Survival of a Phosphate Solubilizing Microorganism in Ion-Sterile Carriers

F. Er, M. Ogut*

Selcuk University, Cumra Meslek Yuksekokulu, Cumra-Konya, Turkey

Abstract

A cold-tolerant phosphate solubilizing bacterium (PSB) was isolated from roots of ryegrass (*Lolium perenne* L.). Studies involving phosphate solubilization in liquid culture and survival of the PSB in non-sterile zeolite, leonardite, peat, rock phosphate, and an organic fertilizer were performed. The PSB was able to dissolve 163 ppm P with a simultaneous fall in pH (from 7.7 to 5.7) in Pikovskaya's medium during a ten-day incubation. The number of PSB declined logarithmically in 28 ^{o}C incubation regardless of the carrier. The rate of decrease in PSB population was less pronounced in zeolite. However, the PSB's population density increased up to 10^9 cfu g^{-1}, and stayed in the range of 10^8 to 10^9 cfu g^{-1} in zeolite and rock phosphate after 13-weeks of storage at +4 ^{o}C. The contaminant microorganisms also grew in the carriers, with population densities ranging between 10^8 to 10^9 cfu g^{-1} at week-9. The suppression of the local microorganisms is required to increase the quality of organic fertilizer by the addition of PSB. Zeolite could be a good carrier, due to its large surface area and porosity, which allow high number of microorganisms to occupy.

Introduction

Phosphorus (P) is an essential plant nutrient. However, most of the soil-P is in hardly available forms to plant, thus water soluble P-fertilizers are commonly used in agriculture. Even then, supplemental ionic phosphate (HPO_4^{-2} and $H_2PO_4^{-}$) undergoes a serious of reaction, finally precipitating as Ca- and Mg-phosphates in alkaline soils; and Fe- and Al-phosphates in acid soils. Furthermore, these ions are adsorbed to surfaces of soil constituents, another way within which the availability of additional-P is reduced.

The isolation of the soil microorganisms with mineral phosphate solubilization ability, and the possibility of using these microbes to enhance P-availability in soil lead to many studies worldwide (Babu-Khan *et al.* 1995; Geonadi *et al.* 2000, Gyaneshwar *et al.* 1999; Kim *et al.* 1997; Kim *et al.* 1998; Krishnaraj & Goldstein 2001; Kumar 1999; Kumar & Narula 1999; Liu *et al.* 1992; Nautiyal *et al.* 2000; Reyes *et al.* 1999; Rodriguez *et al.* 2000; Seshadri *et al.* 2000; Toro *et al.* 1997; Vasssilev *et al.* 1997; Vassileva *et al.* 1998). The mechanism, by which the microorganisms solubilize the hardly soluble-P has generally been recognized as therelease of organic acids and subsequent dissolution of tri-calcium phosphates (Babu-Khan *et al.* 1995; Gyaneshwar *et al.* 1999; Krishnaraj *et al.* 2001; Liu *et al.* 1992; Rodriguez *et al.* 2004). Although many organic acids have been found in microbial cultures, the most pronounced one was gluconic acid.

Following soil- or seed-inoculation, the phosphate solubilizing microorganisms are expected to solubilize P in the rhizosphere (the surrounding of the root, which has a higher microbial activity than the bulk soil due to plant root exudates, usually in couple of mm's in length). Therefore, the solubilized-P in this manner could be readily taken up by plants, without being affected from the soil processes, which decrease the P-solubility. Consequently, the plants, which are inoculated with phosphate solubilizing microbes can accumulate higher amounts of P and dry matter than the uninoculated control plants (Chabot *et al.* 1998; Çakmakçı *et al.* 1999; Gaind & Gaur 2002;

*corresponding author. Email: mogut@selcuk.edu.tr

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Narula *et al.* 2000; Pal 1998; Peix *et al.* 2001; Rojas *et al.* 2001; Sundara *et al.* 2002; Vassileva *et al.*1999).

Production of organic fertilizer is increasing, parallel to expending organic-farming in Turkey. Organic fertilizer may contain organic matters such as peat or leonardite; urea, an organic-N, which is dissociated to ammonium (NH_4^+) following soil application; rock phosphate; aluminosilicate minerals such as zeolite; and trace amounts of humic substances, enzymes and vitamins. Introduction of a phosphate solubilizing microorganism into a non-sterile environment, which could be either organic fertilizer as a whole or a component of it, has almost not been experimented. The objective of this study was to determine the suitability of using non-sterile zeolite, leonardite, peat, rock phosphate and organic fertilizer as a carrier for a phosphate solubilizing microbe.

Materials and methods

Isolation of the phosphate solubilizing bacterium (PSB)

Root samples of ryegrass (*Lolium perenne* L.) under a snow cover were carefully excavated and placed in a sterile bag on ice and transferred to the laboratory in January, 2003. Sampling was done in a mountainous area (40 20'N, 36 36'E) in Tokat-Turkey. The collected root samples were washed with sterile water to remove soil adhering to the roots. The soil-free roots were cut into 1-2 cm pieces by a sterile lancet. The root pieces were surface sterilized by dipping in 95% ethanol for 2 min, then 1% sodium hypochlorite (NaOCl) for 1 min followed by 6 washes in sterile distilled water. They were placed aseptically in a phosphate solubilizing medium containing (1000 ml^{-1}); glucose, 5.0 g; finely ground rock phosphate (31% P_2O_5), 5.0 g; 0.5% alcoholic bromthymol blue, 2 ml; agar, 15 g; pH≈7.0. The cultures were grown at 30 ^oC for 72 h.

Colonies, which rapidly acidified its surrounding were regarded as a phosphate solubilizer. In fact, all colonies in the isolation plate were of the same morphology. The PSB was aerob, gram (-) cocci. It produces green fluorescent pigment under a short wavelength (245 nm) of ultraviolet light and utilizes glucose, malate, and mannitol as the sole source of carbon. The bacterium was able to grow at +4 ^oC, but not at 40^oC.

Mineral phosphate solubilization in liquid culture

A 50 µL of an overnight culture of the PSB was dispensed into each of three 250 ml flask, each containing 50 ml Pikovskaya's medium (Nautiyal, 1999). The cultures were grown at 30 ^oC in a shaking water-bath at 200 rpm for 10 days. The samples were withdrawn aseptically for soluble-P and pH at days 0, 1, 3, 5, 7, and 10.

Survival of the PSB in non-sterile carriers

The selected finely ground carrier materials were *i*.) zeolite, *ii*.) leonardite, *iii*.) peat, *iv*.) rock phosphate, and *v*.) organic fertilizer, which contained (%); leonardite, 58.6; urea, 12.6; rock phosphate, 24.0 (29 % P_2O_5); and in trace amounts of humic substances, vitamins, and enzymes. An overnight bacterial culture and non-sterile materials were mixed in a sterile bag for 2 min in a stomacher at 250 compressions min^{-1}. The bag was then loosely closed. A 5 ml pipet was inserted into a small hole made at the corner of each bag and taped to allow air inlet. The control treatments were run only for rock phosphate and organic fertilizer, which received either sterile water or phosphate solubilizing medium with autoclaved cells of the PSB. The initial moisture contents (v/w) were 45 % for zeolite/rock phosphate and 65 % for leonardite/peat/organic fertilizer. Initial population density of the PSB was 10^9 cfu g^{-1}. Each treatment had three replications.

The sample containing bags were placed in an incubator at 28 ^oC (± 1 ^oC) and a cooler at +4 ^oC (± 1 ^oC). They were removed 1, 5, 9, and 13 weeks post the start of the storage for bacterial/fungal counts and pH determinations. Available-P analyses were performed only at weeks 1, 5, and 9. After 13 weeks, while the storage of the samples at 28 ^oC was discontinued, the storage of the samples at +4 ^oC was followed for additional 31 weeks at +4^oC and further 8 weeks at +8 ^oC, making a full year round. Only bacterial and fungal counts were performed in one year old samples.

Bacterial/fungal counts

A serial dilution of one g sample from each material was aseptically prepared in sterile saline solution (8.5% NaCl). A spread plate technique

(Zuberer, 1994), which was performed by a 48-72 h aerobic incubation at 28 ^{o}C, was used in all counts. The PSB colonies (orange, punctiform, entire, and convex under daylight), which acidified the medium, rapidly changing the medium's color from green to yellow were easily visible after 24 h of incubation. The colonies of the PSB also produce a fluorescent pigment visible under short wavelength (245 nm) of UV-fluorescence light, another criterion that was used to differentiate the PSB from contaminants. Fungal contaminants were counted on potato dextrose agar (PDA).

Soluble-P

Soluble-P analyses were performed principally by the method of Olsen & Sommers (1982). One g sample from each carrier was aseptically transferred into a 50 ml glass container, which contained 25 ml of 0.5 *M* sodium bicarbonate ($NaHCO_3$) solution. After 30 min of shaking at 200 rpm, the content of the container was filtered through Whatman no. 40. A ten ml of the filtrate was dispensed into a 50 ml flask, which then was supplied by a volume of sulfuric acid (5 *N*) enough to bring the pH of 10 ml of 0.5 *M* sodium bicarbonate solution to 5. An eight ml of a reagent containing (1000 ml^{-1}): $(NH_4)_6Mo_7O_{24}.4H_2O$, 6 g; $KSbO.C_4H_4O_6$, 0.1454; ascorbic acid, 5.28 g; H_2SO_4, 74 ml was dispensed into the flask and the volume was brought to 50 ml by distilled water. The absorbance values for the blank, standards, and samples were read 10 minutes after the reagent was added at a spectrophotometer adjusted to 882 nm.

pH

pH-measurements were made on a pH-meter equipped with a pH-electrode principally by the method of Hendershot et al. (1993). The ratio of sample to diluent (water) was 1:2 for mineral materials (zeolite and rock phosphate) and it was 1:5 for organic materials (leonardite, peat, and organic fertilizer). The suspension was mixed with a glass rod shortly for every 10 min for a period of 30 min. The pH of the suspension was read 30 sec after the pH-electrode was immersed into the suspension.

Statistical Analysis

The treatment effects were partitioned by one way-ANOVA. The significant differences among the treatment means were determined by Duncan's Multiple Range Test. All statistical analyses were performed by SPSS (V 10.0).

Results and discussion

Mineral Phosphate Solubilization in Liquid Cultures

The solubilized-P in the shaking flasks increases up to 93 ppm P within 24 h, after which it becomes relatively steady until day-5 (Figure 1). Another rise is observed in available-P after day-5, reaching 163 ppm P at day-7. The pH of the medium falls 5.7 (initially 7.7) within 24 h and it remains relatively constant afterwards (Figure 1). The initial increase in available-P coincided with the decrease in pH, suggesting presence of organic acid(s) produced by the PSB. Generally, the phosphate solubilization trait has been associated with direct oxidation pathway in gram (-) bacteria (Babu-Khan et al., 1995; Krishnaraj et al., 2001; Liu et al., 1992). Glucose is converted to gluconic acid in excess by glucose dehydrogenase in periplasm of the gram (-) bacteria. Gluconic acid released into the bacterial surroundings solubilizes hardly soluble-P in tri-calcium phosphate.

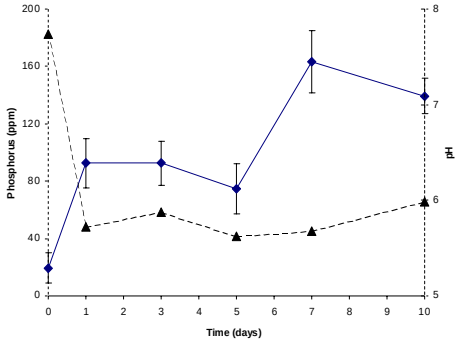

Fig. 1. The solubilized phosphorus (◆) and pH (▲) in the PSB's culture. The values are means of three replicates and the bars represent SE.

Survival of the PSB in non-sterile carriers

The number of PSB slightly increased in zeolite, leonardite and rock phosphate in 28 ^{o}C incubation within the first week (Figure 2). However, the population density initially decreased in the samples stored at +4 ^{o}C. While the initial increases at 28 ^{o}C are attributable to the secondary growth in the carriers, which contain small number of local populations, the larger initial decreases at +4 ^{o}C are attributable to the abrupt change in temperature (from 28 ^{o}C to +4 ^{o}C).

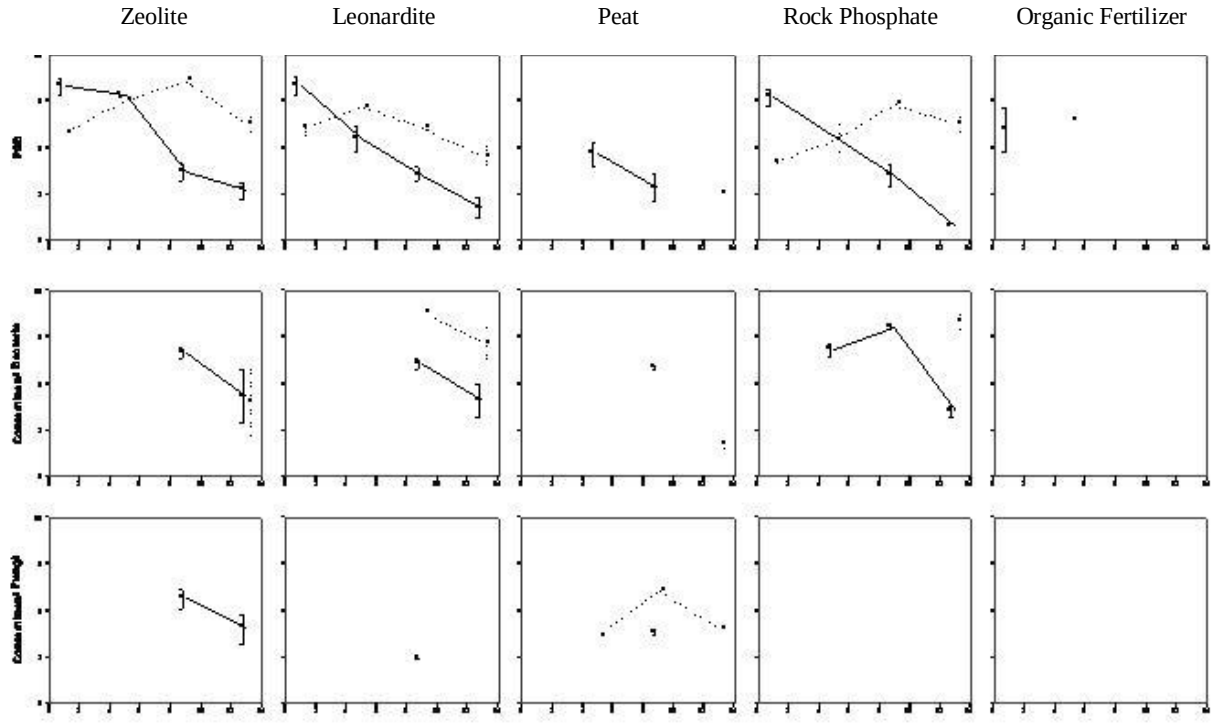

Fig. 2. The numbers of the PSB, contaminant bacteria and fungi in $Log_{(10)}$ during storage at 28 ^oC (O) and +4 ^oC (Δ). Error bars show SE. When there is no data point, the population density is below the detection limit (10^6 cfu g^{-1}).

The numbers of the PSB at 28 ^oC dropped logarithmically after the first week (Figure 2). The rates of decrease in zeolite were not as pronounced as the rates in the other carriers. This is probably related to zeolite's larger surface area, which allows higher number of microorganisms to colonize and survive during the incubation. In addition, the fine porous fabric of this material creates a suitable condition for optimum water regime and aeration for the survival of the microorganisms (Rehakova et al. 2004). Contrarily, the population density of the PSB increased at +4 ^oC after the first week (Figure 2). This is related to the cold tolerance, the ability of the PSB to grow at +4 ^oC. Some *Pseudomonas* mutants have been found to grow and solubilize bi- or tri-calcium phosphate at low temperatures (Das et al., 2003; Katiyar & Goel, 2003).

The increase in the population size of the PSB in the carriers stored at +4 ^oC lasted up to week-5 in leonardite and week-9 in zeolite and rock phosphate (Figure 2). The numbers of the PSB dropped to about 5×10^8 cfu g^{-1} in zeolite/rock phosphate and to about 10^8 cfu g^{-1} in leonardite at the end of the cold storage afterwards. The numbers of the contaminant bacteria and fungi were below the detection limit (10^6 cfu g^{-1}) before week-5 in peat/rock phosphate and before week-9 in zeolite/leonardite. The increases in the population sizes of the contaminants coincided with the reductions in the population sizes of the PSB, after which the numbers of both the inoculant and local microorganisms dropped due probably to the exhaustion of the carbon sources. Temprano et al. (2002) determined that as the moisture content of the non-sterile peat increases from 30 to 50 %, the number of rhizobial cells decline more rapidly. They found that the number of survivers after 15 weeks of storage was close to 10^8 cfu g^{-1} at 30 %, more than 10^7 cfu g^{-1} at 40 %, and less than 10^7 cfu g^{-1} at 50 %. Relatively higher moisture contents (45 % in mineral and 65 % in organic carriers) could be one reason for the rapid declines of the PSB in the current study.

There is almost neither the PSB nor the contaminant in the organic fertilizer. Urea in the organic fertilizer was converted into ammonia (NH_3) and carbon dioxide (CO_2) by the enzyme urease (urea amidohydrolase, EC 3.5.1.5). Ammonia is toxic to microbial cells and volatilization of ammonia is favored when the pH of the solution exceeds 8 (Tisdale et al., 1993). The pH values of the organic fertilizer, both at 28 and +4 ^oC, increased up to 9 at week-5 (Figure 3).

There is almost no microorganism in the organic fertilizer samples, which were not inoculated (control) by the PSB, regardless of sterile water or medium being added (Table 1). However, there are always contaminant bacteria in the rock phosphate control treatments (Table 1).

Table 1.
The number of microorganisms in the control treatments (dead cells of the PSB), which were stored at 28 °C.

MICROORGANISMS (10^8 CFU g^{-1})						
carrier	condition	microbe	weeks			
			1	5	9	13
Rock Phosphate	water	bacterial	4,934	0,597	1,002	0,063
		fungi	<0,010	<0,010	<0,010	<0,010
	medium	bacterial	0,933	0,202	2,948	0,386
		fungi	<0,010	0,702	<0,010	<0,010
Organic Fertilizer	water	bacterial	0,212	<0.010	<0,010	<0,010
		fungi	<0,010	<0,010	<0,010	<0,010
	medium	bacterial	<0,010	<0,010	0,445	<0,010
		fungi	<0,010	<0,010	<0,010	<0,010

The initial density of PSB was adjusted to be the standard (10^9 cfu g^{-1}) for each carrier. However, the number of PSB in the peat incubated at 28 °C was usually less then 10^6 cfu g^{-1} at week-1 (Figure 3). The population density of the PSB increases to 10^8 cfu g^{-1} at week-5 then drops to 10^7 cfu g^{-1} at week-9 in the samples incubated at 28 °C. Contrarily, the number of microorganisms in +4 °C storage is below the detection limit (10^6 cfu g^{-1}). The pH values of peat were the lowest among of all the study carriers during the entire incubation (Figure 3a and 3b). The relatively lower pH values of peat could probably retard the microbial growth.

The PSB was able to persist in the carriers at population densities of 10^4 to 10^5 cfu g^{-1} after one year of cold storage (Figure 4). The number of PSB in the peat was five times and more higher than the number of PSB in the other carriers. The absence of data for initial numbers of the PSB (Figure 2) is simply the result of plating higher dilution levels. Therefore, peat stored at +4 °C could still contain the PSB at population densities below 10^6 cfu g^{-1}. The higher numbers of microorganisms in peat could be related to more optimal moisture content of the peat compared to those of the other carriers (Figure 4). However, the number of fungal contaminant is very close to the number of PSB in not only the peat but also the other carriers. The number of rhizobial survivers in Temprano et al.'s (2002) work ranged between 10^5 and 10^6 cfu g^{-1}, with better survival at lower moisture contents.

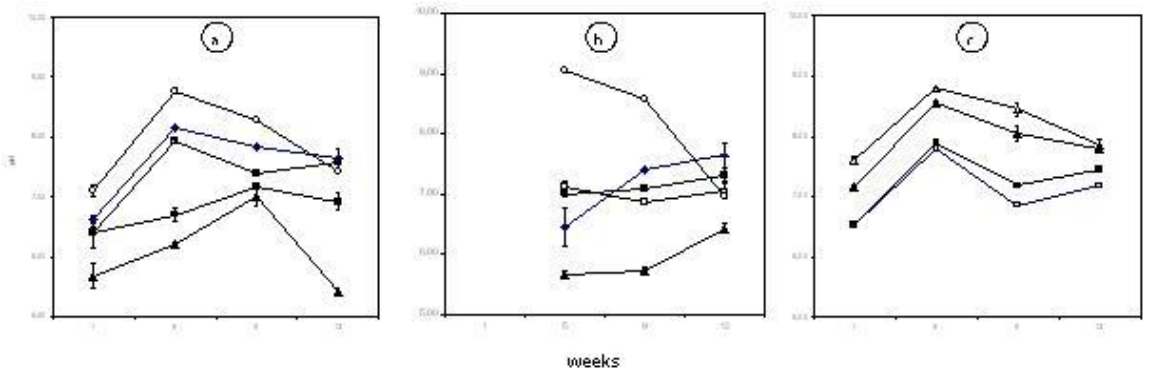

Fig. 3. The pH values in the treatments stored at 28 (a) and +4 (b) °C [zeolite (♦), peat (▲), leonardite (■), organic fertilizer (○), and rock phosphate (□)], compared to the treatments, which contained dead cells (c) at 28 °C [rock phosphate which contained sterile water (□) or medium (■); organic fertilizer which contained water (△) or medium (▲)]. Error bars show SE

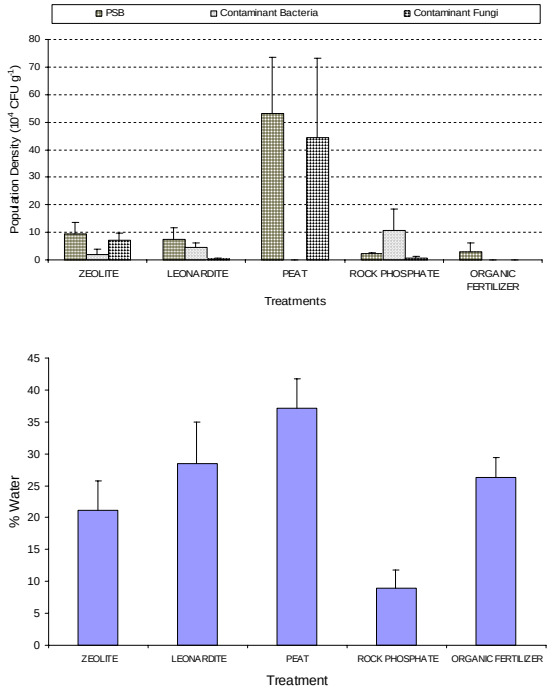

Fig. 4. The survivers at +4 ^{o}C and the moisture contents of the samples after a year. Error bars show SE.

P-solubilization in the carriers

Zeolite and leonardite do not have phosphorus either in soluble or hardly soluble form (Table 2). Olsen-P content of peat tends to increase in both 28 ^{o}C incubation and +4 ^{o}C storage. Since peat was not autoclaved initially, the release of soluble-P from dead cells is not a plausible explanation. Either the PSB-microbe and/or the local microorganisms should have some level of proteolytic activity, so that the mineralization of organically bound phosphorus produces soluble-P. There is almost no soluble-P in the rock phosphate treatments, which have either live or dead cells of PSB (Table 2). Probably, the conditions, under which the experiment was performed, could not favor the P-solubilization from rock phosphate. The air and carbon supplies to the PSB were not sufficient. The growth of local microbes further retarded the persistence of the PSB.

The levels of soluble-P were highest in organic fertilizers (Table 2). The reason for this should be partially abiotic, since the number of microorganism in the organic fertilizer is almost always below 10^6 cfu g^{-1} (Figure 2). Zeolite that was saturated with ammonium (NH_4^+) was shown to increase mineral phosphate solubilization from rock phosphate (Pickering et al., 2001). As noted earlier, urea in the organic fertilizer was converted enzymatically to NH_3 and CO_2. There is equilibrium between NH_3 and NH_4^+ in solution phase. Therefore, NH_4^+ produced in this manner could initially saturate the exchange sites of zeolite. As the volatilization of NH_3 reduces NH_4^+ concentration, calcium (Ca^{+2}) should occupy the exchange sites of zeolite later. This should lower the Ca^{+2} concentration in the solution phase and enhance P-solubilization from rock phosphate by exchange.

Table 2.
The Olsen-P contents of the treatments.

Solube – P (ppm)			
	weeks		
Carrier	1	5	9
	28 ^{0}C		
Zeolite	0	0^{a}	0^{a}
Leonardite	0	0^{a}	0^{a}
Peat	nd	76^{c}	120^{b}
Rock Phosphate	0	0^{a}	1^{a}
Rock Phosphate + Water*	0	6^{a}	26^{a}
Rock Phosphate + Medium*	0	8^{a}	9^{a}
Organic Fertilizer	0	58^{bc}	195^{c}
Organic Fertilizer + Water*	0	43^{b}	200^{c}
Organic Fertilizer + Medium	14	0^{a}	133^{b}
	4 ^{0}C		
Zeolite	0	0a	0^{a}
Leonardite	0	0a	0a
Peat	nd	85^{c}	100^{c}
Rock Phosphate	3	0^{a}	24^{b}
Organic Fertilizer	0	19^{b}	189^{d}

Conclusions

The PSB is able to persist in nonsterile zeolite, leonardite, and rock phosphate at population densities ranging between 10^6 and 10^7 cfu g^{-1} with secondary growth of other microbes for a period of 13 weeks. The suppression of the local microorganisms is required for better survival of the PSB. Zeolite could be a good carrier due to its large surface area and porosity, which allows higher numbers of microorganisms to colonize.

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Received 6 April 2010