Biological Control of Frost Injury: Establishment and Effects of an Isolate of *Erwinia herbicola* Antagonistic to Ice Nucleation Active Bacteria on Corn in the Field

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ABSTRACT

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Isolate M232A of *Erwinia herbicola* lacks ice nucleation activity, and has the ability to decrease both the population of epiphytic ice nucleation active (INA) bacteria and frost damage to plants inoculated with INA bacteria in growth chamber tests. Populations of this isolate and a streptomycin-resistant mutant of the isolate (M232ASR11) were established on corn (*Zea mays* L.) seedlings in the field by applications made shortly after emergence. Total bacterial populations present on corn leaves sprayed with either M232A or M232ASR11 were significantly higher than on control plants until ~20 July, and were composed almost exclusively of bacteria resembling either M232A or M232ASR11. After 20 July, total bacterial populations on leaves sprayed with the antagonists were not different from those on the control plants ($\sim 5 \times 10^7$ colony-forming units (cfu) per gram fresh weight), and M232A or M232ASR11 comprised ~10% of this population. Populations of INA bacteria in the presence of the antagonistic bacteria decreased signficantly throughout the growing season relative to populations on control plants, eg, 30 cfu/g compared to 100–300 cfu/g before 25 July and $\sim 10^4$ cfu/g versus 5×10^5 cfu/g on 20 September for treated and control plants, respectively. Populations of M232A were similar throughout the growing season regardless of whether the plants were treated two or eight times. Populations of M232ASR11 did not increase on plants treated with streptomycin (50 mg/L) relative to plants not sprayed with streptomycin. Exposure to −5 C caused significantly less damage to detached corn leaves colonized with M232A or M232ASR11 than to control leaves throughout the 1976 growing season. Reduction in bacterial populations by the application of these antagonistic bacteria significantly reduced the damage to corn exposed for 2–3 hr to a natural frost on 23 September 1976.

A large reservoir of bacteria, the role of which remains largely unknown, is present on leaf surfaces (2). Certain saprophytic bacteria on leaf surfaces apparently interact with plant pathogenic species and decrease the level of bacterial disease which develops; eg, *Erwinia herbicola* suppresses infection by *Xanthomonas oryzae* on rice (4), saprophytic bacteria suppress infection by *E. amylovora* on pear (10,11) and several pseudomonads suppress infection by *Pseudomonas syringae* pv. *morsprunorum* on cherry (3). Ice nucleation active (INA) strains of *P. syringae* and *E. herbicola* occur commonly as epiphytes on leaf surfaces (8) and incite frost injury to the plants on which they reside by catalyzing crystallization of super-cooled water (1,6). Isolate M232A of *E. herbicola* is not active as an ice nucleus and is antagonistic to INA isolates of both *P. syringae* and *E. herbicola* on leaf surfaces under growth chamber conditions (9). In this study, we investigated the potential usefulness of this antagonist in controlling frost injury to corn (*Zea mays* L.) plants in the field. A preliminary account of this work has appeared (7).

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MATERIALS AND METHODS

The isolation and identification of *E. herbicola* isolate M232A and its streptomycin resistant derivative M232ASR11 are described in the preceding paper (9). Cultures were grown and maintained on nutrient agar containing 2.5% glycerol (NGA). Bacteria for application to corn plants in the field were grown to late log phase at 28 C on a reciprocal shaker in either nutrient broth (NB) (Difco, Detroit, MI) or in minimal salts-glucose broth (5) (MGB; 1 g glucose, 7 g K_2HPO_4, 2 g KH_2PO_4, 0.5 g sodium citrate, 0.1 g $MgSO_4 \cdot 7\ H_2O$, and 1 g $(NH_4)_2SO_4$ per liter of distilled water).

Cells were applied to plants in the field at the rate of $\sim 10^8$ cfu/ml in half-strength nutrient broth (NB diluted with an equal volume of water) until 1 August. Thereafter they were applied in tap water.

Measurement of bacterial populations on leaves. Entire field-grown corn leaf blades (~10–20 g/sample) were cut into 3-cm

squares, placed in 500-ml Erlenmeyer flasks containing 200 ml sterile 0.1 M phosphate buffer supplemented with 0.1% w/v Bacto peptone, and shaken for 2 hr at ~28 C. Appropriate dilutions of these leaf washings were plated on nutrient agar (NA), NGA, or NA containing 50 μg/ml streptomycin sulfate (NSA). The characteristic raised yellow colonies of M232A and M232ASR11 were estimated on dilution plates of NA and NSA, respectively. INA colonies were estimated on NGA plates by the replica freezing technique (8).

Field plot design and treatments. The field experiment on corn (W64A × A632) was conducted at an experimental farm near Arlington, WI, in 1976. Plots were arranged in a randomized complete block design with 11 treatments and three replications. Each plot consisted of 24 rows ~6.5 m long, with 91 cm between rows and 16–18 cm between plants within a row (~61,800 plants per hectare). Individual plots were ~2.5 m apart. Table 1 lists the various treatments and application schedules for this experiment.

Testing detached field-grown corn leaves for frost damage. Leaves from field-grown plants were tested with the same facilities and a similar procedure to that described for seedling corn plants (1,6). Four groups of five leaf blades each were collected at near ear height from individual plants at random in each plot. The five leaf blades of each group were tied together at their tips, hung in a random position in the controlled environmental chamber and cooled at 0.05 C/min to −4.5 C. Each leaf blade was rated immediately after exposure to the low temperature for the presence of water-soaking typical of frost injury. A leaf was scored as damaged regardless of the extent of the frost injury.

TABLE 1. Field plot treatments and treatment schedule in experiments designed to test effects of antagonistic bacteria on ice nucleation active bacteria on corn in the field

Treatment	Concentration[a]	Dates applied
1,11. Controls	...	...
2. RH 6401[b]	0.5 g/L	24 and 30 June; 16, 23, and 28 July; 6, 16, 19, and 28 August; and 5, 6, and 17 September
3. Cupric hydroxide[c]	1.25 g/L Kocide 101	8, 16, 22, and 30 June; 16, 23, and 28 July; 6, 16, 19, and 28 August; and 5, 6, and 17 September
4. Streptomycin 500[d]	0.5 g/L ai 2.5 g/L Agristrep	(Same schedule as #3)
5. Streptomycin 50	50 mg/L ai 0.25 g/L Agristrep	8, 16, 19, and 28 August; 6 and 17 September
6. M232A-2 treatments[e]	$\sim 10^8$ cfu/ml in half-strength NB	9 and 16 June
7. M232A-8 treatments	$\sim 10^8$ cfu/ml in half-strength NB	9, 16, and 30 June; 10, 21, and 29 July; 8 August; and 5 September
8. M232ASR11	$\sim 10^8$ cfu/ml in half-strength NB or in MGB	(Same schedule as #7)
9. M232ASR11 and Streptomycin	(See treatments 5 and 7)	
10. Nutrient broth	in half-strength NB or in MGB	(Same schedule as #7)

[a] Chemical treatments applied until near runoff with a hand-held spray gun from a motorized pump.
[b] Experimental bactericide 6401; Rohm and Haas Co., Philadelphia, PA.
[c] Kocide 101; Kocide Chemical Co., Houston, TX.
[d] Agristrep type D; Merck Chemical Div., Rahway, NJ.
[e] Bacterial suspensions were applied with a hand-held compressed air sprayer before 1 August and with a motorized mist blower after 1 August.

RESULTS

Establishment of M232A and M232ASR11 on corn leaves. Isolates of M232A and M232ASR11 colonized leaves in a quantitatively similar manner in the field. On unsprayed leaves, bacterial colonies resembling M232A in color and morphology comprised ~1–3% of the total bacteria throughout the growing season (Fig. 1). Before 20 July, bacterial populations on M232A treated leaves were almost exclusively M232A based on color and colony morphology (Fig. 2). Thus, early in the season identification of M232A on these leaves was relatively unambiguous. After 2 August, M232A comprised only about 10% of the bacteria present on the leaves. Similarly, nearly all of the bacteria isolated from corn leaves sprayed with M232ASR11 were M232ASR11 (based on color, colony morphology and growth in the presence of streptomycin) until 20 July (Fig. 3); after 1 August about 10% appeared to be this strain.

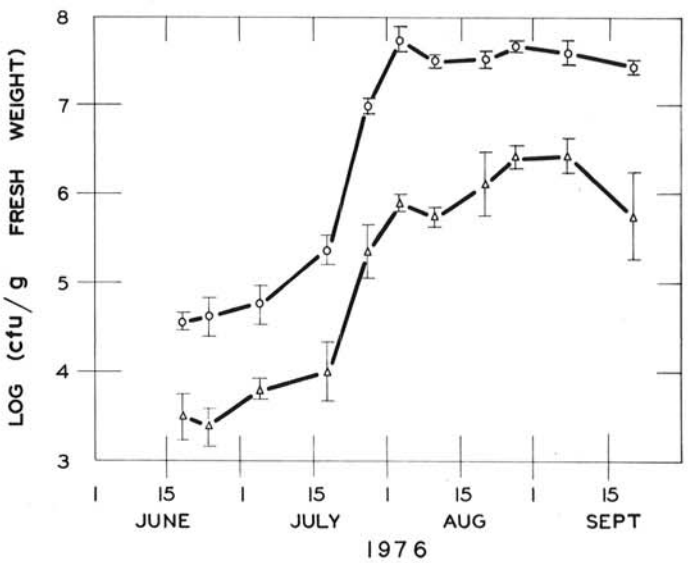

Fig. 1. Total bacteria (o) and yellow pigmented bacteria resembling *Erwinia herbicola* isolate M232A (△) on unsprayed corn leaves during the 1976 growing season. Vertical bars represent the standard error of the mean of log populations from four to 12 separate determinations on each date.

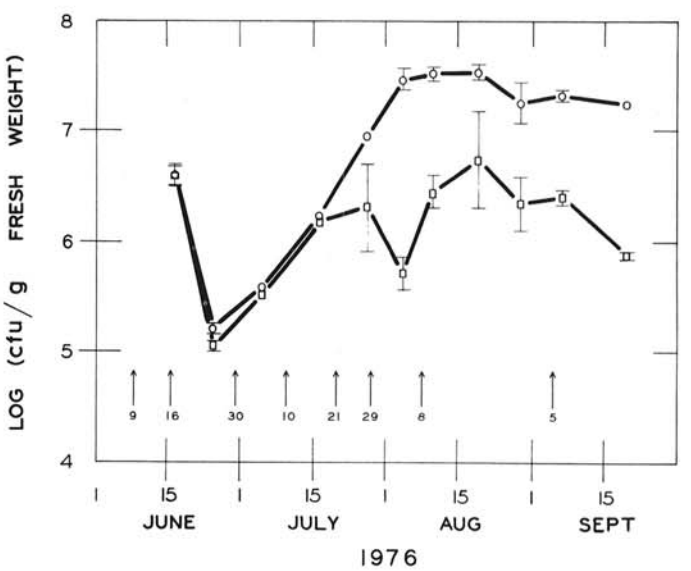

Fig. 2. Total bacteria (o) and bacteria resembling *Erwinia herbicola* isolate M232A (□) on corn leaves sprayed with M232A at the eight times indicated by the arrows. Vertical bars represent the standard error of the mean of log populations from three or four separate determinations on each date.

The total number of bacteria on corn leaves sprayed with M232A or M232ASR11 was significantly higher than on control plants until mid-July (compare Fig. 1 with Figs. 2 and 3). After 1 August, bacterial population sizes on corn leaves sprayed with either M232A or M232ASR11 were not different from those on untreated plants ($\sim 5 \times 10^7$ cfu/g fresh weight). The high bacterial populations (5×10^6 cfu/g) present shortly after treatments began in mid-June, decreased to $\sim 10^5$ cfu/g by 1 July on leaves sprayed with M232A or by 15 July on leaves sprayed with M232ASR11. This decrease followed a period of hot dry weather in late June.

Throughout the growing season plants treated with M232A or M232ASR11 had lower INA bacterial populations than untreated plants (Figs. 4 and 5). Before late July no INA bacteria were detected on the treated plants (level of detection = ~30 cfu/g). Although detectable INA bacterial populations were found on treated leaves after 1 August, populations of INA bacteria were consistently lower than on untreated leaves.

Neither the total number of bacteria, the number of INA bacteria, nor the number of yellow pigmented bacteria resembling *E. herbicola* on plants sprayed with NB or the dilute MGB broth alone differed significantly from those populations on unsprayed control plants at any time during the growing season.

Leaf populations of M232A were not different on plants that were treated eight times with M232A as compared to plants that received only two applications of M232A. Reductions in populations of INA bacteria were similar, whether M232A was applied only early in the growing season or repeatedly. Thus, it appeared that two applications in early summer were sufficient to achieve maximum colonization of corn leaves with this antagonist under the conditions of this test.

The application of streptomycin (50 mg/L), beginning in August, did not affect the size of either established M232ASR11 populations or INA bacterial populations on corn leaves that also supported M232ASR11 populations. The bactericides, streptomycin (0.5 g/L) and Kocide 101, applied frequently beginning on 8 June (Table 1), did reduce the INA bacterial population significantly (6).

Frost damage to corn leaves treated with bacterial antagonists. Since the frost sensitivity of corn leaves has been shown to be directly related to the logarithm of the number of INA bacteria present on those leaves (6), any treatment that can reduce the populations of INA bacteria on leaves should also reduce the susceptibility of those leaves to frost damage. Therefore, field-grown leaves with reduced INA bacterial populations due to treatment with M232A or M232ASR11 or bactericides (Figs. 4 and 5) (6) were tested for injury at −4.5 C in a controlled-temperature chamber. A summary of the results is given in Fig. 6.

Little damage at −4.5 C was sustained by corn leaves when INA bacterial populations were low in early summer. Damage to

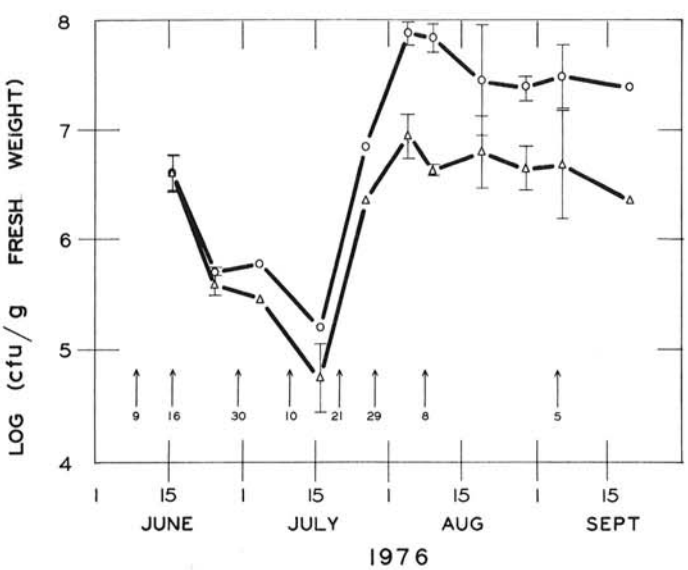

Fig. 3. Total bacteria (o) and *Erwinia herbicola* isolate M232ASR11 (△) on corn leaves sprayed with M232ASR11 at the eight times indicated by the arrows. Vertical bars represent the standard error of the mean of log populations from three to four separate determinations at each date.

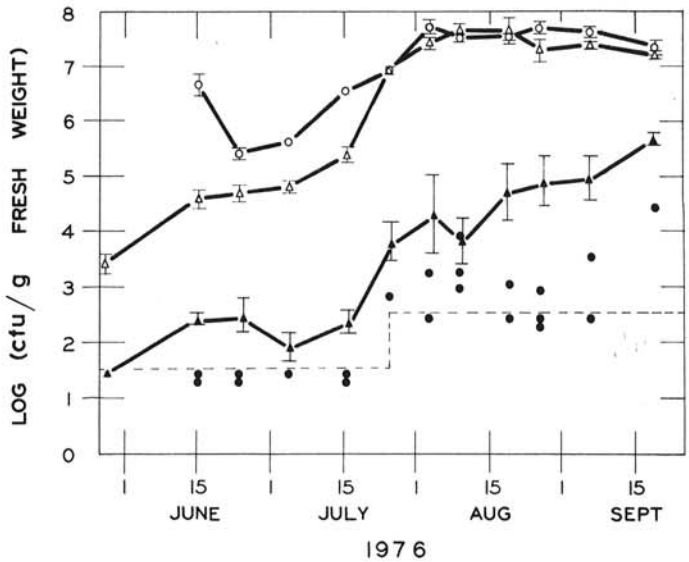

Fig. 4. Total (open symbols) and ice nucleation active INA (closed symbols) bacterial populations on unsprayed (triangles) corn leaves, or those sprayed eight times with *Erwinia herbicola* isolate M232A (circles). The dashed line represents the estimated limits of detection of INA bacteria. Vertical bars represent the standard error of the mean of log populations from two to 12 separate determinations on each date. Samples for which INA bacteria were not detected were assigned the value of the detection limit for that date. Individual data points are shown for INA bacteria on M232A treated plants. Due to the high proportion of samples on which none were detected, mean values were not calculated for this treatment.

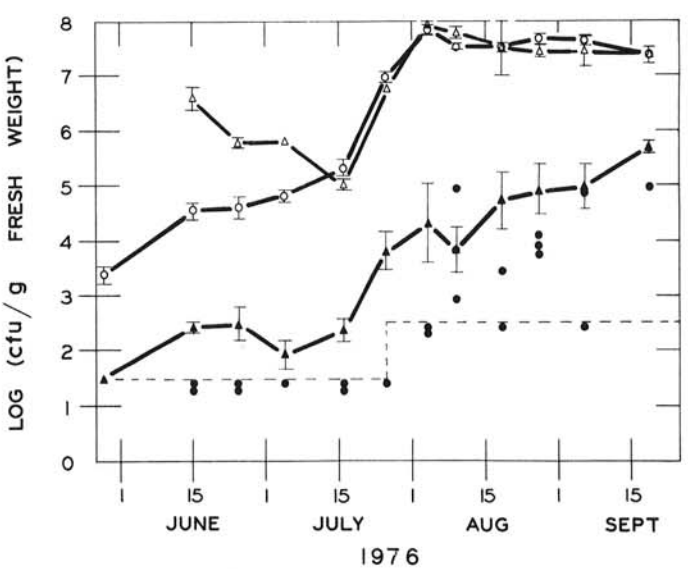

Fig. 5. Total bacteria (△) and ice nucleation active (INA) bacteria (•) on corn leaves sprayed with *Erwinia herbicola* isolate M232ASR11 eight times during the growing season, and total bacteria (o) and INA bacteria (▲) on unsprayed plants. The dashed line represents the estimated limit of detection of INA bacteria. Vertical bars represent the standard error of the mean of log populations from two to four separate determinations on each date. Samples for which INA bacteria were not detected were assigned the value of the detection limit for that date. Individual data points are shown for INA bacteria on M232ASR11 treated plants. Due to the high proportion of samples on which none were detected, mean values were not calculated for this treatment.

untreated plants increased rapidly during August, as did also the bacterial populations (compare Figs. 5 and 6). Leaves from plants sprayed with streptomycin sustained less frost damage than untreated plants on all sampling dates. Leaves from plants treated with M232ASR11 sustained less frost injury than those from untreated plants, but somewhat more damage than leaves from plants treated with streptomycin. Damage to leaves treated with M232ASR11 was consistently about 50% of that on untreated control leaves. Although not shown in Fig. 6, no difference in frost sensitivity was observed between plants that were sprayed with M232A twice in June and those sprayed eight times during the growing season. Similarly, plants treated with M232ASR11 did not differ in frost sensitivity from those M232ASR11-treated plants that were also treated with 50 mg/L streptomycin. Plants treated with NB alone did not differ from untreated plants in frost sensitivity at −4.5 C.

A radiative frost occurred on the morning of 23 September 1976 with minimum air temperature of −1.5 to −2.5 C in the plot area. Ice was observed on leaves in the plots before midnight. Between 0200 and 0300 hours on 23 September, after freezing began, ~30 leaves at about ear height were detached from plants in each plot, transported to a warm building, sealed in plastic bags, and stored at 4 C. Frost damage to these leaves was evaluated about 48 hr after collection, when the water-soaking symptom typical of frost damage remained apparent. Frost damage to leaves from all plants that received treatments effective in decreasing INA bacterial populations were significantly lower than damage to leaves from plots where INA bacterial populations had not been decreased (Table 2). Plants treated with 0.5 g/L streptomycin or Kocide 101 sustained less frost damage than all other treatments. Plants sprayed with M232A (either twice or eight times), M232ASR11 and 50 mg of streptomycin per liter or 50 mg streptomycin per liter alone sustained equivalent damage, but significantly less damage than untreated controls.

A similar order of the extent of frost damage to plants that had received treatments listed in Table 1 was observed on 24 September after the radiative frost. The top six leaves of plants treated with any application schedule of M232A or M232ASR11 sustained significantly less frost damage than either untreated controls or plants sprayed with NB. Damage to all plants was extensive, however. Thus, although protection was significant after 2–3 hr, economic protection was not achieved after plants were exposed to an additional 4–5 hr at −2.5 C.

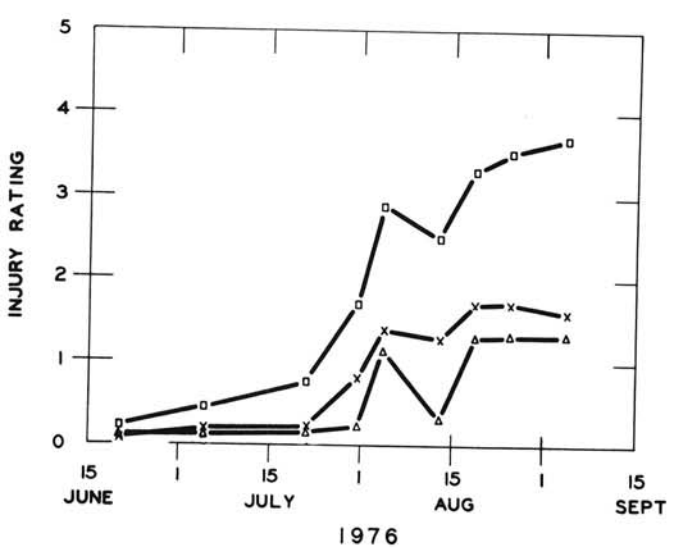

Fig. 6. Frost damage at −4.5 to −5.0 C to detached leaves from corn plants treated with the antagonistic bacterium *Erwinia herbicola* M232ASR11 (×), 0.5 g/L streptomycin (△) or untreated leaves (□) during the 1976 growing season. Injury is represented as the mean number of frost damaged leaves per five leaf bundle.

DISCUSSION

Both M232A and M232ASR11 persisted and grew equally well on corn leaves in the field. Apparently no competitive disadvantage was associated with the spontaneous streptomycin resistant mutation carried by M232ASR11.

Although very dense suspensions of the antagonistic bacteria were applied, populations of the antagonists expressed on a per gram basis either remained approximately constant or declined from ~15 June to after 15 July. The decline in population per gram was probably due to the increase in mass of the leaves, which were expanding rapidly at this time. Populations per leaf probably did not change substantially during this period. The antagonistic bacteria may either have been growing slowly on the leaf surfaces (death rate < growth rate) during this period or merely dying slowly (growth rate ≈ 0). By 1 August the antagonists clearly were established and multiplying. However, by this time they constituted only about 10% of the total bacterial populations on the leaf surfaces, and the total populations on the treated leaves were indistinguishable from those on control leaves. Thus, after growth of M232A is known to have occurred, the total populations were not increased by the presence of these bacteria.

Nonetheless, populations of INA bacteria were decreased by the application of the antagonists during the 15 June–15 July period. Regardless of whether M232A was merely surviving or actively growing, its presence apparently affected the population of at least one other component of the epiphytic bacterial flora—the target organisms.

That additional applications of dense suspensions of M232A after mid-June failed to affect either the total bacterial populations or the population of M232A itself is noteworthy. Perhaps the very hot, dry weather during mid- and late summer created conditions under which the newly applied bacteria had such a low survival rate that they were not detectable among the established populations of M232A and other bacteria. Thus, the additional M232A was not replacing bacteria established on the corn leaves. The fact that total populations did not increase may indicate that the existing populations on the leaves were at or near carrying capacity.

The numbers of INA bacteria present on the corn leaves were decreased by M232A and its mutant; and the extent of frost injury to detached leaves was decreased approximately in proportion to the decrease in numbers of INA bacteria. Thus, the effect of M232A

TABLE 2. Relation of frost injury to population size of ice nucleation active bacteria on corn leaves detached at 0200 hours during a natural radiative frost[a]

Treatment[b]	Log INA bacteria[c]/g	Injury index[d,e]
Streptomycin (0.5 g/L)	ND	0.44
Kocide 101	3	0.47
M232A two treatments		0.83
Streptomycin (50 mg/L)		0.86
M232A eight treatments	4.4	0.96
M232ASR11	5	1.03
M232ASR11 + Streptomycin		1.03
NB Control		1.64
Control 2		1.69
RH 6401	5.5	1.72
Control 1	5.6	2.09

[a] Leaves were collected randomly near ear height between 0200 and 0300 hours on 23 September 1976. Air temperature at the time of collection of samples was approximately −1.0 to −1.5 C.

[b] Treatments are described in Table 1.

[c] Mean populations of ice nucleation active (INA) bacteria on leaves within the plots.

[d] Values represent the mean injury index of 30 corn leaves from each of the three replications in this plot. The average area of the leaf injured was estimated to the nearest quartile (0 = no damage and 4 = >75% of the leaf injured).

[e] $F = 8.09$ (b); LSD 1% = 0.74; LSD 5% = 0.55. Treatment effects were significant at the 99.5% confidence level (F-test).

in decreasing frost damage in the field is apparently an effect on the INA bacterial population on the leaves and not on nucleation efficiency of the INA bacteria present (9).

Isolate M232A of *E. herbicola* was originally isolated from a corn leaf. It has been selected for its ability to colonize corn plants and to decrease populations of INA bacteria on those plants. Data presented above have indicated that this organism can successfully decrease frost injury to corn in the field. Thus, this bacterium shows promise as a possible biological agent for control of frost injury, and illustrates the possibility of use of biological control for this purpose. Isolates with even greater effectiveness may be developed in the future.

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