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Root Colonization of Maize and Lettuce by Bioluminescent *Rhizobium leguminosarum* biovar phaseoli

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Two strains of *Rhizobium leguminosarum* bv. phaseoli and three other plant growth-promoting rhizobacteria (PGPR) were examined for the potential of maize and lettuce root colonization. All of these strains were selected in vitro for their phosphate-solubilizing abilities. Maize and lettuce seeds were treated with derivatives of all strains marked with *lux* genes for bioluminescence and resistance to kanamycin and rifampin prior to planting in nonsterile Promix and natural soil. The introduced bacterial strains were quantified on roots by dilution plating on antibiotic media together with observation of bioluminescence. Rhizobia were superior colonizers compared with other tested bacteria; rhizobial root populations averaged log 4.1 CFU/g (fresh weight) on maize roots 4 weeks after seeding and log 3.7 CFU/g (fresh weight) on lettuce roots 5 weeks after seeding. The average populations of the recovered PGPR strains were log 3.5 and log 3.0 CFU/g (fresh weight) on maize and lettuce roots, respectively. One of the three PGPR was not recovered later than the first week after seeding in Promix. Bioluminescence also permitted visualization of in situ root colonization in rhizoboxes and demonstrated the efficiency of rhizobial strains to colonize and survive on maize and lettuce roots.

The N_2-fixing rhizobia (*Bradyrhizobium* and *Rhizobium* spp.) are generally considered beneficial symbionts specific to legumes. However, many reports suggest that rhizobia have the ability to colonize roots of nonlegumes as efficiently as they colonize their legume hosts (2, 3, 14, 19, 26, 29, 31, 36). In a cereal-legume crop rotation system, the legume-*Rhizobium* symbiosis was improved by inoculating the preceding cereal crop with *Rhizobium* spp. (14), suggesting that the growth of rhizobia could be stimulated in the maize rhizosphere. Another report described the rhizobial attachment and the root hair curling on nonlegumes (31). Nodule-like structures in nonlegume roots were also observed under particular conditions without significant N_2 fixation (2, 3). Shimshick and Hebert (29) reported that attachment of rhizobia to the roots of wheat and rice seedlings can be analyzed in terms of a dynamic equilibrium model such as the Langmuir adsorption isotherm, in which the maximum number of binding sites was 8×10^9/g of fresh root (gfr) at 22°C. More recently, the rhizosphere colonization and growth promotion of nonlegumes by *Rhizobium leguminosarum* bv. trifolii were reported (19, 36). Wiehe and Höflich (34) observed that root colonization of maize by rhizobia ranged from more than log 5 to less than log 3 CFU/gfr according to the year of experiments and time during season.

There is considerable evidence that soil microorganisms can directly increase phosphorus (P) supply to plants through the processes of solubilization and/or mineralization of soil inorganic and organic P (28). Rhizobia are soil microorganisms which possess this potential to solubilize soil P (18, 30) and can also promote the growth of nonlegumes. In fact, a strain of *R. leguminosarum* bv. trifolii stimulated the growth of nonlegumes such as Gramineae and crucifers under field conditions (19).

The survival and root colonization of introduced strains in soil and rhizosphere need to be monitored to understand the mechanisms of action and the potential of these strains to compete with other soil microorganisms. However, to distinguish the introduced strains from the natural flora, a marking system is necessary. In the present work, the bioluminescent marker *luxAB* combined with resistance to kanamycin and rifampin was used to differentiate inoculated strains from indigenous bacteria (5, 8, 21). Previous studies examined root colonization of nonlegumes by rhizobia as a group without regard to specific function other than N_2 fixation (2, 3, 29, 31). The current study was designed to determine if the introduced rhizobial strains P31 and R1 can colonize and survive in the nonsterilized rhizosphere of the nonlegumes maize and lettuce as well as other P-solubilizing microorganisms considered to be plant growth-promoting rhizobacteria (PGPR).

MATERIALS AND METHODS

Bacterial strains. *Enterobacter* sp. strain 22a, *Serratia* sp. strain 22b, and *Pseudomonas* sp. strain 24 were previously selected for their ability to solubilize inorganic P in culture media. All of these bacteria promoted the growth of maize or lettuce in the field (10). Strains P31 (22) and R1 (from our lab) of *R. leguminosarum* bv. phaseoli were isolated from Quebec soils and also selected for their ability to solubilize P. Their rhizobial identity was confirmed by inoculation of seeds of *Phaseolus vulgaris* (cultivars Strike, Sungold, Kentucky Wonder, and Contender) and reisolation from nodules (32). Isolates were compared with their parental strains by using Biolog (Hayward, Calif.) GN Microplates. Spontaneous mutants resistant to 100 μg of rifampin per ml were obtained for all strains by gradually increasing the concentration of rifampin in the tryptic soy agar (TSA; Difco) medium used to cultivate the bacteria. All strains were stored at −80°C in tryptic soy broth (TSB; Difco) plus 20% glycerol.

Marking bacteria with *luxAB* genes. *Escherichia coli* WA803(pDLB30), resistant to chloramphenicol and carrying the Tn*5*-*luxAB* genes of *Vibrio harveyi* with kanamycin resistance (8), was used as the plasmid donor by conjugation with the strains mentioned above. The plasmid donor was grown on TSA supplemented with 25 μg of chloramphenicol per ml and 25 μg of kanamycin per ml, and the recipient strains were grown on TSA medium supplemented with 100 μg of rifampin (TSA-R). After overnight growth at 30°C, the cells were harvested and suspended in sterile 0.85% NaCl solution (saline) with a sterile loop. The optical density at 590 nm was adjusted to 1.1, and 100-μl volumes of both donor and recipient cell suspensions were filtered through a 0.22-μm-pore-size sterile nitrocellulose filter (25-mm diameter) with 2 ml of additional sterile saline. Filters harboring bacteria were placed on TSA for overnight incubation. The cells from

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the filters were resuspended in 10 ml of sterile saline, and the resulting suspension was serially diluted and plated onto TSA supplemented with 100 μg of rifampin and kanamycin per ml (TSA-RK), which did not permit growth of recipient strains or the *E. coli* donor but permitted growth of recombinant bacteria. The dilutions were also plated on TSA-R, which permitted growth of all recipient cells but not of the *E. coli* donor, to calculate the transconjugant frequency. The transconjugants were selected from TSA-RK by their luminescence in the dark, and the retention of P solubilization abilities was checked on dicalcium phosphate medium (17). Selected mutants were also compared with their respective wild types on Biolog GN Microplates and by comparison of the growth curves in 10% TSB medium.

Inoculant preparation. All inoculants were prepared from a 24-h 10% TSA culture. The cells were suspended in sterile saline, and the optical density was adjusted to obtain a minimum of log 8 CFU/ml. A sterile saline solution without bacteria was used for all uninoculated controls. The lettuce seeds were inoculated by adding 5 ml of cell suspension in a 5-cm-diameter petri plate containing 200 seeds and air dried overnight. The lettuce seeds (Paris Island COS; Asgrow Seed Co., Ontario, Canada) were bought with a commercial clay coating which allowed bacteria to adhere and survive on the seeds. Prior to use, maize seeds (hybrid Funk's 4066) were surface disinfected for 2 min in 70% ethanol and for 5 min in 6% sodium hypochlorite. The seeds were inoculated by soaking for 1 h in a sterile plastic bag with a mixture of 10 ml of cell suspension and 30 ml of 1% carboxymethylcellulose solution (Sigma catalog no. C-5013). The nonadhering liquid was decanted, and the seeds were air dried overnight in 15-cm-diameter petri plates. After seeding, the numbers of CFU per seed were calculated by plating on 10% TSA a sample of 10 seeds per treatment, suspended in 10 ml of sterile saline.

Lux$^+$ rhizosphere population. The inoculated seeds of lettuce and maize were grown in a greenhouse with the natural photoperiod of Alabama in February to March and an average temperature of 25°C. The 10-cm-diameter pots contained the nonsterile peat substrate Promix (Tourbière Premier, Rivière du Loup, Québec, Canada). Plants were watered daily with tap water as required to maintain soil at field capacity. The experimental design was a split plot with six bacterial treatments (strains 22a, 22b, 24, P31, R1, and an uninoculated control) as the main plot factor, four sample times (weeks 1, 2, 3, and 4 for maize; weeks 2, 3, 4, and 5 for lettuce) as the subplot factor, and five replicates. At each sampling time, the root systems were separated from the Promix by vigorous shaking to dislodge loosely adhering soil and placed in sterile plastic bags for determination of the rhizosphere populations. The plastic bags containing the root systems were filled with 10 ml of sterile saline and shaken for 30 s in a Lab-Blender 80 stomacher (Tekmar Co.). Dilution plating was performed with a Spiral System Instruments plater (model D) on TSA plus 100 μg of rifampin, kanamycin, and cycloheximide per ml (TSA-RKC) for Lux$^+$ bacterial populations and on 10% TSA for total bacterial populations. The cycloheximide was used to inhibit fungal growth. The plates were incubated at room temperature for 36 h. Bacterial enumeration was performed with a Laser colony counter (model 500 A) and CASBA II software (Spiral System instruments). Bioluminescence was used to confirm that isolated colonies were the inoculated strains. Bioluminescence was observed by adding one drop of *N*-decyl aldehyde (Sigma catalog no. D-7384) to the covers of inverted petri plates in the dark. The bacterial counts were expressed as CFU per gfr by the number of weeks after planting (WAP). The detection level of the recovery method was adjusted by the use of the lower dilution after 2 WAP because the number of bacteria is reduced with time. The time also naturally reduced the detection level because the biomass of roots was increased. It was about log 4 CFU/gfr for the first 2 WAP and about log 2.5 CFU/gfr for the last 2 WAP.

This experiment was repeated with natural soil collected at Ile d'Orléans (Québec, Canada). The soil used was a silty clay loam with a pH of 6.29 (1:1 in water) and 5.75% organic matter content. The P and K available in the soil as determined by the Mehlich III procedure (24) were 629 and 595 kg/ha, respectively. Each 12.5-cm-diameter pot received approximately 1 kg of air-dried soil sieved to a 5-mm pore size. In this experiment, the detection level was reduced to the minimum by plating directly from the stomacher bags containing roots to increase the chances of recovering the lower colonizing strains.

Presence of Lux$^+$ rhizobia on roots. Lettuce and maize seeds inoculated with strains P31 and R1 were grown in a greenhouse as described above in Promix to observe the presence of rhizobial strains on the surface and inside the roots. A randomized block design with three treatments (strains P31, R1, and an uninoculated control) and eight replicates was used. The root systems were harvested at 5 and 16 days after seeding for maize and at 10 and 21 days after seeding for lettuce. The root systems were shaken, washed in water, and air dried for 5 to 10 min. The segments and complete root systems were placed on TSA-RKC plates and incubated for 24 h at room temperature. The spreading of bacteria on the plates was avoided by the root drying, which removed the surplus of water after washing. The plates were imaged in a darkroom with a charge-coupled device camera (Photometrics Ltd., Tucson, Ariz.). The exposure times of 1 to 5 min were used with the camera cooled to −115°C (±5°C) with liquid nitrogen. To study internal (endophytic) root colonization, after the last harvest, a root sample from each treatment was surface disinfected for 2 min for lettuce and 4 min for maize in a solution of 1% sodium hypochlorite and 0.05% of the surfactant Triton X-100 (Sigma catalog no. T-6878) and washed three times in sterile saline to eliminate surface microorganisms (25). Surface-disinfected samples were ground in a sterile mortar with 1 ml of sterile saline and plated on TSA-RKC for recovery of inoculated strains and on 10% TSA for recovery of other endophytic bacteria. Root surface disinfection was checked by placing root subsamples on TSA-RKC and 10% TSA. Any sample indicating growth was discarded.

In situ bioluminescence observation. The lettuce and maize seeds inoculated with strains P31 and R1 were grown in Plexiglas rhizoboxes (6) filled with nonsterile fritted clay. Each rhizobox, containing two seeds, was placed in a plastic bag to avoid cross-contamination and water evaporation. The plants were grown in a growth chamber with a 12-h photoperiod and day-night temperatures of 25°C. A randomized block design with three treatments (strains P31, R1, and an uninoculated control) and three replications was used. The rhizoboxes were observed directly with the charge-coupled device camera in the darkroom 11 days after seeding for maize and 25 days after seeding for lettuce. The covers of the rhizoboxes were removed to add *N*-decyl aldehyde drops to the soil at about 1 cm from the root system. The rhizoboxes were then returned to the plastic bags to maintain decyl vapor and avoid desiccation. Exposure times of 10 to 20 min were used with the camera cooled to −115°C (±5°C). The maize and lettuce roots were harvested after the in situ observation, and samples were processed as described above for Lux$^+$ rhizosphere population.

Data analysis. All data were tested for homogeneity of variances by Hartley's test (20). The $\log_{10}$ transformation was used to calculate bacterial population on root systems (23), and populations were expressed as log (CFU + 1) to avoid zero values (5, 20). The analyses of variance were done with the General Linear Models procedure of PC-SAS (SAS Institute, Cary, N.C.). Comparisons between the means were done by the least significant difference test, and the regression curves of root colonization by each strain according to time were done with the REG procedure of SAS.

RESULTS

Lux$^+$ recombinant bacteria. All recipient strains conjugated with *E. coli* WA803(pDLB30) resulted in production of Lux$^+$ bioluminescent mutants which emitted visual light in the dark after the addition of *N*-decyl aldehyde. The frequency of transconjugants ranged from $1/10^8$ recipient cells (strain P31) to 1/50 recipient cells (strain R1). The introduced *luxAB* genes were stable, on the basis of several repeated transfers on dicalcium phosphate-RK and TSA-RK plates, and P solubilization by the mutants was similar to that by the wild types on dicalcium phosphate plates. All selected mutants had reactions similar to those of their respective wild types on Biolog GN Microplates and had similar growth curves on 10% TSB medium.

Lux$^+$ rhizosphere population. No light-emitting bacteria were detected on plates prior to the addition of *N*-decyl aldehyde, and no spontaneous mutants resistant to antibiotics were found in the uninoculated control in TSA-RK plates. All bacterial strains were recovered on maize roots after 1 week, but after 2 weeks, strain 22b was below the detection level of about log 4 CFU/gfr of maize and lettuce. The importance of the bioluminescent population in the roots of maize and lettuce was significantly ($P \leq 0.05$) affected by the bacterial strains used and by the time of root sampling (Table 1). In general, *R. leguminosarum* P31 and R1 were the best root-colonizing strains, but strain R1 was always the best root colonizer at each sampling date (Table 1; $P \leq 0.05$). The introduced bacterial population decreased significantly ($P \leq 0.05$) by about 1 log unit per week (Table 1), except at 5 WAP on lettuce. The Lux$^+$ bacterial population of maize ranged from log 6.5 to 7.1 CFU/gfr at 1 WAP and decreased linearly ($R^2 = 0.81$) to log 3.4 to 4.4 CFU/gfr at 4 WAP (Fig. 1). The same colonization pattern was observed on lettuce from the second to the fourth week, where the Lux$^+$ population varied from log 5.7 to 6.3 CFU/gfr at 2 WAP and decreased linearly ($R^2 = 0.84$) to log 2.7 to 3.6 CFU/gfr at 4 WAP. However, at 5 WAP, there was an increasing trend in the population of introduced bacteria in the lettuce rhizosphere. The total bacterial rhizosphere populations (indigenous and introduced) ranged from log 7.1 to 7.6 CFU/gfr and from log 7.2 to 7.7 CFU/gfr during the whole experiment for maize and lettuce, respectively.

Similar results were obtained for the natural soil (Fig. 2);

TABLE 1. Colonization of roots of maize and lettuce cultivated in pots containing Promix by the bioluminescent mutants of *Enterobacter* sp. strain 22a, *Pseudomonas* sp. strain 24, and *R. leguminosarum* bv. phaseoli strains P31 and R1[a]

Variable		Mean log CFU/gfr[b]	
Strain	Sampling time (WAP)	Maize	Lettuce
R1		5.73 a	4.75 a
P31		5.40 b	4.43 ab
22a		5.05 c	4.12 bc
24		5.04 c	3.99 c
	1	6.89 a	
	2	5.69 b	6.03 a
	3	4.79 c	4.86 b
	4	3.84 d	3.07 c
	5		3.33 c

[a] *Serratia* sp. strain 22b was not included in this table because it was not recovered later than the first week after seeding (WAP). The strain means include five replications and four sampling times, and the time means include five replications of each strain. The strain and time effects, but not their interaction, were significant ($P \leq 0.05$).

[b] Means followed by the same letter are not significantly different according to the least significant difference test ($P \leq 0.05$). The least significant difference values were 0.2772 and 0.3448 for maize and lettuce, respectively.

rhizobial strain R1 was the best colonizing strain, and *Serratia* sp. strain 22b was the poorest colonizing strain. The reduction of the detection level permitted the recovery of strain 22b until the penultimate week. However, in natural soil, maize root colonization by *Pseudomonas* sp. strain 24 decreased faster than it did in Promix.

Presence of Lux$^+$ rhizobia on roots. Placing the roots on TSA-RK allowed growth of Lux$^+$ rhizobia and permitted the visualization of bioluminescence with the charge-coupled device camera. No luminescent bacteria were found on uninoculated control roots. Strains P31 and R1 were present on the whole 10-day-old root system of lettuce (Fig. 3A to D). At 3 WAP, strain R1 was present on the whole root system, while strain P31 was mostly concentrated close to the seed part of lettuce roots. Strains P31 and R1 were also present on the whole root system of 5-day-old maize as shown for strain R1 (Fig. 3E and F). Sixteen days after seeding, strain R1 was present on the main and adventitious roots (Fig. 3G and H), while strain P31 was present mostly near the seed. Some gaps

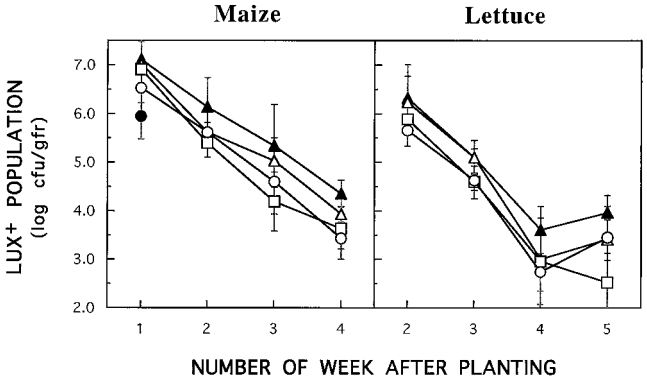

FIG. 1. Colonization of roots of maize and lettuce cultivated in pots containing Promix by the bioluminescent mutants of *Enterobacter* sp. strain 22a (○), *Serratia* sp. strain 22b (●), *Pseudomonas* sp. strain 24 (□), and *R. leguminosarum* bv. phaseoli strains P31 (△) and R1 (▲). *Serratia* sp. strain 22b was not recovered 2 WAP at the detection level of approximately log 4 CFU/gfr.

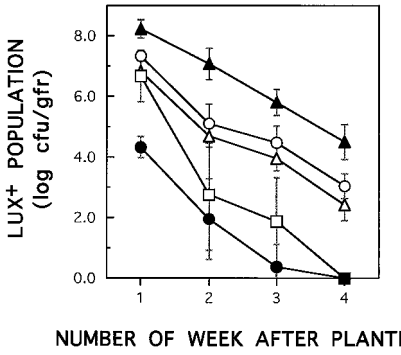

FIG. 2. Colonization of roots of maize cultivated in pots containing silty clay loam from Ile d'Orléans (Québec, Canada) by the bioluminescent mutants of *Enterobacter* sp. strain 22a (○), *Serratia* sp. strain 22b (●), *Pseudomonas* sp. strain 24 (□), and *R. leguminosarum* bv. phaseoli strains P31 (△) and R1 (▲).

in the distribution of bioluminescence visible in Fig. 3H are caused by the abundance of the root system which did not permit full contact between the roots and the agar. The internal root tissues were free of Lux$^+$ rhizobia as endophytes, and no bacteria were detected in the surface-disinfected control roots. However, other endophytes were present.

In situ bioluminescence observation. Growing the plants in rhizoboxes containing nonsterile soil allowed observation of bioluminescence from the Lux$^+$ rhizobia colonizing the lettuce and maize rhizospheres. No luminescence was detected on uninoculated roots. Root colonization by Lux$^+$ rhizobia was abundant on visible roots of 11-day-old maize seedlings (Fig. 4A to D). Strains P31 and R1 survived at levels of log 6.0 and log 5.5 CFU/gfr, respectively, on 11-day-old maize seedlings. For the 25-day-old lettuce seedlings, only a few and very small roots were present at the visible surface of the rhizoboxes. However, bioluminescence was observed on roots throughout the soil, indicating abundant colonization of roots (Fig. 4E and F). Both strains P31 and R1 survived at a level of log 7 CFU/gfr of lettuce.

DISCUSSION

The present results clearly demonstrate that *R. leguminosarum* bv. phaseoli strains P31 and R1 can colonize roots and survive in the rhizospheres of maize and lettuce in a nonsterile soil. The colonization pattern was only on the external parts of these roots, and no nodule-like structures were observed, indicating the inability of the tested rhizobia to penetrate these nonlegumes under natural conditions. Previous studies used enzymes and polyethylene glycol to induce nodule-like structures on rice and oilseed rape seedlings inoculated with rhizobia. This enzyme treatment removed cell walls at the tips of root hairs, providing a port of entry for rhizobia (2, 3). Similar enzymes and polyethylene glycol treatments with legumes can also remove the barrier to *Rhizobium* host specificity (1). Studies involving rhizobial colonization, root hair curling response, and nodule-like structure formation on various nonlegume roots did not examine growth promotion potential (2, 3, 29, 31), and the N_2-fixing activity was nonsignificant when measured by acetylene reduction (2, 3, 29). However, root colonization and growth promotion of rice (36) and other nonlegumes such as wheat, maize, rape, and sugar beet (19, 34) by *R. leguminosarum* bv. trifolii were reported under field and laboratory conditions. Obviously, the growth promotion of nonlegumes was related to mechanisms independent of biological N_2 fixation, and neither root nodules nor N_2 fixation was observed.

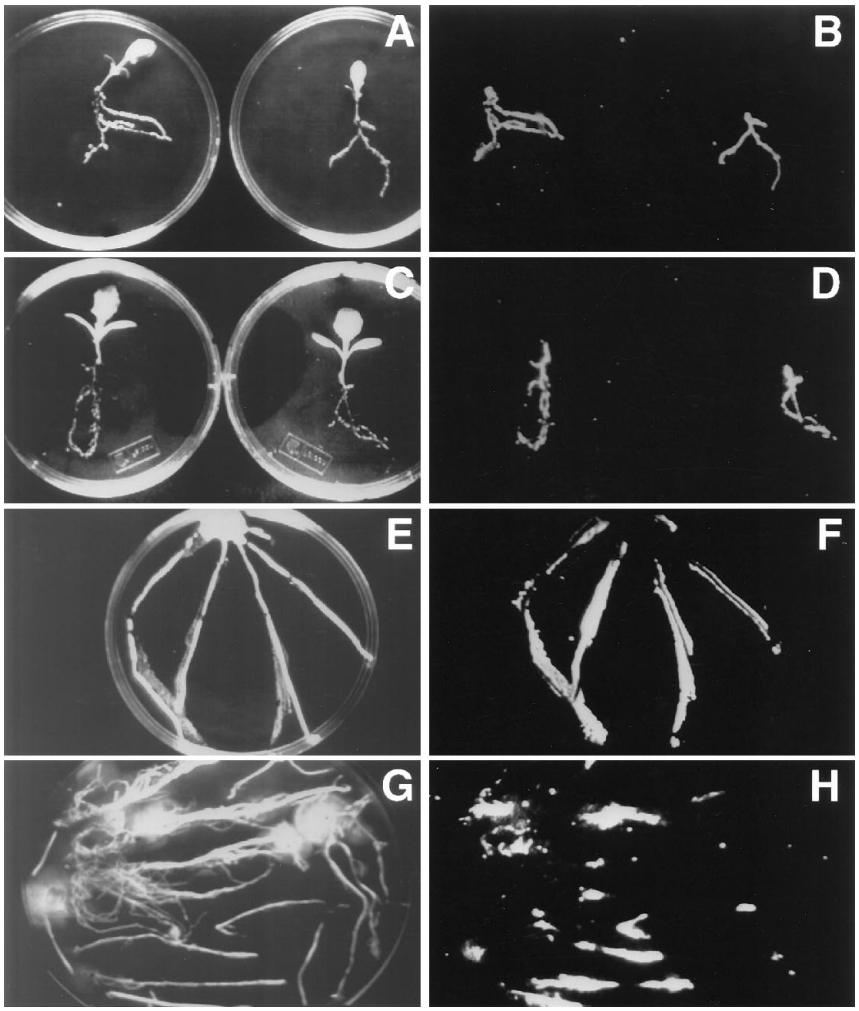

FIG. 3. Root colonization of lettuce and maize cultivated in pots containing Promix by *R. leguminosarum* bv. phaseoli strains P31 and R1 after 24 h of incubation of roots in TSA-RKC plates. (A and B) Strain P31 from 10-day-old lettuce roots; (C and D) strain R1 from 10-day-old lettuce roots; (E and F) strain R1 from 5-day-old maize roots; (G and H) strain R1 from 16-day-old maize roots. The right panels show bioluminescence photographed in the dark; the left panels were photographed in the light.

The recovery of rhizobial strains P31 and R1 at high levels in maize and lettuce roots confirms these previous reports observing rhizobial colonization of nonlegume roots (2, 3, 14, 19, 26, 29, 31, 36). The results can also explain in part the improved legume-*Rhizobium* symbiosis obtained by inoculating the preceding crop of maize with *Rhizobium* spp. (14) and the relatively high *Rhizobium* numbers found in soils of long-term field experiments with cereals without any legumes (34). The significant decrease of root colonization with time, which is a function of the root density, can be explained by the root biomass which increases rapidly during the first 4 WAP in pot experiments (19). The number of rhizobia recovered following 4 WAP on maize roots was comparable to that obtained by Wiehe and Höflich (34).

Rhizobium spp. can promote the growth of legumes by symbiotic N_2 fixation in root nodules and the growth of nonlegumes such as Gramineae and crucifers following root colonization without nodule formation (19). The mechanisms of action of growth promotion of nonlegumes by rhizobia may be similar to other PGPR of nonlegumes and should be investigated further. Biological control and direct growth promotion are the two main strategies of PGPR to increase the yield of plants (4). Rhizobial strains P31 and R1 used in this work were selected for their P-solubilizing ability. This characteristic should be an important factor in plant growth promotion as observed in the field with other P-solubilizing microorganisms (10). The literature frequently reported P solubilization (18) and other PGPR characteristics of rhizobia (9, 11, 13, 18, 33, 35).

The efficiency of *R. leguminosarum* bv. phaseoli strains P31 and R1 in colonizing maize and lettuce roots was generally higher than that of other P-solubilizing microorganisms used in this study. This corroborates previous work in which *Rhizobium* sp. strain R39 was recovered in higher numbers than strain PsIA12 of *Pseudomonas fluorescens* on maize roots (19). Root colonization and survival in the rhizosphere are very important factors for PGPR-mediated growth promotion because the activity of introduced cells is necessary to produce beneficial substances or to compete with deleterious and pathogenic organisms. The intensity of the bioluminescence phenotype is largely a reflection of the metabolic activity of cells since the observed light is the direct result of transcription (8). The visualization of an intense bioluminescence of strains

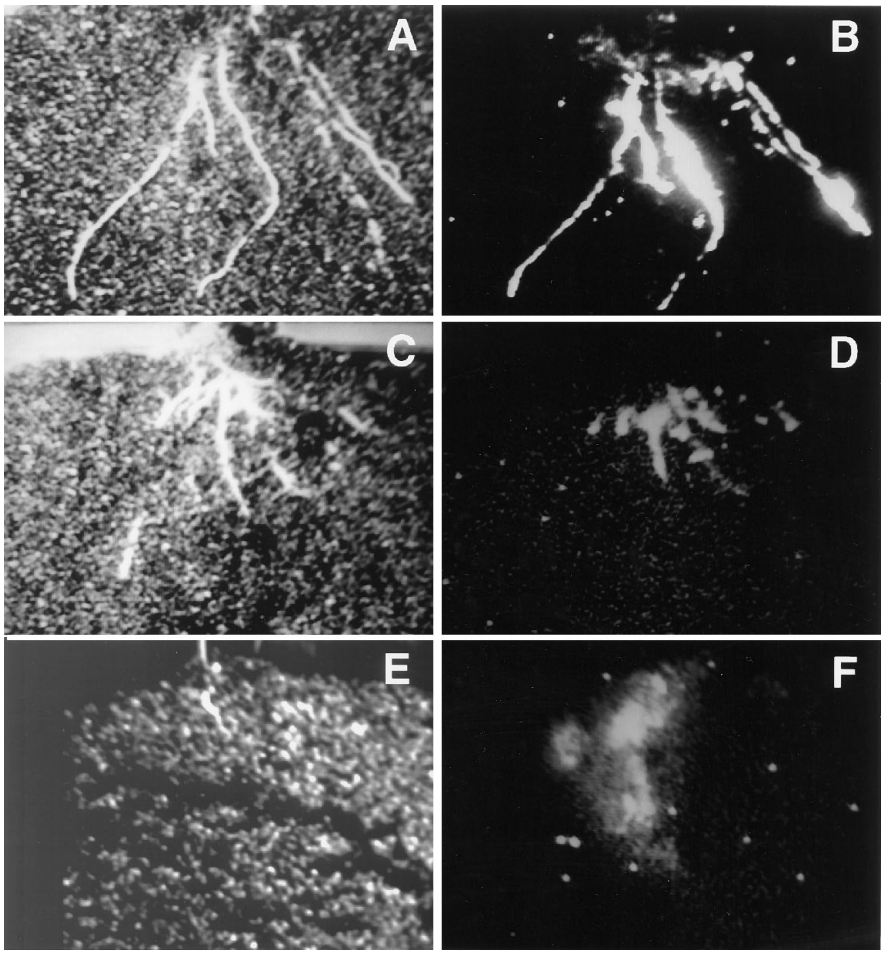

FIG. 4. Observation of in situ root colonization of lettuce and maize cultivated in rhizoboxes containing fritted clay by *R. leguminosarum* bv. phaseoli strains P31 and R1. (A and B) Strain P31 from 11-day-old maize roots; (C and D) strain R1 from 11-day-old maize roots; (E and F) strain P31 from 25-day-old lettuce roots. The right panels show bioluminescence photographed in the dark; the left panels were photographed in the light.

P31 and R1 in the rhizosphere may reflect this high metabolic activity (Fig. 4).

Our results also indicate that antibiotic markers such as rifampin (spontaneous mutant selection) and kanamycin (given by Tn*5*) provided an efficient recovery method for rhizosphere bacteria including rhizobia. No spontaneous resistant mutants were found on uninoculated control plates. Antibiotic resistance, including rifampin or kanamycin resistance, is an efficient tool to recover specific strains from the rhizosphere (15, 16, 21, 34). Furthermore, the bioluminescent phenotype was a very fast and efficient method to control the identity of recovered strains and to observe their in situ activity. Bioluminescence was chosen as a marker because of the stability of the Tn*5* insertion (5, 8, 27) and the absence of indigenous bioluminescent bacteria in the rhizospheres (6, 21). Bioluminescence can also be used in future genetic studies as a genetic marker (7). Luciferase-only synthesis, which occurs with *luxAB* genes, makes a low energy demand on cells in comparison with that of the synthesis of luciferase-aldehyde substrate, which occurs with *luxCDABE* genes (8, 12). The Tn*5*-*luxAB* system was chosen to avoid this high energy demand of mutants in the rhizosphere. However, addition of the substrate *N*-decyl aldehyde to luciferase near the colonies is necessary (21).

Rhizobium is a very important genus of PGPR. The advantage of using rhizobia as PGPR for nonlegumes comes from its safety, as indicated by the absence of any potential risk associated with its use with legumes for many decades. Research using rhizobia as PGPR with nonlegumes can also make rapid progress because of the well-established inoculation technology and the comprehensive genetic studies available for these bacteria. Root colonization is one of the more important properties of efficient PGPR, because introduced strains need to survive and grow in the rhizosphere to give beneficial effects. *R. leguminosarum* bv. phaseoli strains P31 and R1 seem to be particularly efficient and nonspecific root colonizers, since maize and lettuce are members of very different plant genera, representing monocotyledonous and dicotyledonous plants.

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REFERENCES

1. **Al-Mallah, M. K., M. R. Davey, and E. C. Cocking.** 1987. Enzymatic treatment of clover root hairs removes a barrier to *Rhizobium*-host specificity. Bio/Technology **5:**1319–1322.
2. **Al-Mallah, M. K., M. R. Davey, and E. C. Cocking.** 1989. Formation of

nodular structures on rice seedlings by rhizobia. J. Exp. Bot. **40:**473–478.
3. **Al-Mallah, M. K., M. R. Davey, and E. C. Cocking.** 1990. Nodulation of oilseed rape (*Brassica napus*) by rhizobia. J. Exp. Bot. **41:**1567–1572.
4. **Beauchamp, C. J.** 1993. Mode d'action des rhizobactéries favorisant la croissance des plantes et potentiel de leur usage comme agent de lutte biologique. Phytoprotection **74:**19–27.
5. **Beauchamp, C. J., J. W. Kloepper, and H. Antoun.** 1993. Detection of genetically engineered bioluminescent pseudomonads in potato rhizosphere at different temperatures. Microb. Releases **1:**203–207.
6. **Beauchamp, C. J., J. W. Kloepper, and P. A. Lemke.** 1993. Luminometric analyses of plant root colonization by bioluminescent pseudomonads. Can. J. Microbiol. **39:**434–441.
7. **Beauchamp, C. J., J. W. Kloepper, J. J. Shaw, and P. A. Lemke.** 1992. Bioluminescence as a marking system to monitor the fate of *Pseudomonas putida*, strain 61.9A.3L, released in the soybean rhizosphere. Phytopathology **82:**1179.
8. **Boivin, R., F.-P. Chalifour, and P. Dion.** 1988. Construction of a Tn*5* derivative encoding bioluminescence and its introduction in *Pseudomonas*, *Agrobacterium* and *Rhizobium*. Mol. Gen. Genet. **213:**50–55.
9. **Carson, K. C., S. Holliday, A. R. Glenn, and M. J. Dilworth.** 1992. Siderophore and organic acid production in root nodule bacteria. Arch. Microbiol. **157:**264–271.
10. **Chabot, R., H. Antoun, and M. P. Cescas.** 1993. Stimulation de la croissance du maïs et de la laitue romaine par des microorganismes dissolvant le phosphore inorganique. Can. J. Microbiol. **39:**941–947.
11. **Derylo, M., and A. Skorupska.** 1991. Biological activity of rhizobial siderophore. Acta Microbiol. Pol. **40:**265–268.
12. **DeWeger, L. A., P. Dunbar, W. F. Mahafee, B. J. J. Lugtemberg, and G. S. Sayler.** 1991. Use of bioluminescence markers to detect *Pseudomonas* spp. in the rhizosphere. Appl. Environ. Microbiol. **57:**3641–3644.
13. **Ehteshamul-Haque, S., and A. Ghaffar.** 1993. Use of rhizobia in the control of root rot diseases of sunflower, okra, soybean and mungbean. J. Phytopathol. **138:**157–163.
14. **Gaur, Y. D., A. N. Sen, and N. S. Subba Rao.** 1980. Improved legume-*Rhizobium* symbiosis by inoculating preceding cereal crop with *Rhizobium*. Plant Soil **54:**313–316.
15. **Gemmell, L. G., and R. J. Roughley.** 1993. Counting rifampicin-resistant rhizobia when a minor component of the soil rhizobial flora. Soil Biol. Biochem. **25:**539–544.
16. **Glandorf, D. C. M., I. Brand, P. A. H. M. Bakker, and B. Schippers.** 1992. Stability of rifampicin resistance as a marker for root colonization studies of *Pseudomonas putida* in the field. Plant Soil **147:**135–142.
17. **Goldstein, A. H.** 1986. Bacterial solubilization of mineral phosphates: historical perspective and future prospects. Am. J. Altern. Agri. **1:**51–57.
18. **Halder, A. K., and P. K. Chakrabartty.** 1993. Solubilization of inorganic phosphate by *Rhizobium*. Folia Microbiol. **38:**325–330.
19. **Höflich, G., W. Wiehe, and C. Hecht-Buchholz.** 1995. Rhizosphere colonization of different crops with growth promoting *Pseudomonas* and *Rhizobium* bacteria. Microbiol. Res. **150:**139–147.
20. **Kirk, R. E.** 1982. Experimental design: procedures for the behavioral sciences, 2nd ed. Brooks/Cole Publishing Company, Pacific Grove, Calif.
21. **Kloepper, J. W., and C. J. Beauchamp.** 1992. A review of issues related to measuring colonization of plant roots by bacteria. Can. J. Microbiol. **38:** 1219–1232.
22. **Lalande, R., H. Antoun, T. Paré, and P. Joyal.** 1986. Effets de l'inoculation avec des souches du *Rhizobium leguminosarum* biovar *phaseoli* sur le rendement et la teneur en azote du haricot (*Phaseolus vulgaris*). Nat. Can. **113:** 337–346.
23. **Loper, J. E., T. V. Suslow, and M. N. Schroth.** 1984. Lognormal distribution of bacterial populations in the rhizosphere. Phytopathology **74:**1454–1460.
24. **Mehlich, A.** 1984. Mehlich 3 soil test extractant: a modification of Mehlich 2 extractant. Commun. Soil Sci. Plant Anal. **15:**1409–1416.
25. **Misaghi, I. J., and C. R. Donndelinger.** 1990. Endophytic bacteria in symptom-free cotton plants. Phytopathology **80:**808–811.
26. **Pena-Cabriales, J. J., and M. Alexander.** 1983. Growth of *Rhizobium* in unamended soil. Soil Sci. Soc. Am. J. **47:**81–84.
27. **Pillai, S. D., and I. L. Pepper.** 1990. Survival of Tn*5* mutant bean rhizobia in desert soils: phenotypic expression of Tn*5* under moisture stress. Soil. Biol. Biochem. **22:**265–270.
28. **Richardson, A. E.** 1994. Soil microorganisms and phosphorus availability, p. 50–62. *In* C. E. Pankhurst, B. M. Doube, V. V. S. R. Gupts, and P. R. Grace (ed.), Soil Biota, management in sustainable farming systems. CSIRO Australia, Melbourne.
29. **Shimshick, E. J., and R. R. Hebert.** 1979. Binding characteristics of N_2-fixing bacteria to cereal roots. Appl. Environ. Microbiol. **38:**447–453.
30. **Surange, S., and N. Kumar.** 1993. Phosphate solubilization under varying pH by *Rhizobium* from tree legumes. Indian J. Exp. Biol. **31:**855–857.
31. **Terouchi, N., and K. Syōno.** 1990. *Rhizobium* attachment and curling in asparagus, rice and oat plants. Plant Cell Physiol. **31:**119–127.
32. **Vincent, J. M.** 1970. A manual for the practical study of root nodule bacteria. IBP handbook 15. Blackwell Scientific Publications, Oxford.
33. **Wang, T. L., E. A. Wood, and N. J. Brewin.** 1982. Growth regulators, *Rhizobium* and nodulation in peas. Planta **155:**345–349.
34. **Wiehe, W., and G. Höflich.** 1995. Survival of plant growth promoting rhizosphere bacteria in the rhizosphere of different crops and migration to noninoculated plants under field conditions in north-east Germany. Microbiol. Res. **150:**201–206.
35. **Williams, M. N. V., and E. R. Signer.** 1990. Metabolism of tryptophan and tryptophan analogs by *Rhizobium meliloti*. Plant Physiol. **92:**1009–1013.
36. **Yanni, Y. G., R. Y. Rizk, V. Corich, A. Squartini, and F. B. Dazzo.** 1995. Endorhizosphere colonization and growth promotion of indica and japonica rice varieties by *Rhizobium leguminosarum* bv *trifolii*, abstr. O17. *In* Proceedings of the 15th Symbiotic Nitrogen Fixation Conference, North Carolina State University, Raleigh, N.C.