plants was determined by a droplet freezing assay (12). Purified isolates were grown on slants of NAG at 24 C. Suspensions of 48-h cultures were made with ice nucleus-free 0.01 M sterile phosphate buffer (pH 7.0, pretested for nuclei at −10 C) and adjusted to about OD600 = 0.1 (about 10^8 cfu/ml) by visual comparison with standards adjusted spectrophotometrically. Twenty 10-μl drops of each suspension were placed on wax-coated aluminum foil boats and brought to −5 C by floating the boats for 1–3 min on a 9% (w/v) saltwater solution maintained at −5 C ± 0.05 C in an insulated tank cooled in a freezer. Droplet freezing was determined visually. The bath was stirred manually and its temperature was verified with a thermometer (calibrated between 0 and −30 C) immediately before and after each freezing cycle.

INA strains were tested for their Gram and oxidase reactions (5), production of fluorescent pigment on King's medium B, presence of arginine dihydrolase, growth at 41 C, ability to utilize glucose under anaerobic and aerobic conditions, ability to liquefy gelatin, ability to utilize sucrose and D(−)tartrate as single carbon sources, ability to denitrify nitrate (5), and ability to produce levan from sucrose (29), presence of poly-β-hydroxybutyrate inclusions (5) and pectolytic activity (10).

Freezing of plant material and assessment of frost damage and soft rot. In tests to determine cabbage frost sensitivity, the leaf surfaces were allowed to dry before plants were exposed to cold to avoid the effect of leaf wetness on supercooling (2) (about 2 h at room temperature after removing plastic bags). Five 1.8-cm^2 leaf disks were cut from each plant (10 plants per treatment) and were individually placed in tubes of 4.5 ml of sterile distilled water free of ice nuclei at −10 C. Tubes were placed in a constant temperature bath (Peter Huber type HS-40 bath, D7600 Offenburg-Elgersweier, Germany) and exposed to a range of temperatures between −2 and −8 C. The tubes were held at each temperature for 30 min and the cumulative number of tubes frozen per treatment was determined at each temperature (11).

For each freezing trial of whole plants, eight plants were arranged in a styrofoam box (inner dimensions = 48 × 38 × 39 cm; walls = 6 cm thick) such that leaves of adjacent plants did not touch. The closed box was placed in a freezer at −20 C. The box was left in the freezer for about 4.5 h once the air temperature in the box reached 0 C. The air in the box cooled at a rate of 1°/h for temperatures below 0 C as measured by two mercury-soldered copper-constantan thermocouples randomly placed at different locations within the canopy during each trial. Additionally, a thermocouple was attached to a leaf at a random position on four of the plants during each trial and instantaneous leaf temperature was measured at 5 min intervals throughout each trail. The temperature measured by the thermocouples used in these experiments had a maximum difference of 0.07 C under calibration conditions between 0 and −6 C in a constant temperature bath. After the freezing trial, plants were removed from the box and allowed to thaw at room temperature. The percent area of each leaf damaged by frost was determined visually after 12–18 h. Freezing experiments were conducted in five replicate trials with two plants per treatment tested during each trial.

To determine the effect of frost damage on soft rot, leaf disks were cut from plants, placed on aluminum trays, and exposed

to −2.5 C for 90 min in a freezer. Those disks that froze and were subsequently water soaked (about 50% of the disks) were separated from those that did not freeze at −2.5 C. Disks were incubated at 5 C on moistened sterile filter paper in sealed petri dishes for about 2 wk. At each sampling time after freezing, eight to ten disks from each treatment were teased with sterile toothpicks to determine the percent leaf area macerated and then were removed with sterile forceps from the petri dishes for determination of bacterial populations.

RESULTS

Population densities of total and INA bacteria on field-grown plants. INA and total bacterial population sizes on leaves of Chinese cabbage in the field and in storage are presented in Table 1. As found by Hirano et al (13) for epiphytic bacterial populations, the frequency distribution of INA and total bacterial populations on Chinese cabbage leaf surfaces did not differ significantly from a lognormal distribution based on the Shapiro-Wilk statistic (W: for our data, $W > 0.93$) (30). Hence, mean population levels are reported as the mean of $\log_{10}$ values of the size of the population on each leaf. At each sampling date there were no significant differences ($P < 0.05$) among the three field (Tree Village, Xiao Jia He Brigade 3, or Xiao Jia He Brigade 5) means or variances of INA or total bacterial population sizes. Therefore, the values reported in Table 1 are the mean and variance of populations from all leaf pieces sampled at each date.

High populations of INA bacteria were present on cabbage before and at harvest and after 2 mo of storage. After 3 mo of storage, INA bacteria could be detected at population sizes greater than 10^4 cfu/cm^2 of leaf on only 4.4% (2 per 45 leaves) of the Chinese cabbage leaves sampled at this time, making detection of INA bacteria on these leaves improbable, given the size of the total bacterial population (Table 1).

Characteristics of INA isolates. Of 3,023 bacterial isolates from Chinese cabbage in the field and in storage, 555 (18.3%) had detectable activity at −5 C. All of the INA isolates were obligately aerobic gram-negative rods. They were identified as *P. viridiflava* (93% of the total INA bacteria isolated), *P. fluorescens* biovar V (4.2%), *P. fluorescens* biovar III (0.5%), arginine dihydrolase-positive nonfluorescent pseudomonads (1.4%), and arginine dihydrolase-negative nonfluorescent pseudomonads (0.9%) based on characterization of a randomly selected subset of about half of the INA isolates. The nonfluorescent INA isolates were presumed to be *Pseudomonas* spp., considering their strong oxidase reaction, ability to reduce nitrate to nitrite, and presence of poly-β-hydroxybutyrate inclusion bodies in the cells. About 45% of the INA isolates displayed pectolytic activity on medium containing sodium polypectate as a substrate (10).

The INA isolates were tested for pathogenicity to Chinese cabbage because 83% of the *P. viridiflava* isolates and 20% of the nonfluorescent isolates caused a hypersensitive response in tobacco. When bacterial suspensions (about 10^8 cfu/ml) were injected into the base of petioles of Chinese cabbage cultivar 106, 67% of the nonfluorescent and 22% of the *P. viridiflava* INA isolates caused systemic vascular discoloration. However, none of the INA isolates caused symptoms in Chinese cabbage when bacterial suspensions were rubbed onto wounded leaves and plants incubated under high RH. Among the isolates that caused vascular discoloration, production of pectolytic enzymes was detected in all of the *P. viridiflava* isolates but in none of the nonfluorescent isolates.

For each of the 555 isolates considered active as ice nuclei, at least 15 of the 20 droplets froze during the first test of the isolates. For the remaining 2,468 isolates tested, fewer than three droplets froze. These were considered to be inactive as nuclei because, occasionally, up to two out of 20 droplets of nucleus-free sterile buffer froze when subjected to the droplet-freezing test. After three to four cycles of transfer of single colonies over a period of 3 mo, eight out of nine *P. fluorescens* biovar V and all nonfluorescent and *P. fluorescens* biovar III isolates lost detectable levels of ice nucleation activity at −5 and −10 C, but none of the isolates of *P. viridiflava* lost their activity.

P. viridiflava represented over 95% and *P. fluorescens* less than 5% of the INA bacterial populations from Chinese cabbage sampled before or at harvest. During storage, the proportion of *P. viridiflava* in the total INA bacterial population decreased to below 40%, whereas the proportion of *P. fluorescens* increased to 50%. These estimates were based on characterization of a random sample of 23–48% of the INA bacteria isolated at each sampling date (Table 1).

Freezing and frost sensitivity of inoculated plants. Freezing of leaf disks in tubes of water indicated that tissue inoculated with INA bacteria froze at much warmer temperatures than noninoculated tissue. Fifty percent of inoculated disks nucleated water between −2.5 and −3.0 C, whereas 50% of noninoculated disks nucleated water at temperatures below −7.5 C.

All leaves of all whole plants were frozen at the time they

TABLE 1. Population densities of INA and total bacteria on Chinese cabbage in the field and in storage in Beijing

Date	Mean population density (log cfu/cm^2 leaf)[a]		Number characterized[c]	Percent of characterized isolates identified as		
	Total bacteria	INA bacteria[b]		*P. viridiflava*	*P. fluorescens*	Nonfluorescent
In field:						
11 Oct 86[d]	4.36 (0.79)[e]	3.44 (1.08)	47 (48%)	95.8	2.1	2.1
10 Nov 86	7.14 (0.51)	6.39 (0.89)	166 (47%)	96.4	3.0	0.6
In storage:						
17 Dec 86	7.96 (0.59)	6.50 (0.93)	19 (23%)	73.7	15.8	10.5
02 Jan 87	7.24 (0.66)	5.94 (0.85)	8 (38%)	37.5	50.0	12.5
20 Feb 87	6.53 (0.22)	4.92[f] (0.22)	0 (0%)	...	...	...

[a] Data represent the mean of the $\log_{10}$ of populations on 45 leaf samples collected at each sampling date from cabbages from three experimental fields (15 leaves from each field). Data from the three fields were pooled because at none of the sampling dates were there significant differences ($P \leq 0.05$) among the three field population means or variances.

[b] Population sizes of INA bacteria were estimated based on the number of randomly sampled isolates from each leaf that were active as ice nuclei at −5 C.

[c] Numbers in parentheses indicate the percent of the total INA bacteria isolated at each date that were characterized. All randomly selected INA isolates were tested for production of fluorescent pigment on King's medium B (17), oxidase reaction, and presence of arginine dihydrolase. Subsets of the isolates collected at each date were subjected to additional tests for determination of species identity.

[d] Data from this date represent the mean of 15 samples collected from the Tree Village field only.

[e] The standard deviation is indicated in parentheses after each mean.

[f] The limit of sensitivity of the plating assay at this date was 6.46×10^4 total cfu/cm^2, about 100-fold higher than at other dates. Only two out of 45 samples at this date had detectable INA populations. To calculate the mean and variance for these data, the limit of sensitivity was used for the 43 cases where INA bacteria were not detected. Hence, the mean INA population is probably overestimated and the standard deviation underestimated at this date.

were removed from the freezer. Exotherms were detected on all plants to which thermocouples were attached; 95% of the exotherms occurred between −1.75 and −2.5 C, and one occurred at −3.1 C. Thermocouple measurements indicated that the maximum variability in temperature at a given moment among nonfrozen leaves at the same position in the canopy (among outer leaves, for example) was 0.5 C. However, the variability in surface temperature among frozen leaves was greater than among nonfrozen leaves; this seemed to depend on the amplitude and duration of the exotherms detected. Innermost leaves attained the temperature of outer leaves with a delay of 15–30 min at ambient temperatures near 0 C. The length of the delay decreased with decreasing ambient temperature.

After plants thawed and recovered over a period of about 18 h, nonhardened plants had significantly more frost damage than hardened plants (Table 2). However, inoculation with INA bacteria had no significant effect on frost damage to either hardened or nonhardened plants (Table 2). Hardened and nonhardened plants inoculated with INA strain FMu-107 had mean populations of 1.48×10^4 and 2.63×10^4 fluorescent cfu/cm^2 of leaf surface, respectively, at the time of freezing. These means were not significantly different ($P < 0.25$) based on an analysis of variance of the log of the population density on each disk. Noninoculated plants had fewer than 320 total cfu/cm^2 of leaf surface.

Frost damage of cabbage leaves depended on the position of the leaf on the plant; younger leaves close to the growing point of the plant were less damaged than mature leaves (Fig. 1). This difference may have been attributable to the fact that inner leaves cooled more slowly than outer leaves as explained above. The difference in frost damage among leaves at different positions was probably not attributable to differences in colonization by FMu-107; the range in FMu-107 population size among disks sampled from leaves at different positions was less than 10-fold for 75% of the plants. There was no significant effect ($P < 0.1$) of inoculation with INA bacteria on frost damage at any of the individual leaf positions based on analyses of variance of the arcsin[proportion of damaged surface$^{1/2}$] of each leaf.

Effect of frost damage on population dynamics of pectolytic bacteria and on soft rot. Cabbage leaf disks came from a single group of inoculated cabbage plants colonized by, on average, 2.7×10^6 FMa-50 cfu/cm^2 of leaf surface. Bacterial populations, determined immediately after frozen disks thawed, were reduced

TABLE 2. Effect of hardening and inoculation with INA bacteria on frost damage of Chinese cabbage plants

Hardening	Inoculation[a]	Frost damage (%)[b]
Hardened	−	6.5 a
Hardened	+	4.3 a
Nonhardened	−	25.5 b
Nonhardened	+	22.4 b

[a] Plants were sprayed with INA *Pseudomonas viridiflava* strain FMu-108 (+) or phosphate buffer (−).

[b] Frost damage is reported as the mean percent leaf area damaged after thaw and recovery of leaves at positions represented on at least 50% of the plants tested (the first 12 innermost leaves). Means followed by different letters are significantly different ($P \leq 0.001$) based on Tukey's multiple comparison of the arcsin [percent damage$^{1/2}$] for each leaf.

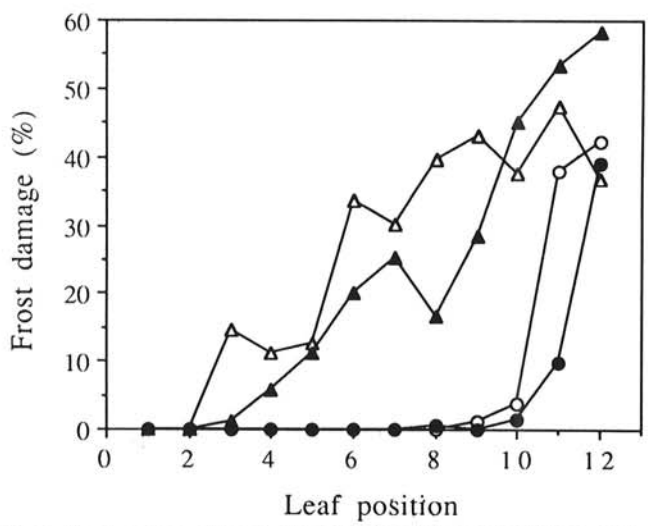

Fig. 1. Frost damage of leaves of whole chamber-grown Chinese cabbage plants expressed as the mean percent of the damaged surface of leaves at each of twelve adjacent positions on the plant (innermost leaf = position 1). Leaves at these positions were represented on at least 50% of the plants tested. Before exposure to freezing temperatures, plants were hardened and sprayed with phosphate buffer (○) or with INA *Pseudomonas viridiflava* strain FMu-107 (●) or not hardened and sprayed with phosphate buffer (△) or with FMu-107 (▲).

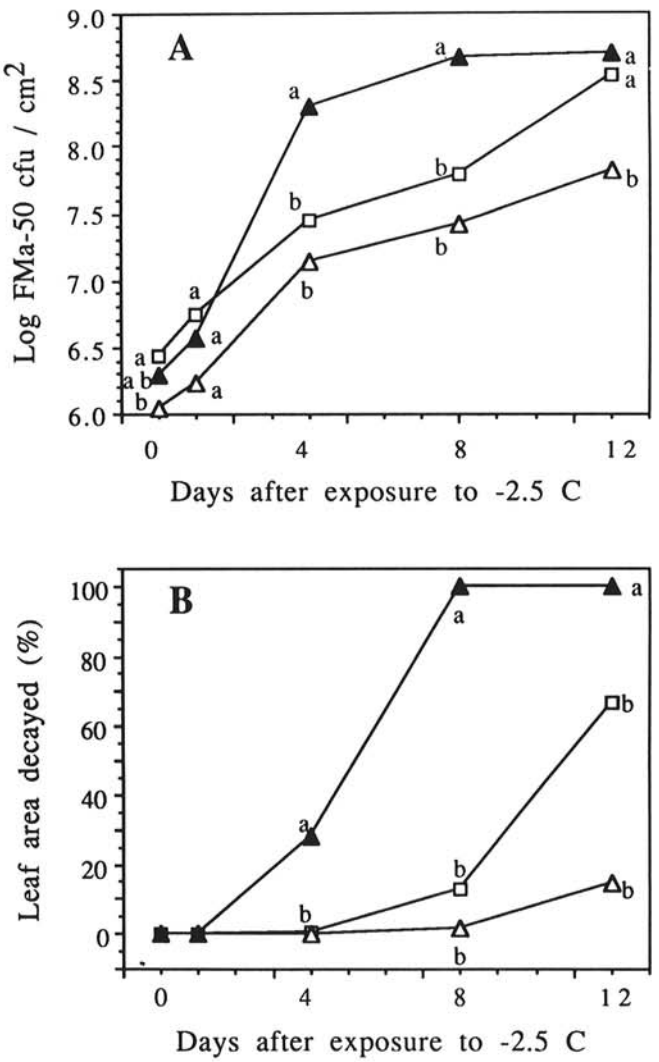

Fig. 2. A, Population dynamics of *Pseudomonas viridiflava* strain FMa-50, and **B,** progress of soft rot of Chinese cabbage leaf disks exposed to −2.5 C and frost-damaged (▲), exposed to −2.5 C but not frozen (△), or left at ambient temperature (□) before incubation at 5 C. Populations are expressed as the mean density and decay is expressed as the mean percent decay for 10 disks for each treatment at each sampling time. Forty-eight hours before cutting leaf disks and exposing them to cold, 70-day-old chamber-grown plants were sprayed with a suspension (10^8 cfu/ml) of strain FMa-50 and incubated in plastic bags. Population densities at 0 days after cold exposure were determined immediately after thawing of frozen disks. At each sampling date, bacterial population means associated with different letters are significantly different ($P \leq 0.01$) based on Tukey's multiple comparison procedure. Mean decay values associated with different letters are significantly different ($P \leq 0.05$) according to confidence intervals calculated from the mean of the arcsin[proportion decay$^{1/2}$] for each treatment and the overall standard deviation (sd) for all treatments for which sd > 0.

significantly ($P < 0.05$) on leaf disks that were exposed to −2.5 C without freezing but not on those that froze at −2.5 C (Fig. 2A). However, at 1 day after cold exposure there were no significant differences in populations among the treatments.

Populations of isolate FMa-50 were significantly greater on frost-damaged disks at 4 and 8 days after cold exposure than on disks not damaged at −2.5 C or those receiving no cold treatment. By 12 days after cold exposure, mean populations on frost-damaged disks were equal to those on disks receiving no cold treatment but were greater than those on disks exposed to −2.5 C but not frozen (Fig. 2A).

At 4 days after cold exposure and thereafter, the percent leaf area macerated of disks frozen at −2.5 C was significantly greater than that for other treatments (Fig. 2B). By 8 days after cold exposure, all of the frozen disks were completely macerated, whereas less than 20% of the disks of other treatments were macerated. By 12 days after cold exposure, maceration of disks not exposed to cold was greater than that of disks exposed to −2.5 C without freezing, but this difference was not significant. These differences in soft rot corresponded to differences in bacterial population sizes among treatments. For all treatments, decay of an individual disk was not observed until the density of FMa-50 on that disk was at least 10^8 cfu/cm^2. Equivalent or greater population densities were observed on 80% of frozen leaf disks by 4 days after cold exposure but on 40% or fewer disks of the other treatments until 8 days after cold exposure.

Effect of bacterial interactions on soft rot of frost-damaged tissue. Chinese cabbage leaf tissue inoculated with nonpectolytic strain S107X 48 h before inoculation with pectolytic strain R50X experienced significantly ($P < 0.05$) less decay after freezing than tissue inoculated with R50X only (Fig. 3B). The reduction in decay of tissue inoculated with S107X corresponded to reduced populations of R50X in comparison with tissue not inoculated with S107X (Fig. 3A). Between 10 and 14 days after freezing, a rapid increase in decay was observed, which corresponded to a decline in the density of R50X populations for two of the treatments (Fig 3A and B). This also corresponded to the period during which the incubation temperature was increased, which may have changed the rate of production of pectolytic enzymes or their activity.

There were no differences in the mean population densities of S107X from 0 to 14 days after freezing of cabbage tissue between disks inoculated with 10^4 and 10^8 cfu S107X per milliliter. The mean population density of S107X at 0 days after freezing was 10^6 cfu/cm^2, and this stabilized at about 3×10^7 cfu/cm^2 between 5 and 14 days after freezing. At the time of inoculation with R50X (48 h after inoculation with S107X; two days before freezing), the mean population density of S107X on disks inoculated with a suspension of 10^4 cfu/ml (2×10^5 cfu/cm^2) was slightly less than on disks inoculated with 10^8 cfu/ml (7×10^5 cfu/cm^2), although this difference was statistically significant ($P < 0.01$). In spite of the nearly equivalent population densities of S107X on disks inoculated with the different doses, at 5 and 10 days after freezing the population density of R50X on tissue inoculated with the higher dose of S107X was less than that on tissue inoculated with the lower dose (Fig. 3A).

DISCUSSION

Previous reports have suggested that populations of INA bacteria on leaves of *Brassica* spp. are very low. On samples taken in October in Wisconsin, there were 8×10^2 or fewer cfu of INA bacteria per gram fresh weight of leaves of *B. rapa* and various members of the *B. oleracea* group, and in Florida there were 2.5×10^5 cfu INA bacteria per gram fresh weight of leaves of *B. hirta* (22). Kaneda (15) could not detect INA bacteria on *B. napus* during a 6-wk sampling period in the summer in Alberta, Canada, although he detected INA bacteria on other plants during the same period. Goto et al (6,7) isolated INA bacteria from *B. oleracea, B. chinensis, B. pekinensis*, and *B. campestris* in Japan but did not report the population sizes on these plants.

Our results indicate that there are high populations of INA bacteria on Chinese cabbage both in the field and in storage facilities in the Beijing area. To compare the population sizes reported here with those reported previously for other *Brassica* spp. we note that 1 cm^2 Chinese cabbage tissue has an average fresh weight of about 0.05 g. Hence, the population of INA bacteria reported on *B. hirta* in Florida (22) (2.5×10^5 cfu/g) is about 0.4% of the maximum population of INA bacteria we detected on Chinese cabbage (3.2×10^6 cfu/cm^2 = 6.4×10^7 cfu/g).

It should be noted that the mean population sizes of INA bacteria on Chinese cabbage reported here might be overestimated and the variances underestimated. Because of the sampling technique used for detecting INA bacteria, populations of INA bacteria present as less than about 1% of the total bacterial population on a leaf were not likely to have been detected. When

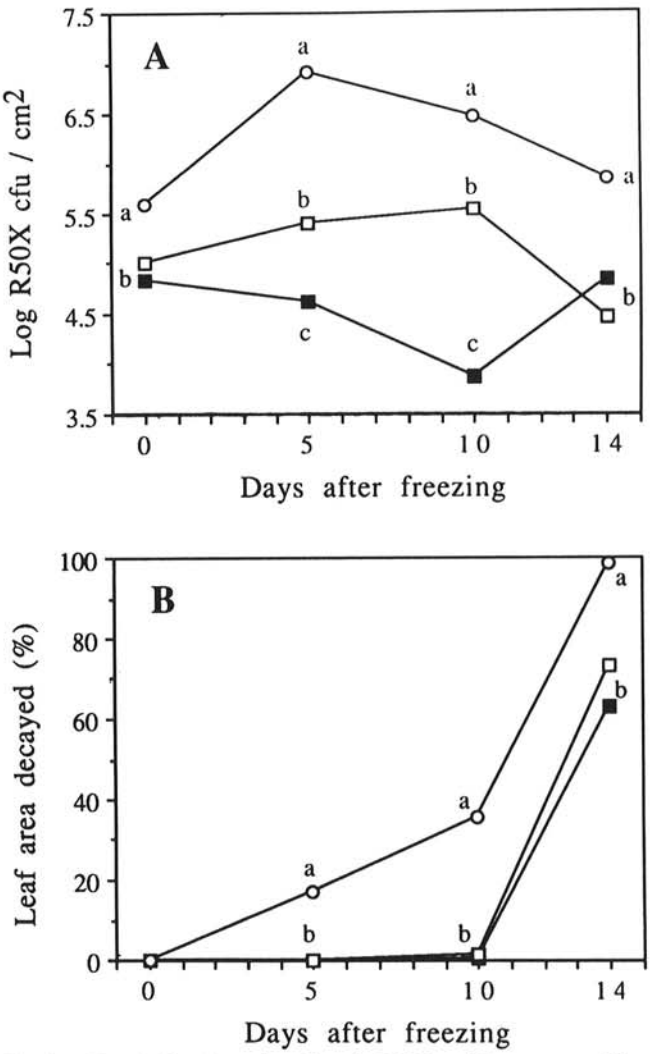

Fig. 3. A, Population dynamics of strain R50X, and **B,** progress of decay of frost-damaged Chinese cabbage leaf disks inoculated with pectolytic strain R50X in combination with nonpectolytic strain S107X. Populations are expressed as the mean density of R50X and decay is expressed as the mean percent decay for eight to ten disks for each treatment at each sampling time. Four days before leaf disks were cut and frozen, plants were sprayed with a suspension of 10^4 (□) or 10^8 (■) cfu/ml of strain S107X or with sterile phosphate buffer (○). Two days later these same plants were sprayed with R50X (10^6 cfu/ml). After all inoculations, plants were incubated in plastic bags. Two days after inoculation with strain R50X, leaf disks were cut, frozen at −2.5 C, and then incubated at 5 C for 12 days and at 25 C thereafter. At each sampling date, bacterial population means associated with different letters are significantly different ($P \leq 0.05$) based on Tukey's multiple comparison procedure. Mean decay values associated with different letters are significantly different ($P \leq 0.05$) based on paired t tests of ranked percent decay values.

INA bacteria were not detected, the limit of sensitivity (i.e., the estimated population size had one colony of INA bacteria been detected in our random sample) was used to represent the INA population on that leaf in calculating the mean and variance. INA bacteria were rarely nondetectable on plants in the field and after the first month of storage. Hence the means and variances of populations of INA bacteria reported for October through December are probably accurate estimates. However, by the end of the storage period, INA bacteria were rarely detectable (on only 2 per 45 leaves), such that the population parameters reported at this date are probably poor estimates.

All of the INA bacteria isolated from Chinese cabbage were *Pseudomonas* spp., and the majority of these were oxidase (−), arginine dihydrolase (−), levan (−) strains able to use sucrose as a single carbon source. We have identified them as *P. viridiflava* based on their ability to use D(−)tartrate as a single carbon source (27). Goto et al (6) reported that the majority of INA bacteria on leaves of frost-damaged *Brassica* spp. isolated in Japan were *P. viridiflava*; however, they also isolated INA *Erwinia* spp. The absence of *Erwinia* spp. from the populations of INA bacteria on Chinese cabbage may be attributable to the fact that we conducted our screening for INA bacteria at −5 C, whereas Goto et al (6) conducted theirs at −10 C. Sun et al (33) detected *E. herbicola* active as ice nuclei at −3 C on crop plants in China, but this species composed less than 5% of the isolates obtained from vegetable crops.

Our results also suggest that the proportion of *P. viridiflava* in the INA population declines during storage and that of *P. fluorescens* increases. The extent of the shift reported here may be exaggerated because of the small number of INA bacterial isolates available for characterization at the end of the season, compared to the larger number at the beginning of the season. Nevertheless, the trend may be real. Eight of the 21 isolates collected on January 2 were identified, of which four were *P. fluorescens*, three were *P. viridiflava*, and one was a nonfluorescent pseudomonad. If we assume that in our random sample of eight isolates we accidentally chose all the *P. fluorescens* and nonfluorescent pseudomonads collected on January 2 and that the remaining 13 were *P. viridiflava*, we can estimate that the real proportions of species at this date were 76% (16 out of 21) *P. viridiflava*, 19% (4 out of 21) *P. fluorescens*, and 5% (1 out of 21) nonfluorescent pseudomonads. These proportions still suggest a change in the balance of *P. viridiflava* and *P. fluorescens* in the population of INA bacteria during storage. However, we do not have information about the relative proportions of these species in the populations of INA bacteria that were below the level of detection.

There are some differences between the INA bacteria we have isolated and those reported by others. Among the pseudomonads isolated from Chinese cabbage were species and biotypes not previously reported to be ice nucleation-active. These include *P. fluorescens* biovar III and nonfluorescent pseudomonads. Goto et al (7) isolated a nonfluorescent INA pseudomonad from Japanese horse radish (*Eutrema wasabi*) petioles, but this bacterium differed from the nonfluorescent pseudomonads reported here in that it did not accumulate poly-β-hydroxybutyrate and did not reduce nitrate to nitrite. The ice nucleation activity of the nonfluorescent pseudomonads and members of the *P. fluorescens* group described here was not as stable over time as that of *P. viridiflava*. We did not detect any contaminants, ruling out the possibility of contamination of inactive cultures by cells of an active species. Hence, we propose that this observation implicates differences in the regulation or loss of the genetic determinants of ice nucleation activity among the pseudomonads we have isolated.

To demonstrate that INA bacteria can increase frost damage to Chinese cabbage we must demonstrate 1) that their presence on Chinese cabbage tissue increases the frequency of freezing events or raises the temperature at which the tissue freezes and 2) that freezing of the leaf tissue leads to subsequent damage. The results of freezing of individual leaf disks in tubes of water indicate that Chinese cabbage tissue colonized by FMu-107 nucleates water at much warmer temperatures than tissue free of INA bacteria. Furthermore, when used in freezing experiments of cabbage tissue, strain FMu-107 had ice nucleation activity detectable at about −3 C. This observation may be surprising, given that the mean population of FMu-107 on the 2 cm^2 disks used in the freezing experiments was about 10^4 cfu, and the frequency of ice nucleation-active NAG-cultured cells at −4.5 C was about 10^{-8}. This suggests that the nucleation frequency of FMu-107 is higher when it grows on plant tissue than when cultured on synthetic medium. Changes in bacterial nucleation frequency due to the medium of culture have been reported previously (24).

Results of freezing of whole plants do not give as clear an indication of the importance of INA bacteria as nucleators for ice formation in Chinese cabbage tissue. The majority of the exotherms detected from intact plants (with or without FMu-107) occurred within a narrow range of temperatures warmer than −2.5 C. Although FMu-107 may have been the cause of the initial freezing of inoculated plants, these results suggest that intact Chinese cabbage tissue harbors endogenous ice nuclei active at temperatures near −2.5 C. Before harvest of Chinese cabbage and during its storage in northern China, the ambient temperature is likely to fall below −3 C or −5 C. At these temperatures, endogenous nuclei as well as bacterial nuclei would be able to initiate ice formation in the plant tissue.

Inoculation of plants with INA bacteria and freezing of intact plants tended to increase frost damage to outer leaves of nonhardened plants only. In fact, the presence of INA bacteria resulted in a decrease in frost damage more frequently than in an increase when damage was evaluated by leaf position. These differences in frost damage among inoculated and noninoculated leaves were considered to be statistically insignificant. However, based on this observation it is tempting to speculate that INA bacteria active at relatively warm temperatures may help partially frost-tolerant plants avoid frost damage by inducing freezing at relatively mild temperatures. Such a mechanism has been suggested for certain alpine plants (18) and frost tolerant insects (38) possessing endogenous nuclei. Endogenous ice nuclei in Chinese cabbage might also function to help the plant avoid damage, hence the reduction in frost damage attributable to INA bacteria would not be dramatic.

We observed that inner leaves of Chinese cabbage had less frost damage than outer leaves. This result may have been due to differences in both temperature and frost sensitivity among leaves at different positions on the plant. Thermocouples indicated that there was a temperature gradient from outer to inner leaves. Although all leaves of all plants were frozen when plants were removed from the freezer, inner leaves may have been less damaged because they were frozen for a shorter period of time and the minimum temperature they experienced was warmer than that of outer leaves. However, it is unlikely that differences in frost damage among leaves at positions 9–12 were attributable to temperature differences. A large variability in frost damage to hardened plants was observed among leaves at these positions. If the site of implantation of the petiole at the base of the stem was not inspected, any of the leaves at positions 9–12 could be considered the outermost leaf based on exposure of the laminae. The two truly outermost leaves, however, could be readily distinguished by the quality of their laminae (thickness, coloration), and they had significantly lower specific dry weights than leaves at other positions. Hence, our results suggest that the cultural conditions (e.g., hardening vs. nonhardening temperatures) or phenological stage of the plant material to be used for evaluating frost sensitivity be carefully considered.

Our results clearly indicate that frost-damaged Chinese cabbage tissue rots more rapidly than non-frost-damaged tissue. Previous workers have noted that frost is a predisposing factor for the development of certain plant diseases (1,28,31,32). Süle and Seemüller (31) proposed that the development of bacterial disease after a freezing event is not necessarily attributable to the ingress of phytopathogenic bacteria through wounds caused by freezing, but rather is attributable to the sucking of bacteria into the tissue

upon thawing. In the case of frozen sour cherry leaves, Süle and Seemüller (31) noted that inoculations with *P. syringae* pv. *syringae* made 30 min after thawing did not result in any lesions, whereas inoculations made earlier did result in lesions. In our tests with Chinese cabbage, leaf tissue was frozen after inoculation with pectolytic bacteria. However, in preliminary experiments (*unpublished*) in which inoculations with pectolytic bacteria were conducted at least 1 h after tissue thawed, results were identical to those reported here. This implies that wounding of Chinese cabbage tissue by ice formation plays a role in the development of soft rot.

Furthermore, our results suggest that frost damage does not necessarily increase the inherent susceptibility of cabbage tissue to soft rot: frost-damaged as well as nondamaged tissue began to decay once pectolytic bacteria attained a population density of about 10^8 cfu/cm^2 in experiments with FMa-50. Based on our results, it seems more likely that frost damage renders the tissue a better environment for colonization by certain pectolytic bacteria. If colonization of damaged tissue is inhibited by, for example, antagonistic interactions with conspecific, nonpectolytic bacteria, soft rot may be reduced. This was demonstrated by the reduction in soft rot observed for tissue inoculated with strain S107X before being inoculated with R50X.

Previous workers have suggested that successful antagonism among conspecific or nearly isogenic epiphytic bacteria may be attributable to competition (20,21). In our experiments, application of two different inoculum doses (10^4 and 10^8 cfu/ml) resulted in populations of equal densities of viable cells of S107X. However, on leaves receiving the higher dose of S107X, the development of R50X was suppressed to a greater extent than on leaves receiving the lower dose. A possible explanation for this observation is that dead cells of S107X, in relative abundance on leaves receiving the higher inoculum dose, had an effect on R50X either in that they released compounds detrimental to R50X or that they effectively occupied space.

Based on the results reported here, we suggest that temperatures below about −2 C will cause freezing of Chinese cabbage leaf tissue. It is likely that Chinese cabbage plants, in the fashion they are cultivated and stored in northern China, would experience such temperatures several times before harvest and during the storage period. The catalyzer of freezing of the water in Chinese cabbage tissue may be INA bacteria, which are abundant on Chinese cabbage in the Beijing area, or may be ice nuclei endogenous to the plant tissue. The relative frequencies of either of these types of nuclei and the conditions under which they are active on Chinese cabbage remain to be elucidated. The majority of the leaves of Chinese cabbage will recover after thawing. However, the outer leaves will probably be severely damaged. If frost damage occurs before harvest it may not have a significant effect on subsequent soft rot in storage because damaged outer leaves are stripped from heads at harvest. However, if frost damage occurs during storage, these leaves will likely decay rapidly given that psychrophilic pectolytic bacteria are abundant on their surfaces. The decay of frost-damaged Chinese cabbage reported here occurred under conditions of high RH. However, under real storage conditions some frost-damaged tissue may dehydrate, which could inhibit the development of soft rot. Dehydration of tissue would depend on the aeration of the storage facility and the way in which the cabbages are stacked.

Although a significant amount of soft rot of Chinese cabbage is attributable to *Erwinia* spp. (3), *Pseudomonas* spp. are the principal bacteria isolated from soft rotting Chinese cabbage during the cool periods of storage (35). Hence, it may be worthwhile to evaluate the effectiveness of field application of nonpectolytic strains of *P. viridiflava*, for example, as a means of controlling soft rot of Chinese cabbage under storage conditions presently realized in China.

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