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Rock Phosphate Solubilization and Colonization of Maize Rhizosphere by Wild and Genetically Modified Strains of *Penicillium rugulosum*

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Abstract

Maize root colonization and phosphate solubilizing activity of the fungus *Penicillium rugulosum* were assessed in a greenhouse trial using soil–plant microcosms. The bacterial gene *hph* conferring resistance to hygromicin B was introduced by electroporation in the wild-type strain IR-94MF1 of *P. rugulosum* and one transformant, w-T3, was selected. Maize plants were grown for 5 weeks in a P-poor soil and fertilized with a Florida apatite mineral, with Navay, an apatite rock deposit from Venezuela, or with simple superphosphate. Inoculation treatments included strain IR-94MF1, transformant w-T3 and two IR-94MF1 UV-induced mutants with enhanced (Mps^{++}) or reduced (Mps^{-}) *in vitro* mineral phosphate solubilizing activity. In the absence of P fertilization, inoculation with any *P. rugulosum* isolate significantly reduced the size of the total and P-solubilizing bacterial community present in maize rhizosphere. The bacterial community significantly increased in maize inoculated with IR-94MF1 and w-T3 when P was added as apatites Navay or Florida. All *P. rugulosum* strains were able to stimulate the growth of maize plants as indicated by 3.6 to 28.6% increases in dry matter yields. In the presence of rock phosphate, P uptake by maize plants inoculated with the two mutants Mps^{++} and Mps^{-} was not always in agreement with their P-solubilizing phenotypes. Strain IR-94MF1 and transformant w-T3 increased P assimilation by the plants fertilized with Navay rock phosphate by 26 and 38%, respectively. In this treatment, w-T3 showed its highest significant maize rhizosphere colonization. With the simple superphosphate treatment, w-T3 increased P uptake in plants by 8% over the uninoculated control and also decreased significantly the community size of total bacteria, total fungi, and P-solubilizing fungi in the rhizosphere.

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Introduction

The availability of phosphate anions in soil has been related to the presence and activity of soil microorganisms. Factors such as the root morphology, the stage of plant growth, and the physical and chemical properties of the soil influence the distribution of microbial communities in soil and rhizosphere. It is well known that microbial biomass contains a large reservoir of P, which is potentially available to plants [2] by a mineralization process, whereas insoluble inorganic P associated with the solid phase can be sorbed to the surfaces of soil constituents or can occur in Ca, Fe, or Al minerals. Mineral P is further released and made available to plants mostly by the action of the phosphate-solubilizing microorganisms [8]. An increase in P availability to plants through the inoculation of phosphate-solubilizing microorganisms has been reported several times in pot experiments [9, 22] and under field conditions [5, 9]. Recently, the addition of rock phosphate significantly increased N, P, total plant biomass, infection by arbuscular mycorrhizal fungi, and the number of nodules of two legume species under field conditions [29]. Nevertheless, little is known about the colonization of the rhizosphere by the introduced mineral phosphate solubilizing (MPS) microorganisms and their impact on the size of rhizospheric microbial communities when different phosphate sources are used in a plant–soil system.

Richardson [21] signaled two strategies to improve the efficiency of P fertilizer utilization: (1) management of existing populations of soil microorganisms to optimize their capacity for P transformation and to synchronize their nutrient mobilization activity with that of plant requirements, and (2) the introduction of a specific inoculant to enhance either the supply and availability of soil P or the uptake of P by plant roots. However, the success of an introduced microorganism for phosphate solubilization has been related to both its capacity to readily colonize plant roots [22] and the activity of the phosphate-solubilizing microbial population already present in soil [8]. Moreover, fertilizer applications to soil also affect the activities of diverse groups of soil microorganisms, directly by supplying nutrients and indirectly through greater carbon availability from root exudates resulting from increased plant growth [24].

Recently, MPS activity in the fungus *Penicillium rugulosum* was characterized in the wild-type strain IR-94MF1 and two UV-induced mutants derived from it with altered phenotypes for phosphate solubilization [18]. The nature and amount of the organic acids secreted by *P. rugulosum* were strongly influenced by the pH and buffer capacity of the culture medium, by the presence or absence of certain metals, and by the nitrogen, phosphate or carbon sources used [18, 19]. Solubilization of a variscite mineral was performed by the production of citric acid, whereas gluconic acid appeared to be responsible for the solubilization of apatite mineral or rock phosphate deposits [20]. The aim of the present study was to determine the effect of soil inoculation with *P. rugulosum* on maize yield and P uptake, and on the size of the rhizospheric microbial communities in microcosms under greenhouse conditions. To assess the competitive ability of *P. rugulosum* in a nonsterile soil–plant system subjected to different P fertilization treatments, a genetically marked transformant, w-T3, was obtained by inserting a hygromycin B resistance marker into the nuclear genome of strain IR-94MF1.

Materials and Methods

Preparation of Protoplasts and Transformation by Electroporation

Protoplasts of *P. rugulosum* strains IR-94MF1 (wild-type), Mps^{-}, and Mps^{++} were prepared using the method of Durand et al. [6] modified as follows. Conidia were inoculated in 50 mL of a complete liquid medium [18] and the cultures were grown for 3 days at 28°C on a gyratory shaker at 120 rev min^{-1}. The mycelium was harvested by filtration, washed with 0.7 M NaCl, and then resuspended in a filter-sterilized osmotic buffer (0.7 M NaCl in 0.1 mM phosphate buffer, pH 5.6) containing 10 mg mL^{-1} lysing enzymes (Sigma, St. Louis, MO) and 10 mg mL^{-1} cellulase Onozuka (Serva, Germany). After 3 h incubation at 30°C with agitation at 100 rev min^{-1}, protoplasts were separated from cell debris by filtration through glass wool. The suspension was transferred to sterile centrifuge tubes, overlaid with an equal volume of NaCl solution, and centrifuged at 2000*g* for 10 min. The pelleted protoplasts were then washed with a sorbitol solution (1 M sorbitol, 50 mM $CaCl_2$) and suspended in 1.2 M sorbitol plus 1% PEG 6000 solution at a final concentration of 1×10^8 protoplasts mL^{-1}. The protoplasts were then mixed with plasmid pPS57, a 5.38 kb construct carrying a chloramphenicol acetyltransferase gene from *E. coli*, the M13 origin of replication, and the isopenicillin N synthetase promoter from *P. chrysogenum* fused to the hygromycin B (HmB) resistance gene, *hph*, of *E. coli* [26].

Between 10 and 30 μg of undigested pPS57 plasmid was mixed with 400 μL of protoplasts suspension and kept on ice. Electroporation was carried out at a voltage of 2.8 kV cm^{-1}, with a capacitance of 25 μF and an internal resistance of 800 ohms [7]. Electric pulses were delivered to the protoplasts and DNA suspension using a Gene Pulser apparatus (Bio-Rad Laboratories,

Hercules, CA). After delivery of the electrical pulse, the protoplasts and DNA suspension were mixed with 5 mL of complete liquid medium plus 1.2 M sorbitol, and incubated for 10 min on ice and for 10 h at 28°C to allow cell wall regeneration and gene expression. Aliquots of 400 μL were plated using an agar overlay on complete solid medium plus 1.2 M sorbitol and 200 μg mL^{-1} of HmB. Plates were incubated at 28°C and HmB resistant colonies were counted within 7–10 days. Colonies were transferred from selective to nonselective medium plates. After 10 days of growth on nonselective medium, monosporic colonies (growing from one aseptically selected spore) were further subjected to at least five additional rounds of subculture in the absence of HmB. All putative transformants expressed the HmB-resistant phenotype when subcultured again on selective medium containing 100 μg of HmB mL^{-1}. Three putative transformants (w-T1, w-T2, and w-T3) were chosen among the eight obtained, and analyzed by Southern hybridization.

Isolation and Analysis of DNA

Strain IR-94MF1 and putative transformants of *P. rugulosum* were grown in complete liquid medium at 28°C. Mycelia were then filtered, quickly frozen in liquid nitrogen, lyophilized overnight, and ground in liquid nitrogen using a mortar and pestle. About 170 μL of ground mycelium was transferred to a 1.5 mL Eppendorf tube and total genomic DNA was extracted as described by Möller et al. [16]. The final DNA pellet was dried, suspended in 35 μL of TE (0.01 M Tris [hydroxymethyl] aminoethane–hydrochloridric acid, pH 8.0, and 0.001 M tetraacetic acid [ethylenedinitrile]) and stored at −20°C.

Genomic DNA was digested with *Bgl*II, which does not cut plasmid pPS57, and *Sal*I, which linearizes pPS57. Approximately equal amounts (6 μg) of DNA were subjected to agarose gel (1% agarose in 0.5× TBE) electrophoresis in 0.5× TBE buffer [23] at 20 V for 10 h. After depurination in 0.25 M HCl for 15 min, denaturation in 0.4 N NaOH, 0.6 M NaCl for 1 h 30 min, and neutralization in 0.1 M Tris HCl, pH 7.5, for 5 min, DNA was transferred onto a Genebind nylon membrane (Pharmacia LKB Biotechnology AB, Qc, Canada), and probed with linearized pPS57. Probe labeling, DNA hybridization against fluorescein-12-dUTP (Renaissance, DuPont, NEN Boston, MA) and chemiluminescent detection of the hybridizing bands with the Renaissance Antifluorescein-HRP Conjugate were all performed according to supplier's recommendations.

Physiological Characterization of the Strains

Growth and phosphate solubilization were measured in solid complete medium [18] and minimal medium (complete medium without KH_2PO_4, yeast extract and malt extract) with hydroxyapatite at 2.5 g L^{-1}. Acidity produced by the strains was determined by the production of a yellow halo when using bromphenol blue as a pH indicator in complete medium, whereas the solubilization of hydroxyapatite was determined by measuring a clarification halo under or around the colony. Petri dishes were incubated in darkness for 1 week at 28°C.

Greenhouse Experiment

A low fertility soil was used and collected at Ile d'Orléans (Québec, Canada). It had a pH of 6.25 (1:1 in water), 3.14% organic matter content, and a texture composed of 50, 31, and 19% sand, loam, and clay, respectively. The P, K, Mg, and Ca available in the soil as determined by the Mehlich III procedure [12] were 16, 104, 65, and 2773 kg ha^{-1}, respectively. A randomized bifactorial design with 20 treatments and three repetitions was used. The five inoculation treatments used were *P. rugulosum* wild-type strain IR-94MF1, transformant w-T3, mutants Mps^{-} and Mps^{++}, and an uninoculated control. Three phosphate sources were used: Florida apatite mineral (Ward Natural Sciences, Rochester, NY), Navay rock phosphate (apatite deposit from Venezuela), and ordinary superphosphate (SSP). According to the guidelines used in Quebec [1] based on soil analysis, phosphorus was added at a concentration of 46 mg P kg^{-1} soil for all P sources used; nitrogen at 60 mg N kg^{-1} soil as ammonium nitrate and potassium at 48 mg K kg^{-1} soil as KCl. A micronutrient solution (Hoagland's solution) was applied at the beginning of the experiment and three weeks after sowing. Approximately 600 g of air-dried soil sieved to a 5-mm pore size was placed in a 13-cm diameter microcosm container. The inorganic fertilizers were spread evenly over the soil surface, and 2 cm of soil was placed to cover the fertilizers. Commercial seeds of maize (*Zea mays* L. hybrid Pioneer 3967), were washed to eliminate fungicide treatment and disinfected (3 min in 70% ethanol, 15 min in a solution of 6% sodium hypochlorite and washed several times with distilled water). Five seeds were then placed on the soil surface and covered with an additional 2 cm of soil. For each microcosm a total of 1000 g of soil was used, moistened and inoculated. Water was added daily as necessary to maintain soil moisture levels near field capacity. Microcosms were placed in a greenhouse under 16 h of light at 25°C and 8 h of darkness at 20°C. Following emergence of all seedlings, the number of seedlings in each microcosm was thinned to three. At 5 weeks, the plants were cut at the soil surface and shoots from each microcosm were oven-dried (80°C for 4 days) and weighed. The material was ground and subsamples of the material were acid digested with 15 ml $HClO_3$ and 5 ml HNO_3, and then analyzed by the vanado-molybdate method for P content [27]. Soil pH was measured in water (1:1 volume).

For the production of fungal inocula, isolates were grown on the complete solid medium until mycelia produced conidia. Spores were suspended in a 0.05 % (v/v) Tween 20 sterile solution, the concentration of conidia was adjusted to 1×10^8 mL^{-1} by adding sterile distilled water, and 10 mL of the conidial suspension was applied to the soil after sowing.

Estimation of Microbial Community Size

At the end of the experiment, the root system was separated from soil by hand shaking to dislodge loosely adhering soil and 5 long

roots containing approximately 1 g d.w. of rhizosphere soil were placed in 100 mL sterile saline solution [17]. After shaking for 30 min at 200 rev min^{-1}, 0.1 ml samples from appropriate dilutions were plated by spreading. Bacteria were counted on 10% TSA (tryptic soy agar, Difco, Detroit, MI) and fungi on PDA (potato dextrose agar, Difco). Communities of phosphate-solubilizing bacteria and fungi were grown on minimal medium containing 2.5 g L^{-1} hydroxyapatite. Media for culturing fungi were always supplemented with 300 mg L^{-1} rose bengal to obtain small colonies and with 30 mg L^{-1} choramphenicol and 100 mg L^{-1} streptomycin sulfate to suppress bacteria. The population size of the transformant w-T3 was also counted in 1 g d.w. of bulk soil, on a selective medium prepared with hydroxyapatite minimal medium supplemented with 200 mg L^{-1} HmB. Plates were incubated at 28°C and total microorganisms were counted after 2 to 4 days and P-solubilizers after 3 to 5 days. Populations of w-T3 were counted after 10 days' incubation due to the slow growth of the colonies. Counts of culturable bacteria and fungi were expressed as CFU g^{-1} of dry rhizosphere soil.

Stability of the Transformant w-T3 in Soil

The genetic stability of transformant w-T3 in the soil–plant microcosms was verified by conducting Southern hybridizations of HmB-resistant *P. rugulosum* colonies recovered randomly from the hydroxyapatite minimal medium supplemented with 200 mg L^{-1} HmB. Total genomic DNA was extracted, digested with *Hind*III or *Bgl*II, and probed with pPS57 as described above. Hybridization patterns of transformants recovered from soil–plant micocosms were compared with patterns from the original transformant prior to its inoculation.

Statistical Analysis

Variance homogeneity determination (ANOVA) and comparison of treatment means (LSD) were conducted with the General Linear Models (GLM) of SAS [25]. Where appropriate, log transformation [log(CFU +1)] was used to normalize the data.

Results

Transformation of P. rugulosum Strain IR-94MF1 and Its Mutants with the hph Gene

The minimal inhibitory concentration of HmB for strain IR-84MF1 was 100 μg mL^{-1}. Approximately 10^7 protoplasts were treated with 10–30 μg of undigested plasmid pPS57. After three trials, electroporation-mediated transformation of *P. rugulosum* IR-94MF1 yielded 0.7 HmB resistant colonies per μg of plasmid DNA in only one experiment (with 12 μg of plasmid DNA). Mutants Mps^{++} and Mps^{-} were able to grow in the presence of >100 μg HmB mL^{-1} and did not yield any potential transformants. Putative transformants obtained from the wild type had similar or very close morphology to that of strain IR-94MF1.

Genomic DNA was isolated from the wild-type strain and from three putative transformants derived from it. Both undigested DNA and DNA digested with *Sal*I, *Hind*III, or *Bgl*II were hybridized with linearized pPS57. There was no hybridization between the probe and DNA of wild-type strain IR-94MF1 and putative transformant w-T1. When undigested DNA from transformants w-T2 or w-T3 was probed with pPS57, hybridization occurred in the high-MW portion of genomic DNA. A similar pattern was observed for genomic DNA digested with *Bgl*II, which does not cleave pPS57. Digestion of DNA from strains w-T2 and w-T3 with *Hind*III, which cuts pPS57 once, resulted in complex and strain-specific hybridization patterns which included the 5.4 kb band corresponding to linearized pPS57, plus six and eight additional bands, respectively. Thus, both transformants w-T2 and w-T3 likely resulted from ectopic integration of tandem arrays of pPS57 at multiple chromosomal loci.

Characterization of Growth and Phosphate Solubilization of the Strains

After 7 days of incubation on complete medium, colonies of strains IR-94MF1, w-T2, and w-T3 exhibited similar radial growth, whereas the highest significant growth was measured with Mps^{-} mutant (Table 1). Growth of all strains on hydroxyapatite medium was statistically similar.

Table 1. Radial growth and phosphorus solubilizing activity of *P. rugulosum* strain IR-94MF1 (wild-type), its hygromycin B-resistant transformants (w-T2 and w-T3), and its mutants (Mps^{++} and Mps^{-}) after 7 days of incubation on complete medium (CM) or on hydoxyapatite medium (HAM)[a]

	CM		HAM	
Strain	Growth[b]	Halo[b]	Growth	Halo
IR-94MF1	7.0^{bc}	29.0^{b}	5.8^{a}	14.3^{b}
w-T2	7.0^{bc}	29.3^{b}	6.0^{a}	14.4^{b}
w-T3	6.7^{c}	28.5^{b}	5.8^{a}	14.5^{b}
Mps^{++}	7.5^{b}	42.5^{a}	5.8^{a}	19.5^{a}
Mps^{-}	8.3^{a}	23.5^{c}	6.0^{a}	nm

[a] Values are mean radius (mm) of three replicates. In the same column according to the LSD test, significant differences between treatments at $P < 0.01$ level are indicated by different letters; nm, not measurable.
[b] Complete medium containing bromphenol blue. Acidity production (pH ≤ 3) was detected under and around the colony by a yellow halo.

All strains were able to acidify the complete medium, as indicated by the production of a yellow halo caused when the pH of the medium was lower than 3. The acidity halo was significantly larger for the Mps^{++} mutant and smaller for mutant Mps^{-}, while no significant differences were observed between the wild-type (IR-94MFl) and the transformed strains (w-T2 and w-T3). When the insoluble phosphate source hydroxyapatite was used, Mps^{++} exhibited the largest solubilization halo and mutant Mps^{-} did not show any measurable one. Growth, production of acidity, and hydroxyapatite solubilization were very similar between the wild-type IR-94MF1 and the two transformants. Since transformant w-T3 produced spores that were morphologically similar to the green conidia produced by the wild-type IR-94MF1, this transformant was used in preference to w-T2 in the maize inoculation study.

Plant Inoculation Under Different Phosphate Sources

In general inoculation with the fungal strains tested affected both maize dry matter yields and P uptake, when a P source was added to the soil (Table 2). In the presence of the phosphate rock Navay, maize dry matter yield and total P uptake were significantly ($P < 0.01$) increased by inoculation with strains IR-94MF1 and w-T3. When P was added as Florida apatite mineral, all fungi used except Mps^{-} caused a significant ($P < 0.01$) increase in maize dry matter yield. With this P-source maize P uptake was similar in noninoculated plants and in plants inoculated with IR-94MF1 or the transformant w-T3, while significant ($P < 0.01$) increase in P uptake was found with both mutants, the Mps^{++} and the Mps^{-}. The application of SSP increased significantly ($P < 0.01$) the yield of maize with all inocula used, but only transformant w-T3 significantly ($P < 0.01$) increased the plant P uptake (Table 2). Control treatments (without the addition of P source) did not show any significant variation due to inoculation.

No significant interactions were observed between the different fungal inoculation treatments and the different P sources used. Regarding P sources, there was an increase of both dry matter production and P uptake in the following order: control < Navay < Florida < SSP, and control < Navay = Florida < SSP, respectively. At the end of the experiment soils pH ranged from 6.1 to 6.4 and were not significantly different.

Effect of Inoculation on Microbial Community Size and Colonization of the Maize Rhizosphere by Transformant w-T3

In the absence of any P-fertilization treatment, soil inoculation with the different strains of *P. rugulosum* significantly decreased the numbers of total and P-solubilizing bacteria in the rhizosphere of 5-week-old maize plants (Fig. 1), but did not affect the numbers of the total and P-solubilizing fungi (Fig. 2). In microcosms fertilized with the Florida apatite mineral or the Navay rock phosphate inoculation with strains IR-94MF1, w-T3 or Mps^{-} significantly enhanced the total rhizospheric bacterial community size (Fig. 1). The P-solubilizing bacterial population was stimulated by inoculation with IR-94MF1 or Mps^{++} when Florida was the P source used or with Mps^{-} with SSP. In microcosms receiving SSP mutant Mps^{-} also stimulated the total and P-solubilizing fungal communities (Fig. 2). Contrary to the observations made with bacteria, the community size of total fungi in maize rhizosphere decreased significantly by inoculation with strain IR-94MF1 in the presence of Florida mineral or SSP (Fig. 2).

Table 2. Effect of different phosphate sources on P uptake and shoot dry matter yield of 5-week-old maize plants growing under greenhouse conditions in microcosms inoculated with *P. rugulosum* wild-type strains IR-94MF1, its transformant w-T3, and its mutants Mps^{++} or Mps^{-}[a]

	P source							
	Control		Florida[b]		Navay[b]		SSP[b]	
Fungal strain	Dry matter (g)	P (mg)	Dry matter (g)	P (mg)	Dry matter (g)	P (mg)	Dry matter (g)	P (mg)
IR-94MF1	2.6[a]	4.3[a]	39[ab]	5.6[abc]	3.5[ab]	5.9[a]	8.6[b]	12.7[b]
w-T3	2.5[a]	4.0[a]	3.9[ab]	5.5[abc]	3.6[a]	6.5[a]	8.9[a]	14.5[a]
Mps^{++}	2.5[a]	3.9[a]	4.0[a]	6.2[a]	2.9[c]	4.8[b]	8.8[ab]	13.5[b]
Mps^{-}	2.7[a]	4.3[a]	3.7[abc]	6.0[ab]	3.1[abc]	4.8[b]	8.8[ab]	13.2[b]
Uninoculated	2.7[a]	4.3[a]	3.4[C]	5.0[c]	2.8[c]	4.7[b]	7.6[c]	13.4[b]

[a] Values are means of three replicates. In the same column, significant differences according to the LSD test at $P < 0.01$ level are indicated by different letters. Dry matter yield is expressed as g per microcosm and P uptake as mg per microcosm.
[b] Florida, apatite mineral; Navay, Venezuelan rock phosphate; SSP, ordinary superphosphate.

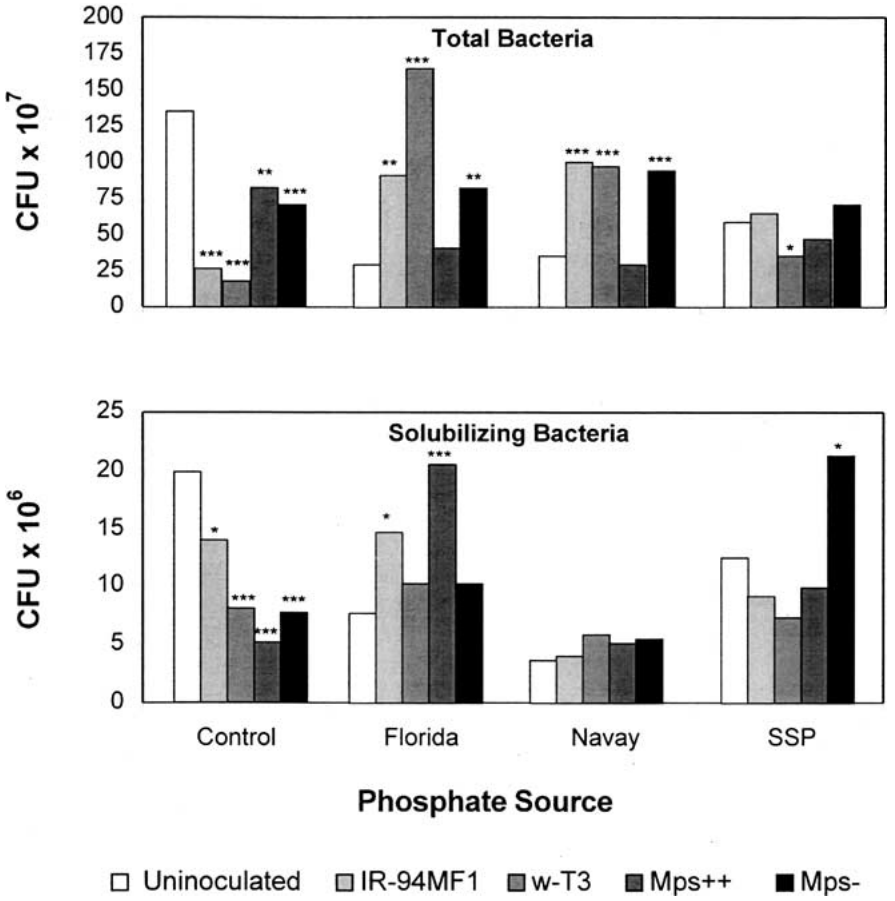

Fig. 1. Effect of inoculation with P. *rugulosum* strains IR-94MF1 (wild-type), w-T3 (transformant), Mps^{++}, and Mps^{-} and of different P sources on the number of total and P-solubilizing culturable bacteria present in the rhizosphere of 5-week-old maize plants. Values are means (CPU per g of dry soil) of three replicates significantly different from the respective uninoculated treatment at (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$, based on the LSD test. SSP, ordinary superphosphate.

Inoculation with the transformant w-T3 decreased significantly the total rhizosphere bacterial community size and both total and solubilizing fungi in microcosms fertilized with SSP. Inoculation with mutant Mps^{++} increased significantly the population of P-solubilizing fungi only when Florida mineral was used but it decreased significantly the size of the total rhizosphere fungal population in Florida and SSP treatments (Fig. 2).

The population size of the transformant w-T3 in maize rhizosphere soil was significantly ($P < 0.01$) higher when Navay rock phosphate was used as fertilizer as compared to the other P sources used (Table 3). In bulk soil, the addition of any P fertilizer did not affect ($P < 0.01$) the number of w-T3 transformant present. The highest R/S ratio for w-T3 was observed with Navay rock phosphate. In comparison to the nonfertilized control, addition of SSP reduced substantially the R/S ratio of w-T3.

Mitotic Stability of Transformant w-T3

Before its use for soil inoculation, *P. rugulosum* transformant w-T3 was grown vegetatively for at least five rounds under nonselective conditions and was found to have retained its resistance to HmB, as well as the multiple insertions of plasmid pPS57 (Fig. 3, lane 6). However, when two colonies of transformant w-T3 recovered randomly from the Navay and SSP treatments were analyzed, while they were still HmB resistant, they did not display the same hybridization pattern as the original transformant. Both colonies had lost copies of high molecular weight DNA (between 6 and 19 kb) of the tandemly integrated plasmid pPS57 (Fig. 3, lanes 7 and 8).

Discussion

Colonization of a soil by a nonindigenous microorganism depends both on its interactions with indigenous microbial populations associated with plants and its ability to utilize diverse substrates in the soil [3, 13]. The increase in the number of bacteria colonizing maize rhizosphere observed when the plant is inoculated with some fungal strains may be attributed, in addition to the P solubilized, to the release of other elements. Phosphate fertilization of uninoculated maize at a rate equivalent to 100 kg P/ha

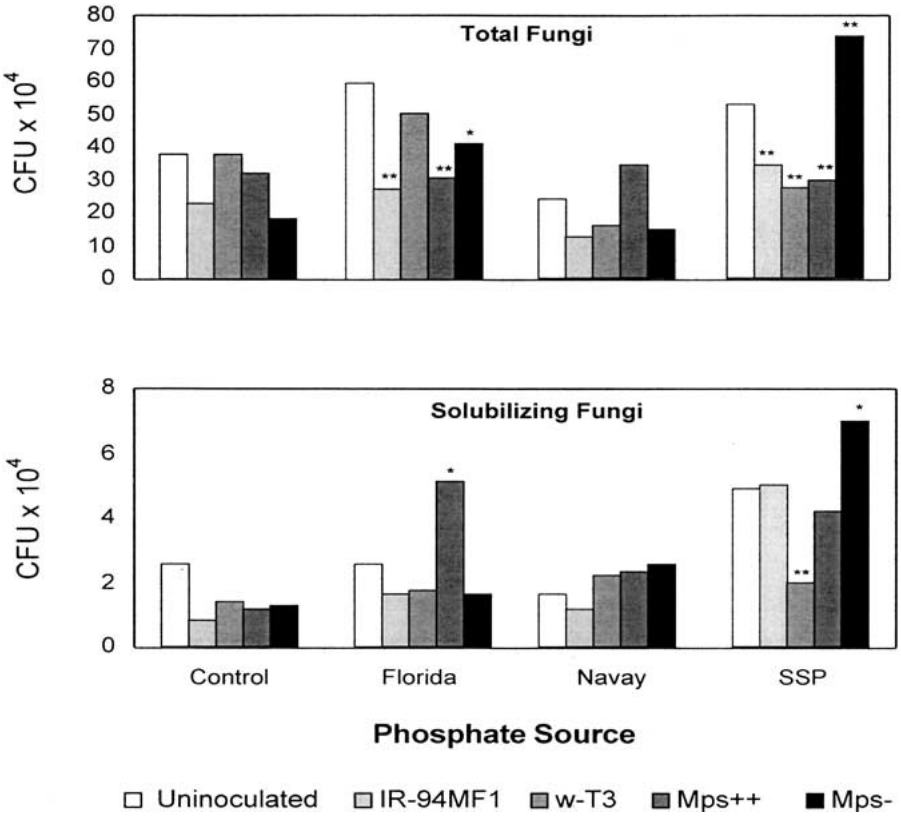

Fig. 2. Effect of inoculation with *P. rugulosum* strains IR-94MF1 (wild-type), w-T3 (transformant), Mps^{++}, and Mps^{-} and of different P sources on the number of total culturable fungi and P-solubilizing culturable fungi present in the rhizosphere of 5-week-old maize plants. Values are means (CPU per g of dry soil) of three replicates significantly different from the respective uninoculated treatment at (*) $P < 0.05$; (**) $P < 0.01$; based on the LSD test. SSP, ordinary superphosphate.

Table 3. Population of *P. rugulosum* transformant w-T3 in the rhizosphere and bulk soil of 5-week-old inoculated maize plants fertilized with different phosphate sources and growing in microcosms under greenhouse conditions[a]

Phosphorus source[b]	Rhizosphere	Soil w-T3 CFU × 10^3 g^{-1}	R/S ratio
Control	8.7[b]	1.9[a]	4.6
Florida	12.3[b]	5.4[a]	2.3
Navay	93.0[a]	11.7[a]	7.9
SSP	8.6[b]	9.9[a]	0.9

[a] Values are means of three replicates. Means in the same column followed by different letters are significantly different ($P < 0.01$) based on the LSD test.
[b] Florida, apatite mineral; Navay, Venezuelan rock phosphate; SSP, ordinary superphosphate.

showed a tendency to decrease the size of the rhizosphere bacterial communities (Fig. 1), but in some instances it had a positive effect on the size of fungal communities (Fig. 2). Fluctuations in soil bacterial or fungal counts following soil fertilization or amendments were previously observed. When a pastoral field in New Zealand was fertilized with North Carolina phosphate rock at rates up to 120 kg P/ha, both total bacterial and fungal counts declined significantly in fertilized plots [24]. Under greenhouse conditions, the bacterial populations of a dark red latosol used for tomato cultivation increased as a function of triple superphosphate application, while the fungal populations were not affected [10]. Inoculation of maize with the different *P. rugulosum* strains used in this study increased the dry matter yield of maize by 3.6 to 28.6% in comparison to the uninoculated treatments (Table 2). Other phosphate-solubilizing *Penicillium* species were also reported as plant growth-promoting fungi, such as *P. radicum* with wheat [30] and *P. bilaii* with wheat and canola [8, 9]. Stimulation of maize growth was also obtained by inoculation with phosphate-solubilizing bacteria under hydroponic [4] and field conditions [5].

Inoculation of maize with mutants Mps^{-} or Mps^{++} in the presence of the Florida apatite mineral increased plantp P uptake significantly (Table 2). Earlier work conducted *in vitro* showed that mutant Mps^{-} conserved at least the proton-extrusion mechanism for P solubilization, whereas mutant Mps^{++} had an enhanced production of citric and gluconic acids [19]. We have also previously observed [20] that the Florida apatite allowed the highest specific P solubilization activity (P solubilized g^{-1} fungal biomass) with both mutants. In fact, since Mps^{++} grow poorly on this P source, excess soluble P accumulates in

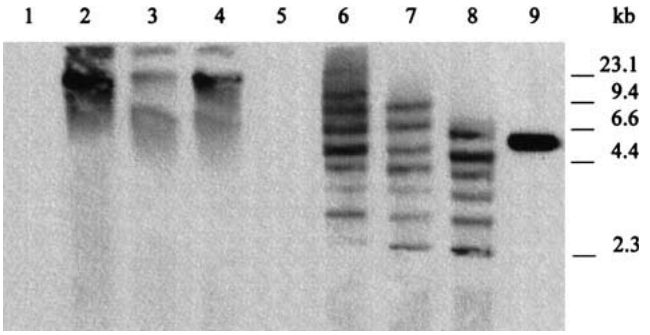

Fig. 3. Southern hybridization analysis of total genomic DNA extracted from *P. rugulosum* and digested with *Bgl*II (lanes 1–4) or *Hind*III (lanes 5–8). Wild-type strain IR-94MF1 (lanes 1 and 5); transformant w-T3 after at least five rounds of vegetative growth on non-selective medium (lanes 2 and 6); two colonies of the transformant w-T3 isolated from maize rhizospheric soil (lanes 3, 4, 7 and 8); and the pPS57 plasmid linearized with *Hind*III (lane 9). Molecular sizes are indicated in kb.

the culture filtrate of that mutant. On the other hand, even with its low P-solubilization phenotype the Mps^- mutant grows much better than the Mps^{++}, when the Florida material is the sole P source, and this higher biomass of Mps^- is accompanied with more P-solubilization activity and accumulation of excess P in the culture filtrate [20]. These observations and the significant increase of the P-solubilizing bacterial and fungal populations observed in the rhizosphere of maize fertilized with the Florida apatite and inoculated with the Mps^{++} mutant (Figs. 1, 2) can explain in part the increase of P uptake by maize plants inoculated with Mps^{++} and Mps^-. Fertilization with Navay rock phosphate and inoculation with Mps^- or Mps^{++} did not affect P uptake by maize plants (Table 2), which is in accordance with the lower specific P-solubilizing activities observed previously with these two mutants when compared to the Florida mineral [20].

The highest significant increase in P-uptake by maize (38%) was obtained by inoculation with the transformant w-T3 in the presence of the Navay rock phosphate (Table 2). This was associated with a dramatic and significant increase in the extent of rhizospheric colonization by this fungus (Table 3). As the P-solubilizing activity of w-T3 is comparable to that of the wild type strain IR-94MF1 (Table 1), which produced a 26% increase in P-uptake, the observed differences in P-uptake might be the result of the ability of w-T3 to colonize maize rhizosphere in high number in the presence of the Navay rock. Indigenous soil phosphate-solubilizing microbiota and arbuscular mycorrhizal fungi could also have played an important role in P uptake by maize, as indicated by the absence of difference in P uptake between the uninoculated control and some fungal inoculation treatments (Table 2).

Transformant w-T3 showed its highest degree of competitiveness toward the indigenous microbiota in the presence of SSP which resulted in a significant decrease in community size of total bacteria (Fig. 1), total fungi, and solubilizing fungi (Fig. 2). Moreover, for the SSP treatment, distribution of strain w-T3 was similar in both the rhizosphere and bulk soil, yielding a R/S ratio of 0.9. We suggest that production of organic acids by transformant w-T3 might have had a negative effect on the indigenous microfloral population rather than playing a role in phosphate solubilization, and that this allowed more P (+ 8.2%) to be assimilated into maize plants (Table 2). Other P-solubilizing microorganisms, such as *Aspergillus* sp. and *Penicillium* sp., were found to increase P uptake by sorghum in a calcareous soil treated with both a rock phosphate and triple superphosphate [22]. As soluble phosphates are already available to plants, the chelating action of organic acids could be directed to other soil substrates. In this sense, organic acids in soil have also been associated with biological control [11], as they may sequester Ca^{2+} and other important cations from microbial cell walls affecting pH of soil at the microsite level.

Addition of extra genes in microorganisms can affect their fitness under some conditions. The competitiveness of an organism may thus be increased or reduced by unknown effects on the genotype and also by disruption of the normal physiological processes as a result of the expression of novel traits [28]. Thus, the introduction of heterologous genes coding for extracellular lytic enzymes could improve fitness in HmB-resistant transformants through enhanced competitive ability against natural HmB-producing microbiota [14]. Here, the transformant w-T3 was more competitive than its wild-type progenitor, strain IR-94MF1, as evidenced by the fact that inoculation of strain w-T3 led to a significant decrease of the indigenous microbiota, as well as increased growth and P uptake by plants fertilized with SSP. However, the relative instability of the w-T3 genome, as evidenced by the loss of high-MW DNA sequences in HmB-resistant colonies reisolated from the rhizosphere of maize, makes this transformant unsuitable for more detailed analysis of MPS activity in soil. Unsuitability of this transformant comes from both unknown genotype and phenotype effects of revertants or the possible release of antibiotic resistance

genes in the environment. Instability of the integrated sequences was probably due to a lack of homology between the plasmid and the fungal genome, as it has already been reported by other authors [15]. Different vectors, harboring *P. rugulosum* sequences in addition to heterologous marker genes, may be required for further assessing the fate and activity of *P. rugulosum* in the soil and rhizosphere.

This work shows that it is possible, under greenhouse conditions, to benefit from the phosphate-solubilizing activity of *P. rugulosum* when an appropriate phosphate rock such as Navay is used. More field tests, particularly under tropical conditions, are required before any recommendation can be made on the beneficial use of *P. rugulosum* IR-94MF1 as an inoculant for maize fertilized with the Navay rock phosphate.

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