A note on the presence of ice nucleation-active bacteria in roots of alfalfa grown in Québec

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Bacteria capable of ice nucleation activity were readily isolated from the xylem of alfalfa roots at four sites in Québec. Their number ranged from 3.5 to 5.3 × 10^4 cells per gramme of fresh tissue. These bacteria were identified as *Erwinia herbicola*, *Pseudomonas fluorescens*, *P. syringae* and *Pseudomonas* spp. Ice nucleation-active strains of *E. herbicola* and two species of *Pseudomonas* were also isolated from the neighbouring soil.

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On a isolé des bactéries glaçogènes à partir du xylème de racines de luzerne provenant de quatre endroits au Québec. Leur nombre a varié de 3,5 à 5,3 × 10^4 cellules par gramme de matière fraîche. Ces bactéries ont été caractérisées et identifiées aux espèces suivantes: *Erwinia herbicola*, *Pseudomonas fluorescens*, *P. syringae* et *Pseudomonas* spp. On a aussi isolé du sol environnant des souches glaçogènes de *E. herbicola* et de deux espèces de *Pseudomonas*.

The study of the internal microflora of alfalfa roots has revealed the presence of bacteria in diseased as well as in healthy plants (Richard 1981; Sandford 1948). Among isolated bacteria, many have been identified as fluorescent pseudomonads (Rousseau *et al.* 1984). Strains of some species of *Pseudomonas*, known to be ice nucleation-active (INA) bacteria, have been found on plant surfaces (Butera *et al.* 1984; Lindow *et al.* 1978) and in the air (Schnell and Vali 1972). In this work, we report on the presence of INA bacteria in the xylem of alfalfa roots in Québec.

Two-year-old plants of alfalfa (*Medicago sativa* L.) cv. Saranac and Iroquois were sampled at four field sites (Table 1) in the autumn of 1985. The cultivar Primal replaced Iroquois at one site. The roots from 10 plants of each cultivar were washed with tap water, and 5-cm segments were cut below the crown, surface-sterilized in sodium hypochlorite (6%) for 2 min followed by a 15-s rinse in ethanol (95%), and flamed. The cortex and both ends of the root segments were removed aseptically and the remaining 2-cm long portion of the xylem was immersed in 5 mL sterile 0.01 M phosphate buffer (pH 7.0) in a sterile Whirl-Pak bag and crushed during 4 min in a Stomacher Lab-Blender 400. Bacteria were isolated from xylem extracts on nutrient agar (Difco) supplemented with 2.5% glycerol (NAG) and *Pseudomonas* isolation agar (PSI) (Difco).

After 3 to 7 days of incubation at 21°C, a loopful of each bacterial colony was suspended in 1 mL of sterile distilled water in sterile test tubes, and assayed for ice nucleation activity by the tube nucleation test (Paulin and Luisetti 1978). The ice nucleation tests were carried out by placing the tubes containing the bacterial suspension at room temperature in a Lauda refrigerating circulator bath (Model RCS-6, Brinkmann Instruments Co., Rexdale, Ontario) at −5°C for Normandin site and −8.0°C for the three other sites. The time required for ice nucleation to occur in a tube was recorded within the first 10 min. If nucleation occurred after this time, the test was

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Table 1. Ice nucleation activity of bacteria isolated from the root xylem of 2-year-old alfalfa plants at four sites in Québec

Site	Cultivar	Isolation medium §	Root samples		Bacterial isolates	
			No. tested	Percentage positive †	No. tested	Percentage which were INA ‡
Saint-David-de-l'Auberivière	Saranac	NAG	13	100	13	85
	Iroquois	NAG	13	100	13	46
Normandin	Saranac	PSI	10	60	7	43 ¶
	Iroquois	PSI	10	90	10	50 ¶
Sainte-Anne-de-Bellevue	Saranac	NAG	10	80	17	6
		PSI		60	6	0
	Primal	NAG	10	90	13	23
		PSI		20	3	0
La Pocatière	Saranac	NAG	10	100	55	31
		PSI		60	11	9
	Iroquois	NAG	10	100	52	21
		PSI		90	17	6

§ NAG = Nutrient agar + 2.5% glycerol; PSI = Pseudomonas isolation agar.

† Percentage of root samples from which bacteria were isolated.

‡ Tested in tubes in a refrigerating circulator bath adjusted to −8.0°C.

¶ Tested at −5.0°C.

considered negative. Control samples of sterile distilled water were negative after 90 min.

To determine the temperature at which an ice nuclation event had occurred, the temperature of suspensions of 10 different bacterial isolates (INA-) was measured at 15-s intervals with an alumel-chromel thermocouple and a digital thermometer for 10 min. The data were pooled and a curve of temperature versus time was drawn on logarithmic graph paper. It was then possible to determine, from the curve, the nucleation temperature of any INA bacteria for which the time required for ice nucleation is known.

INA bacterial isolates were grouped by colony appearance and characterized using the procedures described for plant pathogenic bacteria (Schaad 1980), the criteria used for nonpathogenic *Pseudomonas* (Stolp and Gadkari 1981), the API System Rapid NFT kit (DMS Laboratories, Darts Mill, Flemington, NJ) for gram-negative nonfermentative bacteria and according to Bergey's Manual of Systematic Bacteriology (Lelliott and Dickey 1984).

On NAG, 80 to 100% of the xylem samples yielded bacterial isolates while fewer isolates were obtained on PSI (Table 1). INA bacteria were found in both cultivars at all four sites. At Saint-David and Normandin, percentages of INA bacterial isolates were higher than at Sainte-Anne-de-Bellevue and La Pocatière. The low frequency of ice nucleation events at Sainte-Anne-de-Bellevue and La Pocatière may have been caused by a longer delay between the isolation of bacteria and the ice nucleation test (7 days). Ice nucleation activity may considerably decrease or be lost after 24 to 48 h storage in buffer (Kozloff *et al.* 1983) or culture (Maki and Willoughby 1978). Most of the INA isolates gave a positive ice nucleation event within 45 to 180 s which corresponds to temperatures between 0 and −6.5°C.

Among INA bacteria isolated, different species were identified: *Erwinia herbicola* (Lohnis) Dye, *Pseudomonas fluorescens* Migula, and *P. syringae* van Hall. Other INA pseudomonads were tentatively identified as belonging to the species *P. facilis* (Schatz and Bovell) Davis, *P. maltophilia* (*ex* Hughand Ryschenkow) Hugh, and *P. putida* (Trevisan) Migula.

Quantitation of the INA bacterial population in the xylem was performed on a sample of 10 roots of 3-year-old plants of each

cultivar Saranac and Iroquois from Saint-David. Dilution plating was done on NAG in six replicates and bacterial colonies counted after 4 days of incubation at 18°C. The number of INA bacteria was estimated by the replica freezing method of Lindow *et al.* (1978) modified as follow: a sterilized Whatman N° 2 filter paper (instead of velvet cloth) was pressed against the surface of the dilution plate and then pressed against the inside bottom surface of an empty plastic Petri plate. A drop (10 μL) of sterile distilled water was placed on each replicated colony and the plate was floated on the surface of the refrigerating bath. After 5 min, the number of frozen drops was determined. The number of INA bacteria ranged from 3.5 to 5.3 $\times$ 10^4 cells per gramme of fresh tissue for Saranac and Iroquois respectively representing 11.3% and 7.0% of the total number of bacterial cells in the root. These results demonstrate that INA bacteria form a significant component of the bacterial flora in plant roots.

To determine if soil harbors the same INA bacteria as were found in roots, we isolated bacteria from soil taken from under alfalfa plants (cvs. Saranac and Iroquois) at Saint-David, and tested them for ice nucleation activity. All extracts of soil taken from Saranac and Iroquois sites yielded INA bacteria. Some were identified as *E. herbicola*, *P. putida* and *P. syringae*. This is not surprising as soil is known to be a source of ice nuclei (Schnell and Vali 1973, 1976) and a source of *Pseudomonas* (Curl and Truelove 1986). Moreover, INA bacteria are found airborne over fields (Lindemann *et al.* 1982) and on alfalfa leaf surfaces (Lindow *et al.* 1978).

This study shows that INA bacteria, mainly *Pseudomonas* spp. and *E. herbicola*, are present in the xylem of alfalfa roots. These INA bacteria may possibly prevent super-cooling of water in the root tissue and affect cold resistance of plants under freezing conditions, and consequently the winter survival of alfalfa, a major problem of this crop in temperate climates.

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