SHORT COMMUNICATION

EFFECT OF PHOSPHORUS ON ROOT COLONIZATION AND GROWTH PROMOTION OF MAIZE BY BIOLUMINESCENT MUTANTS OF PHOSPHATE-SOLUBILIZING *RHIZOBIUM LEGUMINOSARUM* BIOVAR *PHASEOLI*

ROCK CHABOT,[1] CHANTAL J. BEAUCHAMP,[2] JOSEPH W. KLOEPPER[3] and HANI ANTOUN[1]*

[1]Département des Sols et de Génie Agroalimentaire et Centre de Recherche en Horticulture, Université Laval, Pavillon Charles-Eugène Marchand, Quebec, Que., Canada G1K 7P4, [2]Département de Phytologie et Centre de Recherche en Horticulture, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Pavillon Paul-Comtois, Quebec, Que., Canada G1K 7P4 and [3]Department of Plant Pathology, Biological Control Institute, Life Science Bldg., Auburn University, Auburn, AL 36489, U.S.A.

Several P-solubilizing microorganisms (PSM), are able to solubilize unavailable soil P and increase the yield of crops (Richardson, 1994). Moreover, some strains of rhizobia are PSM and like other plant growth promoting rhizobacteria (PGPR) they can colonize the roots and increase the yield of non-legume crops (Höflich *et al.*, 1995; Chabot *et al.*, 1996a,b). A major barrier to the successful agronomic use of bacterial inocula is the need to establish high population densities of the introduced bacterium in the root environment (Kloepper *et al.*, 1989). The nutrient status of the rhizosphere determines the nature of the root exudates and it can have a direct effect on the composition of the rhizosphere microbial community (Curl and Truelove, 1986; Klein *et al.*, 1990; Lynch, 1990). Therefore, available nutrients can also probably affect the ability of an introduced PGPR, to colonize roots and to perform their beneficial activity. Goldstein (1986) demonstrated that P-solubilization by *Erwinia herbicola* was repressed by increasing amounts of soluble P in the culture medium. In our work, we found that maize growth promotion and root colonization by the PSM strain R1Lux$^+$ of *Rhizobium leguminosarum* bv. *phaseoli* were not affected by soil P-fertilization and that P-solubilization is an effective mechanism of growth promotion of this strain.

Bacterial maintenance and culture, evaluation of phosphate solubilization activity and isolation of Lux$^+$ transconjugants of the PSM strain R1 were carried out as described by Chabot *et al.* (1996b). To isolate mutants altered in their P-solubilizing activity, transconjugants were suspended in liquid dicalcium phosphate (DCP) medium (Goldstein, 1986) and exposed for 24 h to carbenicillin (100 mg l^{-1}). This enrichment method was based on the hypothesis that mutants altered in P-solubilization could not grow in DCP and would not be affected by carbenicillin. Mutants were selected by screening for colonies exhibiting solubilization haloes smaller than those produced by strain R1 on DCP plates. The P-solubilizing activities of the selected mutants were compared with the wild type strain R1, in liquid hydroxyapatite medium (HAP). This medium is similar to the DCP medium, but 4 g HAP (Sigma No C-5267) l^{-1} replaced DCP precipitate. The 250 ml Erlenmeyer flasks containing 50 ml HAP were inoculated with 0.1 ml of bacterial suspension adjusted to 0.25 optical density at 590 nm. Following 16 and 24 h incubation, culture samples were harvested aseptically, passed through a 0.22 μm filter, and soluble P was measured by the spectrophotometric vanadate–molybdate method (Tandon *et al.*, 1968).

Inoculant preparation and inoculation of maize seeds (hybrid Funk's 4066) were performed as described by Chabot *et al.* (1996b). The two soils used for the experiments came from agricultural fields on Île d'Orléans (Quebec, Canada). Each pot (12.5 cm diameter) received 1 kg of soil. The soils were selected for their respective low and high available-P content (poor and rich soils) to determine

*Author for correspondence.

Table 1. Some properties of the surface soils (0–15 cm) used in the greenhouse experiments selected for their high or low P content

Soil	Texture	pH	Organic matter (%)	Total P* (kg ha^{-1})	Available elements† (kg ha^{-1})			
					P	K	Ca	Mg
Rich	silty-clay loam	6.25	4.71	4092	646	548	6197	289
Poor	silty loam	5.32	4.47	1124	8	162	3232	87

*Measured by the spectrophotometric vanadate–molybdate method (Tandon et al., 1968) following soil digestion (1 g) in HClO$_4$ and HNO$_3$ (O'Halloran, 1993).
†Extracted by the Mehlich III procedure (Mehlich, 1984).

the P-fertilization effect on root colonization. Some physical and chemical properties of these soils are presented in Table 1 and were determined as described by Chabot et al. (1996a). Basic N, P and K fertilization was, respectively, 180 mg N–NH$_4$NO$_3$, 65.5 mg P-superphosphate and 167 mg K–KCl kg^{-1} of soil. The P-rates applied were 0, 1 and 2 times the basic P-fertilization (0, 65.5 and 131 mg P kg^{-1}). Six seeds were sown 2 cm deep in each pot. The experiments were carried out in a greenhouse with 16 h photoperiod and average temperature of 25°C. After emergence, plants were thinned to three plants of uniform appearance per pot. The experimental design was a split plot with four inoculation treatments as main plot, three P-rates as subplot, with four replications. Plants were grown for 21 d and shoots were harvested and dried to obtain the dry weight (average of 3 plants pot^{-1}). The rich and poor soils were used separately in identical experiments and each of these experiments was duplicated. Data from repeated experiments were compiled and used for statistical analysis. Statistical analysis, data transformations and the evaluation of the Lux$^+$ rhizosphere populations were performed as described by Chabot et al. (1996b).

Forty mutants (MP-1 to MP-40) were selected after screening of several hundred transconjugant colonies obtained from three different conjugations
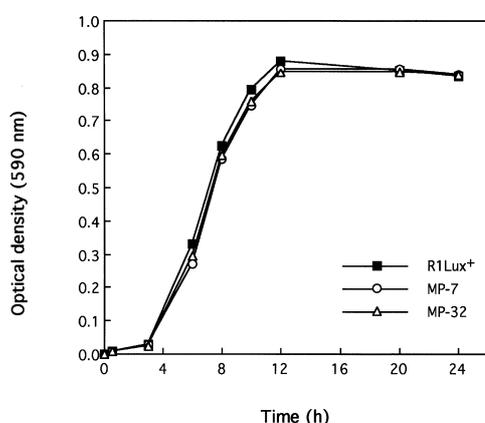
of strain R1rif$^+$ with the E. coli donor. The mutants emitted visual light in the dark after addition of N-decyl aldehyde (Sigma No D-7384) and appeared to solubilize less P on DCP plates. No completely altered mutants in P-solubilization were found. The frequency of transconjugants was about 1 Lux$^+$ cell per 50 recipient cells. All selected mutants had similar reactions to the wild type strain R1rif$^+$ on Biolog GN Microplates$^{®}$. Mutants MP-7 and MP-32, which were obtained from two different conjugations, appeared to be the most altered in P-solubilization on DCP plates and were selected for the following experiments. The growth curves in 5% tryptic soy broth, of the mutants altered in P-solubilization and strain R1Lux$^+$ were similar (Fig. 1). In liquid HAP, MP-7 and MP-32 mutants solubilized significantly less P than the wild type strain R1 or its bioluminescent mutant R1Lux$^+$ (Table 2). The shoot dry weight of maize increased significantly ($P \leq 0.01$) with the rate of P-fertilizer applied in the two soils tested [Fig. 2(A)–(B)]. In the rich soil, for all amounts of added P, dry matter of maize was significantly increased by bacterial inoculation, compared to the uninoculated control [Fig. 2(A), $P \leq 0.01$]. In the absence of P-fertilization, the altered mutant MP-32 induced the least increase in shoot dry matter, but addition of P increased the dry matter production considerably [Fig. 2(A)]. Maize responded differently to inoculation with mutants MP-7, MP-32 or R1Lux$^+$ in the soil rich in P, but no difference was found in the less fertile soil where all treatments showed a linear effect under P-fertilization [Fig. 2(B)]. Lux$^+$ mutants were recovered from all maize root samples except from the uninoculated controls. Strain R1Lux$^+$ colonized roots to a higher degree than the altered mutants, especially under no

Fig. 1. Growth of R1Lux$^+$ and two of its mutants (MP-7 and MP-32) altered in P-solubilization in 5% tryptic soy broth (Difco). All cultures were grown at 28°C on a rotary shaker (150 rpm)

Table 2. Solubilization of hydroxyapatite by mutants of R. leguminosarum bv. phaseoli strain R1

Strain	HAP dissolved (mg P l^{-1})	
	16 h incubation	24 h incubation
R1	81.1 a*	212.9 a
R1Lux$^+$	66.1 b	216.2 a
MP-32	39.2 c	201.0 b
MP-7	39.2 c	183.3 c
LSD value	13.7	10.8

*Means followed by the same letter are not significantly different according to LSD test at the 95% level.
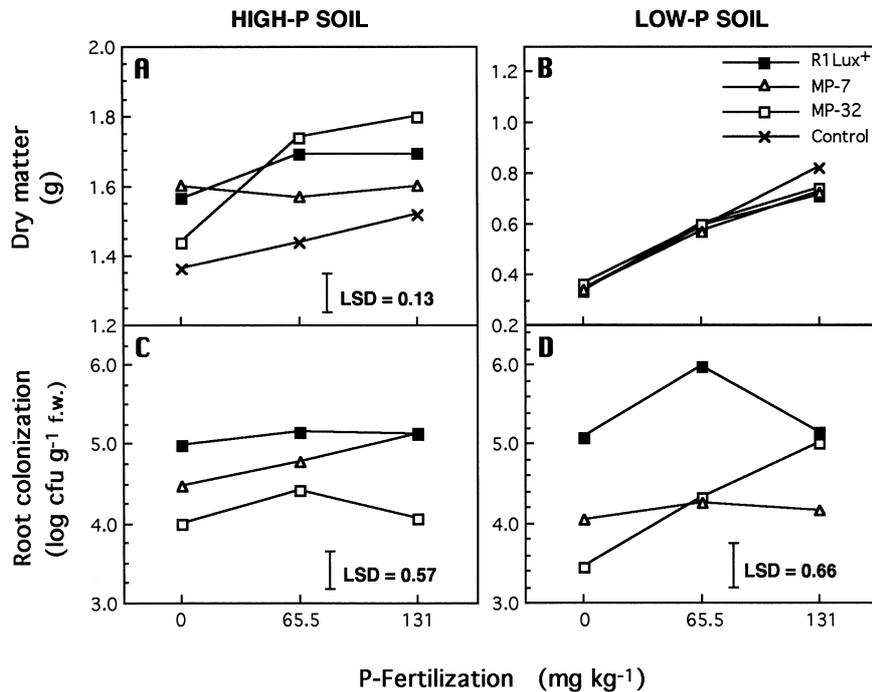
Fig. 2. Effect of P-fertilization and inoculation of maize with strain R1Lux$^+$ and its mutants MP-7 and MP-32 altered in P-solubilization, on plant dry matter production and on root bacterial colonization. Vertical bars give the least significant differences (LSD) at the 95% level. In the low-P soil, inoculation treatments did not show any significant effect on maize dry matter yield (B)

added P or with 65.5 mg kg^{-1} [Fig. 2(C)–(D)]. On average the population of strain R1Lux$^+$ was log 5.12 cfu g^{-1} f.w. in the rich soil and log 5.38 cfu g^{-1} f.w. in the poor soil. There was no significant effect of inoculation or P-fertilization on the total bacterial rhizosphere community in either rich and poor soils. In fact, the total rhizosphere community averaged log 7.68 cfu g^{-1} f.w. in the rich soil and log 7.89 cfu g^{-1} f.w. in the poor soil. Strain R1Lux$^+$ represented approximately 0.36% of the total bacterial rhizosphere community while altered strains MP-7 and MP-32, represented approximately 0.04% of the total.

The competitiveness of P-solubilizing rhizobia in rhizosphere colonization of maize was not affected by increasing P-fertilization in the poor and rich soils tested. Similarly, the proportion of introduced strains from the total rhizosphere community was unchanged by the increased P-fertilization. According to Klein et al. (1990), inadequate root colonization is a major problem in potential biotechnological applications of beneficial strains. The use of highly efficient root-colonizing bacteria like rhizobial strain R1, may therefore reduce the chances of failed inoculation. The observation that strain R1Lux$^+$ colonized roots in both soils better than the altered mutants, suggests that P-solubilization might have an important role to play in rhizosphere competitiveness of P-solubilizing rhizobia. This phenotype might influence the ability of a strain to colonize plant roots. The increase of dry matter of maize by the inoculation with the P-solubilizing rhizobia R1Lux$^+$ in the rich soil and its higher root colonization ability support the relationship between P-solubilization, root colonization and growth promotion. The use of mutants completely negative in P-solubilization would have allowed more precise conclusions about the role of P-solubilization in root colonization and growth promotion. However, the presence of several genes that control P-solubilization (Goldstein and Liu, 1987) decreases the probability of obtaining completely negative mutants.

Goldstein (1986) found that P-solubilization activity of Erwinia herbicola was negatively controlled by the amount of soluble P in the media. Addition of 30 mM (approximately 0.1% or 1000 mg l^{-1}) soluble P was necessary to completely repress visible P-solubilization in DCP plates. In our work a complete repression was obtained in the presence of 0.5% soluble P with rhizobial strain R1 and its mutant R1Lux$^+$ (results not shown). In cultivated soils, soluble P ranges from 0.3 to 1 mg ml^{-1} in the solution phase (Stevenson, 1986), and extreme values ranged from less than 0.01 to 8 mg l^{-1} (Foth and Ellis, 1988). Therefore, it would be expected that these P concentrations are largely insufficient to inhibit solubilization activities of strain R1.

The increased dry matter obtained with inoculation of maize in the rich soil demonstrated the

high potential of P-solubilizing rhizobia to stimulate plant growth, but no stimulation was found in the poor soil. Plant growth promotion by microbially increased P-availability, in short-term greenhouse experiment in small pots, may not be obvious because total P is already limited in that small soil quantity (1 kg), and because maize seedlings utilize seed reserves. The total P content of the poor soil seemed too low to allow a sufficient increase of P availability by PSM. The total P contained in the pots of poor soil (560 mg kg^{-1}) was approximately one-fourth (22%) that of rich soil (2050 mg kg^{-1}). Moreover, the bio-available P in the poor soil, according to the Mehlich III procedure (Mehlich, 1984), was extremely low (4 mg kg^{-1}) compared to the rich soil (320 mg kg^{-1}). These results support previous observations indicating that growth promotion by P-solubilization is less effective in poor soils under field conditions (Chabot et al., 1996a). Furthermore, because of the large populations of strain R1 Lux^{+} recovered from the maize roots, it is possible that in addition to P-solubilization, other mechanisms of action generally found in PGPR (Kloepper et al., 1989), were involved in the rich soil but were absent from the poor soil.

REFERENCES

Chabot R., Antoun H. and Cescas M. P. (1996a) Growth promotion of maize and lettuce by phosphate-solubilizing *Rhizobium leguminosarum* biovar *phaseoli*. *Plant and Soil* **184,** 311–321.

Chabot R., Antoun H., Kloepper J. W. and Beauchamp C. J. (1996b) Root colonization of maize and lettuce by bioluminescent phosphate-solubilizing *Rhizobium leguminosarum* biovar *phaseoli*. *Applied and Environmental Microbiology* **62,** 2767–2772.

Curl, E. A. and Truelove, B. (1986) *The Rhizosphere*. Springer-Verlag, Berlin.

Foth, H. D. and Ellis, B. G. (1988) *Soil Fertility*. John Wiley, New York.

Goldstein A. H. (1986) Bacterial solubilization of mineral phosphates: historical perspective and future prospects. *American Journal of Alternative Agriculture* **1,** 51–57.

Goldstein A. H. and Liu S. T. (1987) Molecular cloning and regulation of a mineral phosphate solubilizing gene from *Erwinia herbicola*. *Bio/Technology* **5,** 72–74.

Höflich G., Wiehe W. and Hecht-Buchholz C. (1995) Rhizosphere colonization of different crops with growth promoting *Pseudomonas* and *Rhizobium* bacteria. *Microbiological Research* **150,** 139–147.

Klein, D. A., Salzwedel, J. L. and Dazzo, F. B. (1990) Microbial colonization of plant roots. In *Biotechnology of Plant-Microbe Interactions*, eds. J. P. Nakas and C. Hagedorn, pp. 189–225. McGraw-Hill, New York.

Kloepper J. W., Lifshitz R. and Zablotowicz R. M. (1989) Free-living bacterial inocula for enhancing crop productivity. *Trends in Biotechnology* **7,** 39–44.

Lynch, J. M. (1990) Some consequences of microbial Rhizosphere competence for plant and soil. In *The Rhizosphere*, ed. J. M. Lynch, pp. 1–10. Wiley Interscience, New York.

Mehlich A. (1984) Mehlich 3 soil test extractant: a modification of Mehlich 2 extractant. *Communications in Soil Science and Plant Analysis* **15,** 1409–1416.

O'Halloran, I. P. (1993) Total and organic phosphorus. In *Soil Sampling and Methods of Analysis*, ed. M. R. Carter, pp. 213–229. Canadian Society of Soil Science, Lewis Publishers, Boca Raton.

Richardson, A. E. (1994) Soil microorganisms and phosphorus availability. In *Soil Biota, Management in Sustainable Farming Systems*, eds. C. E. Pankhurst, B. M. Doube, V. V. S. R. Gupts and P. R. Grace, pp. 50–62. CSIRO Australia, Melbourne.

Stevenson, F. J. (1986) *Cycles of Soil Carbon, Nitrogen, Phosphorus, Micronutrients*. John Wiley, New-York.

Tandon H. L. S., Cescas M. P. and Tyner E. H. (1968) An acid-free vanadate–molybdate reagent for the determination of total phosphorus in soils. *Soil Science Society of America Proceedings* **32,** 48–51.