Proceedings
of the
Third SHIFT-Workshop
Manaus
March 15 - 19, 1998

Studies on Human Impact on Forests and Floodplains in the Tropics

Germany

SHIFT

Brazil

A German - Brazilian Research Program

Solubilization of Al- and Fe-phosphates by bacteria isolated from the rhizosphere of *Theobroma grandiflorum* (Willd. ex Spreng.) Schum.

Wolfgang T. Marino*, José Pereira da Silva Junior#, Petra Marschner* and Reinhard Lieberei*

* Institut für Angewandte Botanik, Universität Hamburg, Germany
# EMBRAPA Amazônia Ocidental, Manaus, AM, Brazil

ABSTRACT

Increasing ecological awareness in agricultural practice lead to criticism of intensive use of fertilisers. The high costs of mineral fertiliser demands additional strategies in nutrient management, particularly in tropical regions. In oxisols, phosphate availability is low due to poor solubility of Fe- and Al-phosphates. Therefore mobilisation processes play an essential role for the P-supply of tropical plants. Both roots and microorganisms produce organic acids and phosphatases which solubilize P from Al- and Fe-phosphates and organic phosphates, respectively.

One hundred sixty-eight bacterial isolates from the rhizoplane and rhizosphere (2-10 mm away from the root surface) of *Theobroma grandiflorum* (WILLD. ex SPRENG.) SCHUM. were isolated in spring 1997 in an experimental polyculture site (SHIFT ENV-23) near Manaus and their capacity to grow on media with poorly soluble $AlPO_4$ and $FePO_4$ was determined. Seventy-four percent of the isolated bacteria were found to be effective in solubilizing hardly-soluble $AlPO_4$ and $FePO_4$. Two strains were selected because of their ability to solubilize high amounts (> 50 µg P/ml) of $AlPO_4$ and $FePO_4$ in former experiments. Their capacity to solubilize inorganic phosphates was investigated in liquid culture. Both strains solubilized similar amounts of P. The amount of P solubilization was highest after 3 days of growth while cell numbers were maximal after 13 days. During the solubilization process one strain show a decrease in pH while the other strain increased the pH. This indicates that the solubilization of inorganic phosphates is not necessarily related to the acidification.

RESUMO

O aumento da consciência ecológica nas práticas agrícolas conduz a uma forte crítica ao uso intensivo de fertilizantes. O alto custo de fertilizantes minerais exige estratégias adicionais no manejo de nutrientes, particularmente em regiões tropicais. Em Oxisois, a disponibilidade de fosfato é baixa devido a reduzida solubilidade dos Fosfatos de Al e Fe. Portanto, processos de mobilização possuem um papel essencial no suprimento de P para as plantas tropicais.

Ambos, raízes e microrganismos, produzem ácidos orgânicos e fosfatases que solubilizam o P presentes nos Fosfatos de Al e Fe e, Fosfatos orgânicos. Foram isoladas do rizoplano e rizosfera (entre 2-10 mm da superfície da raiz) de cupuaçu (*Theobroma grandiflorum* (WILLD. ex SPRENG.) SCHUM) cento e sessenta e oito isolados bacterianos em um experimento de policultivo (SHIFT ENV-23) próximo a Manaus durante o no período chuvoso e , determinou-se a sua capacidade de crescer em meio de cultura com AlPO4 e FePO4 pouco solúvel. Setenta e quatro porcento das bactérias isoladas foram efetivas em solubilizar esses fosfatos. Duas estirpes foram selecionadas e sua capacidade para solubilizar fosfatos inorgânicos foram investigadas em meio de cultura líquido. Ambas estirpes solubilizaram igual quantidade de P. A quantidade de P solubilizado foi maior após 3 dias de crescimento, enquanto o número de céluas atingiu o máximo ao 13 dias. Durante o processo de solubilização uma estirpe diminiu o pH do meio, enquanto a outra aumentou. Isto indica que a solubilização de fosfatos inorgânicos não é necessariamente relacionada à acidificação.

ZUSAMMENFASSUNG

Die zunehmende Beachtung ökologischer Gesichtspunkte in der Landwirtschaft führte zu Kritik an einer intensiven Düngungspraxis. Wegen der hohen Kosten für Mineraldünger, besonders in den tropischen Regionen, sind zusätzlichen Strategien in der Düngepraxis notwendig.

In Oxisolen ist die Phosphatverfügbarkeit wegen des geringen Löslichkeitsproduktes von Eisen- und Aluminiumphosphaten sehr gering. Hier spielen Mobilisierungsprozesse eine wichtige Rolle bei der P-Versorgung tropischer Pflanzen. Sowohl von Pflanzenwurzeln als auch von Mikroorganismen werden organische Säuren und Phosphatasen ausgeschieden, die sowohl aus schwerlöslichen Eisen- und Aluminiumphosphaten als auch aus organischen Phosphatverbindungen P mobilisieren können.

Einhundertachtundsechzig Bakterienisolate wurden im Frühjahr 1997 auf der Experimental-fläche (SHIFT ENV-23) in der Nähe von Manaus von der Rhizoplane und aus der Rhizosphäre (2-10 mm von der Wurzeloberfläche entfernt) von *Theobroma grandiflorum* (WILLD. ex SPRENG.) SCHUM. isoliert. Die Fähigkeit dieser Isolate auf Medien mit schwerlöslichem $AlPO_4$ und $FePO_4$ zu wachsen wurde bestimmt. 74% der Bakterienisolate waren imstande auf dem $AlPO_4$ und $FePO_4$ enthaltenen Nährmedium zu wachsen. Die Fähigkeit in Flüssigkultur Phosphat zu solubilisieren wurde an zwei ausgewählten Bakterienisolaten untersucht. Beide Isolate konnten in diesem Versuch die gleichen Mengen an Phosphat solubilisieren. Während die Menge an gelösten P nach drei Tagen in Kultur am höchsten war, erreichte die Zellzahl erst am Ende des Versuchs nach 13 Tagen ihr Maximum. Während des Solubilisierungsprozesses verursachte das Bakterienisolat CR 156 eine Abnahme des pH-Wertes während das andere Isolat CRP326 den pH-Wert erhöhte. Dies deutet darauf hin, daß die Solubilisierung von anorganischen Phosphatverbindungen nicht an eine Ansäuerung gebunden sein muß.

INTRODUCTION

The P content of average soils is about 0.05% (w/w) but as only about 0.1% of this phosphorus is available to plants (Scheffer and Schachtschabel 1989), intensive fertilization is often indispensable in agricultural soils. In order to avoid ecological and economical disadvantages of fertilization, a great number of scientists have tried to shift the balance between total and plant available phosphorus in the soil to the latter fraction by means of phosphate solubilizing microorganisms (PSMs) (Alagawadi and Gauer, 1992). Microorganisms capable of solubilizing poorly soluble phosphates may increase P uptake of plants (Fernandez et al. 1985, Banik and Dey 1981), however this is not always the case (Badr El-Din et al. 1986, Illmer and Schinner, 1995). The aim of our experiment was to determine the number of P-solubilizers in the rhizosphere of *Theobroma grandiflorum*. Two strains were selected for a detailed study of the solubilization process in vitro. The strains were grown in liquid medium with $AlPO_4$ and $FePO_4$. During incubation, P concentration, pH of the medium and cell numbers were determined periodically.

MATERIALS AND METHODS

The sampling area is located 28 km to the north of Manaus (2°51`south, 59°52`west). The soil is characterized as a xanthic ferralsol. The amount of plant available P in the rhizosphere is low (2.4 mg/kg soil). The pH varies between 5.4 on the rhizoplane and 4.7 in the rhizosphere. Rhizosphere samples (rhizoplane and rhizosphere (2-10 mm) were collected from *Theobroma grandiflorum* in the upper mineral soil layer (0-5 cm).

Dilution series were plated on minimal C source media (medium 1) containing [g l^{-1}]: 0.8 K_2HPO_4, 0.2 KH_2PO_4, 0.05 $CaSO_4$ x 2 H_2O, 0.5 $MgSO_4$ x 7 H_2O, 0.01 $FeSO_4$ x 7 H_2O, 1.0 $(NH_4)_2SO_4$, 2.0 glucose, 15.0 agar. The pH was adjusted to the soil pH from which the strains were isolated. The occurring colonies were transferred to medium 2 (Illmer and Schinner 1994, modified), containing 3.5% $AlPO_4$ and 3.5% $FePO_4$ and [g l^{-1}], 0.373 NH_4NO_3, 0.410 $MgSO_4$ x $7H_2O$, 0.295 KCl, 0.200 NaCl, FeIICl trace, 12.5 ml soil extract, 2.0 glucose, 22.0 agar, pH adapted to soil and rhizoplane conditions . After mixing the medium thoroughly with the sterilized P sources ($AlPO_4$ and $FePO_4$), 15 ml were dispensed into each of the Petri dishes while stirring the medium. Turbidity was due to the insoluble Al- and Fe-phosphates. Phosphate-solubilizing bacteria can be identified by their rapid growth on this medium and in some cases by the formation of a clearing zone around their colonies. Two strains CRP 326 and CR 156 were selected for further studies in liquid culture.

Erlenmeyer flasks containing 250 ml of medium 2 (without agar) with 6% $AlPO_4$ and 6% $FePO_4$ were inoculated with two single colonies. The Erlenmeyer flasks were placed on a rotary shaker with 150 rev min^{-1} and 25° C.

P was analysed using the molybdenum-blue method of Olsen and Sommers (1982). The P concentration of the uninoculated controls was substracted from that of the flasks containing bacteria.

The cell growth was determined by using MPN on agar plates. All values shown are averages of 3 or 5 replicates.

RESULTS AND DISCUSSION

Seventy-six percent of oligotrophic bacteria in the *Theobroma*-rhizosphere are able to grow on medium containing poorly soluble phosphates (Table 1). However, only 4 % of the isolates formed a clearing zone around their colonies, indicating strong P solubilization.

Table 1: Capacity of rhizosphere bacteria of *Theobroma grandiflorum* to grow on Fe- and Al-phosphate medium.

Growth (%)	no growth (%)	Appearance of clearing zones around colonies (%)
76	24	4

n= 168 bacterial isolates (=100%)

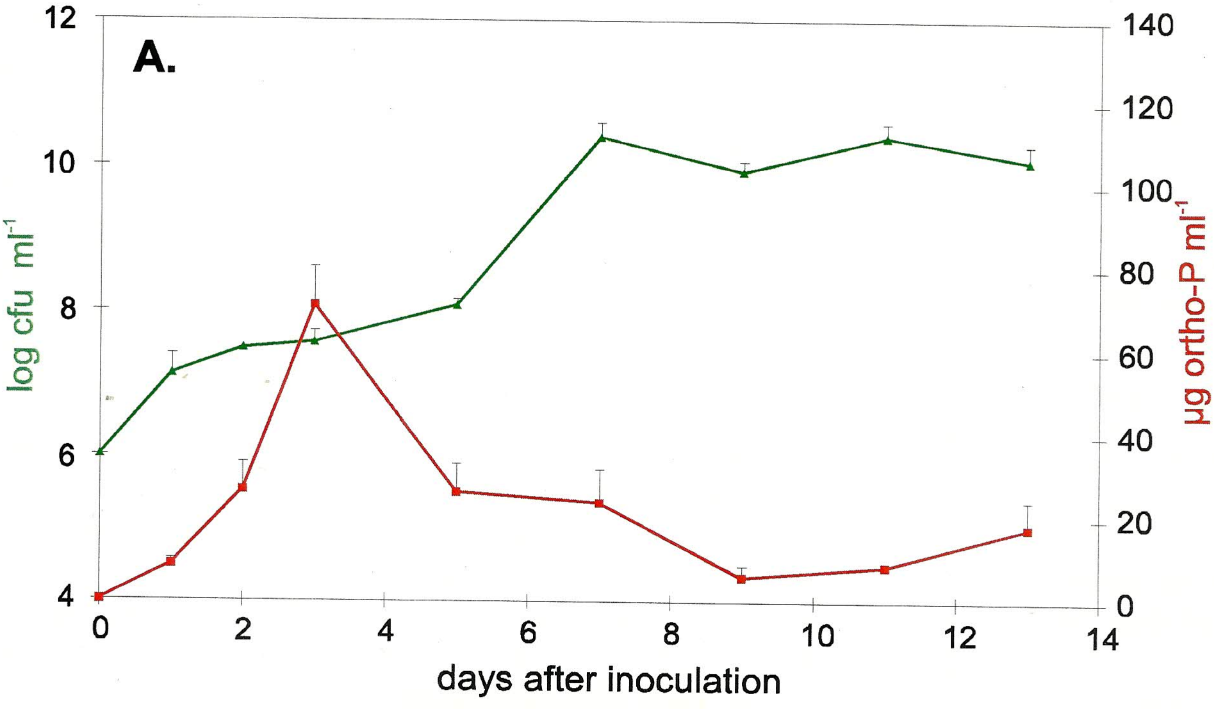
It is well known from former studies that bacteria which did not form a clearing zone around their colonies are able to solubilize poorly soluble phosphate (Illmer, 1993, Kucey, 1983, Thomas, 1985, Kucey 1987).

Two bacterial strains which were capable to grow on this medium but which did not form these clearing zones were selected for further studies.

Changes in cell growth and P-concentration in the solutions are shown in Fig. 1 A and B.

In the initial incubation period (until day 3), when cell density increased only slightly the amount of mobilized P was greater than after day 6 when cell density increased strongly . Thus, strong P mobilization occured before cell numbers increased. Increased cell growth was associated with low amounts of soluble P, indicating that soluble P was taken up by the growing population or that P reprecipitated as Al- or Fe-phosphate (Illmer and Schinner 1994). The initial low cell density appears to have mobilized enough P to support high growth rates after 6 days. At day 3 when maximum soluble P concentrations were measured, it can be calculated that CR 156 solubilized 4.1 µg P/(10^6 cfu)$^{-1}$ while CRP 326 solubilized only 2 µg P/(10^6 cfu)$^{-1}$.

In isolate CRP 326, P-solubilization was accompanied by an initial pH increase to about pH 6 (Figure 2 A). After 5 days, the pH value decreased to about pH 4. In isolate CR 156 the pH decreased during the whole process of P-solubilization. Thus in this study, P mobilisation from Fe and Al phosphates was not necessarily related to pH. This is in accordance with Hinsinger and Gilkes (1996) who also found that dissolution of Al-phosphate was not related to pH while dissolution of Ca phosphates increased with decreasing pH.



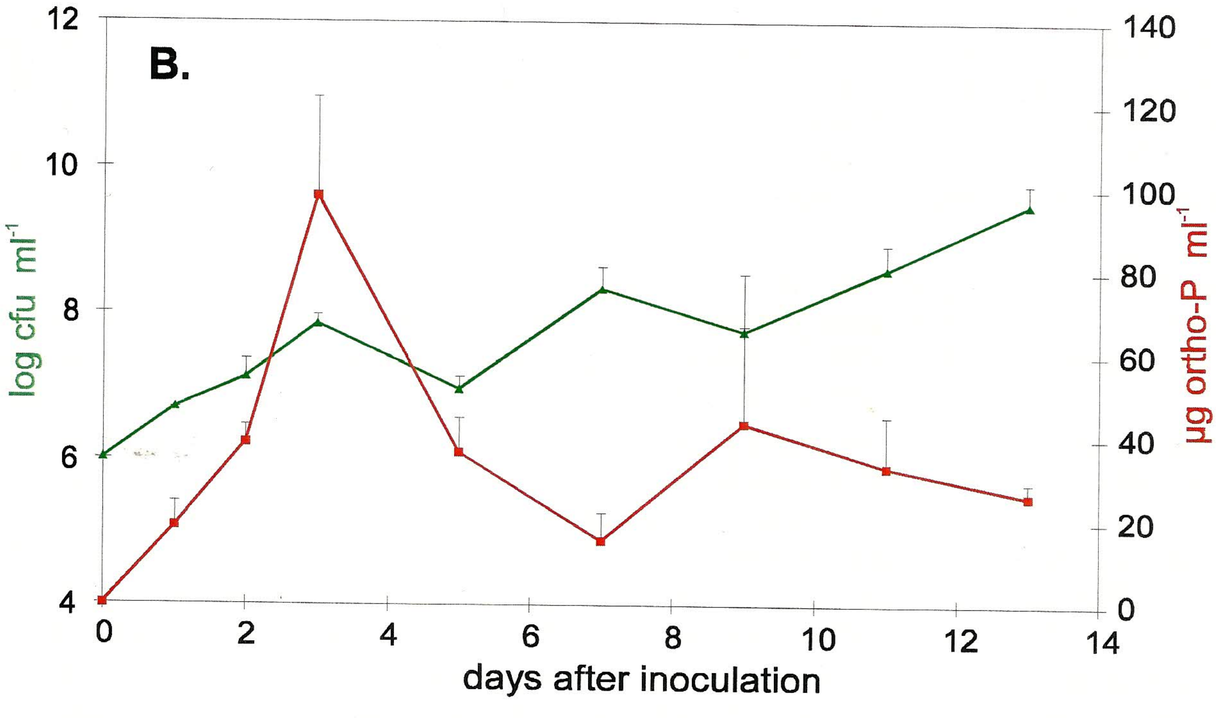
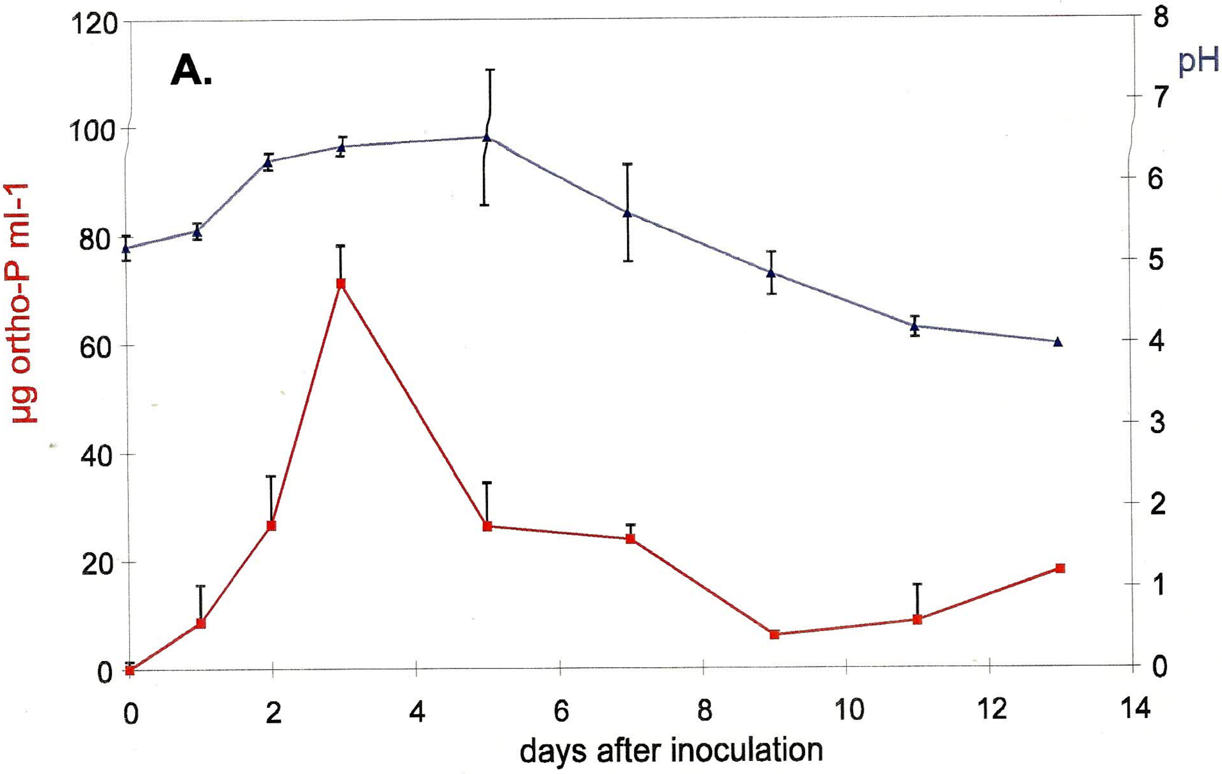


Figure 1: P-mobilisation and cell growth of bacterial isolates A. CRP 326 (bacterial strain from the rhizoplane) and B. CR 156 (bacterial strain from the rhizosphere).
▲ = log cfu ml^{-1}; ■ = µg ortho-P ml^{-1}. Bars indicate standard deviation.



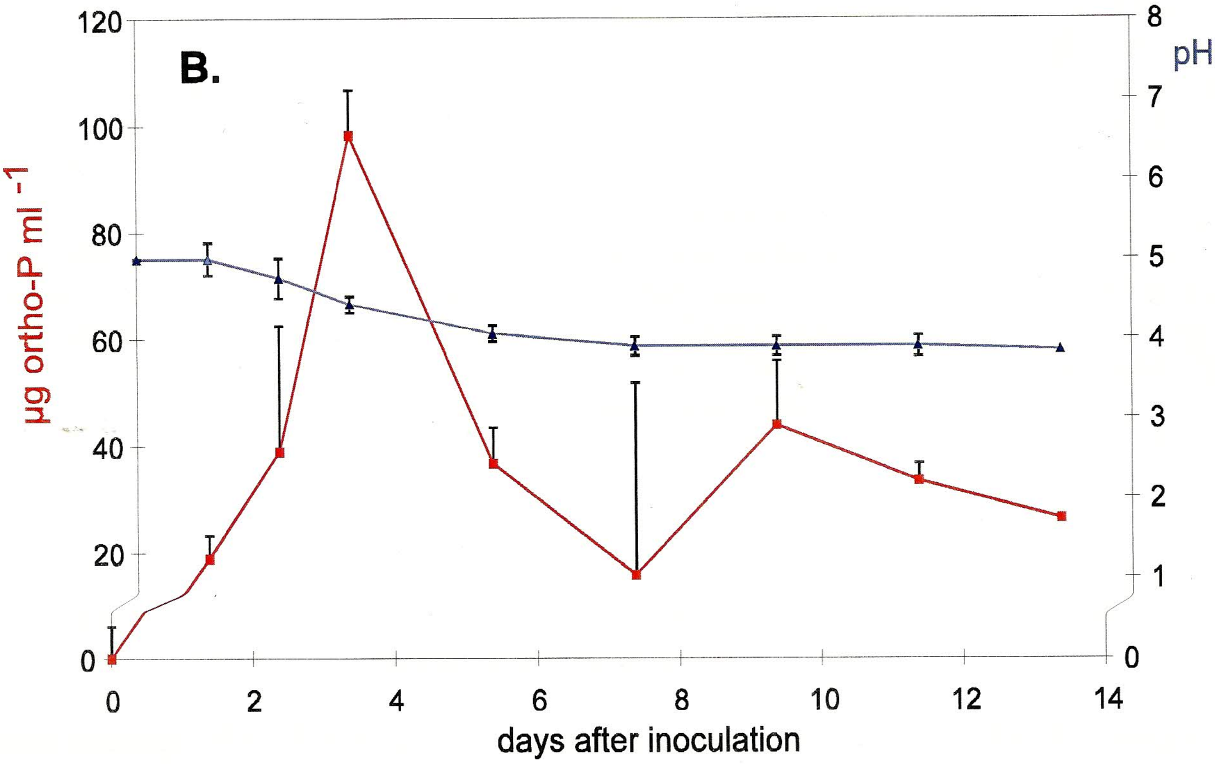


Figure 2: Changes in P-concentration and pH during the solubilization of $AlPO_4$ and $FePO_4$ by 2 bacterial strains. A. CRP 326, B. CR 156.

▲= pH; ■ = µg ortho-P ml^{-1}. Bars indicate standard deviation.

The mechanism of P solubilisation by microorganisms is still unclear. According to the sink theory of Halvorson et al. (1990), P solubilisation is due to the uptake of P by the microorganisms which shifts the equilibrium towards further solubilisation. However, this would not explain the increase in available P in the presence of P solubilizing bacteria (Fig. 2, Fernandez et al. 1985). It is more likely that P solubilizing bacteria increase P solubility by other mechanisms.

One proposed mechanism is the extrusion of H^+ from direct oxidation of glucose or other aldose sugars ("non-phosphorylating oxidation") which leads to acidification and dissolution of Ca-phosphates (Goldstein et al. 1993). Another mechanism is the excretion of organic acids. These may also lead to a pH decrease by dissociation. Additionally, organic acids can complex Al, Fe and Ca and compete with P for the same binding sites (Cunningham and Kuiack 1992, Illmer and Schinner 1995, Kim et al. 1997). Therefore, organic acids are more effective in P dissolution than H^+ (Lopez-Hernandez et al. 1979). P solubilizing microorganisms produce different organic acids, such as oxalate, citrate, malate and keto-glutarate (Banik and Dey 1983, Cunningham and Kuiack, 1992).

In the present study, the slight pH decrease in CR 156 may have contributed to P solubilization (Fig. 2). However as mentioned above, the dissolution of Al- and Fe-phosphates appears not to be affected by pH (Hinsinger and Gilkes, 1996).

We therefore conclude that the observed P dissolution is mainly due to the excretion of organic acids by the P solubilizing bacteria. After inoculation, the P deficient cells probably produced large amounts of organic acids. Thus, less carbohydrates were available for growth. When the soluble P concentration had reached a sufficient level, metabolism of the cells could be shifted towards cell growth and cell division. Additionally, free organic acids could be taken up again. This, together with reprecipitation of Al and Fe phosphate and the utilization of soluble P by the growing cells, lead to the observed decrease in the amount of soluble P in the medium (Illmer and Schinner, 1994).

The contribution of P solubilizing microorganisms to plant nutrition is still controversial. In some cases, inoculation with P solubilizers increased yield and P uptake of plants (Fernandez et al. 1985, Gaind and Gaur 1991, Belimov et al. 1995), while in other cases they had no effect on P uptake (Azcon-Aguillar et al. 1986, Badr-el-Din et al. 1986).

The two P solubilizing bacteria in the present study are unlikely to contribute substantially to plant P uptake as they do not appear to solubilize more P than needed for subsequent growth. However in the rhizosphere, they may solubilize more P if plant uptake decreases the P concentration in the soil solution. Additionally, root exudates would provide easiliy available carbohydrates, thus decreasing the reuptake of organic acids by the bacteria. On the other hand, microorganisms in the rhizosphere will compete with the plant for P.

However, increasing P availability may be one mechanism by which plant growth is enhanced by P solubilizing bacteria. Other beneficial effects include production of phytohormones (Sattar et al. 1987, Leinhos 1994, Chabot et al. 1996) and increased arbuscular mycorrhizal infection as well as N_2 fixation (Kundu, 1980, Azcon-Aguilar et al. 1986).

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