Xylem-residing bacteria in alfalfa roots[1]

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Bacteria were consistently isolated from the root and crown xylem of symptomless field-grown alfalfa plants. Most of the plants tested contained more than one bacterial genus. *Pseudomonas* spp. accounted for 52% of the 387 isolates identified and the fluorescent pseudomonads were the most frequent bacteria isolated. About 23% of the isolates were *Erwinia*-like bacteria. Bacterial population ranged from 6.0×10^3 to 4.3×10^4 CFU/g of fresh xylem, and was not affected by plant age or cultivar or by the sampling locations. The surface-sterilized seeds of the cultivars Iroquois and Titan were bacteria free and only 3 and 5% of the seeds of the cultivars Apica and Saranac, respectively, contained bacteria. In a greenhouse experiment, double antibotic resistant bacteria were inoculated into the soil of artificially wounded and intact roots of alfalfa plants, and on the stubble. The highest incidence of bacteria in the root xylem occurred when the roots were wounded. The highest numbers of bacteria (CFU/g fresh weight) were found when plants were wounded (stubble or roots) as compared with intact plants. The results suggest that bacteria are normal residents of the root xylem and that their main avenues of entrance to the xylem are natural root wounds and plant stubbles remaining after harvest.

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Nous avons isolé des bactéries à partir du xylème de racines et de collets de plants de luzerne d'apparance saine prélevés au champ. La plupart des plantes ont révélé la présence de plus d'un genre bactérien. Les *Pseudomonas* spp. ont compté pour 52% des 387 isolats identifiés et les bactéries isolées le plus fréquemment furent des pseudomonades fluorescentes. Environ 23% des isolats ont été identifiés comme *Erwinia* ou genres semblables. La population bactérienne a été évaluée entre $6{,}0 \times 10^3$ et $4{,}3 \times 10^4$ UFC/g de xylème frais et des valeurs semblables ont été obtenues pour des plantes d'âges ou de cultivars différents ou encore prélevées à des endroits différents. Le taux de contamination bactérienne de graines désinfectées en surface s'est avéré nul pour les cultivars Iroquois et Titan et seulement de 3 et 5% pour les cultivars Apica et Saranac respectivement. Dans une expérience en serre, des bactéries résistantes à deux antibiotiques furent inoculées soit au sol chez des plantes intactes ou blessées aux racines, soit sur les chaumes lors de la coupe. La fréquence des bactéries dans le xylème des racines fut la plus élevée chez les plantes dont les racines avaient subi des blessures. Les bactéries furent isolées en plus grands nombres (UFC/g de xylème frais) chez les plantes blessées aux racines ou inoculées par les chaumes que chez les plantes intactes. Les résultats suggèrent que les bactéries sont des résidents permanents du xylème des racines de la luzerne et qu'elles y pénètrent principalement par les blessures naturelles sur les racines et par les tiges lors de la coupe.

Introduction

The internal parts of apparently healthy plants are frequently colonized by bacteria. In fact, bacteria belonging to several genera and species were isolated from surface-sterilized ovules and seeds of different species of plants (Mundt and Hinkle 1976) and in seed pieces and aerial stems of healthy potato plants (Hollis 1951). Bacteria were also isolated from inner taproot tissue of clover (Philipson and Blair 1957) and were found to normally reside inside the xylem tissues of citrus roots (Gardner *et al.* 1982). Some reports indicated the presence of bacteria in nodules (Evans *et al.* 1972), in alfalfa seeds (Evans *et al.* 1972; Gulash *et al.* 1984; Handelsman and Brill 1985; Mundt and Hinkle 1976), and in roots of healthy alfalfa plants (Lukezic 1979; Richard 1981; Sanford 1948). However, little is known about their number, importance, and source. We report on the frequency of occurrence and number of bacteria in the xylem of alfalfa roots. The possible route of entrance into roots was also investigated with some double antibiotic resistant xylem isolates. Our results indicate that bacteria normally reside inside the xylem of alfalfa roots and that wounds are probably their main avenue of entrance.

Materials and methods

Isolation of bacteria from the xylem of alfalfa roots

Symptomless alfalfa plants (*Medicago sativa* L.) of the cultivar Saranac (4, 16, and 28 months old) were randomly collected from plots established on the Agriculture Canada experimental farm at La Pocatière, Québec. The plants were placed in a cooler immediately after digging and kept at 4°C until processed in the laboratory. Roots were washed with soapy water, rinsed thoroughly with tap water, and 1 cm long pieces of tap root were taken at the crown and 2 cm below the

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crown. The degree of rot of the pieces was assessed using an adaption of the Horsfall–Barratt scale (Horsfall and Cowling 1978). The pieces were surface sterilized with 0.2% $HgCl_2$ in 50% ethanol for 4 min, followed by 1-min rinses in three changes of sterile water. Surface sterility of the root pieces was checked by shaking the pieces for 1 min in test tubes containing 10 mL of nutrient broth (Difco Laboratories, Detroit, MI). The tubes were incubated at 21°C for 14 days, and in the rare cases where growth occurred, the tests were rejected and the bacteria isolated discarded. The bark and sections from both ends were aseptically removed and discarded. The remaining vascular cylinder was cut into five sections and each section was placed in tubes containing 10 mL of the following media (all supplemented with 40 μg of cycloheximide per mL): nutrient broth (NB) (Difco); nutrient broth – yeast extract (NBY); yeast extract – dextrose – $CaCO_3$ (YDC) (Schaad 1980); King's B (KB) (King *et al.* 1954); and medium D_1 (Kado and Heskett 1970).

Bacteria were isolated from the tubes showing growth on the same media solidified with agar. Bacterial isolates were randomly selected from single colonies and restreaked until pure cultures were obtained for further characterization. The genera of the bacteria isolated were determined according to the procedures described for plant pathogenic bacteria (Schaad 1980) including the following tests: shape of the cells and Gram staining, catalase and oxidase reactions, oxidation–fermentation of glucose (Hugh and Leifson 1953), growth on YDC medium and on media selective for *Agrobacterium* (D_1) and *Pseudomonas* (D_4) (Kado and Heskett 1970), growth and fluorescence on KB medium (King *et al.* 1954), and growth on a medium selective for *Erwinia* (Miller and Schroth 1972).

Enumeration of xylem-residing bacteria in roots

Symptomless alfalfa plants of different cultivars (5 or 17 months old) were randomly collected from plots established at three different locations in Québec. Plants were placed in a cooler immediately after digging and were processed the same day or the day following collection. The roots of 15 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, dipped for 15 s in ethanol (95%), and flamed. The cortex and a 1-cm section from both ends of the root segments were removed aseptically and the remaining 3 cm long portions from 15 segments were weighed and homogenized in 100 mL of 0.01 M phosphate buffer (pH 7.0) with a Sorvall Omni-Mixer for 3 min. The xylem suspensions were plated on NBY solid medium and colonies were counted after 6 days of incubation at 25°C.

To appraise the variation in the number of bacteria in roots, 37 plants of the cultivar Iroquois were collected from plots established at Saint-David. Root segments were sampled, surface sterilized, and decorticated, and bacteria were counted as described earlier with the following modification. The remaining 3 cm long portion of the xylem was immersed in 10 mL of 0.01 M phosphate buffer (pH 7.0) in a sterile Whirl-Pak bag and crushed for 5 min in a Stomacher Lab-Blender 400.

For every assay all the procedures were also performed with autoclaved root, and no bacterial contamination occurred.

Incidence of bacteria in alfalfa seeds

Alfalfa seeds were soaked in ethanol (95%) for 20 s and in sodium hypochlorite (6%) for 2 min, followed by several rinses in sterile distilled water. The seeds were allowed to dry for 24 h under a laminar flow at room temperature, and 200 seeds from each cultivar tested (Iroquois, Titan, Apica, and Saranac) were germinated on NBY agar plates (10 seeds/plate) supplemented with 20 μg of benomyl per mL. The plates were incubated in darkness at 25°C for 7 days and observed for bacterial growth.

Alternatively, seeds of the cultivar Saranac were surface sterilized by washing in sodium hypochlorite (1%) for 10 min and rinsing several times in sterile distilled water. Seeds were germinated as previously described (Lafrenière *et al.* 1984) in plates containing 1.5% water agar. Each 3-day-old seedling was aseptically crushed in test tubes containing 10 mL NBY broth with 40 μg of cycloheximide per mL and bacteria were isolated from tubes showing growth; their genera were identified as described earlier. Surface contamination of the seeds of the cultivar Saranac was also determined by allowing 100 seeds to germinate in plates (four seeds/plate) of NBY agar with 40 μg of cycloheximide per mL and checking for bacterial growth after 7 days incubation at 21°C. Bacteria were isolated from contaminated seeds and their genera were identified.

Penetration of bacteria into the root xylem

Five randomly selected xylem-residing bacteria were further identified according to the criteria used with nonpathogenic *Pseudomonas* (Stolp and Gadkari 1982) and according to *Bergey's Manual of Determinative Bacteriology* (Lelliott and Dickey 1984), and by using the API rapid NFT system for gram-negative nonfermentative bacteria (DMS Laboratories, Darts Mill, Flemington, NJ). The ability of the five selected isolates to rot potato tubers or lettuce leaves was also determined (Lukezic 1979). Bacteria resistant to 100 μg of nalidixic acid per mL and 2000 μg of streptomycin sulfate per mL were isolated by sequential cultivation in NBY broth containing increasing concentrations of each antibiotic.

Alfalfa plants were grown and inoculated with the effective strain of *Rhizobium meliloti* A_2 as previously described (Antoun *et al.* 1984) with the following modifications. Pots were filled with nonsterile potting medium (Redi-Earth, F. Hyde & Co. Ltd., Montréal) and thinned to four uniform seedlings 10 days after sowing. The experimental design was a randomized complete block with five replicates. After the first harvest, taken 6 weeks after sowing, plants were inoculated (*i*) by simply adding to the soil 150 mL of a bacterial suspension containing approximately 10^8 cells per mL or, (*ii*) by wounding the roots with a knife and adding the bacterial suspension to the soil, or (*iii*) by infecting the stubble with a sterile cotton swab impregnated with bacteria. Control pots were treated with autoclaved bacteria. Roots were dug 5 weeks after inoculation and bacteria in the xylem were enumerated as described earlier on NBY agar plates containing antibiotics.

Results

Incidence of bacteria in alfalfa roots

The mean degree of rot ranged from 0.21 to 3.56 in the crown of the 28-month-old alfalfa cv. Saranac, indicating that the plants sampled were relatively healthy (Table 1). Bacteria were consistently isolated from the xylem of alfalfa root and crown and the frequency of isolation of bacteria from the root xylem increased slightly with plant age. The crown xylem is readily colonized by bacteria regardless of plant age. However, the occurrence of *Erwinia*-like bacteria in the root and crown xylem was higher in older plants (Table 1). The fluorescent *Pseudomonas* spp. were isolated more frequently from the crown than from the root. In fact, the fluorescent pseudomonads were present in 36 and 63% of the root and crown xylem, respectively.

Most of the samples tested (63%) contained more than one bacterial genus. Fluorescent *Pseudomonas* spp. were the most frequent bacteria isolated from the xylem and, in general, pseudomonads accounted for 52% of the 387 isolates identified and about 23% of the isolates were *Erwinia*-like bacteria (Table 2). Gram-positive bacteria represented 6% of all isolates and 19% were other unidentified nonpathogenic gram-negative bacteria.

Bacterial counts in root xylem

A large number of bacteria was present in the root xylem of the field-grown alfalfa plants sampled. Plant age and cultivar, and the sampling location had no effect on the bacterial counts (Table 3). The number of bacteria observed ranged from 6×10^3 to $4.3 \times 10_4$ CFU/g of fresh xylem. As these figures were obtained from a pool of 15 root segments, we further estimated the number of bacteria present in a single root segment. From 37 root segments obtained from different 5-month-old alfalfa

TABLE 1. Frequency of isolation of bacteria from root and crown xylem of apparently healthy field-grown alfalfa cv. Saranac and incidence of *Pseudomonas* and *Erwinia* in plant samples

Part of the plant	Plant age (months)	Degree of rot*	No. of samples tested	No. of positive samples	% of samples with: *Pseudomonas* Fluorescent	% of samples with: *Pseudomonas* Nonfluorescent	% of samples with: *Erwinia*
Root	4	0.21	30	22	36.4	13.6	18.2
	16	1.06	40	35	34.3	31.4	27.3
	28	2.43	40	37	37.8	21.6	51.4
Crown	4	0.18	40	39	61.5	20.5	25.6
	16	2.13	24	22	54.5	31.8	45.4
	28	3.56	40	37	73.0	18.9	51.4

*According to the Horsfall–Barratt scale; 0 = absence of rot and 11 = 100% rot.

TABLE 2. Frequency of isolation of *Pseudomonas*, *Erwinia*, and other groups of bacteria from root and crown xylem of apparently healthy field-grown alfalfa cv. Saranac

Group	No. identified	% of total
Fluorescent *Pseudomonas*	138	36
Nonfluorescent pseudomonads	62	16
Erwinia-like bacteria	90	23
Other gram-negative bacteria	74	19
Gram-positive bacteria	23	6
Total	387	100

TABLE 3. Enumeration of xylem-residing bacteria in roots of apparently healthy field-grown alfalfa

Sampling site	Cultivar	Plant age (months)	CFU/g of fresh xylem ($\times 10^4$)*
La Pocatière	Saranac	5	2.8
	Saranac	17	0.6
	Iroquois	5	3.7
Saint-David	Apica	5	1.3
	Iroquois	5	1.5
	Titan	5	3.3
Normandin	Iroquois	17	4.3
	Titan	17	0.6

*Mean of five replicates of a sample of 15 roots.

plants of the cultivar Iroquois, only one segment was free of bacteria. Bacterial counts in other segments varied from 2×10^2 to 3.5×10^4 CFU/g of fresh xylem. However, 49% of the root segments tested contained at least 1×10^4 CFU/g of fresh xylem, and the average bacterial count for all segments was 1.2×10^4 CFU/g of fresh xylem.

Incidence of bacteria in seeds

No bacteria were detected in the surface-sterilized seeds of the cultivars Iroquois and Titan and only 3 and 5% of the seeds of the cultivars Apica and Saranac, respectively, contained bacteria. To ascertain that the low incidence of bacteria in alfalfa seeds is not due to technique used, we alternatively sterilized the seeds of alfalfa cv. Saranac with 1% sodium hypochlorite for 10 min and the presence of bacteria was verified on 3-day-old seedlings. Bacteria were isolated from only 8% of the seedlings confirming the low incidence of bacteria in the seeds. The surface contamination of the seeds was also determined. Only 25% of the seeds were surface contaminated with bacteria, mainly *Erwinia*-like organisms (88% of the bacteria isolated and identified).

Avenue of entrance of bacteria into the root xylem

Isolate no. 472 was identified as a denitrifying fluorescent *Pseudomonas* sp. and did not exhibit any rot on potato tubers or lettuce leaves. Severe rots on potato and lettuce were observed with isolate no. 488 identified as *P. syringae*. Mild rot on lettuce leaves was recorded with *E. herbicola* isolates no. 473 and B_4, while *E. herbicola* isolate no. 485 caused a light rot on potato tubers.

Regardless of the method of inoculation used, *E. herbicola* 485 was not recovered in the root xylem of alfalfa and might have lost its infectivity during the antibiotic resistance marking process. In general, the highest incidence of bacteria in the root xylem occurred when bacteria were introduced into the soil around artificially wounded roots (Table 4). Higher numbers of bacteria were also found in wounded plants. *Pseudomonas syringae* 488 and *E. herbicola* B_4 appeared to have greater abilities to colonize the xylem of alfalfa roots. For *P. syringae*, this can be explained in part by its high phytopathogenic potential as indicated by the severe root rot observed on potato tubers and lettuce leaves. *Pseudomonas* sp. 472 was found in higher number than other bacteria studied but the consistency of isolation was not uniform. *Erwinia herbicola* 473 colonized also the xylem of alfalfa but to a lesser extent.

Discussion

The consistent isolation of bacteria from the root and crown xylem of apparently healthy field-grown alfalfa plants is an indication that bacteria are normal residents of the xylem. The substantial number of bacteria observed in alfalfa is comparable with the number recorded from the xylem of citrus roots (Gardner *et al.* 1982). The root xylem of alfalfa is colonized by a large number of bacteria right from the year of stand establishment and the total bacterial count was not influenced by plant age or by the location where alfalfa is grown. As in many cases in the rhizosphere (Curl and Truelove 1986), xylem is mainly colonized by *Pseudomonas* spp. and the fluorescent pseudomonads were predominant. This is an indication that xylem-residing bacteria are probably rhizosphere bacteria (rhizobacteria).

This work shows also that, as previously reported (Gulash *et al.* 1984; Mundt and Hinkle 1976), very few alfalfa seeds were

TABLE 4. Recovery of Nal^r str^r bacteria from the root xylem of 11-week-old alfalfa cv. Saranac grown in a growth chamber and inoculated by three different methods

	Inoculation method					
	Soil		Soil and wounded roots		Stubble	
Bacteria	No. of positive plants	CFU* ($\times 10^3$)	No. of positive plants	CFU ($\times 10^3$)*	No. of positive plants	CFU ($\times 10^3$)*
Denitrifying						
Pseudomonas sp. 472	1	0.08	3	60–>300	1	400
P. syringae 488	3	0.2–0.6	5	7–188	2	25–55
Erwinia herbicola 473	0	—	4	0.08–20.3	1	1.3
Erwinia herbicola 485	0	—	0	—	0	—
Erwinia herbicola B4	3	0.4–7.5	5	5–20	4	1.8–2.7
Control	0	—	0	—	0	—

NOTE: After the first harvest, 6-week-old alfalfa plants were inoculated by adding to the soil 150 mL of a bacterial suspension containing 10^8 cells/mL, by adding the bacterial suspension and wounding the roots with a knife, or by infecting the stubble with bacteria. Five plants were used for each test.

*CFU per g of fresh xylem.

colonized by bacteria suggesting that the root xylem inhabiting bacteria are not seed borne. However, since very high frequencies of isolation of bacteria from alfalfa seeds were also observed (Handelsman and Brill 1985), further investigation is required to determine the effect of age, source, and storage conditions of the seeds on the incidence and survival of bacteria.

Double antibiotic resistant bacteria colonized more plants and occurred in higher numbers when plant roots were artificially wounded or when bacteria were inoculated through the stubble. Thus, it appears that the main avenues of entrance of bacteria are probably natural wounds on the roots and the cut end of the stubble after plant harvest. Alfalfa seeds also contain facultative anaerobic bacteria which are also found on the root surface and in nodules (Evans *et al.* 1972), and which are probably seed borne. In this study, no attempt was made to isolate anaerobic fastidious bacteria which are seldom found in the xylem (Gardner *et al.* 1982). The exact role played by xylem-residing bacteria in the roots of alfalfa has yet to be elucidated. These bacteria might be plant growth promoting (Burr and Ceaser 1984; Kloepper *et al.* 1980), deleterious, or pathogenic (Lukezic 1979; Suslow and Schroth 1982) rhizobacteria. *Erwinia*-like bacteria were the second most frequently isolated bacteria from alfalfa root xylem. These bacteria could play a role in the nodulation of alfalfa by *Rhizobium meliloti* (Handelsman and Brill 1985) or in the development of some alfalfa diseases (Shinde and Lukezic 1974). Finally, the presence of a substantial number of bacteria in the roots of alfalfa plants might influence the symbiotic nitrogen fixation process by competing with *R. meliloti* bacteroids for the energy derived from photosynthesis.

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