Abundance, diversity and prospecting of culturable phosphate solubilizing bacteria on soils under crop-pasture rotations in a no-tillage regime in Uruguay.

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Abstract

Phosphate solubilizing bacteria (PSB) abundance and diversity were examined during two consecutive years, 2007 and 2008, in a crop/pasture rotation experiment in Uruguay. The study site comprised five treatments with different soil use intensity under a no-tillage regime. In the first year of sampling, abundance of PSB was significantly higher in Natural Prairie (NP) and Permanent Pasture (PP) than in Continuous Cropping (CC); rotation treatments harbored populations that did not differ significantly from those in the others. The percentage of PSB relative to total heterotrophic bacteria ranged between 0.18% and 13.13%. PSB diversity also showed

statistical differences among treatments, with PP populations more diverse than those present in CC. In the second year sampled no differences were found in PSB abundance or diversity. Two hundred and fifty PSB were isolated in 2007 and classified according to their phosphate solubilization activity *in vitro*. Twelve of these isolates showing the greatest solubilization activity were selected for 16S rDNA sequencing. Ten isolates presumably belong to the genus *Pseudomonas* and two isolates showed high similarity with members of the genera *Burkholderia* and *Acinetobacter*.

Key words

Phosphate solubilizing bacteria (PSB); soil bacterial diversity; crop-pasture rotations; soil management; no-tillage; biofertilizer

1. Introduction

Phosphorus (P) is an essential element found in all living beings as part of proteins, nucleic acids, membranes and energy molecules such as ATP, GTP and NADPH. Usually, it is the second element limiting plant growth preceded by nitrogen, but depending on some environmental and biological factors it can be the main growth-limiting nutrient (Hinsinger, 2001). Even though some soils may have high levels of total P, they can still be P-deficient due to low levels of soluble phosphate available to plants (Gyaneshwar et al., 2002). Available P concentrations for maximum pasture production are estimated to be between 20 and 50 µg/g; higher concentrations are typically required for sandy soils because of slower diffusion rates (Mathews et al., 1997). Soil phosphate deficiency has been traditionally overcome by adding P-fertilizers (Tate, 1984; Khan et al., 2006). One of the drawbacks of fertilization is that only a fraction of the P added is eventually assimilated by plants, the rest becomes unavailable by forming complexes with either, Al, Fe, Ca or Mn depending on soil type (Rodríguez & Fraga,

1999). Generally a few days after fertilization, available phosphate levels can reach similar values to those before application (Sharpley, 1985). Overcoming these effects by adding fertilizers in excess is a practice that can lead to environmental problems such as water eutrophication (Correll, 1998) or accumulation of toxic elements present in the fertilizers (e.g. As and U) (Yamazaki & Geraldo, 2003). In order to meet current demands of food production, enhancement of plant growth through fertilization has provoked an intense scavenging of phosphorus mines worldwide; it is estimated that by 2060 these mines could be depleted (Gilvert, 2009). Many agricultural soils represent a phosphate sink where this element is not readily available to plants but may still be recovered.

Phosphate solubilizing bacteria (PSB) are a diverse group of unrelated bacteria able to readily solubilize sparingly soluble forms of P (Khan et al., 2006). This bacterial group is vital to the P cycle in soil and some of them may be used in order to enhance the availability of P in soil. Although there are numerous studies regarding the ecology of this group of soil bacteria (Kämpfer, 2002), information about the effect of agricultural practices on abundance and diversity of PSB is scarce. Understanding the effect of agricultural practices over soil populations of PSB is crucial to develop strategies aimed to guarantee productivity and sustainability of agroecosystems.

Crop/pasture rotation is a characteristic farming practice in Uruguay and Argentina, where it is more used than continuous cropping (García-Préchac et al., 2004). In this study, we analyzed the impact of continuous cropping and crop/pasture rotations on the abundance and diversity of PSB populations. We used a culture based approach to count both PSB and total heterotrophic bacteria. We isolated nearly 60 PSB per treatment and used two primers random amplified polymorphic DNA (TP-RAPD) as a fingerprinting method to study diversity. Available P levels in the soil from the field site where the

samples were taken was below those recommended for maximum pasture production. We also selected and characterized a group of PSB isolates that showed the greatest solubilization potential. Our hypothesis was that crop/pasture regime has an impact on both abundance and diversity of PSB and this could be depicted by differences of PSB numbers and diversity along a gradient of crop/pasture rotations.

2. Material and methods

2.1 Study site and soil sampling

In fall 2007 (May, 3) and 2008 (June, 3) soil samples were collected from a site belonging to the Instituto Nacional de Investigación Agropecuaria (INIA), located at Treinta y Tres, Uruguay (33°15'S, 54°29'W), in which a long term study is in progress since 1995. Soil type of the plots is a Typic Argiudoll, loam (41% sand, 35% lime, 24% clay), with 2.2% organic C, 0.22% N, pH=5.7 and 2.2-12.4 µg/g P Bray I. The experiment consists on 5 different regimens of no-tillage crop/pasture rotation which are: natural prairie (NP), consists of a previous agricultural field that was restored to a natural grassland; continuous cropping (CC), consists in two crops per year, *Avena sativa* (oat), *Lolium multiflorum* (Italian ryegrass) and *Trifolium alexandrinum* (berseem clover) in winter and *Sorghum bicolor* (sorghum) in summer; long rotation (LR), consists in 2 years identical to CC followed by 4 years of pasture composed of *Trifolium repens* (white clover), *Lotus corniculatus* (bird's foot trefoil), *Dactylis glomerata* (orchard grass) and *Festuca arundinacea* (tall fescue); short rotation (SR), consists in 2 years identical to CC followed by 2 years of pasture composed of *Trifolium pratense* (red clover) and *L. multiflorum*; and permanent pasture (PP), composed by *T. repens*, *L. corniculatus*, and *L. multiflorum*. All treatments are subjected to cattle grazing. Plots were arranged in a complete randomized design; three plots per treatment were sampled and two samples per plot were collected. Samples consisted on 15 randomly collected

cores (2x10 cm) mixed together. They were air-dried, sieved to 2 mm, and stored in plastic bags for no longer than two months at 4 °C.

2.2 Culturable heterotrophic bacteria and PSB counting

For each soil sample, 5g of soil were placed into 45 ml of 0.1% sodium pyrophosphate (NaPPi) (wt/vol) in 125-ml bottles and mixed for 30 min at 200 rpm in a gyratory shaker at 25 °C before being serially diluted. Aliquots of three dilutions were spread on 10-fold diluted tryptic soy agar (1/10 TSA) (Smit et al., 2001) and National Botanical Research Institute's Phosphate (NBRIP) (Nautiyal, 1999) media to enumerate culturable heterotrophic bacteria (CHB) and PSB, respectively. Both media contained 100 µg ml⁻¹ of cycloheximide to inhibit fungal growth. Plates were incubated at 28 °C for 8 days. Fast growing heterotrophic bacteria were counted at day 2 and slow growing heterotrophic bacteria were determined by counting those colonies that appeared up to day 8 but were not present at day 2. The number of PSB was obtained by counting colonies that formed a solubilization halo in NBRIP at day 8.

In order to express the bacterial populations as colony forming units (CFU) per g of dry soil, the moisture content of the samples was estimated by drying them at 100 °C during 48 h, to calculate the loss of weight.

2.3 Isolation and TP-RAPD analysis of PSB isolates

From the 2007 NBRIP plates we randomly picked 20 halo forming colonies per plot and streaked them on 1/10 TSA. These isolates were cultured in 10-fold diluted tryptic soy broth (1/10 TSB) at 30 °C in a gyratory shaker (200 rpm). Cultures were stored at -80 °C suspended in 25% glycerol (vol/vol). To extract DNA, a loopfull of culture was resuspended in 100 µl of 0.05 M NaOH and heated at 100 °C for 4 min. Samples were then placed in an ice bath and 900 µl of sterile water were added. After a centrifugation at 5000 g for 2 min, 700 µl of the supernatants were harvested and stored at -20 °C.

Four µl of these supernatants were used as DNA source for PCR reactions. PCR was performed using 1 U of GoTaq Taq polymerase (Promega, Belgium), 1× GoTaq buffer, 25 mM MgCl₂, 0.2 mM of each dNTP, 0.004% BSA (wt/vol), and 2 µM of each primer for a final reaction volume of 25 µl. The primers used were 879F (5'-GCCTGGGGAGTACGGCCGCA-3') and 1522R (5'-AAGGAGGTGATCCANCCRCA-3') which correspond to 16S rDNA *E. coli* positions 879-898 and 1509-1522 respectively (Valverde et al., 2006). PCR cycling program was as described by Rivas et al. (2001) except that the initial denaturation step lasted 3 min. Five µl of PCR product were electrophoresed in 2% agarose (wt/vol) in TAE buffer (40 mM Tris, 0.02 mM acetic acid, 1 mM EDTA, pH 8.0) at 6 V/cm during 2 h. Gels also included 0.04% ethidium bromide (wt/vol), and were photographed while exposed to UV light. At least one lane of each gel was loaded with 2 µl of molecular size markers according to manufacturer instructions (Standard VI; Boehringer-Roche, IN, USA). In order to facilitate comparison of TP-RAPD patterns, all samples from the same plot were casted in a single gel. Each TP-RAPD pattern obtained was considered an operational taxonomic unit (OTU) with which Shannon diversity (*H*) and evenness (*E*) indexes were calculated for every plot (Shannon & Weaver, 1949).

2.4 16S rDNA sequencing

Among those isolates exhibiting the largest solubilization halo, one representative strain from each TP-RAPD group was selected for taxonomic identification through 16S rRNA gene sequencing. The PCR amplification of 16S rDNA was performed using primers 8F (5'-AGAGTTTGTATCTGGCTCAG-3) and 1522R (Rivas et al., 2002). PCR mix content was identical to the one used for TP-RAPD except from primers concentrations which were 0.2 µM. PCR cycling program differed from TP-RAPD only in the annealing step which was controlled at 58 °C. Products were electrophoresed as

described previously and bands of the expected size were cut and purified using PureLinkTM gel extraction kit (InvitrogenTM) according to manufacturer instructions. Purified PCR products were sent to Macrogen Inc. (Seoul, Korea) for sequencing. Forward and reverse sequences of each sample were edited and pasted using the program DNA Baser V2.91.5.1292 (Heracle Software); the contigs were analyzed by BLAST at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> to identify the closest relatives of the isolates.

2.5 Semi-quantitative and quantitative phosphate solubilization assays

Initial inocula were grown overnight in 1/10 TSB at 30 °C in a gyratory shaker (200 rpm). Cultures were diluted with 0.9% (wt/vol) NaCl to 0.2 OD units (wavelength= 620 nm) and a drop of 10 µl of the adjusted inocula was plated on NBRIP plates and incubated at 28 °C for 6 days. The halo magnitude (*h*) was determined by subtracting the diameter of the colony from the diameter of the halo. Isolates were grouped into 5 classes according to their *h* score; class 0: lack of halo, class 1: *h*= 0-2 mm, class 2: *h*= 2-4 mm, class 3: *h*> 4 and class 4: *h*> 4 and a perfectly transparent halo. Class 4 includes a qualitative aspect of the halo, as halos of class 3 were as large as class 4 but rather diffuse.

Quantitative solubilization assay was conducted to the best solubilizers. Flasks containing 50 ml of NBRIP medium were inoculated with 50 µl of the adjusted inocula; one uninoculated flask served as control. They were incubated at 30 °C in a giratory shaker (200 rpm) for 7 days. Each isolate was tested in triplicate. Aliquots of 1 ml of the cultures were harvested at: 0 h, 12 h, 24 h, 48 h, 72 h, and 7 days after inoculation. Aliquots were centrifuged at 10.000 g for 2 min, and phosphate amounts in the supernatants were determined spectrophotometrically by the vanado-molybdate method (Bertramson, 1942).

2.6 Statistical analysis

Data were analyzed with the Statistica 8.0 (Statsoft) software. Normal distribution of the data was verified with Shapiro-Wilk's test. Homogeneity of variances were assessed by Hartley's, Cochran's and Bartlett's tests. Bacterial counts, phosphate levels, and Shannon's indexes means were compared by ANOVA and the Tukey's honestly significant difference (HSD) test, considering a significance level of $p < 0.05$.

3 Results and discussion

3.1 Culturable heterotrophic bacteria populations

Population ranges of slow and fast growing heterotrophic bacteria were similar for both years with minima of 2.0×10^6 CFU and 2.7×10^6 CFU g^{-1} dry soil, respectively; maximum values were 5.6×10^7 and 6.6×10^7 CFU g^{-1} dry soil for fast and slow growing bacteria, respectively. Fast growing heterotrophic bacteria populations were significantly higher in PP compared with CC, SR and LR, but only in the first year of sampling (Fig. 1); in that year NP had an intermediate value with no statistical difference with any treatment. The size of slow growing bacteria populations showed no significant differences between treatments. In the second year of sampling, the abundance of heterotrophic bacteria showed no statistical differences between treatments for both slow and fast growing bacteria.

The number of culturable heterotrophic bacteria, both fast and slow growing, found in all our samples fell well within the theoretical and practical limits reported in the literature (Kennedy, 1999). The samples from the first year showed differences between treatments for fast growing bacteria, those treatments with a more intensive soil use harbored less abundant populations of heterotrophic bacteria. However no differences were found in the second year. These results are in accordance with those obtained by Crecchio et al. (2004) who analyzed total heterotrophic bacteria in a study comparing

intensive horticulture vs. organic farming and found differences only in one out of three sampling dates. However other reports show no differences between the treatments studied. Schutter et al. (2001) using direct counting by microscopy did not find differences between crop rotation systems, although they did find them between seasons. Other studies support the idea that the total number of heterotrophic bacteria is not sensitive to crop rotations, whereas both mycorrhizal fungi and protozoa populations as well as enzymes activities can be affected by rotations (Pankhurst et al., 1995). The number of CHB is only one aspect of the population, other parameters such as structure and diversity may be affected by the crop/pasture rotations as other works on the effect of soil management on bacteria conclude (Ceja-Navarro et al., 2010). It is noteworthy that the cultivation approach to study bacterial populations has a strong bias since only a small proportion of the total bacteria present in a soil sample can be cultivated (Ward et al., 1990). Thus, the results obtained are certainly distorted by the fact that the majority of the bacteria present in the samples are unaccounted for. Even though this bias is unquestionable, it has been proposed that many unculturable bacteria may not be ecologically relevant and that the culturable proportion of the population represents most of the biomass and activity (Ellis et al., 2003). Slow growing bacteria population sizes were not statistically different between any treatments. This group of bacteria is considered by some authors as k-strategists (van Elsas et al., 2002), and as such, they should be more sensitive to changes in the environmental conditions. Our result, challenge the idea of recognizing slow growing bacteria as k-strategists, at least with the culture conditions used.

3.2 Phosphate solubilizing bacteria populations

Populations of PSB ranged from 6.5×10^4 to 6.2×10^6 CFU g⁻¹ dry soil. For the first year, populations in CC were significantly lower than those in NP and PP, while SR and LR

harbored population sizes that did not differ significantly from those in any other treatment (Fig. 2). On the second year no significant differences between treatments were observed.

The percentage of PSB relative to total heterotrophic bacteria (fast and slow growing) ranged between 0.33% and 8.65% with a mean of 2.90% in 2007. Whereas there were no statistical differences between treatments, the highest mean percentage was observed in NP. In 2008 the range of percentages was wider, from 0.18% to 13.13% and with a mean of 3.80%; both extreme values occurred in LR which also showed the highest mean. There were also no statistical differences between treatments.

All isolates were tested for semi-quantitative phosphate solubilization activity. In 2007, we classified 250 isolates according to the diameter of their solubilization halo. Most of the isolates (91) were classified as class 1, whereas 24 belonged to class 4 (i.e., those exhibiting the largest and clearer solubilization halo). Sixty eight isolates were categorized as class 2 and 47 as class 3; only 20 showed no apparent halo. The next year we obtained 242 isolates; surprisingly only 14 of them were classified as class 1 accounting for the less abundant class that year. Seventy four isolates were classified as class 2 and 41 as class 3. There were 71 isolates within the class of greatest solubilization potential, three times more than the previous year. The remaining 42 isolates were classified as those with the least solubilizing potential.

We observed PSB in all of our samples, and their number were always well above the detection limit (i.e. $>1 \times 10^2$ CFU g⁻¹ dry soil). On the contrary, Fallah (2006) reported numbers below the detection limit in 6% of their samples. Nonetheless, in their remaining samples the numbers of PSB were similar to those found in this study.

The percentage of PSB found are well below the maximum (40%) reported by van der Heijden et al. (2008). Nevertheless, other works registered percentages of PSB similar

to ours (Fallah, 2006). Undoubtedly, the medium used to count PSB selected for those bacteria that can grow on glucose as sole carbon source and solubilize $\text{Ca}_3(\text{PO}_4)_2$, omits PSB unable to use these nutrient sources but that may still play an important role in phosphate solubilization in soils. Using hydroxyapatite as an insoluble phosphate source Reyes et al. (2006) found an average of 19% of PSB. The difference with our results can be explained by the fact that their study site was an abandoned phosphate mine where soil P concentration was higher than in unmined soil. On the other hand, the total numbers of PSB found by Reyes et al. (2006) are similar to those found by us. Our results indicate that population sizes of PSB were statistically different between treatments only in one year. Santa Regina et al. (2003) found differences in PSB abundance on soils with different plant cover composition in three seasons but did not find so in fall, the season in which we sampled. Also, Hu et al. (2009) found differences in the abundance of PSB in plots under different fertilization regimes and concluded that P input increased PSB population size. This increase was more pronounced in plots where organic manure was added than where inorganic fertilizer was used; the same result was obtained for organic phosphorus mineralizing bacteria.

3.3 Diversity of Phosphate solubilizing bacteria

Since there is no existing, culture independent technique to study PSB, we used TP-RAPD, a simple and tested fingerprinting method, to characterize isolates of PSB in a taxonomically meaningful way. TP-RAPD fingerprints have an advantage for grouping purposes because strain dependent variations are minimal. Thus, strains belonging to the same bacterial taxon (species/genus) share a unique band pattern, which, in turn, is different from that of other bacterial taxa (Rivas et al., 2001). Therefore, for identification purposes, this technique is a good tool for grouping bacteria in order to select representative strains for 16S rRNA gene sequencing, as demonstrated with a

broad range of soil eubacteria (Rivas et al., 2002; Valverde et al., 2006; Velázquez et al., 2008).

Considering each TP-RAPD pattern as a distinctive OTU, we were able to compute Shannon diversity and evenness indexes for every plot (Fig. 3). In this way, we obtained three values of each index for every treatment. For the samples collected in 2007 we found statistical differences between PP and CC for both H and E , both being higher for PP (Table 1). SR diversity index was also significantly lower than PP in that year; evenness, however, was not statistically different between this and any other treatment. LR and NP indexes showed intermediate values and did not differ significantly with any treatment.

Results from samples taken the second year showed a similar trend but showed no significant differences, both for H and E indexes. The highest H mean was obtained for PP and the lowest for SR, whereas for E PP showed the highest value and LR the lowest.

Our results for the first year of sampling showed that PP had the highest diversity and evenness indexes, while the lowest values for both indexes were found in CC. Thus, the treatment with less intensive soil use had the highest values and vice versa.

Nevertheless, that was not the case for the second year, when there were no significant differences between any treatments. Although we were looking for long term effects of crop rotation on PSB diversity, it is impossible to disregard short term effects. Among short term effects on bacterial diversity, the rhizosphere effect plays an important role in which different plant species create different conditions mainly through their exudates (Smalla et al., 2001). This could have been verified if we had found the lowest indexes values in plots with only one plant species present, but that was not the case. Other works show that there can be little or no correlation at all between plant composition

and bacterial soil community (Jangid et al., 2011). It is probable that long term effects are playing an important role in defining PSB diversity. Since the structure of the PSB population was not elucidated in this work, it is possible that the community of PSB had undergone changes in its composition not apparent in the diversity index.

To our knowledge, there are not many articles addressing the effects of agricultural practices in PSB diversity. Kundu et al. (2009), using colony description, antibiotic resistance profile and biochemical tests, studied the diversity of PSB in the rhizosphere of three plant species. They found significant differences both in diversity and abundance of PSB between the three rhizospheres analyzed.

The absence of significant differences on the second year is in concordance with the other biological parameters measured in this study; i.e. abundance of CHB and PSB.

From the agrometeorological data available, we observed that in the 30 days prior to sampling, rainfall was similar for both years (153mm in 2007 and 176mm in 2008) but cardinal temperatures were lower in 2008 (13.5, 18.6 and 23.7 °C for average minimum, medium and maximum temperatures in 2007, and 8.3, 14.3 and 20.2 °C in 2008). Lower temperatures could have affected populations in a way that lowered them to similar values.

In a study conducted by Zerbino et al. (2008) in this same field experiment, the authors found that the richness of morphoespecies of Oligochaeta was significantly higher in PP than in any other treatment but significantly lower in NP. Even though we also found the highest value of diversity for PSB in PP, the relationship of these results remain unclear.

3.4 16S rDNA sequences of selected isolates

Twelve isolates with the greatest solubilization potential and with different TP-RAPD fingerprint were selected to determine their 16S rDNA sequences. When those

sequences were compared with the GeneBank (NCBI) database, 10 out of the 12 isolates belong to the genus *Pseudomonas*. The remaining two isolates showed highest similarities with sequences of *Burkholderia* sp. and *Acinetobacter calcoaceticus* (Table 2). Isolates G10.5 and E8.7 showed 100 and 99% of similarity, respectively, with the same sequence of *Pseudomonas* sp. (Accession no. EU003535.1), indicating that this two isolates are very closely related. Isolates E3.6 and A10.1 also showed 100 and 99% of similarity, respectively, with the same sequence of *Pseudomonas rhodesiae* (Accession no. FJ462694.1), evidencing the relatedness of these two isolates. The genus *Pseudomonas* has been extensively studied and used as a plant growth promoting rhizobacteria (PGPR) (Walsh et al., 2001), and it is known for having members able to synthesize phytohormones, act as biocontrol agents, and solubilize phosphate (Jha et al., 2009). The primacy of this genus in our isolates could be due to the amenability of pseudomonads to grow on culture. Other bacteria not cultivated could still be of paramount importance in phosphate solubilization in our soils. Nevertheless, *Pseudomonas* appears as a very promising group for its proven suitability as a potential inoculant. Another bacterial species with a well-known record of phosphate solubilization activity is *Acinetobacter calcoaceticus*, which has been mainly isolated from activated sludge (Ohtake et al., 1985). Also, genes from *A. calcoaceticus* have been used to complement phosphate solubilizing activity in *E. coli* (Liu et al., 1992). *Burkholderia* spp. have also been extensively cited as PGPR and phosphate solubilization is a common feature of many *Burkholderia* strains (Kämpfer, 2002). Thus, all the PSB isolates whose 16S rDNA have been sequenced in this work are members of genera with well-studied PSB.

3.5 Quantitative phosphate solubilization assay

Five isolates, among those classified as the best solubilizers in 2007, were tested quantitatively for solubilization ability in liquid culture. At 12 h after inoculation the concentration of soluble phosphate was significantly lower in the C10.6 culture than in any other (Table 3). Twenty four hours after inoculation the concentration of soluble phosphate was significantly higher in the E2.2 culture than in any other, and significantly lower again in C10.6 compared to the rest. The amount of phosphate in solution in E2.2 culture dropped at 48 h but was still significantly higher than that in C10.6 and F4.6 cultures. At 72 h the values obtained remained basically constant compared to those at 48 h, although the slight increase of P in C10.6 was enough to render it statistically equal to the other cultures except from F4.6. Seven days after inoculation P in solution was significantly higher in A10.1 compared to all other isolates except from E3.6; surprisingly, E2.2 showed the minimum value at this time being statistically significant lower than A10.1 and E3.6.

It has been reported that solubilization halo measurements is not always the best way to discriminate those isolates that would eventually have the greatest solubilization potential. Even more, there is not always a correspondence between solubilization ability in plate and in liquid culture (Baig et al., 2010). With that in mind, we still used solubilization plate assay to narrow our selection of candidates as best solubilizers. The values of P in solution obtained for our isolates are in accordance with those of other studies such as Son et al. (2006), who obtained concentrations of about 200 µg/ml of soluble P after 5 days of incubation at 30 °C. Vázquez et al. (2000), when analyzed PSB isolates from mangroves, found concentrations of soluble P between 60 and 450 µg/ml after 24 h of incubation. The similarities in the soluble P ranges with these two cited works are difficult to interpret since other media were used to test the isolates.

The observed decline in soluble P after 24 h of incubation for all isolates but C10.6 and the following stasis of its level for all treatments, are consistent with growth in batch culture and have been observed in other works (Malboobi et al., 2009). Although we did not count cells at each point, it is assumable that when the cell growth rate was the highest they were depleting the P in solution. Other possible explanation for this effect is that observed by Delvasto et al. (2006); they found that concentration of soluble P reaches a point when it can recrystallize and became insoluble again. This situation is unlikely to happen in the rhizosphere where continuous plant demand for P will keep concentrations of soluble P low enough to prevent recrystallization.

4 Concluding remarks

Both PSB and CHB populations were affected by crop rotations only in one of the two years sampled. In 2007 they had a tendency towards being more abundant and diverse in those treatments with less intensive use of soil (NP and PP). Differences between soil management were not significant in 2008, perhaps due to interannual variation in other environmental effects such as temperature. These results illustrate the need for multi-year studies to more fully assess the interaction of environmental variation and soil management.

The five isolates tested for solubilization in liquid media showed promising potential for future use as inoculants. Growth parameters such as dry weight or P content of plant tissues inoculated with PSB should be assessed in order to determine if these strains can indeed improve P acquisition by plants. In case at least one isolate is capable of promoting plant growth, many other aspects should be taken into account before its use as inoculant can be recommended; these include industrial amenability, environmental safety and compatibility with other soil beneficial microflora such as rhizobia.

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Legends

Figure 1. Culturable heterotrophic bacteria counts from soil samples of different treatments. Slow and fast growing heterotrophs are shown for both years. Values are the means (n=6) and error bars indicate standard deviation. On each group of bars, different letters indicate significant differences ($p < 0.05$, Tukey's HSD test).

Figure 2. Phosphate solubilizing bacteria counts from soil samples of different treatments. Values are the means (n=6) and error bars indicate standard deviation. For each year, different letters indicate significant differences ($p < 0.05$, Tukey's HSD test).

Figure 3. TP-RAPD fingerprints of the isolates from one replicate plot of NP. From left to right, lanes are: molecular weight ladder, isolates A8.2, A9.5, B6.2, B9.5, C6.2, C9.5, D6.2, D9.5, E6.2, E8.5, E9.5, F6.2, F8.5, F9.5, G5.2, G6.2, G8.5, H6.2 and H8.5. Only samples lanes are numbered. Those isolates with a common fingerprint are: A8.2 (lane 1) and B6.2 (lane 3), C6.2 (lane 5) and D6.2 (lane 7), and G8.5 (lane 17) and H8.5 (lane 19). The high intensity band present in every fingerprint corresponds to the 16S rRNA gene fragment which also provides reference.

Table 1. Shannon diversity (H) and evenness (E) indexes for each treatment at each year. Means of three values per treatment are shown. Numbers in parenthesis indicate standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$, Tukey's HSD test).

Table 2. 16S rRNA gene sequences, and their closest relative, of selected isolates from the most promising phosphate solubilizers group. The columns show: the name of our isolates; the NCBI accession number assigned to the sequences of our isolates; the length of the sequence obtained and contrasted; the closest organism to our isolates according to the NCBI database, and its accession number in parenthesis; and the percentage of similarity found.

Table 3. Amount of soluble P (µg/ml) in liquid media after inoculation with PSB.

Five strains were selected to evaluate their ability to solubilize phosphate in liquid media. Values shown are the means of three replicates. Numbers in parenthesis indicate standard deviation. Different letters in the same column indicate significant differences (p<0.05, Tukey's HSD test).

569 **Tables**

570 **Table 1.**

Treatment	<i>H</i>		<i>E</i>	
	2007	2008	2007	2008
Continuous Cropping	0.872 (0.105) b	0.946 (0.095) a	0.901 (0.042) b	0.938 (0.027) a
Short Rotation	0.874 (0.083) b	0.777 (0.036) a	0.910 (0.042) ab	0.888 (0.053) a
Long Rotation	1.006 (0.130) ab	0.879 (0.189) a	0.963 (0.017) ab	0.885 (0.073) a
Permanent Pasture	1.175 (0.018) a	1.007 (0.125) a	0.983 (0.011) a	0.959 (0.037) a
Natural Prairie	1.068 (0.047) ab	0.971 (0.128) a	0.970 (0.006) ab	0.941 (0.014) a

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572

573 **Table 2.**

Isolate	NCBI accession no.	Sequence length (bp)	Closest organism (NCBI accession no.)	Similarity (%)
A10.1	HQ412501	1399	<i>Pseudomonas rhodesiae</i> (FJ462694.1)	99
D5.2	HQ412502	1407	<i>Pseudomonas fuscovaginae</i> (AB021381.1)	99
D7.2	HQ412503	1398	<i>Pseudomonas fluorescens</i> (GU726880.1)	99
E2.2	HQ412504	1404	<i>Pseudomonas</i> sp. (AY236959.1)	99
E3.6	HQ412505	1385	<i>Pseudomonas rhodesiae</i> (FJ462694.1)	100
E8.5	HQ412506	1399	<i>Pseudomonas fluorescens</i> (HM439651.1)	99
E8.7	HQ412507	1402	<i>Pseudomonas</i> sp. (EU003535.1)	99
F4.2	HQ412508	1222	<i>Pseudomonas cedrina</i> (GU784931.1)	99
F4.6	HQ412509	1397	<i>Pseudomonas</i> sp. (EF427849.1)	99
G10.5	HQ412510	1396	<i>Pseudomonas</i> sp. (EU003535.1)	100
H5.7	HQ412511	1390	<i>Burkholderia</i> sp. (FJ791164.1)	99
E8.6	HQ412512	1219	<i>Acinetobacter calcoaceticus</i> (AY346313.2)	99

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575

576 **Table 3.**

Isolate	12 h	24 h	48 h	72 h	168 h
A10.1	91.7 (4.2) a	262.8 (4.0) b	228.2 (3.5) ab	232.1 (1.7) a	238.5 (16.3) a
C10.6	40.6 (1.4) b	180.6 (15.0) c	219.0 (3.3) b	222.9 (1.9) a	204.3 (2.9) bc
E2.2	71.4 (13.1) a	410.4 (13.0) a	236.0 (12.0) a	228.0 (9.2) a	200.2 (11.0) c
E3.6	93.9 (16.3) a	257.9 (3.0) b	231.8 (1.6) ab	233.7 (7.2) a	230.7 (9.2) ab
F4.6	78.8 (8.6) a	246.2 (3.5) b	216.7 (4.9) b	217.0 (6.8) b	206.9 (5.6) bc

577

Figure 1
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Figure 2
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