Erwinia herbicola: A Bacterial Ice Nucleus Active in Increasing Frost Injury to Corn

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

Department of Plant Pathology, University of Wisconsin, Madison, WI 53706; Third author, Plant Disease Resistance Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Department of Plant Pathology, University of Wisconsin, Madison, WI 53706.

Cooperative research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and the Agricultural Research Service, U.S. Department of Agriculture.

We thank A. Kelman for assistance in the identification of the *E. herbicola* isolate, B. M. Lund for many helpful suggestions throughout this study, and S. Vicen for preparation of figures.

Mention of companies or commercial products does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

Accepted for publication 8 August 1977.

ABSTRACT

LINDOW, S. E., D. C. ARNY, and C. D. UPPER. 1978. Erwinia herbicola: a bacterial ice nucleus active in increasing frost injury to corn. Phytopathology 68: 523-527.

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 3×10^3 cells/g fresh weight of leaf at 6 hr after inoculation had increased to about 5×10^6 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.

Ice nuclei play a major role in frost damage to plants (3, 11,14). Ice nuclei active at temperatures warmer than −5 C have been found on or near the surface of plants; e.g., a peeled lemon (surface nuclei removed) supercooled to about −9 C before freezing, whereas an intact lemon froze at about −4 C (11). Ice nuclei have been presumed to be deposited on plant leaves from the atmosphere (11). However, the nature, the source, and the abundance of ice nuclei on plant leaves has not been reported until recently (1, 7, 8). Since ice nuclei are necessary for the initiation of ice formation even at temperatures much colder than −5 C, and since any ice formation is damaging in frost-sensitive plants such as corn (14), ice nuclei are important determinants of frost damage to these plants.

In an attempt to identify natural sources of atmospheric ice nuclei, Schnell and Vali (15) found a correlation between the content of ice nuclei and the content of organic matter in soils. Autoclaved soil lost all of its ice nucleation activity at temperatures warmer than −10 C (16). Decaying leaf debris also contained many ice nuclei active at temperatures as warm as −5 C (15, 17). However, the most active ice nuclei that have been identified from terrestrial sources were the bacteria, *Pseudomonas syringae* van Hall (12) and *P. fluorescens*, biotype G Migula (13). [Biologically derived ice nuclei, including some from living marine phytoplankton, also are available from oceanic sources (2, 19).] Thus, living microorganisms, particularly a few species of bacteria, apparently are abundant sources of very active ice nuclei.

We have reported recently that the presence of *P. syringae* on leaves of frost-sensitive plants prevents supercooling and thus increases frost damage (1, 7, 9). This report identifies another bacterial ice nucleus, *Erwinia herbicola* (Löhnis) Dye, and demonstrates that it, too, is active in promoting frost damage of frost-sensitive plants. Preliminary accounts of this work have appeared (7, 10).

MATERIALS AND METHODS

The isolate of *E. herbicola* used in this study, obtained in July, 1975, from a corn leaf grown near Marxville, 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.

00032-949X/78/000 087$03.00/0
Copyright © 1978 The American Phytopathological Society, 3340 Pilot Knob Road, St. Paul, MN 55121. All rights reserved.

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 (>10^8 cells/ml). Five 10-μliter 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-μliter 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, WI, during the summer of 1975. Washings of leaves sampled after mid-July were plated on Crosse's medium (4). From most samples numerous (10^5-10^6/gm 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 (>10^8 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* ($\simeq 5 \times 10^8$ 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 (1.1×10^7 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 6×10^4 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 6×10^4 to 10^7 cells/ml. At applied cell densities of 6×10^5 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 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 10^4 cell/ml of *P. syringae* (see Fig. 2, Ref. 1) and 6×10^4 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?

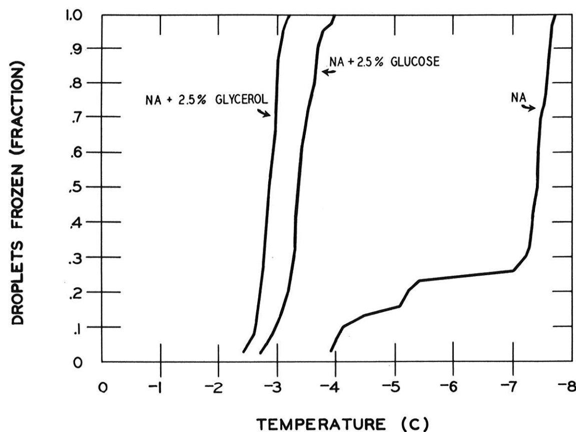

Fig. 1. Effect of growth-medium composition on ice nucleation activity of cell suspensions of *Erwinia herbicola*. Cells (≃5×10^8 cells/ml) were suspended in distilled water after being harvested from nutrient agar (NA) or from nutrient agar fortified with 2.5% glucose or glycerol as indicated. Data plotted are the cumulative fraction of the droplets that had frozen vs. temperature.

Growth of E. herbicola on corn leaves.—The numbers of *E. herbicola* cells recovered from corn plants sprayed at different times with suspensions of 1.1×10^7 cells/ml and then incubated in a mist chamber until harvest are shown in Fig. 4. Only about 3×10^3 *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 5×10^6 cells/g fresh weight after 36 hr of incubation. Between 6 and 36 hr, *E. herbicola* grew exponentially on the plants with a

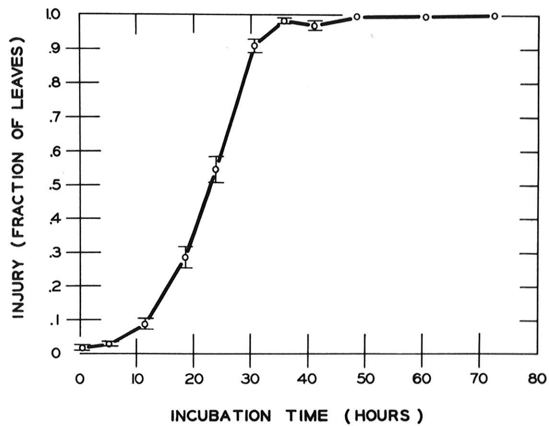

Fig. 2. The effect of time of incubation between application of *Erwinia herbicola* and freezing on frost damage to corn seedlings. Plants were sprayed with a cell suspension of 1.1×10^7 cells/ml in 0.1 M phosphate buffer, pH 7.0 (about 0.5 ml/plant) and incubated in mist chamber for the times given on the abscissa prior to exposure to −4 C. The vertical bars represent the standard error of the mean.

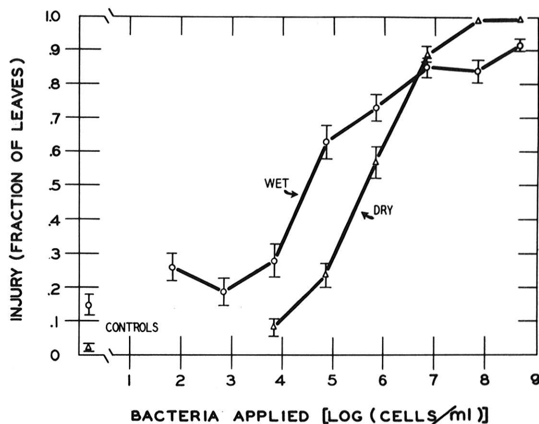

Fig. 3. The effect of *Erwinia herbicola* cell densities applied to corn on the extent of frost damage. Suspensions of *E. herbicola* of cell densities given on the abscissa were sprayed on seedling corn plants (≃0.5 ml/plant) 48 hr prior to freezing. Plants represented by the curve labeled "wet" were 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 10^3 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.

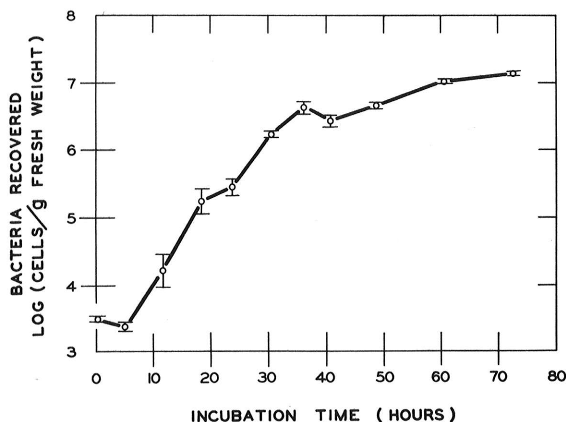

Fig. 4. Multiplication of *Erwinia herbicola* on seedling corn plants. Plants were sprayed with suspensions of about 1.1×10^7 cells/ml ($\simeq$0.5 ml/plant) and incubated in a mist chamber for the times shown before assay. Each point is the mean of the log population from four determinations.

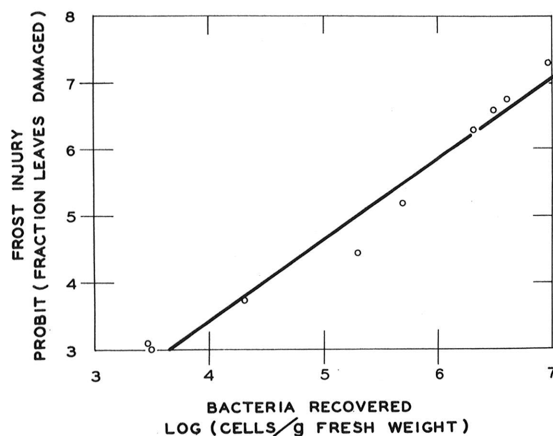

Fig. 5. Relationship between leaf populations of *Erwinia herbicola* and frost damage to seedling corn plants. The probit of the fraction of leaves injured at each time point was calculated from the data shown in Fig. 2. The logarithm of the population of *E. herbicola* found on these leaves at each time point is from Fig. 4. The calculated linear regression is: $y = 1.21\,x - 1.38$; coefficient of correlation = 0.98 ($P < 0.001$).

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 1.1×10^7 cells/ml suspension per 1.5 g plant was applied and only about 3.5×10^3 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 10^6/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

1. ARNY, D. C., S. E. LINDOW, and C. D. UPPER. 1976. Frost sensitivity of Zea mays increased by application of Pseudomonas syringae. Nature 262:282-284.
2. CARNEY, J. F., R. C. SCHNELL, and C. E. CARTY. 1975. Active ice nuclei associated with viable bacteria in Nova Scotia marine fogs. Trans. Am. Geophys. Union. 56:994 (Abstr.).
3. CHANDLER, W. H. 1954. Cold resistance in horticultural plants: a review. Proc. Am. Soc. Hortic. Sci. 64:552-572.
4. CROSSE, I. E. 1959. Bacterial canker of stone-fruits. IV. Investigation of a method for measuring the inoculum potential of cherry trees. Ann. Appl. Biol. 47:306-317.
5. DYE, D. W. 1969. A taxonomic study of the genus Erwinia. III. The "herbicola" group. N. Z. J. Sci. 12:223-236.
6. LELLIOTT, R. A. 1974. Erwinia. Pages 332-339 *in* R. E. Buchanan and N. E. Gibbons, eds. Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins Co., Baltimore, Maryland. 1,268 p.
7. LINDOW, S., D. C. ARNY, W. R. BARCHET, and C. D. UPPER. 1975. The relationship between populations of bacteria active in ice nucleation and frost sensitivity in herbaceous plants. Trans. Am. Geophys. Union. 56:994 (Abstr.).

8. LINDOW, S. E., D. C. ARNY, R. W. BARCHET, and C. D. UPPER. 1976. Bacterial ice nuclei as incitants of warm temperature frost damage. Proc. Am. Phytopathol. Soc. 3:224 (Abstr.).
9. LINDOW, S., D. C. ARNY, and C. D. UPPER. 1975. Increased frost sensitivity of maize in the presence of Pseudomonas syringae. Proc. Am. Phytopathol. Soc. 2:57 (Abstr.).
10. LINDOW, S. E., D. C. ARNY, and C. D. UPPER. 1976. Erwinia herbicola isolates active in ice nucleation incite frost damage to corn (Zea mays L.). Proc. Am. Phytopathol. Soc. 3:224 (Abstr.).
11. LUCAS, J. W. 1954. Subcooling and ice nucleation in lemons. Plant Physiol. 29:245-251.
12. MAKI, L. R., E. L. GALYON, M. CHANG-CHIEN, and D. R. CALDWELL. 1974. Ice nucleation induced by Pseudomonas syringae. Appl. Microbiol. 28:456-459.
13. MAKI, L. R., and D. M. GARVEY. 1975. Bacterially induced ice nucleation. Trans. Am. Geophys. Union. 56:994 (Abstr.).
14. OLIEN, C. R. 1967. Freezing stresses and survival. Annu. Rev. Plant Physiol. 18:387-408.
15. SCHNELL, R. C., and G. VALI. 1972. Atmospheric ice nuclei from decomposing vegetation. Nature 236:163-165.
.16. SCHNELL, R. C., and G. VALI. 1973. World-wide source of leaf-derived freezing nuclei. Nature 246:212-213.
17. SCHNELL, R. C., and G. VALI. 1976. Biogenic ice nuclei: Part I. Terrestrial and marine sources. J. Atmos. Sci. 33:1554-1564.
18. VALI, G. 1971. Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. J. Atmos. Sci. 28:402-409.
19. VALI, G., and R. C. SCHNELL. 1975. Biogenic sources of atmospheric ice nuclei: a review. Trans. Am. Geophys. Union. 56:994 (Abstr.).