**ABSTRACT**


Cell suspensions of an isolate of *Erwinia herbicola* from a corn leaf were active in ice nucleation at -2.3 C and below. Ice nucleation activity was affected by the medium on which the cells were grown. Nucleation at -2.3 and -2.5 C was detected in suspensions of cells that had been grown on nutrient agar supplemented with 2.5% glycerol or glucose, respectively, but was detected only at temperatures below -4 C in cells grown on nutrient agar alone. Corn seedlings sprayed with suspensions of this isolate of *E. herbicola* were severely damaged at -4 C, whereas control plants lacking leaf populations of *E. herbicola* or other bacteria active in ice nucleation were not injured. If plants were frozen 6 hr or less after they were sprayed with suspensions (10^7 cells/ml) of *E. herbicola*, frost damage was not significantly different from that to controls. The amount of damage increased greatly with increase in time of incubation between spraying and freezing, from 12 to about 36 hr. The amount of frost damage measured 48 hr after application of *E. herbicola* suspensions increased as applied bacterial cell densities were increased. *Erwinia herbicola* began rapid multiplication about 6 hr after the cells were sprayed onto leaves; populations of about 3 X 10^6 cells/g/12 hr of inoculation had increased to about 5 X 10^7 cells/g after 36 hr. Probit frost injury was directly proportional to the logarithm of *E. herbicola* population (P <0.001).

*Additional key words: Zea mays, L., maize.*

**MATERIALS AND METHODS**

The isolate of *E. herbicola* used in this study, obtained in July, 1975, from a corn leaf grown near Marathon, Wisconsin, was selected on the basis of its ice nucleation activity at -5 C. This yellow pigmented bacterium was identified as *E. herbicola* on the basis of its bacteriological characteristics (5, 6). Unless otherwise specified, cultures were grown and maintained on nutrient agar fortified with 2.5% glycerol.
Testing for ice nucleation activity of bacterial colonies at -5 C. A -5 C surface was prepared by spraying aluminum foil with a 1% solution of paraffin in xylene; the xylene was removed at 55 C in a circulating oven, and the foil was folded into a flat-bottomed "boat," which was floated on a methanol-water solution maintained at -5 C in a refrigerated constant temperature bath. Discrete 4- to 6-day-old colonies from agar plates were removed with a toothpick and suspended in 0.1 ml of distilled water to yield a turbid suspension (>10^7 cells/ml). Five 10-μl droplets of suspension from each colony were placed on the -5 C test surface. A colony was considered to contain nuclei active at -5 C if one or more of the five droplets froze within 30 sec.

Ice nucleation activity spectra of bacterial suspensions. Cells were removed from discrete colonies, suspended in sterile, glass-distilled water, and diluted to the desired cell densities (subsequently determined by dilution plating). The ice nucleation spectrum of each suspension was determined by a procedure similar to that described by Vali (18). The top of a hollow aluminum block was the controlled-temperature working surface. The block was surrounded by styrofoam for insulation. The working surface was coated with paraffin by spraying it with a 1% solution of paraffin in xylene, and removing the xylene with a stream of warm air. Thirty 10-μl droplets of a test suspension were placed on the working surface. Two plexiglass covers separated by 15 mm of air were supported on the styrofoam above the droplets to furnish thermal insulation and to prevent evaporation. The temperature of the block was decreased at approximately 0.3 C/min by circulating methanol through the block from a controlled temperature bath. The temperature of the surface of the block was measured continuously with a thermodiode and recorded as a function of time on a stripchart recorder. Freezing of droplets was observed visually, and the time of each freezing event was marked on the stripchart temperature record.

Measurement of frost injury to corn seedlings. Frost injury to three-leaf-stage corn seedlings at -4 C was measured by a method similar to that reported earlier (1, 9). Plants were sprayed with suspensions of E. herbicola in 0.1 M phosphate buffer pH 7.0, or buffer alone (about 0.5 ml/plant) at various times before freezing. Plants were incubated in a mist chamber (mist treatment) or in ambient air (dry treatment) at about 24 C in the dark until immediately before freezing. After incubation, plants were cooled to about -2 C at about 0.2 C/min, then to -4 C at about 0.03 C/min, and finally allowed to warm to 30 C. Each of the three leaves of every corn seedling was rated for frost injury. Sixty to 80 plants were included in each treatment. A leaf was scored as damaged regardless of the extent of injury. Damage is expressed as the fraction of leaves that showed frost damage in each treatment.

Populations of E. herbicola on leaves. Samples of growth chamber-grown plants consisted of four entire three-leaf-stage corn plants (total sample about 5 g). Individual samples were stored in plastic bags at 4 C for not more than 6 hr before assaying. Each sample was homogenized in 100 ml 0.1 M phosphate buffer, pH 7.0, in a blender. Dilutions of this leaf homogenate were plated on nutrient agar and incubated for two days at 28 C. Yellow colonies with raised centers characteristic of the isolate of E. herbicola used were counted. Similar methods were used with leaves from field-grown corn plants.

RESULTS AND DISCUSSION

To determine whether bacteria active in ice nucleation were commonly present as epiphytes on corn leaves, leaves were sampled from a field near Marxville, W1, during the summer of 1975. Washings of leaves sampled after mid-July were plated on Crosse's medium (4). From most samples numerous (10^5 - 10^7/g fresh wt) light blue mucoid colonies with patches of yellow appeared after 2-3 days at 28 C. Most colonies of this type contained ice nuclei active at -5 C. Two separate isolates with ice nucleation activity and appropriate morphology were identified as E. herbicola (5, 6). One of these, designated isolate #26, was selected for the remainder of the studies reported here.

Ice nucleation activity of E. herbicola. Dense suspensions (>10^7 cells/ml) from cultures grown for 2 days on nutrient agar were active as ice nuclei at temperatures warmer than -4 C only if the medium had been supplemented with relatively high concentrations of a suitable carbon source. This is illustrated by the comparison of ice nucleation activities (Fig. 1) of suspensions of E. herbicola (= 5 X 10^6 cells/ml) grown on nutrient agar or nutrient agar supplemented with 25 g/liter of glycerol or glucose. Cultures supplemented with glycerol were more active in ice nucleation (i.e., ice formation was catalyzed at warmer temperatures) than those supplemented with glucose, but both were highly active at temperatures warmer than -4 C. Even though the bacterium grew well on nutrient agar without an additional carbon source, it had very little ice-nucleation activity at temperatures warmer than -7 C (Fig. 1). Several other isolates tentatively identified as E. herbicola on the basis of colony morphology and color also were active ice nuclei, and were affected similarly by the carbon source in the growth medium. Thus, composition of the growth medium can markedly affect the ice-nucleation activity of bacterial suspensions.

The effect of E. herbicola on frost injury to corn seedlings. Since the presence of P. syringae, another bacterial ice nucleus, on leaves of frost-sensitive plants prevents supercooling and thus increases the extent of frost injury at temperatures only a few degrees below freezing (1, 9), we examined the effect of E. herbicola on frost injury to growth-chamber-grown corn plants. In preliminary experiments, the presence of E. herbicola on corn leaves resulted in badly damaged plants at -4 C; comparable leaves without E. herbicola were not injured. Plants sprayed with an E. herbicola cell suspension (1 X 10^7 cells/ml) and incubated for 0 or 6 hr before freezing did not sustain significantly greater frost damage than nontreated controls (Fig. 2). After 12 hr of incubation, however, the amount of damage to E. herbicola-sprayed plants was significantly greater than that to the controls. From 12 to 36 hr of incubation, the amount of damage increased with time. After 36 hr of incubation, nearly all of the leaves were damaged, so additional injury could not
be measured.

Frost damage increased when the densities of *E. herbicola* cell suspensions sprayed on corn leaves were increased (Fig. 3). In this experiment, treatments were applied 48 hr prior to freezing. Plants sprayed with suspensions as dilute as $6 \times 10^4$ cells/ml sustained significantly more frost damage than plants sprayed with buffer alone regardless of whether incubation was "wet" or "dry". Frost damage increased as cell densities of *E. herbicola* applied to the plants were increased from $6 \times 10^4$ to $10^6$ cells/ml. At applied cell densities of $6 \times 10^5$ cells/ml or less, plants incubated in a mist chamber sustained greater frost damage than plants left dry prior to freezing.

The increase in frost sensitivity at $-4 \, ^\circ C$ observed in corn seedlings sprayed with *E. herbicola* is qualitatively similar to that observed with seedlings sprayed with *P. syringae* (1, 7). However, *P. syringae* appears to be quantitatively more effective than *E. herbicola* in increasing frost injury to corn plants. Damage is maximal after 24 hr of incubation for *P. syringae* (see Fig. 1, Ref. 1) but only after 36 hr for *E. herbicola* (Fig. 2). Detectable increases in frost injury required application of about $10^5$ cell/ml of *P. syringae* (see Fig. 2, Ref. 1) and $6 \times 10^4$ of *E. herbicola* (Fig. 3).

These results show that: (i) *E. herbicola* is an active bacterial ice nucleus; (ii) application of *E. herbicola* will substantially increase the frost damage to growth-chamber-grown corn plants; (iii) the extent of frost damage increases with the number of bacteria applied; (iv) increased frost damage was not detected if the plants were frozen within the first few hours after application of *E. herbicola*, but was detectable if the plants were frozen after 12 hr of incubation, and was nearly maximal after 36 hr. The question arises: Are the time dependence and applied cell density dependence of frost injury both a reflection of the same variable—the total number of *E. herbicola* cells on the plant at the time of freezing?

**Growth of *E. herbicola* on corn leaves.**—The numbers of *E. herbicola* cells recovered from corn plants sprayed at different times with suspensions of $1.1 \times 10^7$ cells/ml and then incubated in a mist chamber until harvest are shown in Fig. 4. Only about $3 \times 10^4$ *E. herbicola* cells/g fresh weight were recovered from the corn plants 6 hr after spraying. After 12 hr of incubation, however, the *E. herbicola* populations had increased significantly, and continued to increase rapidly to nearly $5 \times 10^6$ cells/g fresh weight after 36 hr of incubation. Between 6 and 36 hr, *E. herbicola* grew exponentially on the plants with a

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**Fig. 1.** Effect of growth-medium composition on ice nucleation activity of cell suspensions of *Erwinia herbicola*. Cells ($=5 \times 10^6$ cells/ml) were suspended in distilled water after being harvested from nutrient agar (NA) or from nutrient agar fortified with 2.5% glucose or glycerol as indicated. Data plotted are the cumulative fraction of the droplets that had frozen vs. temperature.

**Fig. 2.** The effect of time of incubation between application of *Erwinia herbicola* and freezing on frost damage to corn seedlings. Plants were sprayed with a cell suspension of $1.1 \times 10^7$ cells/ml in 0.1 M phosphate buffer, pH 7.0 (about 0.5 ml/plant) and incubated in mist chamber for the times given on the abscissa.

**Fig. 3.** The effect of *Erwinia herbicola* cell densities applied to corn on the extent of frost damage. Suspensions of *E. herbicola* of cell densities given on the abscissa were sprayed on seedling corn plants ($=0.5$ ml/plant) 48 hr prior to freezing. Plants represented by the curve labeled "wet" were placed in a mist chamber for 24 hr and then in ambient air until freezing. Plants represented by the curve labeled "dry" were left in ambient air for the entire 48 hr. The vertical bars represent the standard error of the mean.
mean doubling time of about 2.9 hr. Increases in *E. herbicola* populations occurred more slowly after 36 hr, but populations $>10^7$ cells/g fresh weight had developed after 72 hr of incubation.

The increase in the amount of frost damage with incubation time after application of *E. herbicola* (Fig. 2) appears to be related to the increase of actual populations of *E. herbicola* on the plants (Fig. 4). *Erwinia herbicola* populations were static for at least 6 hr and sensitivity to frost injury did not increase during this period. Populations of *E. herbicola* on plants had increased by 12 hr after application and reached a near maximum level (about $10^7$ times greater than the initial population) within 36 hr after application. Frost sensitivity of these plants also increased significantly by 12 hr and increased sharply between 12 and 36 hr of incubation. After 36 hr, near-maximum frost sensitivity was observed. A linear relationship ($P < 0.001$) was found when the probit of injury was regressed on log *E. herbicola* population (Fig. 5) using data from each time point in the experiment illustrated in Fig. 2 and 4. Thus, the frost injury sustained by corn leaves appears to be directly related to the number of bacteria active in ice nucleation present on those leaves.

Immediately after spraying, less than 0.1% of the total number of *E. herbicola* cells that could have been deposited on the plants were reisolated (about 0.5 ml of a $1.1 \times 10^7$ cells/ml suspension per 1.5 g plant was applied and only about $3.5 \times 10^4$ cells/g were isolated). This low recovery is due, apparently, to a low efficiency of deposition of droplets of bacterial suspensions on the waxy seedling corn leaves. Because of this low efficiency, the initial leaf surface population of *E. herbicola* was small as compared with the population of $10^7$ cells/g of plant tissue attained after multiplication of the bacteria upon the plant. Therefore, both the number of cells applied, and the growth of *E. herbicola* upon the plants are major factors in determining the population of *E. herbicola* on the plants. Frost sensitivity is determined by the size of the population of *E. herbicola*.

We have shown that an isolate of *E. herbicola* active in ice nucleation at $-4$ C also is active in increasing the frost damage to corn upon exposure to $-4$ C. Populations of bacteria active in ice nucleation (chiefly *E. herbicola*) on leaves of field-grown corn typically ranged from $10^5$ to $10^7$/g fresh weight of corn tissue (8). Corn seedlings with similar leaf populations of *E. herbicola* at the time of freezing were substantially damaged at $-4$ C (Fig. 2 and 4). It appears, therefore, that if the isolate of *E. herbicola* used in this study is representative of the bacteria active in ice nucleation on leaves of field-grown corn, there should be sufficient ice nuclei on these leaves to explain the observation that corn does not supercool to temperatures colder than about $-4$ C in the field.

**LITERATURE CITED**


