Biological Control of Frost Injury: An Isolate of *Erwinia herbicola* Antagonistic to Ice Nucleation Active Bacteria

S. E. Lindow, D. C. Arny, and C. D. Upper

First and second authors, Department of Plant Pathology, University of Wisconsin, Madison 53706; third author, Plant Disease Resistance Research Unit, Agricultural Research Service, USDA, Department of Plant Pathology, University of Wisconsin, Madison 53706.
Present address of first author: Department of Plant Pathology, University of California, Berkeley 94720.
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ABSTRACT

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Treatment of corn (*Zea mays* L.) seedlings in a growth chamber with an *Erwinia herbicola* isolate (M232A) that was not active in ice nucleation decreased the amount of frost damage incited by ice nucleation active (INA) isolates of *Pseudomonas syringae* and *E. herbicola* at −5 C. The percentage of leaves damaged by frost decreased significantly if M232A was applied at any time before, and up to 12 hr after, application of an INA *E. herbicola* isolate. Frost damage decreased with increased densities of M232A (10^5 to 10^9 cfu/ml) applied 24 hr before challenging with an INA isolate of *E. herbicola* (8×10^5 or 8×10^6 cfu/ml). Plants sprayed with 3×10^8 colony-forming units (cfu) per milliliter M232A 24 hr prior to application of INA isolates of *E. herbicola* or *P. syringae* sustained less damage than did plants without M232A populations, regardless of the cell densities of INA bacteria applied. From 88 to 100% reduction in frost damage was observed in the presence of M232A on plants challenged by less than $\sim 10^6$ cfu/ml of INA *E. herbicola* or 10^5 cfu/ml of INA *P. syringae* 48 hr before exposure to freezing temperatures. Populations of an INA *E. herbicola* isolate and a streptomycin-resistant mutant of M232A (M232ASR11) were estimated on seedlings treated with M232ASR11 at different times both before and after treatment with the INA *E. herbicola*. Total populations of bacteria at the time of freezing were nearly constant ($\sim 10^7$ cfu/g fresh weight), but the fraction that was ice nucleation active decreased with increasing time of pretreatment with M232ASR11. A significant linear correlation was found between the logarithm of INA populations of *E. herbicola* present on leaves at the time of freezing and frost injury to those leaves. M232A significantly reduced frost injury incited by six different *E. herbicola* and two different *P. syringae* isolates.

There is growing evidence for significant interactions among the various bacteria present on the aerial plant surfaces and that these interactions can affect disease development. For example, reductions in the incidence of fireblight caused by *Erwinia amylovora* have been produced by the application of certain epiphytic bacteria to pears and apples (15,17), the development of bacterial blight of soybean was inhibited by the presence of an epiphytic bacterium (16), and crown gall caused by *Agrobacterium tumefaciens* can be controlled by the application of a closely related nonpathogenic bacterium (3). We have shown recently that large (up to 10^7 colony-forming units [cfu] per gram) epiphytic populations of ice nucleation active (INA) isolates of *Pseudomonas syringae* van Hall and *Erwinia herbicola* (Löhnis) Dye exist on leaves in nature, and that these are responsible for inciting frost injury to the plants on which they reside (1,9–14). A log-linear relationship exists between the size of the population of INA bacteria on plants and the amount of frost damage (13); therefore, any reduction in the populations of INA bacteria will necessarily result in reduced susceptibility to frost injury at a given temperature. Since many other bacterial residents on plant surfaces are not INA (10), it appears probable that there are interactions of INA and non-INA bacteria on leaf surfaces.

This report deals with the effects of populations of a non-INA isolate of *E. herbicola* on populations of INA isolates of both *P. syringae* and *E. herbicola* on corn seedlings, and on the modifications of frost injury to those plants by the presence of the non-INA isolate. A preliminary account of this work has appeared (8). The isolate and methods of its use are also described in patents (6,7).

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MATERIALS AND METHODS

The antagonistic non-INA bacterial isolate (designated M232A) was obtained in late July 1975, from washings of a field-grown corn leaf. This motile (four to six peritrichous flagella), yellow pigmented, facultative anaerobic isolate was identified as a member of the Enterobacteriaceae belonging to the genus *Erwinia* by the following tests. Biochemical tests were preformed with API 50E units (Analytical Products Inc., Prairieview, NY). Important characters of M232A included positive reaction for catalase; production of indole, acetoin, and β-galactosidase; growth in 5% NaCl and 0.1% 2,3,5-triphenyl-2H-tetrazolium chloride; and production of acid from glucose, fructose, sucrose, galactose, arabinois, xylose, lactose, mannitol, rhamnose, ribose, salicin, and melibiose. Negative reactions were found for oxidase, arginine dihydrolase, urease, pectolytic enzymes; for production of H_2S, levan, lysine decarboxylase, and ornithine decarboxylase; for production of acids from sorbose, methyl D-glucoside, dextrine, adonitol, amylose, and glycogen; and in the tobacco hypersensitivity test (4). On the basis of these tests, M232A most closely matched the description of *Erwinia herbicola* var. *ananas* (5). The appearance of spontaneous colorless variants arising from M232A was also characteristic of *E. herbicola* (2). The origins of the challenging INA *E. herbicola* isolate 26 (Eh 26) and *P. syringae* isolate 31 (Ps 31) from corn leaves have been described (1,9). Unless otherwise specified, cultures were grown (ambient room temperature) and stored (5 C) on nutrient agar containing 2.5% glycerol (NGA).

Bacterial cells for application to plants were grown on NGA plates for 2 days at 24 C, harvested with a loop, suspended in 0.1 M phosphate buffer (pH 7.0) or nutrient broth (NB), and diluted to

desired cell densities that were verified by dilution plating. Plants were either sprayed with these bacterial suspensions or with buffer or NB at the rate of ~0.5 ml/plant and were incubated in a mist chamber at 24 C in the dark until immediately before exposure to −4.5 to −5 C.

Measurement of frost injury to corn seedlings. Frost injury to three-leaf-stage corn (*Zea mays* L.) seedlings at −4 to −5 C was measured by a method similar to that reported earlier (1,9,11). Eighty to 100 growth-chamber-grown plants were included in each treatment. Each of the three leaves of each corn seedling was rated for frost injury and was scored as damaged regardless of the extent of frost injury.

To test the effectiveness of M232A in reducing frost damage, corn seedlings were sprayed with suspensions of M232A and held in a mist chamber at ~24 C for 1 or 2 days until sprayed with the challenging INA bacteria. After applying the INA bacteria, plants were again placed in the mist chamber, usually for 2 days, before they were subjected to −4.5 to −5 C.

Selection of streptomycin resistant mutants of isolate M232A. Spontaneous mutants of M232A resistant to 50 mg of streptomycin sulfate per liter were compared with the parent isolate for effectiveness in reducing frost damage. Suspensions of 3×10^8 cfu/ml in NB of each mutant and M232A were sprayed on corn seedlings 72 hr before exposure to −5 C. *E. herbicola* 26 (10^7 cfu/ml) was applied 48 hr before placing at −5 C. All 11 mutant isolates significantly decreased frost damage relative to plants that had not been pretreated, but were not significantly different among themselves. One mutant, designated M232ASR11, was similar to M232A in all pertinent characteristics except sensitivity to streptomycin and was selected for use in experiments requiring a "marked" strain of M232A.

Measurement of bacterial populations on seedlings. Samples consisted of the aboveground portions of four three-leaf-stage corn seedlings. Seedlings were cut into 3-cm lengths and washed in 100 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.1% Bacto peptone in 500-ml Erlenmeyer flasks on a reciprocal shaker for 2 hr. Appropriate dilutions of these washings were plated on NA, NGA, and NA containing 50 mg of streptomycin sulfate per liter. Total bacterial populations were determined on NA. Only bacteria resembling M232ASR11 grew on NA plates containing streptomycin, allowing unambiguous quantitation of this isolate. *E. herbicola* 26 could be quantitated on NA due to its distinct colony morphology.

RESULTS AND DISCUSSION

Effect of time of application of M232A. Application of M232A at any time before and up to 12 hr after the application of Eh 26 effectively reduced frost damage to corn seedlings (Fig. 1). Eh 26 was applied to all plants 2 days before subjecting the plants to freezing temperatures. Damage to M232A treated plants, compared with those treated with NB alone, increased sharply with delay in application of M232A after treatment with Eh 26. Because a 1-day pretreatment with M232A was as effective in reducing damage as a 2-day pretreatment, M232A was applied 24 hr before the application of INA bacteria in subsequent experiments.

Effect of bacterial cell densities. When different cell densities of M232A were applied 24 hr before application of Eh 26, frost damage decreased with increasing M232A cell density (Fig. 2). Highly significant reductions, compared to pretreatment with Eh 26 alone, were obtained with cell densities of M232A as low as $\sim 10^6$ cfu/ml. Damage to plants challenged with 8×10^6 cfu of Eh 26 per milliliter was significantly greater than to those challenged with 8×10^5 cfu of Eh 26 per milliliter for all densities of M232A except 10^9 cfu/ml. Thus, the cell density of M232A applied to corn seedlings appears to be important (at least when applied only 24 hr before Eh 26) in determining the degree to which frost damage is reduced by M232A. Applied cell densities of greater than 10^8 cfu of M232A per milliliter appeared to be required for near maximum reduction in frost damage.

The cell densities of Eh 26 also affected damage. Thus, although frost damage was significantly reduced by M232A at all cell densities of Eh 26, the percent reduction in injury provided by M232A, compared with Eh 26 alone, decreased as the density of Eh 26, which was applied, increased (Fig. 3).

M232A was found to reduce frost damage incited by Ps 31 as well as by Eh 26 (Fig. 4). Damage to plants pretreated with M232A was substantially reduced at most cell densities of either Eh 26 or Ps 31 applied compared to plants with Eh 26 or Ps 31 alone. The largest reductions in damage to plants challenged with Ps 31 occurred when less than $\sim 10^6$ cfu of Ps 31 per milliliter were used. The

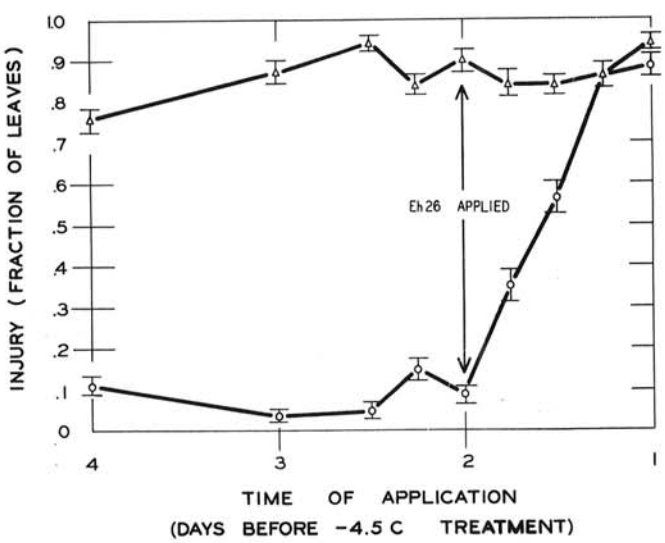

Fig. 1. Effect of time between application of the antagonistic isolate, *Erwinia herbicola* M232A, and exposure to −4.5 C on the reduction of frost damage to corn leaves incited by the ice nucleation active (INA) isolate, *E. herbicola* 26. Corn seedlings were sprayed with either nutrient broth (Δ) or with suspensions of M232A (~7×10^8 cfu/ml) in nutrient broth (o). All plants were challenged by spraying with ~5×10^6 cfu of Eh 26 per milliliter in phosphate buffer 2 days before testing at −4.5 C. All plants were held in a mist chamber until just before exposure to −4.5 C. The vertical bars represent the standard errors.

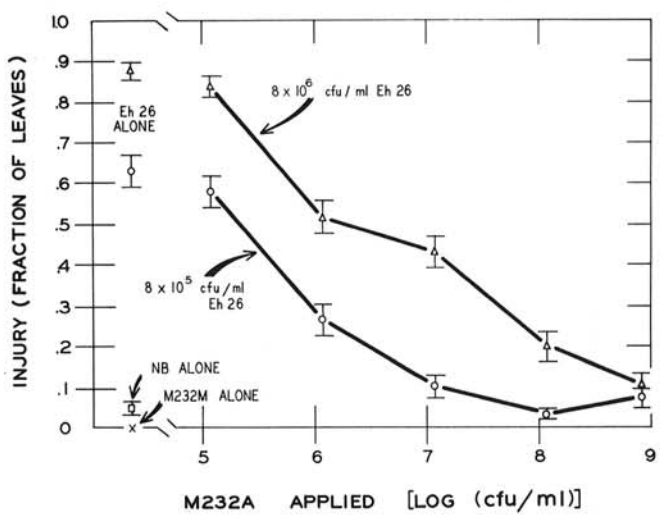

Fig. 2. The effect of antagonist M232A *Erwinia herbicola* cell density on frost injury to corn seedlings incited by the ice nucleation active (INA) isolate, *E. herbicola* 26. Corn seedlings were sprayed with M232A suspensions (in nutrient broth) at the cell densities shown on the abscissa and held in a mist chamber for three days before exposure to −4.5 C. Two days before exposure to −4.5 C plants were sprayed with Eh 26 cell suspensions in phosphate buffer at 8×10^6 cfu/ml (Δ) or 8×10^5 cfu/ml (o) and returned to the mist chamber until −4.5 C treatment. The vertical bars represent the standard errors.

M232A-mediated reduction of frost damage was considerably less with challenges of Ps 31 $>10^6$ cfu/ml. Since Ps 31 has been shown to have higher in vitro INA than Eh 26 (9), the greater extent of frost injury observed on plants sprayed with Ps 31 compared to plants sprayed with Eh 26 (Fig. 4) may represent a higher number of ice nuclei produced per cell in vivo and/or to the development of a higher cell population on plants sprayed with *P. syringae*. Accordingly, a greater degree of reduction of the population of *P. syringae* by M232A would have been required to achieve a comparable reduction in frost injury.

Growth of M232ASR11 and Eh 26 on corn plants. When applied separately, isolates M232ASR11 and Eh 26 grew at nearly identical rates on corn leaves (Fig. 5). For the first 12 hr, the population of M232ASR11 decreased, whereas the population of Eh 26 increased slightly. Between 12 and 30 hr, both isolates grew logarithmically, with a mean doubling time of ~3.8 hr. By 48 hr, Eh 26 had reached a somewhat higher population than had M232ASR11.

The growth of the bacteria in mixed epiphytic populations is shown in Fig. 6. When M232ASR11 was applied 12 hr earlier than Eh 26, lag periods of ~6 and 24 hr were seen for M232ASR11 and Eh 26, respectively (Fig. 6A). M232ASR11 populations were consistently higher than those of Eh 26 at each sampling time. Although the growth rate of M232ASR11 was slightly lower in this mixture than when grown alone, the more notable effect of the prior application of M232ASR11 was the apparent extension of the lag period of Eh 26 (cf, Fig. 5). In the mixture, populations of Eh 26 did not increase significantly until ~24 hr after the application. Although growth rates of Eh 26 between 24 and 36 hr in the mixture were similar to those where it was growing alone (ie, generation time of ~3.4 hr), the total population at the end of the incubation period was lower than when alone.

A similar phenomenon was observed when M232ASR11 was applied only 6 hr before Eh 26 (Fig. 6B). The growth rate of M232ASR11 in the mixture was low relative to its growth alone, whereas Eh 26 exhibited a nearly normal growth rate (doubling time ~3.5 hr between 24 and 36 hr). The maximum stationary population of Eh 26 in the mixture on leaves at the end of the incubation period was lower than on plants without M232ASR11.

The lag period of Eh 26 on plants was only 12 hr when M232ASR11 was applied at the same time as Eh 26 (Fig. 6C). Both grew nearly exponentially between the 12th and 36th hr of incubation with generation times of ~3.6 and ~4.7 hr, respectively. Again, final populations of Eh 26 incubated with M232ASR11 were less than those of plants with Eh 26 alone. M232ASR11 and Eh 26 each comprised nearly equal fractions of the total bacterial populations after all incubation periods.

When M232ASR11 was applied 12 hr after Eh 26 (ie, at the end of the Eh 26 lag period) populations of Eh 26 were much higher than M232ASR11 after all incubation periods except immediately after application of M232ASR11 (Fig. 6D). In this mixture, Eh 26 grew exponentially until approximately the 36th hr, with a generation time of ~4.2 hr, and a final population approaching those on plants without M232ASR11.

At the end of the experiment illustrated in Fig. 6 (48 hr after application of Eh 26), populations of Eh 26 decreased as populations of M232ASR11 increased (Fig. 7). Populations of Eh

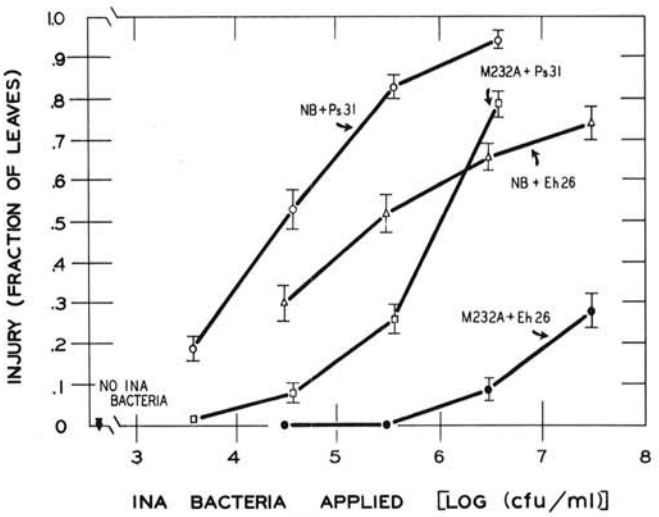

Fig. 4. Frost damage to corn seedlings pretreated with the antagonist (*Erwinia herbicola* M232A) and nutrient broth (NB) challenged with a range of cell densities of the INA isolates *E. herbicola* 26 and *P. syringae* 31. Three days before exposure to −4.5 C three sets of seedlings were sprayed with M232A (3×10^8 cfu/ml) in nutrient broth (●, □, and ▲) and two sets with nutrient broth alone (o, and Δ) and then incubated in a mist chamber. One day later plants were sprayed with Ps 31 (o and □) or Eh 26 (● and Δ) in phosphate buffer and were returned to the mist chamber. Controls (▲) were not treated with either Eh 26 or Ps 31. The vertical bars represent the standard errors.

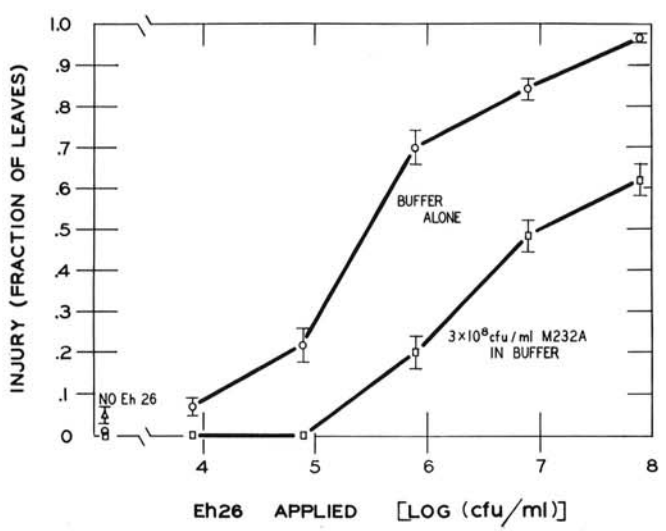

Fig. 3. The effect of cell density of the INA isolate (*Erwinia herbicola* 26) on frost damage to corn seedlings pretreated with the antagonist (*E. herbicola* M232A). Seedlings were sprayed with M232A (3×10^8 cfu/ml) in phosphate buffer (□) or with phosphate buffer alone (o) and were incubated in a mist chamber for 3 days before exposure to −4.5 C. Two days before exposure to −4.5 C all plants were sprayed with Eh 26 in phosphate buffer and then returned to the mist chamber. The vertical bars represent the standard errors.

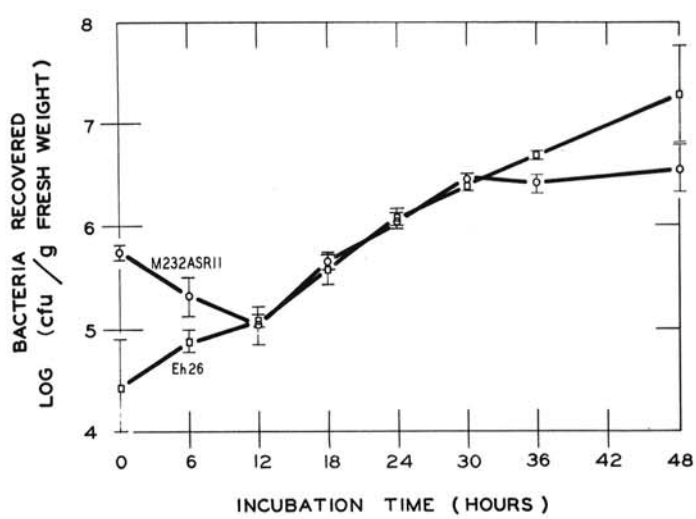

Fig. 5. Multiplication of *Erwinia herbicola* isolates 26 and M232ASR11 on corn seedlings. Plants were sprayed at different times before assay with suspensions of either ~1.2×10^8 cfu of Eh 26 per milliliter (□) or 9×10^7 cfu of M232ASR11 per milliliter (o) in phosphate buffer and were incubated in a mist chamber until assay. Each value represents the mean of the log of three determinations. The vertical bars represent the standard errors.

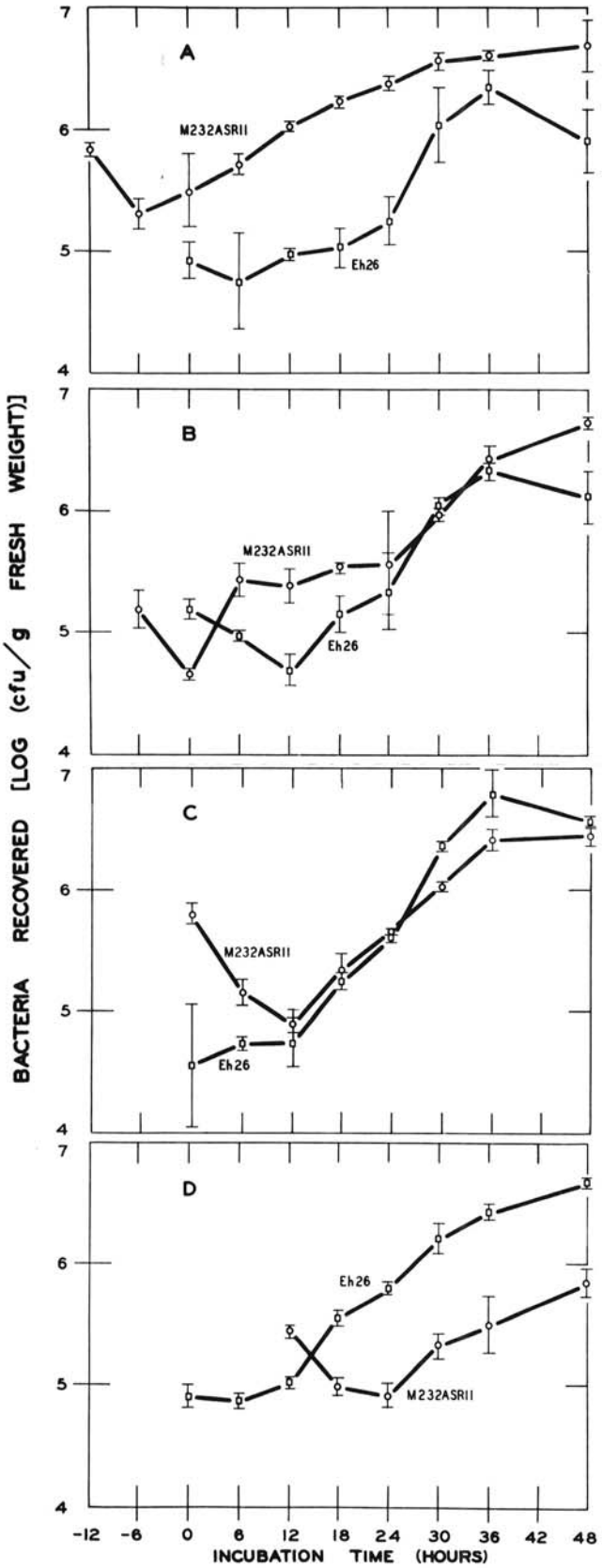

Fig. 6. Growth of mixed populations of *Erwinia herbicola* isolates M232ASR11 and 26 on corn seedlings and the effect of time of application of M232ASR11 relative to the application of Eh 26. Eh 26 (□, 1.2×10^8 cfu/ml) in phosphate buffer was applied to all plants at incubation time marked "0". Plants were sprayed with M232ASR11 (9×10^7 cfu/ml) in phosphate buffer at the relative times indicated by the first data point for each curve, (ie, 12 hr before Eh 26 in the set pictured in "A," 6 hr before Eh 26 in "B", ···, etc) and incubated in a mist chamber. Each value represents the mean log of three determinations of bacterial population. The vertical bars represent the standard errors.

26 were lowest and M232ASR11 highest when application of M232ASR11 preceded application of Eh 26. The total population of bacteria on the plants was nearly constant at $\sim 10^7$ cfu/g regardless of the time of application of M232ASR11 in relation to the application of Eh 26. When logarithm of the final Eh 26 population was plotted against logarithm of the M232ASR11 population, the relationship shown in Fig. 8 was observed. If only those times when both bacteria had been on plants for at least 30 hr were used, the relationship was significant ($r = -0.988$; $P < 0.05$). Thus, M232ASR11 may be functioning as a competitor with Eh 26, reducing the number of cells of Eh 26 harbored by the plants.

At the end of the experiment described in Figs. 6 and 7, the plants were subjected to −5 C. A significant positive linear correlation was found between the logarithm of the Eh 26 population present on the leaves at the time of exposure to the freezing temperature and frost

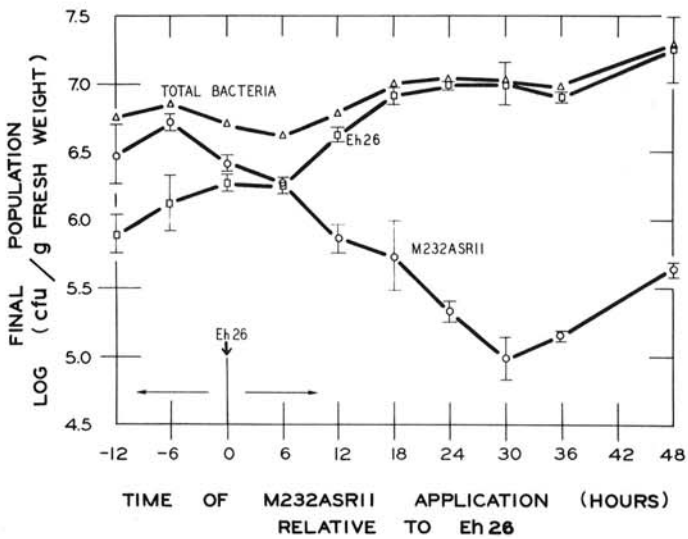

Fig. 7. Effect of time of application of the antagonist (*Erwinia herbicola* M232ASR11) relative to the ice nucleation active (INA) isolate (*E. herbicola* 26) on final bacterial populations (48 hr after application of Eh 26) in the experiment described in Fig. 6. After Eh 26 had been on the plants for 48 hr, populations of M232ASR11 (o), Eh 26 (□), and total bacteria (Δ), were determined. The vertical bars represent the standard errors.

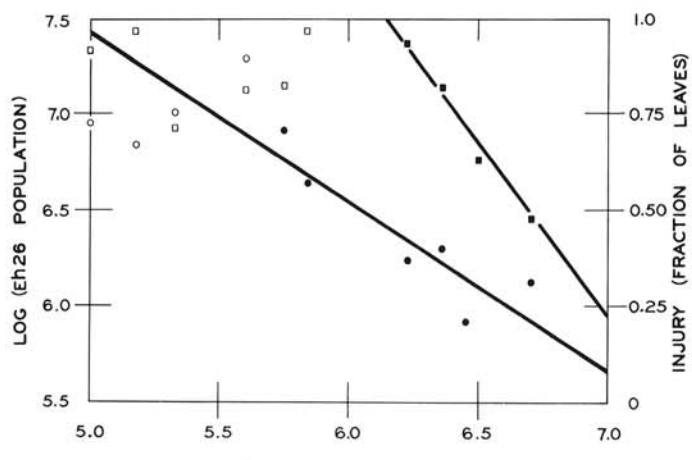

Fig. 8. Relationships of leaf populations of the INA isolate (*E. herbicola* 26) (o, •) at the time of freezing and of frost injury to corn leaves (□, ■) to populations of the antagonist (*E. herbicola* M232ASR11) on corn leaves. Regression lines represent plots of the logarithm of the Eh 26 population against the logarithm of the M232ASR11 population for points up to 18 hr after application of Eh 26 (•; $r = -0.898$; $P < 0.05$) and injury against the logarithm of the M232ASR11 population for points up to 6 hr after application of Eh 26 (■; $r = -0.97$; $P < 0.05$).

TABLE 1. *Erwinia herbicola* isolate M232A-mediated reduction of frost damage incited by several isolates of ice nucleation active (INA) bacteria

INA isolate	cfu/ml[b]	Fraction of leaves damaged ± S. E.		Ice nuclei per leaf[e]		Reduction of frost damage[f] (%)	Reduction of ice nuclei[g] (%)
		NB pretreated[c]	M232A pretreated[d]	NB pretreated	M232A pretreated		
E. herbicola 10[a]	2.6×10^7	0.86 ± 0.03	0.34 ± 0.05	1.96	0.41	60	79
E. herbicola 30B[a]	2.1×10^7	0.87 ± 0.03	0.20 ± 0.04	2.04	0.22	77	89
E. herbicola MXAC[a]	2.0×10^7	0.85 ± 0.04	0.33 ± 0.04	1.89	0.40	61	79
E. herbicola 2[a]	1.5×10^7	0.95 ± 0.01	0.31 ± 0.04	2.99	0.37	67	88
E. herbicola M5532[a]	2.8×10^7	0.86 ± 0.02	0.37 ± 0.04	1.96	0.46	57	77
E. herbicola 26	2.0×10^7	0.95 ± 0.01	0.32 ± 0.04	2.99	0.38	66	87
P. syringae T4D	1.5×10^7	0.99 ± 0.01	0.85 ± 0.02	4.60	1.89	11	59
P. syringae 31	4.8×10^6	0.94 ± 0.02	0.78 ± 0.03	2.81	1.51	17	46
No INA bacteria	0	0.31 ± 0.05	0.03 ± 0.02	0.37	0.03	90	92

[a] These isolates were tentatively identified as *E. herbicola* on the basis of: ice nucleation, characteristic colony morphology, color, and odor, and growth in 5% NaCl and in 0.1% TZC.
[b] Cell density of INA bacteria applied to corn seedlings 2 days before exposure to −5 C.
[c] Each plant was sprayed with approximately 0.5 ml of nutrient broth (NB) 3 days before freezing and incubated in a mist chamber.
[d] Each plant was sprayed with approximately 0.5 ml of a suspension of 5×10^8 cfu/ml M232A in NB 3 days before exposure to −5 C and incubated in a mist chamber.
[e] Ice nuclei per leaf calculated as $\ln (1-f)^{-1}$, where f = fraction of leaves damaged (cf references 14,18).
[f,g] Reduction of frost injury (g) ice nuclei of plants treated with *E. herbicola* isolate M232A 3 days prior to freezing compared to plants sprayed with NB 3 days prior to freezing at −5 C.

damage to those leaves ($r = 0.759$; $P<0.05$). Above M232ASR11 populations of $\sim 10^6$ cfu/g, damage appeared to decrease linearly ($r = -0.97$; $P<0.05$) with increasing logarithm of the M232ASR11 populations on the plants at the time of exposure to −5 C (Fig. 8).

M232A-mediated reduction of frost damage when challenged by other INA bacterial isolates. Several INA isolates, in addition to Eh 26 and Ps 31, were used to challenge M232A-treated plants to determine how generally effective M232A is in reducing frost damage incited by INA bacteria. Pretreatment of corn seedlings with M232A significantly reduced the frost damage incited by the five additional INA isolates of *E. herbicola* and the one additional isolate of *P. syringae* (Table 1). M232A reduced the injury incited by all six INA isolates of *E. herbicola* to a similar extent. Reduction of the injury incited by the isolates of *P. syringae* was smaller, but still significant. Thus, M232A appears to be effective against more than just one or two INA isolates of these bacterial species.

Assuming that a single nucleation event is sufficient to initiate ice formation, and hence injury, the number of effective nuclei per leaf can be estimated (14,18). In the presence of M232A, ice nucleus concentrations on leaves inoculated with INA *E. herbicola* isolates were reduced 77–88% and on leaves inoculated with INA *P. syringae*, 46–59% (Table 1).

Previous reports have shown that ice nuclei are necessary for the freezing of plant tissues, that INA bacteria are the principle source of nuclei on plants, and that the number of nuclei on the plant is directly related to the number of INA bacteria present. The mechanism by which INA bacteria incite frost injury appears to be the production of ice nuclei by the bacteria; the ice nuclei, in turn, catalyze ice formation in or on plant tissues and frost injury results from the ice formation. Indeed, a log-linear relationship has been demonstrated between the size of the populations of INA bacteria and the amount of frost injury (11,13). This report indicates that at least one member of the epiphytic non-INA flora can have a very significant role in limiting colonization of INA bacteria, and thus in limiting frost damage. Isolate M232A of *E. herbicola* limits the potential of many INA bacteria to incite frost injury (Table 1). The available evidence suggests that the mechanism by which M232A decreases frost injury to plants is by competition with, or exclusion of, INA bacteria. The decrease in frost injury achieved by extending the period between application of M232A and Eh 26 (Fig. 1), or increasing the cell densities of M232A applied, (Fig. 2), are probably due to greater establishment of competitive populations of M232A before the challenge inoculation with INA bacteria.

The log-linear relationship between populations of Eh 26 and frost injury in the presence of M232ASR11 is more consistent with the primary influence of M232A as limiting the number of co-resident INA bacteria than with an effect on the nucleation activity of INA bacteria (Fig. 8). We have found no evidence to suggest that M232A can change the ice nucleation activity of *E. herbicola* or *P. syringae* in vitro. Also, no evidence of in vitro antagonism via antibiotic or bacteriocin production by M232A against either of the INA strains of the two species has been found.

This work has been done in growth rooms and mist chambers, where populations of natural epiphytic bacteria on corn seedlings are very low and an antagonist such as M232A becomes established readily. Under field conditions, where populations of natural epiphytic bacteria are very diverse and can be relatively high (10), M232A might have more difficulty in becoming established. The question of whether it will be possible to establish a sufficiently high population of this antagonist on leaves of plants in the field to inhibit the colonization of these plants by INA bacteria, and thus achieve frost protection, is addressed in a companion paper (12).

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