# Phosphate Solubilization Potentials of *Acinetobacter* Strains and their Relations with Soil Properties

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## Abstract

Phosphate solubilizing bacteria can be used as soil or seed inoculum to increase soil phosphorus (P) availability for agricultural purposes. There is also a possibility of using these microorganisms to biotechnologically dissolve phosphate ores for the production of phosphorus fertilizers. Twenty-one soil samples were collected along a highway in Turkey to isolate phosphate solubilizing bacteria. A total of 20 phosphate solubilizers were isolated from the rhizosphere of wheat and maize grown in the pots, which contained the collected soil samples. The isolates were distributed among the genera, *Acinetobacter* (7), *Pseudomonas* (7), *Enterobacter* (2), *Enterococcus* (1), *Escherichia* (1), *Photorhabdus* (1), and *Bacillus* (1) as determined by the 16S rDNA gene sequence analysis. Since the *Acinetobacter* species were most effective in Pikovskaya's agar, which contained tricalcium phosphate for the sole P-source, they were further experimented for the phosphate solubilization in batch cultures. The mean phosphorus dissolved in 5 day incubation ranged between 167 and 1022 ppm P. The initial pH of 7.8 dropped below 4.7 in six isolates with a gluconic acid production in the concentrations ranging between 27.5 and 37.5 mM. *Acinetobacter* isolates have some potential as an inoculum both for soil and biotechnological P-solubilization.

## Introduction

Phosphorus (P) is an essential nutrient, which is taken up by plants in relatively large quantities, hence it is considered as a macro-nutrient. Plants can only benefit from phosphate ions ($HPO_4^{=}$; $H_2PO_4^{-}$), which represent a minute fraction of total-P in soil. Theoretically, two processes reduce the availability of P to cultivated plants in most soils with basic pH and high calcium carbonate ($CaCO_3$) content: (i) precipitation as calcium (Ca) and magnesium (Mg) phosphates; (ii) adsorption to surfaces of $CaCO_3$ and clays. The benefit of fertilizer-P is limited in alkaline soils because supplemental phosphate ions also undergo precipitation and adsorption. Besides, the production of phosphorus fertilizers is a high energy demanding process. It involves sulfuric acid treatment of rock phosphates, which increases solubility of ore contaminants in the final product.

Mineral phosphate solubilizing microorganisms isolated from rhizosphere or bulk soil have been the subject of many researches worldwide (Kim et al., 1997; Vasssilev et al., 1997; Kumar and Narula, 1999; Nautiyal et al., 2000; Reyes et al., 1999; Toro et al., 1997; Vassileva et al., 1998). Inoculation with a phosphate solubilizer increased plant phosphate uptake and dry matter accumulation (Chabot et al., 1998; Gaind and Gaur, 2002; Kucey, 1987; Kumar et al, 2001; Narula et al., 2000; Pal, 1998; Peix et al., 2001a-b; Sundara et al., 2002; Vassileva et al, 1999).

It is generally accepted that organic acid(s) secreted by phosphate solubilizing microorganisms are responsible for the partial dissolution of hardly soluble-P (Banik and Day, 1982; Illmer and Schinner, 1992). The most common organic acid determined in the microbial cultures in earlier works (Babu-Khan et al., 1995; Goldstein et al., 1999; Gyaneshwar et al., 1999; Krishnaraj and

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Goldstein, 2001; Liu et al., 1992; Illmer and Schinner, 1992; Rodriguez et al., 2001) was gluconic acid. Liu et al. (1992) showed that glucose is oxidized into gluconic acid by glucose dehydrogenase (GDH; EC 1.1.99.17), a membrane bound quinoprotein, the active side of which is on the periplasmic space. Pyrroloquinoline-quinone (PQQ) is a cofactor, which is used by the GDH in direct (non-phosphorylating) oxidation. It is accepted that gluconic acid is then taken inside of the cell to be used in Entner-Doudoroff pathway (Adamowicz et al., 1991; Fliege et al., 1992). Goldstein et al. (1993) reviewed that excess gluconic acid in the periplasmic space acidifies the surroundings of the cell, mobilizing phosphorus trapped in tri-calcium phosphate.

Many bacterial species were shown to produce apoquinoprotein (apoGDH), but, not the cofactor PQQ (Hommes et al., 1984; van Kleef and Duine, 1989; van Schie et al., 1984), requiring external-PQQ for aldose sugar utilization. Van Kleef and Duine (1989) reported that some other bacteria produce PQQ in excess of apoGDH. Therefore, the apoGDH synthesis and PQQ-production are independent. Many microorganisms with the ability to produce gluconic acid and solubilize mineral phosphate in well known Pikovskaya's medium, which is a putative evidence for the synthesis of apoGDH have been isolated. However, the information on the presence of PQQ-synthesis in these microorganisms is almost lacking, as PQQ contamination can be carried out from the original isolation culture to the study subcultures. The synthesis of PQQ could be as important as the synthesis of apoGDH in a phosphate solubilizer because of two practical reasons. Firstly, when used as a bio-fertilizer, the phosphate solubilizing microorganism will have to compete with the local micro-flora of the rhizosphere for PQQ to assemble PQQGDH. Secondly, phosphate solubilization trait can also be used in biotechnological solubilization of rock phosphate, where PQQ would also be scarce. Consequently, the objectives of this study are (i) to assess the extend and nature of P-solubilization by *Acinetobacter calcoaceticus* isolates from the rhizosphere, which do not require external growth factors for phosphate solubilization; (ii) to determine the relationships between the extent of P-solubilization in the liquid culture and the properties of the soil, from which a specific strain was isolated.

## Materials and methods

### *Soil Sampling*

Soil samples were collected along a highway in northern part of Turkey with about 5 km intervals between the sampling locations (Figure 1). The field moist soils were sampled from a depth of 15 cm and brought to a glasshouse, where they were loosely packed into brand-new pots (2 L) without any treatment. A portion of soil from each sample was air dried and used for routine soil analyses. Table 1 shows the statistics for the properties of those soils, from which the phosphate solubilizing strains were isolated.

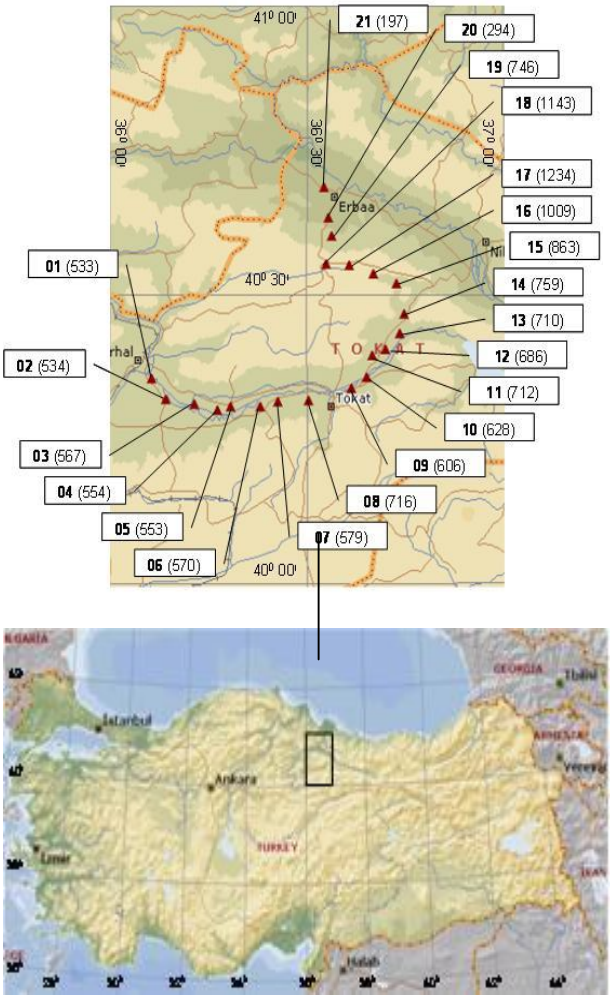


Fig. 1. The points of the collected soil samples. The numbers in paranthesis show the altitudes above see level in m's

**Table 1**
Some Chemical and Physical Properties of Soils.

| Property | Range | Mean | Std.Dev. |
|---|---|---|---|
| Organic Matter (%) | 0.3-7.9 | 2.2 | 2.0 |
| $CaCO_3$ (%) | 0.7-52.1 | 7.4 | 11.0 |
| Sand (%) | 23.7-67.7 | 48.0 | 13.0 |
| Silt (%) | 6.6-50.0 | 28.0 | 8.7 |
| Clay (%) | 8.3-50.3 | 24.1 | 9.3 |
| pH | 6.2-8.7 | 7.7 | 0.5 |
| EC (dS $m^{-1}$) | 0.1-1.6 | 0.4 | 0.4 |
| P (mg $kg^{-1}$) | 0.2-11.7 | 3.6 | 3.4 |
| Fe (mg $kg^{-1}$) | 0.4-17.2 | 3.7 | 3.7 |
| Mn (mg $kg^{-1}$) | 1.8-21.5 | 9.7 | 5.0 |
| Zn (mg $kg^{-1}$) | 0.0-0.04 | 0.1 | 0.009 |
| Cu (mg $kg^{-1}$) | 0.1-0.7 | 0.4 | 0.18 |

ND: Not determined

### *Plant Growth in Glasshouse*

The wheat (*Triticum aestivum*) and corn (*Zea mays*) seeds were surface sterilized by consecutive immersion in 95 % ethanol for two minutes; 1 % sodium hypochlorite (NaOCl) for one minute; and six rinses in sterile distilled water. They were germinated in sterile agar (1.5 %) in dark at 30 $^oC$ for 72 h. The seedlings were sown into the pots containing the soils (one seedling per pot for corn and three seedlings per pot for wheat) and were grown for 25 days. There were two replications for each plant and soil.

### *Isolation and selection of Phosphate Solubilizing Bacteria (PSB)*

The plants were carefully removed from the soil and were gently shaken to remove soil particles loosely adhering to the plant roots. The plants were cut at the base of the stem by a sterile forceps and the whole roots with attached soil were placed in 50 ml centrifuge tubes containing each 20 ml sterile saline solution (0.85 % NaCl). The tubes were shaken in a horizontal shaker at 200 rpm for 30 min. This soil suspension represented rhizospheric soil. The remaining root sample in the tube was cut into small pieces by a sterile forceps and was surface sterilized as mentioned before. A total of 507 strain with growth-promotion potentials were isolated from the rhizosphere and roots using media containing malate and glucose as the sole carbon sources. All isolates were stored at 8 $^oC$ in tryptic soy agar slants under liquid paraffin, in alginate beads in 0.1 M $CaCl_2$ solution at 8 $^oC$, and in 20 % glycerol solution at –24 $^oC$.

Glucose utilizing isolates were further screened for the dissolution of mineral phosphate. Briefly, a 25 µl overnight culture of each isolate was dispensed into the center of tri-calcium phosphate (TCP) agar plate, which then was incubated at 30 $^oC$ for 7 days. The TCP agar contained (g 1000 $ml^{-1}$): glucose, 10; yeast extract, 0.5; $MgSO_4.7H_2O$, 0.25; $CaCl_2$, 0.1; tricalcium phosphate, 5; pH≈7.8. The diameters of the colony and the colony+clearing halo were recorded at 1, 3, and 7 days after the start of the incubation. The solubilization index was the ratio of the diameter of colony+clearing halo to the diameter of the colony. All phosphate solubilizing strains were subcultured consecutively six times in TCP agar to determine the stability of phosphate solubilization trait.

### *Identification of the Phosphate Solubilizing Strains*

The phosphate solubilizing strains were identified by 16S rDNA gene sequence analysis.

### *Phosphate Solubilization in Liquid Cultures*

The seven PSB's were tested for the extent of mineral phosphate solubilization in liquid culture. A strain, which showed growth, but, produced no visible clearing in TCP agar was added for comparison purposes. This isolate was identified as *Bacillus pumilis* by the 16S rDNA gene sequence analysis. A fifty µl portion of an overnight grown culture was dispensed into a 250 ml Erlenmeyer flask, which contained 50 ml of TCP medium. The cultures were grown aerobically in water-bath shakers adjusted to 200 rpm and 30 $^oC$. The flasks were sampled at days 0, 1, 3, and 5 to determine pH, available phosphorus, and gluconic acid. The number of replication per treatment was three.

Available phosphorus was determined by the molybdo-phosphoric blue method of Olsen and Sommers (1982). Gluconic acid determinations in liquid cultures were made by an HPLC (Shimadzu, Prominence ISO-UV) equipped with a $C_{18}$ column heated to 40 $^oC$. The mobile phase was a mixture of acetonitrile (5%) and a solution (95%; pH=6.5) containing 50 mM sodium phosphate and 5 mM tetrabutylammonium hydrogen sulfate. The isocratic flow rate was 0.5 ml $min^{-1}$. The injection volume was 50 µl. Gluconic acid concentrations in the cultures were determined by comparing the peaks from unknown samples and the ones from gluconic acid standards.

***Determination of the effect of PQQ on phosphate solubilization***

A 50 μl portion of an overnight culture from each PSB was dispensed into the center of TCP agar plate. Pyrroloquinoline-quinone (PQQ), was added to the plates at concentrations of 0, 0.01, 0.1, 1 and 10 mM. The diameters of the colony and the colony+clearing halo were recorded at 2, 3, and 5 days of the incubation performed at 35 $^{o}$C. The bacterial growth at the center of the plates was scraped and the whole agar was placed into a centrifuge tube containing distilled water after it was cut into small pieces at day 5. The tube was centrifuged at 5000 ×g for 10 min after an overnight storage at +4 $^{o}$C. The aliquot from the tube was analyzed for soluble-P as mentioned before. There were three replications for each PSB.

***Statistical Analysis***

An attempt was made to determine whether a relationship exists between a PSB's ability to solubilize phosphate in liquid culture and properties of soil, from which this specific PSB was isolated. This was determined by a backward-elimination regression procedure of SPSS (13.0), using the soil properties in Table 1, gluconic acid concentration and pH in a bacterial culture as independent variables to predict soluble-P in the culture at a sampling time (a total of 144 case). All independent variables entered the first regression, while a variable with the highest P-value was omitted on the second regression. This method is followed until all the predictors in a regression had significant P-values ($P<0.05$). The backward regression procedure of SPSS 13.0 automatically detects and excludes the predictor, which has a significant ($P<0.05$) collinearity statistics. One-way ANOVA was used to determine significant treatment effects at 5% significance level. Duncan's Multiple Range Test was used to determine significant ($P<0.05$) differences among the treatment means.

## Results and discussion

Some chemical and physical properties of the study soils are given in Table 1. Most of the soils have low organic matter content and high $CaCO_3$ contents with alkaline reactions, typical of Anatolian soils. This resulted the deficiencies of many plant nutrients especially that of phosphorus. The level of Olsen-P ranged between 0.2 to 11.7 ppm P in this study. Generally, an Olsen-P higher than 8 ppm is considered sufficient for the cultivation of plants in the field.

We isolated a total of 21 isolates which are able to dissolve phosphate in well known Pikovskaya's agar with an initial pH of 7.8 (Table 2). Most of the strains were isolated from the rhizosphere of wheat plant. The halo formations around the colonies were visible mostly after day 3. Only the halos formed by the *Acinetobacter* strains were visible after 24 h of incubation (Table 2). All the *Acinetobacter* strains kept both growth and halo formation in Pikovskaya's agar after 6 successive cultures. Conversely, other isolates were only able to grow until the third subcultures. There were only two Gram (+) microbes and the rest belong to the Gram (-) families.

The phosphate solubilization abilities of the *Acinetobacter* strains in batch cultures were given in Figure 2. All *Acinetobacter* strains with the exception of the strains M.R.21.2.2. and M.R.21.2.2 (a Gram + isolate selected for comparison) were able to decrease the pH of the culture below 5.0. This is in line with the production of gluconic acid, which was determined by HPLC. Consequently, the levels of available-P rose up in the cultures. The strain most effective in the solubilization of tricalcium phosphate, increasing the level of phosphorus to 1022 ppm P (Table 3). This was significantly ($P<0.05$) different from all the strains selected for liquid culturing.

Summary of the models produced by stepwise regression model of the SPSS were given in Table 4. The levels of gluconic acid produced was the first predictor to enter the model with a regression coefficient of 0,769. The second regression model included the predictors gluconic acid and soil pH with standardized coefficients of 0,731 and 0,346, respectively. This indicates that the 73.1% of the variability in the solubilized-P is accounted by gluconic acid while 34.6% of the variability is accounted by gluconic acid. The third and the last model included the predictors gluconic acid, soil pH and soil available-P level, with partial coefficients of 0.707, 0.3016, 0.306, respectively with a resulting regression coefficient of 0.895. The PQQ addition at levels up to 10 mM to the Pikovskaya's agar significantly ($p<0.05$) increased the solubilization index, however, it did not have a significant ($P>0.05$) effect on the solubilized-P (Table 4).

**Table 2**

Solubilization index and growth/halo formation during consecutive subculturing in the strains that are able to form clearing halo in Pikovskaya's agar[†].

| Soil | Microorganism ID | Solubilization index | | | Growth (G) and halo formation (HF) during consecutive subculturing | | | | | | | | | | | | Identification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | |
| | | Day-1 | Day-3 | Day-7 | G | HF | G | HF | G | HF | G | HF | G | HF | G | HF | |
| 1 | W.R.1.2.3. | | | 1,46 | + | + | + | - | | | | | | | | | *Pseudomonas fluorescens* |
| 2 | W.R.2.2.2. | <1,15 | 1,36 | 1,55 | + | + | + | + | + | + | + | + | + | + | + | + | *Acinetobacter calcoaceticus* |
| | W.Rt.2.1.1. | | | 1,20 | + | + | + | + | + | - | | | | | | | *Enterobacter sp.* |
| | M.Rt.2.1.4. | | 1,40 | 1,90 | + | + | + | - | + | - | | | | | | | *Pseudomonas mandelii* |
| 3 | W.R.3.1.1. | | 1,18 | 1,20 | + | + | + | + | + | - | | | | | | | *Pseudomonas thivervalensis* |
| | W.R.3.1.3. | | | 1,45 | + | + | + | - | | | | | | | | | *Pseudomonas fluorescens* |
| | W.R.3.1.4. | | | 1,55 | + | + | + | - | | | | | | | | | *Escherichia coli* |
| | W.R.3.2.6. | <1,15 | 1,78 | 2,00 | + | + | + | + | + | + | + | + | + | + | + | + | *Acinetobacter calcoaceticus* |
| 5 | M.R.5.2.2. | | 1,50 | 1,50 | + | + | + | + | + | + | + | + | + | + | + | + | *Acinetobacter calcoaceticus* |
| | M.Rt.5.1.1. | | 1,50 | 1,90 | + | + | + | + | + | - | | | | | | | *Photorhabdus luminescens* |
| 7 | W.R.7.1.2. | | | 1,82 | + | + | + | - | | | | | | | | | *Pseudomonas thivervalensis* |
| 9 | W.R.9.2.2. | <1,15 | 1,40 | 1,60 | + | + | + | + | + | + | + | + | + | + | + | + | *Acinetobacter calcoaceticus* |
| | W.R.9.2.3. | <1,15 | 1,88 | 1,78 | + | + | + | + | + | + | + | + | + | + | + | + | *Acinetobacter calcoaceticus* |
| 11 | M.Rt.11.2.1. | | 1,33 | 1,83 | + | + | + | - | | | | | | | | | *Pseudomonas sp.* |
| 12 | W.R.12.2.2. | <1,15 | 1,67 | 1,60 | + | + | + | + | + | + | + | + | + | + | + | + | *Acinetobacter calcoaceticus* |
| 13 | M.Rt.13.2.1. | | 1,40 | 1,64 | + | + | + | + | + | - | | | | | | | *Enterobacter ludwigi* |
| 14 | W.R.14.2.1. | <1,15 | 1,67 | 2,00 | + | + | + | + | + | + | + | + | + | + | + | + | *Acinetobacter calcoaceticus* |
| | W.R.14.2.2. | | 1,60 | 1,60 | + | + | + | + | + | - | | | | | | | *Enterococcus hirae* |
| | M.R.14.2.2. | | 1,22 | 1,20 | + | + | + | + | + | - | | | | | | | *Bacillus subtilis* |
| 15 | W.R.15.2.2. | <1,15 | 1,33 | 1,33 | + | + | + | - | + | - | | | | | | | *Enterobacter sp.* |
| 21 | W.R.21.1.2. | | 1,22 | 1,40 | + | + | + | + | + | - | | | | | | | *Pseudomonas putida* |
| | W.R.21.2.2. | | 1,00 | | + | - | | | | | | | | | | | *Bacillus pumilis* |

[†] W:wheat; R:rhizosphere; Rt:root.

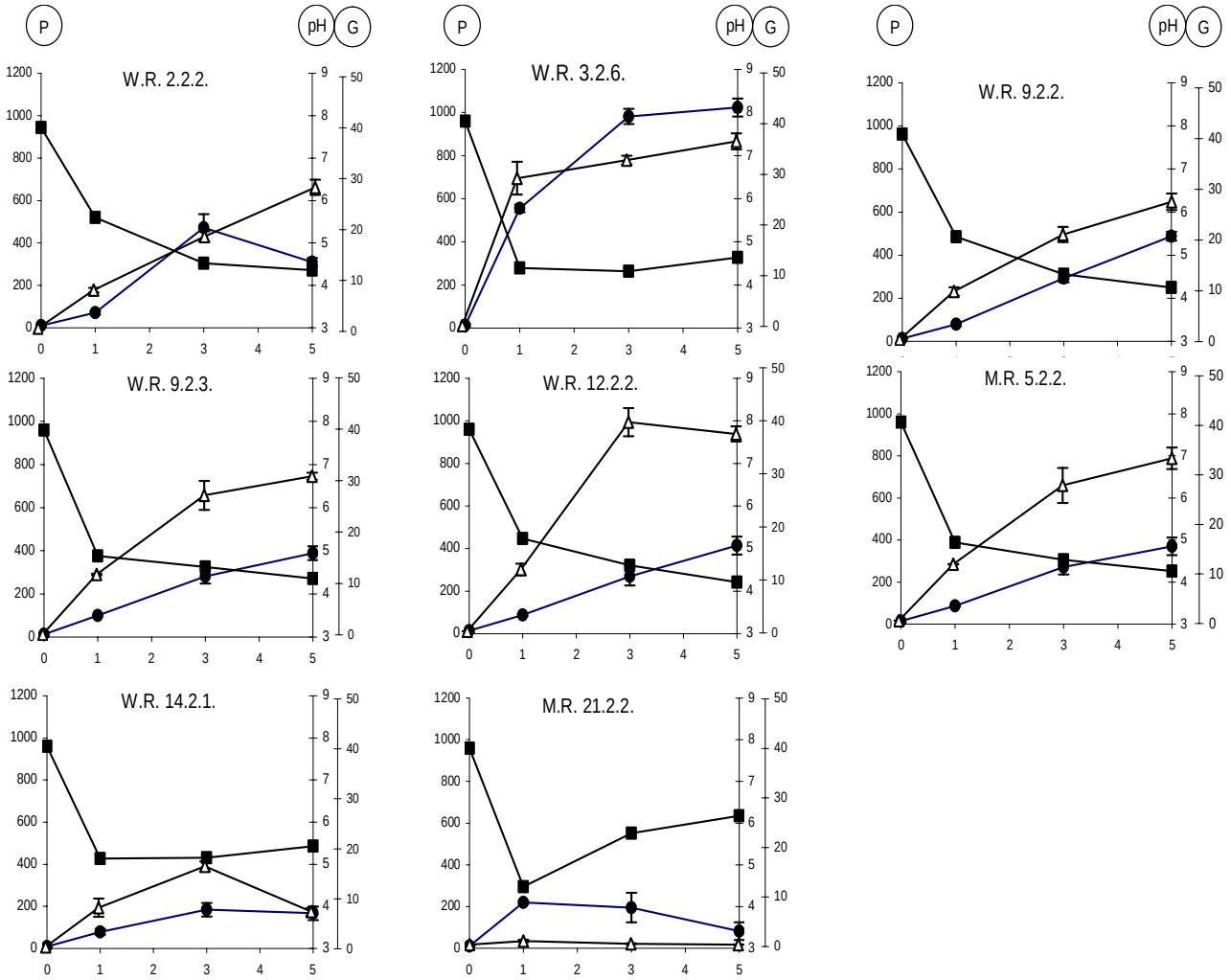


Fig. 2. The abilitiy of the strains to solubilize tricalcium phosphate in liquid culture. W: wheat; M: Maize; R; Rhizosphere. Soluble-P (ppm), (♦), P-axis; pH, (●), pH-axis; Gluconic asid (mM), (△), G-axis. The bars represent SE. The strain B.R.21.1.2. is unlike the other strains Gram (+). It represents microorganisms, which are able to grow, but, not able to produce clearing halo on Pikovskaya's agar.

**Table 3**

The mean values for pH, phosphorus (ppm), and gluconic acid (mM) in the liquid cultures[†].

| | ICOLATES | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mesurement | W.R. 2.2.2. | W.R. 3.2.6 | W.R. 9.2.2. | W.R. 9.2.3 | W.R. 12.2.2 | M.R. 5.2.2 | W.R. 14.2.1. | W.R. 21.2.2 |
| pH | 4.35 | 4.63 | 4.25 | 4.35 | 4.21 | 4.26 | 5.43 | 6.17 |
| Phosphorus (ppm) | 309c | 1022a | 488b | 389bc | 414bc | 37c | 167d | 82d |
| Gluconic asit (mM) | 28.3c | 36.5a | 27.5a | 30.8bc | 37.5a | 33.3ab | 7.4d | 0.4e |

[†] The means followed by the same letters are not significantly different at 5 % significance level according to Duncan's multiple range test. W: Wheat; M: Maize; R: Rhizosphere.

**Table 4**
Solubilization index and soluble-P in the Pikovskaya's agar plates†

| | Solubilization index | | | Soluble P (ppm) |
|---|---|---|---|---|
| | Day 2 | Day 3 | Day 5 | |
| -Strains- | | | | |
| W.R.3.2.6. | 1.272 b | 1.247 c | 1.334 d | 111 ef |
| W.R.9.2.2. | 1.216 c | 1.348 ab | 1.445 bc | 121 de |
| W.R.9.2.3. | 1.328 a | 1.332 b | 1.496 ab | 127 cd |
| W.R.12.2.2. | 1.336 a | 1.389 a | 1.541 a | 135 c |
| M.R.5.2.2. | 1.320 ab | 1.232 c | 1.382 cd | 188 a |
| W.R.14.2.1. | 1.318 ab | 1.306 b | 1.339 d | 105 f |
| W.R.21.2.2 | 1.191 c | 1.082 d | 1.074 e | 148 b |
| -PQQ Level (mM)- | | | | |
| 0.00 | 1.248 c | 1.240 c | 1.334 b | 133 |
| 0.01 | 1.230 c | 1.225 c | 1.331 b | 137 |
| 0.10 | 1.248 c | 1.219 c | 1.299 b | 128 |
| 1.00 | 1.301 b | 1.300 b | 1.434 a | 131 |
| 10.00 | 1.388 a | 1.398 a | 1.466 a | 138 |

**Table 5**
Summary of the Models produced by the stepwise regression procedure of SPSS

| | | | | |
|---|---|---|---|---|
| 1 | | R=0.769 | F=118,473 | P=0,000 |
| | | β† | t | Sig. |
| | Gluconic acid | 0,769 | 10,885 | 0,000 |
| 2 | | R=0,842 | F=98,661 | P=0,000 |
| | | β | t | Sig. |
| | Gluconic acid | 0,731 | 12,132 | 0,000 |
| | Soil pH | 0,346 | 5,731 | 0,000 |
| 3 | | R=0,895 | F=107,398 | P=0,000 |
| | | β | t | Sig. |
| | Gluconic acid | 0,707 | 14,051 | 0,000 |
| | Soil pH | 0,316 | 6,276 | 0,000 |
| | Soil Phosphorus | 0,306 | 6,087 | 0,000 |

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