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Phosphate solubilizing bacteria from runner bean rhizosphere and their mechanism of action

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Abstract

Phosphorus is one of the most important nutrients for biological growth and development but also a limiting one because of the low availability of soluble forms. Microorganisms, especially rhizobacteria, have the ability to convert insoluble forms of phosphate to an accessible form for plants using different mechanisms. Therefore, the main goal of this study was to identify the mechanisms used by runner bean (Phaseolus coccineus L.) rhizospheric bacteria for inorganic phosphate solubilization. Ten out of twenty five isolated bacterial strains solubilized $Ca_3(PO_4)_2$ in qualitative P-solubilization assays, with solubilization indices varying from 1.33 to 3.19. The strains that exhibited the highest potential to solubilize $Ca_3(PO_4)_2$ in a quantitative assay were selected for further studies of the mechanisms involved in the process. The pH of the medium was monitored, the organic acids exported in the culture medium were identified by HPLC analysis and the acid and alkaline phosphatase activities were determined. An inverse relationship between pH and P solubilization was evidenced. The main mechanisms involved are the lowering of pH due to the production of organic acids such as tartaric and isocitric acids. No positive correlation between the soluble P and the phosphatase activity could be inferred.

Keywords: inorganic phosphate solubilization; organic acid production; phosphatases activity; rhizobacteria.

1. Introduction

The excessive use of phosphorus (P) fertilizers in agricultural practices in order to achieve optimum crop yields is considered an important environmental problem around the world. Almost 75-90% of the P fertilizers added in the soil are rapidly immobilized soon after application and becomes unavailable to plants (H. RODRíGUEZ, R. FRAGA [1]). As a result, the efficiency of P fertilizers is estimated to be around 10-25% (K.F. ISHERWORD [2]).
Bean is one of the most important legume used in human nutrition which requires high amounts of P for a high productivity (A.M. BONSER & al. [3]). Thus, the application of P fertilizers is absolutely necessary. Besides common bean, runner bean (*Phaseolus coccineus* L.) is the second *Phaseolus* species grown in America and Europe (H. LABUDA [4]) as annual crop for dry seeds or immature pod production and also as ornamental vine (A. RODIÑO & al. [5]). In contrast to common bean, runner bean has a high adaptability to low temperature, a stronger resistance to pathogen attack and greater potential for high yield in organic farming conditions. Due to its high ecological plasticity it is a suitable species for a sustainable agriculture system (N. MUNTEANU & al. [6]). Therefore, finding some strategies that can replace the application of P fertilizers is absolutely necessary.

A friendly alternative to chemical P fertilizers are microorganisms, especially bacteria, which represent up to 50% of the soil population capable of phosphate solubilization (A.A.

KHAN & al. [7]). A high proportion of phosphate solubilizing bacteria (PSB) belongs to the rhizosphere, the region of contact between root and soil, where the microorganisms are exposed to the specific influence of the plant roots (G. ZARNEA [8], P. JOSHI, A.B. BHATT [9]). The use of PSB as inoculants for crop plants to increase P soil mobilization and therefore to improve plant nutrition has received great attention over the past years (M. KHAN & al. [10]). Yet, the widespread use of PSB inoculants remains limited because of the controversial results obtained and insufficient exploration of the phosphate solubilization mechanisms.

Knowledge of the mechanisms involved in phosphate solubilization is the key to find viable P biofertilizers which can replace conventional fertilizer to achieve optimum yields. Although the phosphate solubilizing process is not fully understood, several mechanisms have been described. The production of low molecular weight organic acids such as gluconic and keto-gluconic acids seems to be the major mechanism used by phosphate solubilizing microorganisms for solubilization of inorganic P (H. RODRIGUEZ & al. [11]). Through their hydroxyl and carboxyl groups, organic acids chelate the cations bound to phosphate, thereby converting it into soluble forms. Other mechanisms that have been involved in solubilization of inorganic phosphate are: the production of acid and alkaline phosphatases, the release of H^+ and inorganic acids as well as the synthesis of exopolysaccharides (H. RODRíGUEZ, R. FRAGA [1], E. GAMALERO, B.R. GLICK [12], S. ALAM & al. [13]). To the authors' knowledge, this is the first report dealing with the mechanisms involved in inorganic phosphates solubilization phenomenon that occur in the rhizosphere of runner bean plants.

2. Material and methods

Isolation of phosphate solubilizing bacteria

Bacteria were isolated from the rhizosphere of field-grown runner bean from The Experimental Farm, University of Agriculture Sciences and Veterinary Medicine, Iasi County, Romania. After the roots were separated from the bulk soil, rhizospheric soil and root samples were blended in a sterile Waring blender at high speed for 1 min and serial dilutions (1/10) were made in PBS (phosphate buffer saline, pH 7.4). Aliquots (0.1 ml) were plated on Bunt-Rovira nutrient medium (J.S. BUNT, A.D. ROVIRA [14]) and incubated at 28°C for 7 days. Isolates were re-streaked on the same nutrient medium, checked for purity and stored on slants at 4°C.

Phosphorus solubilizing plate assay

The ability of the rhizobacterial isolates to solubilize P was qualitatively assessed using Pikovskaya's agar medium (R.I. PIKOVSKAYA [15]) containing $Ca_3(PO_4)_2$ as an insoluble inorganic form of phosphate. Each bacterial strain was streaked in the center of a Pikovskaya's agar plate and incubated at 28°C. After 7 days, phosphate solubilization was assessed visually observing clear zones (halos) around the colonies. The ratio of the total diameter (colony + halo zone) to the colony diameter (mm) was calculated and used as an indicator of phosphate solubilization. Three replicate plates were used for each isolate.

P-solubilization in liquid culture

Bacterial strains that exhibited the largest zones of clearing in the qualitative screening assay were further analysed for their ability to solubilize the $Ca_3(PO_4)_2$ in Pikovskaya liquid medium (PVK). Bacteria were grown in 50 ml centrifuge tubes containing 15 ml of nutrient broth on a gyratory shaker (190 rpm) at 28°C for 24 h. After incubation, the optical density of the cultures (OD_{600}) was assessed using a DU 730 spectrophotometer (Beckman Coulter, Nyon, Switzerland) at 600 nm. The cell density was further adjusted to an OD_{600} of approximately 0.100 and 1 ml of each culture was transferred to 25 ml PVK medium (C. NAUTIYAL, S. [16]). The same protocol was used to inoculate 25 ml of PVK liquid medium

without $Ca_3(PO_4)_2$. Control flasks containing 25 ml PVK medium were not inoculated with bacteria. Three replicate flasks for each isolate were incubated on a gyratory shaker (190 rpm) at 28°C and sampled at 0, 3, 5 and 7 days. A 2 ml aliquot was aseptically removed from each flask (control, PVK with and without $Ca_3(PO_4)_2$) and centrifuged at 3756 *g* for 30 min. An aliquot (2 ml) of the supernatant was further centrifuged at 17,968 *g* for 30 min and assessed for pH using a Hanna HI 2211 pH meter (Cluj Napoca, Romania) and the amount of P released into solution was measured as an indicator of the efficiency of the selected isolates (K.A. EL-TARABILY & al. [17]). Water-soluble P was analyzed by the colorimetric procedure of Murphy and Riley using molybdophosphoric acid blue complex (J. MURPHY, J.P. RILEY [18]). The amount of solubilized P was calculated as a difference between the amount assayed for PVK cultures and uninoculated PVK controls and expressed as μg P ml^{-1}. The isolates cultivated in Pikovskaya liquid medium lacking $Ca_3(PO_4)_2$ were included for comparison concerning the pH drop and HPLC analysis. Three experimental replicates for each supernatant sample were used.

Determination of acid and alkaline phosphatase activities and organic acid production

Only the phosphate-solubilizing isolates providing the greatest increase of P concentration in Pikovskaya liquid medium were selected for the acid and alkaline phosphatase activities, and organic acids production assays. Acid and alkaline phosphatase activities were determined using the method presented by De Freitas with following modifications (J.R. DE FREITAS & al. [19]): 300 μl of culture supernatant was incubated at 28°C with 100 μl 25 mM ρ-nitrophenyl phosphate and 200 μl modified universal buffer (J.L. THORNTON & al. [20]) pH 6.5 or pH 11. After 3 h the reaction was terminated by adding 100 μl 0.5 M $CaCl_2$ and 400 μl 0.5 M NaOH. The assay mixtures were centrifuged for 10 min at 17,968 *g* and the yellow color measured at 410 nm. A calibration curve was constructed using ρ-nitrophenol (0-100 nmoles) as standard. The enzyme activity was expressed as nmoles ρ-nitrophenol (pNP) ml^{-1} of culture medium h^{-1}. The experiment was repeated three times.

For organic acids identification, 1 ml of culture was centrifuged at 17,968 *g* for 20 min and filtered through a 0.2 μm PES syringe filter (Roth, Karlsruhe, Germany). Culture filtrates (20 μl) were injected on a PRP-x300 PSDVB-Sulfonic acid column (part number 79475) with 7 μm particle size (Hamilton, Reno, U.S.A) and organic acids were monitored using a Bischoff Lambda 1010 UV detector (Bischoff, Leonberg, Germany) at 220 nm. The mobile phase consisted of 1 mN sulphuric acid with a flow rate of 1 ml min^{-1} (P. WALSER [21]). Qualitative and quantitative data were obtained by comparing the retention times and peak areas of the query compounds with that of known standards. The isolates cultivated in Pikovskaya liquid medium lacking $Ca_3(PO_4)_2$ were used as controls.

Statistical analysis

The experimental data (solubilization index, activity of acid and alkaline phosphates) were statistically processed using two-factor analysis of variance with replication and Student (t) test. All results are expressed as mean ± SEM. F values for which $p < 0.05$ were considered significant (L. FERNÁNDEZ & al. [22]). Pearson correlation analysis was used to explore the relationships between solubilized P, phosphatase activity and pH values (D. MULETA & al. [23]).

3. Results and discussions

Plate assay

Bacteria isolated from runner bean rhizosphere were first assayed for their ability to solubilize insoluble phosphate by using a plate screening method. Ten out twenty five isolated bacterial strains showed clearly visible haloes around their colonies on PVK agar medium after 7 days of incubation. The solubilization index based on colony diameter and halo zone ranged from 1.33 to 3.19. The highest solubilization index was recorded for R4 (3.19 ± 0.10) followed by R1 (2.89 ± 0.43) and R2 (2.87 ± 0.57) (Table 1). The production of clear zones around the microbial colonies on PVK medium containing $Ca_3(PO_4)_2$ as sole P source indicated the presence of phosphate-solubilizing capabilities (S. MEHTA, C.S. NAUTIYAL [24]). Bacterial strains that exhibited a clear halo on PVK plates were tested further for P solubilization in PVK broth.

Table 1. Solubilization index of rhizobacteria isolates after 7 days of incubation.

Bacterial strain	Solubilization index
R1	2.89 ± 0.43^{b}
R2	2.87 ± 0.57^{b}
R3	2.26 ± 0.10^{ab}
R4	3.19 ± 0.10^{b}
R5	2.7 ± 0.32^{b}
R6	1.33 ± 0.33^{a}
R7	1.5 ± 0.5^{ab}
R8	2.62 ± 0.11^{b}
R9	1.33 ± 0.33^{a}
R10	1.33 ± 0.88^{ab}

The values are means ± SEM. Values followed by the same letters are not significantly different according to Tukey's means comparison test ($p < 0.05$).

Quantitative P determination

The phosphate solubilization potential of the strains that exhibited solubilization haloes was further assessed using a quantitative method.

Phosphate solubilization in liquid media showed that all the isolates had the potential to solubilize the inorganic form of P as indicated by a gradual increase in the amount of soluble

P in liquid medium during the 7 days of incubation. However, the strains with the highest potential to solubilize $Ca_3(PO_4)_2$ in liquid media were not the same as the ones that exhibited the greatest halos. These confirm the fact that the plate method is reliable only for isolation and preliminary characterization of phosphate-solubilizing microorganisms. The highest solubilized P concentrations were recorded for strains R8 (19.84 ± 1.89 μg P ml^{-1}) on the 5th day, R10 (19.12 ± 0.35 μg P ml^{-1}) on the 7th day and R3 (17.51 ± 1.21 μg P ml^{-1} on the 5th day (Figure 1). The three strains were identified as : R8 – *Pseudomonas lini* (using 16sDNA analysis) (S. MIGNARD, J.P. FLANDROIS [25]), R3 – *Bacillus mycoides* and R10 – *Bacillus pumilus* (using API 50 CHB system and Apiweb software, Biomerieux, France) (L.V. KUZINA & al. [26]) and used for further tests in order to asses the mechanisms involved in P solubilization. All three strains were deposited in the UAIC Iasi culture collection.

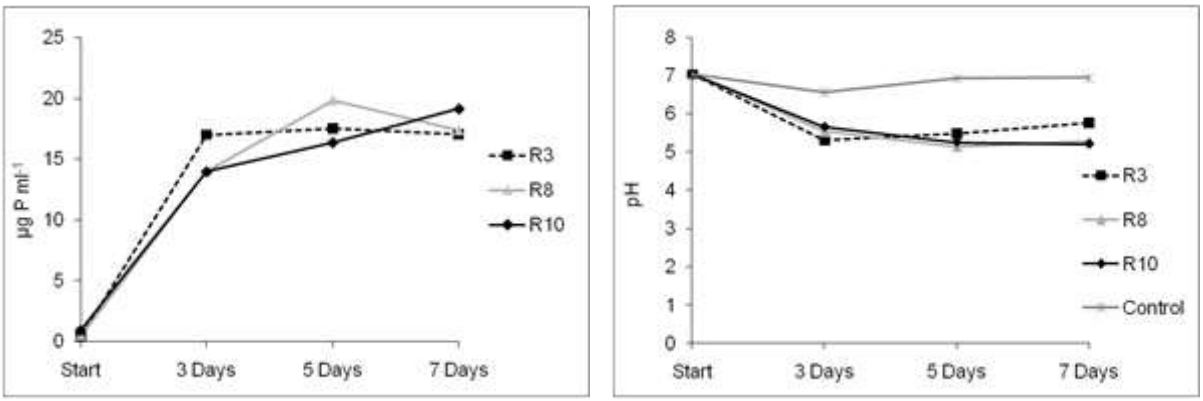

Figure 1. Changes in P-solubilization (left) and pH value (right) during 7 days of incubation in PVK broth.

Tricalcium phosphate solubilization was followed by a significant pH decrease of PVK broth, from an initial value of 7.03 ± 0.05 registered at the beginning of the experiment to 5.13 ± 0.025 (strain R8) after 7 days of inoculation. The pH value remained almost the same in the uninoculated control flasks (Figure 1). Moreover, a correlation between the pH and the amount of solubilized P could be established for all the tested strains. Thus, for R10 strain the accumulation of P in PVK medium gradually increased until the last day of incubation when the lowest pH value (5.21 ± 0.025) was measured. The statistical analysis showed a strong negative correlation between the soluble P concentration and pH ($r = 0.986$; $p < 0.05$). The $Ca_3(PO_4)_2$ solubilization assessed for the strains R3 and R8 reached the maximum value on the 5th day of incubation, decreasing slightly afterwards, simultaneously with the increase of medium pH. As in case of R10 strain, a significant negative correlation between P concentration and pH value (strain R3 - $r = 0.941$, $p < 0.05$; strain R8 - $r = 0.992$, $p < 0.05$) was set.

Organic acid production

Tricalcium phosphate solubilization usually involves the production of organic acids followed by a decrease in the pH of the medium (H. RODRíGUEZ, R. FRAGA [1], J.R. DE FREITAS & al. [19], S. MEHTA, C.S. NAUTIYAL [24]).

HPLC analysis of the culture filtrates was performed in order to identify the organic acids produced by R3, R8 and R10 strains in the PVK medium supplemented or not with the inorganic P source.

Albeit the most frequent organic acids produced by phosphate solubilizing rhizobacteria are gluconic acid (H. RODRíGUEZ, R. FRAGA [1], A. GULATI & al. [27], P. VYAS, A.

GULATI [28]), oxalic and citric acid (S. ALAM & al. [13], M. RASHID & al. [29]), 2-ketogluconic acid (D. MULETA & al. [23]), succinic acid (Q.A. PANHWAR & al. [30]), the present results indicate that the main acids produced by the strains isolated from runner bean rhizosphere are different.

The growth of R10 strain in the presence of $Ca_3(PO_4)_2$ resulted in the gradually accumulation of isocitric acid in comparison with the control (Figure 2). Moreover, it has been found that during the 7 days incubation the accumulation of isocitric acid increased along with the amount of solubilized P.

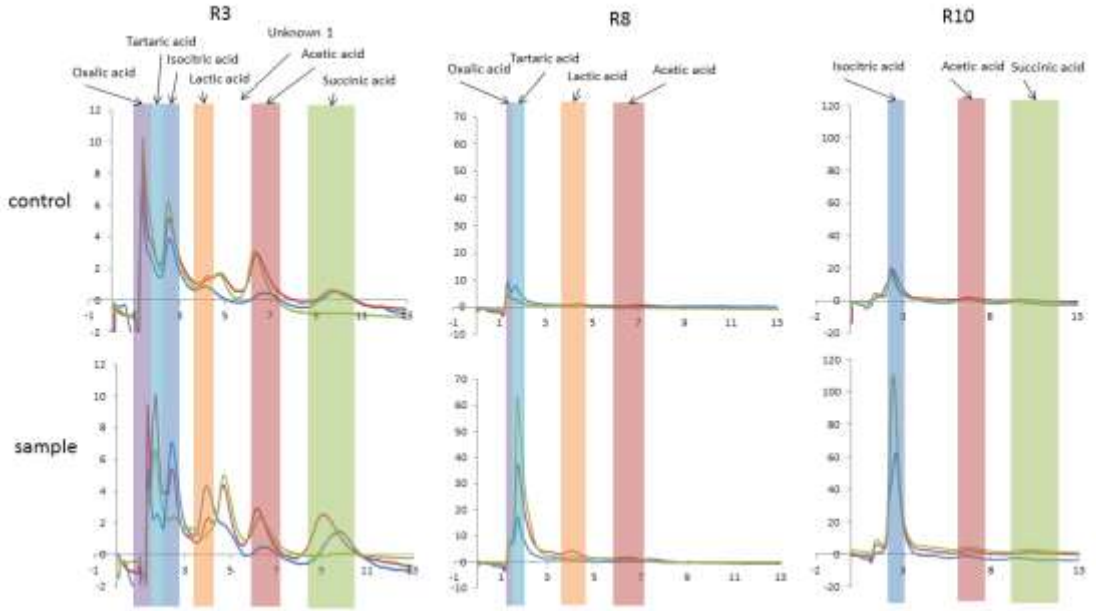

Figure 2. HPLC profiles of the culture filtrates for R3, R8 and R10 strains grown on PVK with (sample) and without $Ca_3(PO_4)_2$ (control) at various times: blue line - day 3, red line - day 5, green line - day 7.

The growth of R8 strain in PVK supplemented with $Ca_3(PO_4)_2$ also resulted in a gradual accumulation of tartaric acid (Figure 2). However, the greatest accumulation of the tartaric acid was recorded on the 7th day while the greatest amount of solubilized P was found on the 5th day of incubation.

In the case of R3 strain, several organic acids accumulated during the culture growth but the differences between cultures grown with or without phosphate source were not significant. Notable differences were recorded only in the case of tartaric, lactic and succinic acids (Figure 2). Also, an unidentified acid accumulated during the incubation in PVK supplemented with the inorganic P source compared with the control. The tartaric acid was first identified on 3rd day and the chromatographic analysis showed an important accumulation up to the 7th day of incubation.

On the last day of incubation an increase in the pH of the medium was observed for two strains (R8 and R3) when the amount of solubilized P decreased. This could be explained by the reutilization of available P by the growing bacterial population via P precipitation of organic metabolites or the formation of organo-P compounds with secreted organic acids, used subsequently as an energy or nutrient source as suggested by Muleta et al. (2013).

Phosphatase activity

Several authors proposed the phosphatase activity as an important mechanism involved in mineral phosphate solubilization (S. PANTUJIT, N. PONGSILP [31], J.H. PARK & al. [32]).

The analysis performed for the R3 strain cultivated in PVK medium supplemented with $Ca_3(PO_4)_2$ showed a gradual increase of acid (from 6.44 ± 0.35 to 60.89 ± 6.76 nmoles pNP ml^{-1} h^{-1}) and alkaline (from 7.34 ± 1.22 to 70.82 ± 12.78 nmoles pNP ml^{-1} h^{-1}) phosphatase activities, during the whole experiment (Figure 3). Significant differences were recorded between sample and control for both enzymes only on the 7th day ($p<0.05$), although no significant positive correlation between the amount of solubilized phosphorus and the acid ($r = 0.10$; $p = 0.405$) or alkaline phosphatase activity ($r = 0.17$; $p = 0.260$) was found.

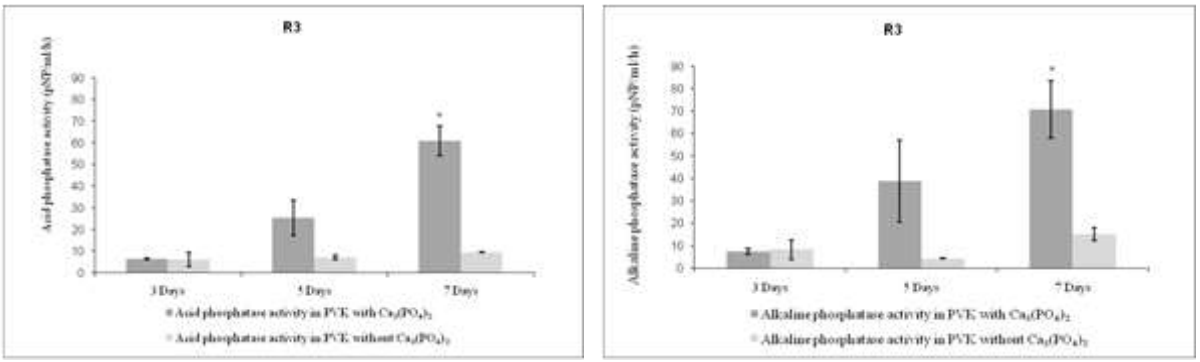

Figure 3. Acid and alkaline phosphatase activity in PVK broth with and without $Ca_3(PO_4)_2$.
* - significant differences between sample and control.

Both acid and alkaline phosphatase recorded for R8 strain cultivated in PVK medium supplemented with inorganic P source showed the highest activity on the 7th day of incubation (29.5 ± 11.08 and 30.56 ± 11.52 nmoles pNP ml^{-1} h^{-1}, respectively) (Figure 3). However, no significant differences between cultures cultivated in PVK medium supplemented with $Ca_3(PO_4)_2$ and control were registered. Also, no significant positive correlation between the amount of solubilized P and the acid ($r = 0.008$; $p = 0.823$) and alkaline phosphatase activity ($r = 0$; $p = 0.991$) could be found.

R10 strain exhibited low levels of acid and alkaline phosphatase activity, which ranged from 10.11 ± 0.78 to 14.06 ± 1.15 and 9.14 ± 1.64 to 13.48 ± 0.86 nmoles pNP ml^{-1} h^{-1}, respectively, but the recorded differences were not significant. The correlation between the amount of solubilized P and the phosphatase activity was not significant ($r = 0.06$; $p = 0.843$ for acid phosphatase; $r = 0.03$; $p = 0.616$ for alkaline phosphatase).

Taking into account the lack of correlation between the amount of solubilized P and the level of acid and alkaline phosphatase activity, it may be presumed that the two enzymes are not involved in the mechanisms used by these isolates to solubilize inorganic P. De Freitas et al. (1997) explained that the synthesis of these phosphatases is stimulated when the level of inorganic P in the growth medium is limited. However, the present results do not support this hypothesis since the amount of available P increased gradually during the incubation time, along with the increase of phosphatase activities. More likely the high acid and alkaline phosphatase activity could be related to the culture growth. For instance, the data recorded for R3 strain showed that the phosphatase activity gradually increased during the 7 days of incubation (Figure 3) along with the CFU number (data not shown). Even if this study showed no relationship between P solubilization and phosphatase activity, the two enzymes may indirectly participate in this process by lowering the pH of the culture medium via dephosphorylating action and the production of acids (J.H. PARK & al. [32]).

4. Conclusions

This study highlights the role of tartaric and isocitric acid in phosphate solubilization processes that occur in the runner bean rhizosphere. The relationship between acid and alkaline phosphatase activity and phosphate solubilization can be considered only coincidental. The results confirm the potential of *Pseudomonas lini* R8, *Bacillus mycoides* R3 and *Bacillus pumilus* R10 strains, isolated from the runner bean roots, to be used for the development of biofertilizer formulae based on phosphate solubilizing bacteria in the context of organic agriculture. However, practical application of these results should be further evaluated in field experiments.

References

1. H. RODRíGUEZ, R. FRAGA, Phosphate solubilizing bacteria and their role in plant growth promotion, *Biotechnol. Adv.*, 17 (4–5): 319-339 (1999).
2. K.F. ISHERWORD, Fertilizer use and environment, N. AHMED, A. HAMID, NFDC eds., *Plant Nutrition Management for Sustainable Agricultural Growth*, Islamabad, 1998, 57-76.
3. A.M. BONSER, J. LYNCH, S. SNAPP, Effect of Phosphorus Deficiency on Growth Angle of Basal Roots in Phaseolus vulgaris, *New Phytologist*, 132 (2): 281-288 (1996).
4. H. LABUDA, Runner bean *(Phaseolus coccineus* L.) – biology and use, *Acta Sci. Pol., Hortorum Cultus*, 9 (3): 117-132 (2010).
5. A. RODIÑO, M. LEMA, M. PÉREZ-BARBEITO, M. SANTALLA, A. DE RON, Assessment of runner bean (*Phaseolus coccineus* L.) germplasm for tolerance to low temperature during early seedling growth, *Euphytica*, 155 (1): 63-70 (2007).
6. N. MUNTEANU, L.D. POPA, G.C. TELIBAN, Suitability of *Phaseolus coccineus* L. species for cultivation in sustainable agriculture systems, *Lucrări Stiintifice, seria Horticultură*, 50 (1): 501-504 (2007).
7. A.A. KHAN, G. JILANI, M.S. AKHTAR, S.M.S. NAQVI, M. RASHEED, Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production, *J. Agric. Biol. Sci.*, 1 (1): 48-58 (2009).
8. G. ZARNEA, Tratat de microbiologie generală eds., Ed. Academiei Romane (1994).
9. P. JOSHI, A.B. BHATT, Diversity and function of plant growth promoting rhizobacteria associated with wheat rhizosphere in North Himalayan Region, *International Journal of Environmental Sciences*, 1 1135-1143 (2011).
10. M. KHAN, A. ZAIDI, P. WANI, Role of phosphate-solubilizing microorganisms in sustainable agriculture — A review, *Agronomy for Sustainable Development*, 27 (1): 29-43 (2007).
11. H. RODRIGUEZ, T. GONZALEZ, I. GOIRE, Y. BASHAN, Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium Azospirillum spp, *Naturwissenschaften*, 91 (11): 552-555 (2004).
12. E. GAMALERO, B.R. GLICK, Mechanisms Used by Plant Growth-Promoting Bacteria, D.K. MAHESHWARI eds., *Bacteria in Agrobiology: Plant Nutrient Management*, Springer, Berlin, Heidelberg, 2011, 17-46.
13. S. ALAM, S. KHALIL, A. NAJMA, M. RASHID, In vitro Solubilization of Inorganic Phosphate by Phosphate Solubilizing Microorganisms (PSM) from Maize Rhizosphere, *Int J Agric Biol*, 4 454-458 (2002).
14. J.S. BUNT, A.D. ROVIRA, Microbiological studies of some subantarctic soils., *Journal of Soil Science*, 6 (1): 119-128 (1955).
15. R.I. PIKOVSKAYA, Mobilization of phosphorous in soil in connection with vital activity of some microbial species, *Mikrobiologya*, 17 362-370 (1948).
16. C. NAUTIYAL, S., An efficient microbiological growth medium for screening phosphate solubilizing microorganisms, *FEMS Microbiology Letters*, 170 (1): 265-270 (1999).
17. K.A. EL-TARABILY, A.H. NASSAR, K. SIVASITHAMPARAM, Promotion of growth of bean (Phaseolus vulgaris L.) in a calcareous soil by a phosphate-solubilizing, rhizosphere-competent isolate of Micromonospora endolithica, *Appl. Soil Ecol.*, 39 (2): 161-171 (2008).
18. J. MURPHY, J.P. RILEY, A modified single solution method for the determination of phosphate in natural waters, *Analytica Chimica Acta*, 27 (0): 31-36 (1962).
19. J.R. DE FREITAS, M.R. BANERJEE, J.J. GERMIDA, Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.), *Biol. Fertil. Soils*, 24 358-364 (1997).

20. J.L. THORNTON, D. CRIM, A.D. MCLAREN, Enzymatic characterization of soil evidence, *J Forensic Sci*, 20 (4): 674-692 (1975).
21. P. WALSER, New ion-exclusion phase for organic acids, *J. Chromatogr. A*, 439 (1): 71-81 (1988).
22. L. FERNÁNDEZ, P. ZALBA, M. GÓMEZ, M. SAGARDOY, Phosphate-solubilization activity of bacterial strains in soil and their effect on soybean growth under greenhouse conditions, *Biology and Fertility of Soils*, 43 (6): 805-809 (2007).
23. D. MULETA, F. ASSEFA, E. BÖRJESSON, U. GRANHALL, Phosphate-solubilising rhizobacteria associated with Coffea arabica L. in natural coffee forests of southwestern Ethiopia, *Journal of the Saudi Society of Agricultural Sciences*, 12 (1): 73-84 (2013).
24. S. MEHTA, C.S. NAUTIYAL, An Efficient Method for Qualitative Screening of Phosphate-Solubilizing Bacteria, *Curr. Microbiol.*, 43 (1): 51-56 (2001).
25. S. MIGNARD, J.P. FLANDROIS, 16S rRNA sequencing in routine bacterial identification: a 30-month experiment, *J Microbiol Methods*, 67 (3): 574-81 (2006).
26. L.V. KUZINA, J.J. PELOQUIN, D.C. VACEK, T.A. MILLER, Isolation and identification of bacteria associated with adult laboratory mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae), *Current Microbiology*, 42 (4): 290-294 (2001).
27. A. GULATI, N. SHARMA, P. VYAS, S. SOOD, P. RAHI, V. PATHANIA ,R. PRASAD, Organic acid production and plant growth promotion as a function of phosphate solubilization by Acinetobacter rhizosphaerae strain BIHB 723 isolated from the cold deserts of the trans-Himalayas, *Arch Microbiol*, 192 (11): 975-983 (2010).
28. P. VYAS, A. GULATI, Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*, *BMC Microbiology*, 9 174-174 (2009).
29. M. RASHID, S. KHALIL, N. AYUB, S. ALAM , F. LATIF, Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under in vitro conditions, *Pak. J. Biol. Sci.*, 7 (2): 187-196 (2004).
30. Q.A. PANHWAR, R. OTHMAN, A. RAHMAN, S. MEON, R.I. MOHD, Isolation and characterization of phosphate-solubilizing bacteria from aerobic rice, *Afr. J. Biotechnol.*, 11 (11): 2711-2719 (2012).
31. S. PANTUJIT, N. PONGSILP, Phosphatase activity and affects of phosphate-solubilizing bacteria on yield and uptake of phosphorus in corn, *World Applied Sciences Journal*, 8 (2): 429-435 (2010).
32. J.H. PARK, N. BOLAN, M. MEGHARAJ, R. NAIDU, Isolation of phosphate solubilizing bacteria and their potential for lead immobilization in soil, *J. Hazard. Mater.*, 185 (2–3): 829-836 (2011).