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Identification of rhizobacteria from maize and determination of their plant-growth promoting potential*

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Abstract

During the growing season of 1986, the rhizobacteria (including organisms from the ectorhizosphere, the rhizoplane and endorhizosphere) of 20 different maize hybrids sampled from different locations in the Province of Quebec were inventoried by use of seven different selective media. Isolates were characterized by morphological and biochemical tests and identified using the API20E and API20B diagnostic strips. *Pseudomonas* spp. were the prominent bacteria found in the rhizoplane and in the ectorhizosphere. *Bacillus* spp. and *Serratia* spp. were also detected, but in smaller numbers. In the endorhizosphere, *Bacillus* spp. and *Pseudomonas* spp. were detected in order of importance. Screening for plant growth-promoting rhizobacteria was carried out in three soils with different physical and chemical characteristics. The results depended on the soil used, but two isolates (*Serratia liquefaciens* and *Pseudomonas* sp.) consistently caused a promotion of plant growth.

Introduction

The soil adjacent to the surface of roots is a zone of intense microbial activity (Whipps and Lynch, 1986). Katznelson (1965) pointed out evidence that the rhizosphere phenomenon can have a profound effect on the growth and survival of plants. The rhizosphere interaction between roots and microbes is not apparent unless the microbes weaken or kill the plant through disease; in this case, the fragile balance in between the different microorganisms of the rhizosphere is moved toward the harmful bacteria. There are, however, beneficial interactions that may occur and be enhanced if actively managed (Becker, 1984).

Previously confined to inoculation of seeds of legumes by Rhizobium, management of the rhizosphere population has since advanced toward the concept of plant growth promoting rhizobacteria (PGPR). These bacteria cause a growth stimulation of plants in field soil and have been studied on commercially important crops (Burr and Caesar, 1983). The exact mechanism by which PGPR stimulate plant growth is not clearly established, although several hypotheses such as production of plant-growth substances, suppression of deleterious organisms, promotion of the availability and uptake of mineral nutrients are usually believed to be involved (Kloepper *et al.*, 1980). Although variations in the plant response to PGPR in laboratory and field assays are evident, the full potential of rhizobacteria and other microorganisms to promote plant growth remains to be investigated (Schippers, 1988). There is a need for a better understanding of the factors affecting their ecology and establishment of PGPR associated with various crops. The aim of the present study was to isolate rhizobacteria from maize (*Zea mays* L.) and appraise their plant growth promoting activity.

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Material and methods

Sampling of the roots

The samples were taken from seventeen different hybrids of maize grown for grain: Pioneer 3851, 3881, 3906, 3925, 3949, NK 9055, 9144, 9151, Pride 1122, 1144 A, 1169, PAG 123, 1120, Asgrow 398, Coop 2645, Dekalb XL8, Jacques 47 and three hydrids of maize grown for silage: Funk 4083, Pioneer 1114, Pride 1111. The plants were sampled from different locations in the province of Quebec during the growing season of 1986. Two-month-old plants were carefully removed from the soil and the roots with adherent soil were put in a plastic bag and kept at 4°C. The isolation of rhizospheric and endorhizospheric bacteria was performed the day after.

Isolation of rhizospheric bacteria

For the isolation of rhizospheric bacteria, the roots were shaken to remove excess soil and two pieces of 10-cm length were aseptically cut and shaken for 10 min on a mechanical gyratory shaker in 100 mL of sterile phosphate buffer (PB) containing, per liter: peptone, 1.0 g; K_2HPO_4, 1.21 g; KH_2PO_4, 0.34 g. Ten-fold dilutions were made and plated onto 7 selective media: Nutrient agar (Difco) + penicillin G (1.0 mg L^{-1}), Nutrient Agar + polymyxin B (5.0 g L^{-1}), Pseudomonas Agar F (Difco), selective media for Agrobacterium (D_1) and Pseudomonas (D_4) (Kado and Heskett, 1970), selective medium for Erwinia (Miller and Schroth, 1972) and the Rennie's medium for the nitrogen fixers (Rennie, 1981). All the media were supplemented with benomyl 20 ppm (Benlate, 50% W.P. Dupont, Canada, Inc.) to avoid the growth of fungi. For each medium, one sample of each different colony was isolated, purified and kept on yeast extract-dextrose-$CaCO_3$ medium (YDC) (Schaad, 1980) at 4°C until their identification.

Isolation of endorhizospheric bacteria

The surface of the roots were sterilized by soaking in ethyl alcohol 70% for 5 min and in sodium hypochlorite (6.25%) for 10 min followed by five rinses in sterile distilled water. The washed roots were spread on nutrient agar supplemented with glycerol (1%) to verify the surface sterility of the roots. They were cut length-wise, placed onto the enrichment medium of Bunt and Rovira (Bunt and Rovira, 1955) and incubated at 25°C. After 2 weeks, 0.1 mL of the growing medium was spread onto the 7 selective media and incubated at 25°C. For each medium, one sample of each different colony was isolated, purified and kept on YDC medium at 4°C until their identification.

Strain identification

The following features were examined: colonial and cellular morphology, pigmentation and gram stain. Gram-negative bacteria were further identified with API 20E diagnostic strips (Analytab Products, Division of Sherwood Medical, Plainview, NY). Seven supplemental tests for type of metabolism in OF glucose, oxidase, reduction of nitrates to nitrites or to dinitrogen, catalase, motility and growth on MacConkey's bile salt medium (Difco) are also performed to identify the bacteria with the API 20E system. Gram-positive bacteria were identified using the API 20B (API System SA, La Balme Les Grottes, 38390 Montalieu, Vercieu, France) as described by Rennie (1987). Presence of spores, and oxidation-fermentation of glucose may also be performed to identify the gram-positive bacteria with the API 20B system.

Screening of isolates as plant growth promoting rhizobacteria for maize

In the first screening, the soil used was a greenhouse mixture (Table 1). Bacteria were grown in petri plates of Nutrient agar at 25°C for 5 days. Maize seeds (Pride 1122) were inoculated by contact with the bacterial lawn and three seeds were sown in a plastic cone (Super Cell 160 cm^3, Ray Leach 'Cone-Tainer' Nursery, Oregon, USA) and transferred to a greenhouse where the day and night temperatures were 22°C and 15°C respectively and with a daylength of 16 hours. Each cone was fertilized with 50 ml of a potassium nitrate solution (NO_3^--N, 15 ppm) once a week. Seedlings were thinned to one per tube after emergence. After 8

Table 1. Description of the different soils used in the screening for plant growth promoting potential of maize bacterial isolates

Analysis[a]	Soil		
	Greenhouse mixture	Saint-Aimé (sandy loam)	Blouin (clay loam)
pH	6.2	6.1	5.6
Organic matter (%)	30	3.9	0.9
Calcium (ppm)	4220	1250	1062
Magnesium (ppm)	1630	86	197
Phosphorus (ppm)	380	35	66
Potassium (ppm)	2650	117	46

[a] Analyses were performed according to Bremner (1965).

weeks, the plants were cut, dried for 48 h at 70°C and weighed. The experimental plan was a randomized complete block design with 5 blocks of 100 bacteria. The control (plant inoculated with saline water) and treatments were replicated 4× within each block. The variance of plant dry weight means was analyzed according to a normal distribution.

Bacteria with plant growth promoting potential in the first screening were then tested in 2 different soils: a sandy loam, St-Aimé Serie, and a clay loam, Blouin serie (Table 1). The bacteria were grown in Nutrient broth with glycerol for 48 h at 25°C, washed three times and concentrated in saline water (NaCl 0.85%) to obtain more than 10^9 cells/mL. Four seeds of maize were sown in a 5-inch pots containing 550 g of soil, and were inoculated with 30 mL of washed cells. The plants were transferred in the greenhouse and fertilized with a complete Hoagland solution (Hoagland and Arnon, 1938) containing 15 ppm of NO_3^--N once a week. After 8 weeks, the plants were cut, dried for 48 h at 70°C and weighed. The experimental plan was a randomized complete block design with 20 blocks of 20 bacteria. The control and treatments were replicated 8× within each block. The variance of dry weight was analyzed according to the experimental plan. Plant dry weight means were compared to the control plant means with Dunnett's procedures (Steel and Torrie, 1980).

Results

Strain identification

A total of 477 bacteria were isolated from the rhizosphere and the endorhizosphere of 20 different maize hybrids representing respectively 73% and 27% of the isolates. In Table 2, the differentiation in specific genera with the frequency of their isolation indicated that in the rhizospheric population, the Pseudomonas genus was the most prominent group with 63% followed by the Bacillus and Serratia genera with 27% and 5% respectively. The remainder of the identified bacteria (5%) belonged to different genera of microorganisms such as Enterobacter, Acinetobacter, Klebsiella, Agrobacterium, Corynebacterium and finally Pasteurella. Of the Pseudomonas group, 27% were *Ps. fluorescens* while 2% of the *Serratia* were the species *Serratia liquefaciens*.

Table 2. Distribution of the bacterial population from the rhizosphere and endorhizosphere of 20 different hybrids of maize sampled in the province of Quebec in 1986

Bacteria	Percentage of the bacterial population from	
	Rhizosphere (%)	Endorhizosphere (%)
Pseudomonas	63	11
Bacillus	27	88
Serratia	5	ND[a]
Enterobacter	2.5	ND
Acinetobacter	0.8	ND
Klebsiella	0.7	ND
Agrobacterium	0.6	ND
Corynebacterium	0.3	1
Pasteurella	0.1	ND

[a] ND = Not detected.

With the endorhizospheric population, the major genus was the Bacillus with 88% followed by the Pseudomonas genus with 11%. The remaining belonged to the Corynebacterium genus (1%).

Screening of isolates as plant-growth promoting rhizobacteria for maize

In the first experiment, all 477 strains were tested and only the 24 strains giving the highest yield were retained for further tests. Of these strains, 17 were classified as *Pseudomonas* sp., 4 as *Bacillus* sp. and 3 as *Serratia* sp. The percentage increase obtained in the yield of maize with these strains when compared to uninoculated control was between 9% and 14%.

Results of the second assay (Table 3) indicated that there was variation in the behaviour of the

Table 3. Significant yield and percentage increases in maize inoculated with some rhizobacteria

Bacteria	Isolate #	Saint-Aimé		Blouin	
		Dry weight (g)	Increase in dry weight (%)	Dry weight (g)	Increase in dry weight (%)
S. liquefaciens	566	7.071 (0.644)[a]	10	4.316 (0.452)	12
Pseudomonas sp.	264	7.005 (0.578)	9	3.995 (0.131)	3
Pseudomonas sp.	271	6.935 (0.508)	8	4.262 (0.398)	10
Bacillus sp.	910	6.867 (0.440)	7	3.979 (0.115)	3
Control		6.427		3.864	

[a] Values in parentheses are the difference between the mean of treatments and the means of controls. Values higher than the Dunnett critical value are significant at $P > 0.025$.
Dunnett critical value for Saint-Aimé: 0.439.
Dunnett critical value for Blouin: 0.294.

strains tested. Within the Saint-Aimé soil, the Dunnett critical value was of 0.439 and 4 strains were exceeded, while in the Blouin soil, with a critical value of 0.294, only 2 strains resulted in a significant effect. These results indicate that there was less variability in the Saint-Aimé soil than in the Blouin soil. As seen in Table 3, one isolate (# 566), identified as *S. liquefaciens*, came out as the strain with the greatest effect in both types of soil with 10% and 12% increase in the mean dry weight. Another isolate (# 271), identified as *Pseudomonas* sp., ranked second in the Blouin soil and third in the Saint-Aimé with 10% and 8% respectively. The isolates (# 264 and # 910), identified as *Pseudomonas* sp. and *Bacillus* sp., respectively, ranked second and fourth in the Saint-Aimé soil with 9% and 7% increase in the mean of maize dry weight. These two isolates were not classified in the Blouin soil since the value of their dry weight was less than the critical value of Dunnett.

Discussion

The results of this study demonstrate that several bacterial genera were located in the rhizospheric zone of maize roots. As expected, *Pseudomonas* sp. were prominent in the rhizosphere, rhizoplane and ectorhizosphere (Curl and Truelove, 1986). *Bacillus* sp. and *Serratia* sp. were also present in substantial number. The endorhizosphere of maize was mainly colonized by the *Bacillus* followed by the *Pseudomonas* species. Studies published with PGPR involved different genera of bacteria. However, most of the PGPR belong to the Pseudomonad because of their siderophore complexes (Baker *et al.*, 1986) and production of antibiotic compounds (Howell and Stipanovic, 1980).

Serratia liquefaciens, Bacillus sp. and *Pseudomonas paucimobilis* are regularly present in the rhizosphere of maize (Lambert *et al.*, 1987). Our work also shows that the endorhizosphere of maize was mainly colonized by *Bacillus*-followed by *Pseudomonas* species. Since some members of the Bacillus genus are known to be nitrogen fixers and that there is a sink for oxygen inside the root, this could explain their predominence inside the roots. However, our experimental procedure does not necessarily allow the isolation and identification of some important bacteria which might be present like Azospirillum. *Bacillus* spp. are able to control some diseases of corn (Kommedahl and Mew, 1975) and have also been frequently used as seed inoculants. The importance of the bacillus population in the root of maize is noteworthy and identification of the population, its mode of entry and the colonization of the root are presently under investigation.

The 24 PGPR selected in the first screening gave different responses to the bacterization of maize seeds during the second trial. Certain strains that were classified good in the first screening behaved differently in this second one. However, one isolate (# 566), identified as *Serratia liquefaciens*, showed the highest stimulation in the two different soils used. Another isolate identified as *Pseudomonas* sp. (# 271) came out third and second in the Saint-Aimé and Blouin soils respectively. Since the soils used are physically and chemically different, this

reflects the capacity of these two strains to be competitive in different environments.

The results presented in this study indicate the presence of a diverse population of microorganisms in the rhizosphere of maize roots. It also indicated the possibilities of manipulating the root microflora in favour of improved plant growth. Subsequent field studies on seed inoculation, colonization of the roots and persistence in the soil are required.

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References

Baker R, Elad Y and Snel B 1986 Physical, biological and host factors in iron competition in soils. *In* Iron Siderophores, and Plant Diseases. Ed. T R Swinburne. pp 77–84. Plenum Publishing Corporation, New-York.

Becker J O 1984 Isolation and characterization of antimycotic bacteria for rhizosphere. Pests and Diseases 1, 365–370.

Bremner J M 1965 Organic forms of nitrogen. *In* Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties. Eds. C A Black, D D Evans, J L White, L E Ersminger and F E Clark. pp 1236–1255. American Society of Agronomy, Madison, Wis.

Bunt J S and Rovira A D 1955 Microbiological studies of some sub-antarctic soils. J. Soil Sci. 6, 119–128.

Burr T J and Caesar A 1983 Beneficial plant bacteria. Crit. Rev. Plant Sci. 2, 1–20.

Curl E A and Truelove B 1986 The Rhizosphere. Springer-Verlag, Berlin, 288 pp.

Hoagland D R and Arnon D L 1938 The water-culture method for growing plants without soil. Univ. of California, Coll. Agr. Exp. Sta., Berkeley, California, Circ. 341, 1–39.

Howell C R and Stipanovic R D 1980 Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings of *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology 70, 712–715.

Kado C I and Heskett M G 1970 Selective media for isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas and Xanthomonas. Phytopathology 60, 969–976.

Katznelson H 1965 Nature and importance of the rhizosphere. *In* Biological Control in Crop Production. Ed. G C Papavizas. pp 187–209. Allanheld Asmun, London.

Kloepper J W, Schroth M N and Miller T D 1980 Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato development and yield. Phytopathology 70, 1078–1082.

Kommedahl T and Mew C M 1975 Biocontrol of corn root infection in the field by seed treatment with antagonists. Phytopathology 65, 296–300.

Lambert B, Leyns F, Van Rooyen L, Gosselé F, Papon Y and Swings J 1987 Rhizobacteria of maize and their antifungal activities. Appl. Environ. Microbiol. 53, 1866–1871.

Miller T D and Schroth M N 1972 Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62, 1175–1182.

Rennie R J 1981 A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. 27, 8–14.

Rennie R J 1987 The API 20B microtube system to aid in the identification of N_2-fixing *Bacillaceae*. Can. J. Microbiol. 33, 504–509.

Schaad N W 1980 Initial identification of common genera. *In* Laboratory Guide for Identification of Plant Pathogenic Bacteria. Ed. N W Schaad. pp 1–11. American Phytopathological Society, St. Paul.

Schippers B 1988 Biological control of pathogens with rhizobacteria. Phil. Trans. R. Soc. Lond. B 318, 283–293.

Steel R G and Torrie J H 1980 Principles and procedures of statistics. *In* A Biometrical Approach. pp 188–189. McGraw-Hill, New-York.

Whipps J M and Lynch J M 1986 The influence of the rhizosphere on crop productivity. Adv. Microbiol. Ecol. 9, 187–244.