Chapter 10
Agricultural Development in Tropical Acidic Soils: Potential and Limits of Phosphate-Solubilizing Bacteria

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10.1 Introduction

Freedom from hunger is a fundamental human right. As such, it is intrinsically linked to, as well as essential for, the full enjoyment of other rights such as health, education, and work, and everything that emanates from them. Nevertheless, according to recent estimates from the Food and Agriculture Organization of the United Nations (FAO), more than 923 million people are chronically hungry, most of them in rural areas of poor countries (FAO 2005). The number of hungry people is steadily rising, having increased by about 50 million in 2007 alone. If world population continues to grow at the mean estimated annual rate of 1.1%, it will reach 7.4 billion by 2017. In 2050, it will head towards 9.3 billion people (FAO 2005). Therefore, the world urgently needs a second Green Revolution – sometimes referred to as the "Gene Revolution" – in order to increase food production by at least 50% in the next 20 years.

The challenge we are facing is enormous. Two of the requirements for producing additional food are of paramount importance. First of all, agriculture productivity must significantly increase, particularly in the lesser developed, tropical regions of the world. The second condition is that the agricultural frontier be expanded into regions considered as marginal lands, consisting of fragile soils with a lower productive capacity and a higher risk of degradation (Zapata and Roy 2004).

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10.2 Acidic Soils and Agriculture in the Tropics

Acid soils occupy about 3.95 billion ha and account for 30% of the world's ice-free land area (von Uexküll and Mutert 1995). Approximately 43% of the world's tropical land area is classified as acidic, comprising about 68% of tropical America, 38% of tropical Asia, and 27% of tropical Africa (reviewed in Herrera Estrella 1999). Thus, acid soils represent the largest potential land area for future agricultural development. These soils are highly weathered because of warm temperatures and high rainfall. They are also inherently infertile. Many reasons account for this infertility: a strongly acid (5.5–4.5) to extremely acid (<4.5) pH; a low cation-exchange capacity; a low base saturation; a high phosphorus-fixing (P-fixing) capacity; and high to toxic levels of Al^{3+} cations (reviewed in He et al. 2003; see Chap. 1). Intensive weathering of tropical soils removes nutrient cations, leaving behind more stable materials rich in Fe and Al oxides. Human intervention on soils through fertilization, irrigation, mining, and long-term legume cultivation also contributes to land degradation and acidification.

The dominant acid soils in the tropics and subtropics include Ultisols and Oxisols (termed Ferralsols in the FAO taxonomy scheme). These soils are widely cultivated, and are most important to world agriculture. However, they generate low crop yields due to a combination of factors that includes their extreme nitrogen (N) and phosphorus (P) deficiency (reviewed by He et al. 2003). Since not all acid soils can be used for agriculture (the tropical forest soils, for example, should be left unexploited), efforts to develop acid soils for agriculture should be directed to the acid savannas of the world, which have an enormous potential for food production and cover an area of over 700 million ha (which represents approximately 50% of the global area that is currently under cultivation) (Herrera Estrella 1999). Even though there are some good examples of successful conversion of acid savannas into productive lands for agriculture, new, efficient, and environmentally friendly technologies should be developed for this productivity to be increased in a sustainable way and without causing major harm to the environment.

10.3 Phosphorus and Acid Soils

After nitrogen, phosphorus is the most important nutrient for plant growth and development. Unfortunately, while most mineral nutrients in soil solution are present in millimolar amounts, the free soluble P concentration is in the range of micromolar or lesser quantities even at pH 6.5, where P is most soluble (He et al. 2003). This is paradoxical, since tropical soils contain total P at concentrations which are orders of magnitude greater than those of plant-available P. A large proportion of this total P corresponds to organic forms, traditionally considered as relatively unavailable to plants, although this view has been challenged (reviewed by Turner et al. 2006). On the other hand, inorganic P is either “occluded” within, or else strongly fixed at the surface of soil minerals, mainly Fe and Al hydrous oxides (sesquioxides). Indeed, free Al and Fe cations react readily with phosphate, leading to relatively insoluble precipitates. This reversible process is known as “P fixation” (Johnson and Loeppert 2006).

To cope with such an extreme deficiency, most developing countries import chemical fertilizers, which are often in limited supply and represent a major expenditure for resource-poor farmers. In addition, intensification of agricultural production in these regions necessitates the addition of large P inputs, not only to increase crop production but also to improve soil P status in order to avoid further soil degradation. However, a significant proportion of this soluble P is either rapidly fixed by soil components or else is leached in the short term, causing eutrophication of surface waterbodies (Zapata and Roy 2004). While there are some efficient ways of improving soil N status (by applying crop residues and other organic sources or by inoculating crops with N-fixing bacteria, NFB), soil P status is more difficult to ameliorate. Therefore, it is imperative to explore alternative methods for improving the status of P in acidic soils. Very attractive in this regard is the possibility of enhancing P uptake of crops by the inoculation of P-solubilizing bacteria (PSB).

10.4 The Use of Soil Bacteria as Biofertilizers

Soil micro-organisms play a significant role in the major global biogeochemical cycles, regulating the dynamics of organic matter decomposition and the availability of plant nutrients such as N, P and S. As such, soil micro-organisms play a fundamental role in promoting plant growth. In the rhizosphere, (i.e. the zone surrounding and influenced by roots) micro-organisms interact with plants in several ways. Bacteria that act positively on plant growth and development, through direct or indirect mechanisms, are collectively known as “plant growth-promoting rhizobacteria (PGPR). While some PGPR act by inhibiting or antagonizing plant pathogens (these are termed “bioprotectants” or “biocontrollers”) others promote plant growth by releasing phytostimulators (in the case of the “biostimulants”) or by providing nutrients, such as N and P (for “biofertilizers”) (reviewed by Zahir et al. 2004). Today, the use of N-fixing bacteria (NFB) to enhance crop yield, particularly through the improvement of nodulation and biological N_2 fixation in legumes, is widespread. By comparison, the use of PSB as biofertilizers is much less common. As we will review in this chapter, even though literally thousands of PSB have been isolated, identified, characterized, and proposed as potential biofertilizers, their effectiveness in the soil-plant system is still unclear, particularly under field conditions.

10.4.1 The Phosphate-Solubilizing Bacteria

Rhizospheric micro-organisms may affect P supply to plants in different ways: (1) by immobilizing P in microbial biomass, (2) by enzymatic decomposition of organic P compounds, and/or (3) by promoting the solubilization of insoluble phosphates. The last two mechanisms release soluble inorganic orthophosphate ($H_2PO_4^-$) into soil solution, from which it can be absorbed by plant roots. The ability of some soil-borne bacteria to dissolve mineral phosphates was noticed by researchers more than a century ago (Goldstein and Krishnaraj 2007). Most of the early studies were centered on the isolation of P-solubilizing micro-organisms (PSM) from the rhizospheric soil and the characterization of their phosphate-solubilizing activity under in vitro conditions. Research into solubilization of P under field conditions and its uptake by plants began much later (Gerretsen 1948).

Even though fungi are among the best studied PSM, this chapter will focus on the activity and potential use of P-solubilizing bacteria (PSB) for agricultural development. In his pioneer work, Gerretsen concluded that the "solvent action of bacteria" (today called mineral phosphate solubilizing ability, MPS) is a general character of several types of micro-organisms. Since then, the so-called MPS$^+$ phenotype has been identified in numerous bacterial isolates (for review see Rodriguez and Fraga 1999). Strikingly, PSB can also enhance growth and development of plants by mechanisms other than P solubilization. Indeed, they can release phytohormones that promote root growth, enhance availability of other nutrients (e.g. Mn and Fe), and control plant pathogens (reviewed by Vassilev et al. 2006, Nehl and Knox 2006, Avis et al. 2008). Additionally, some PSB may protect plants against the detrimental effects of heavy metals (Ma et al. 2008) and induce plant systemic resistance (Han et al 2008).

According to some authors, PSB occur in most soils and may represent up to 40% of the culturable population (Richardson 2001), even though this proportion has been said to be overestimated (see below). PSB started to be massively applied to soils during the 1950s in the former Soviet Union, with rather spectacular results. However, serious concerns about the reliability and reproducibility of these results arose very soon (Mishustin and Naumova 1962).

Although thousands of PSB have been isolated in the past, these belong to only a few bacterial genera, namely: *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Rahnella*, *Escherichia*, *Enterobacter*, *Burkholderia*, *Serratia*, *Pantoea*, and some other members of the *Enterobacteriaceae* family. Species belonging to the genus *Rhizobium* and related organisms have also been identified as PSB (Kämpfer 2007).

Considering the diverse mechanisms which are involved in the solubilization of P (see below), it is no surprise that knowledge of the genetics of the MPS$^+$ phenotype remains scanty (Goldstein 1995; Rodriguez and Fraga 1999; Rodríguez et al. 2007). Only a few genes, mainly encoding proteins related to the production of organic acids, have been isolated and characterized. Almost nothing is known about the regulation of the expression of these genes, and results of efforts at improving P-solubilizing ability through genetic modification of PSB remain modest.

10.4.2 Mechanisms of Inorganic Phosphate Mobilization by PSB

As we will review in Sect. 10.5 of this chapter, it is widely agreed that soil bacteria participate actively in P solubilization from sparingly soluble P-containing minerals by producing and excreting organic acids (e.g. see review by Rodriguez and Fraga 1999). However, other microbially based mechanisms have also been proposed as contributing to soil P mobilization, often on the basis of an observed lack of correlation between acidification of PSM culture supernatants and P solubilization (Sperber 1958b; Halvorson et al. 1990; Illmer and Schinner 1992 and 1995a). These alternative mechanisms of P solubilization are briefly summarized below.

10.4.2.1 Sink Theory

The "sink theory," proposed by Halvorson et al. (1990), considers that the very effective P uptake systems of rhizospheric micro-organisms would enable the assimilation of large amounts of P from the soil solution, thus disturbing the equilibrium between insoluble and soluble P. Sparingly soluble phosphates would then be dissolved indirectly by the continuous removal of P from the soil solution.

10.4.2.2 Acidification by H^+ Excretion Theory

This mechanism was proposed to explain P solubilization, dependent on the production of microbial biomass and accompanied by a decrease in pH, *in the absence* of organic acid or chelating agent production (Illmer and Schinner 1995a). The H^+ release is thought to be associated, under these circumstances, with ammonium ion (NH_4^+) assimilation. Protons may then act as agents for P solubilization. Plants evolved a similar strategy to release P from sparingly soluble minerals (reviewed in Hiradate et al. 2007).

10.4.2.3 The Bacterial Cell Wall and P Solubilization

Due to their negative charge, bacterial surfaces can adsorb a wide range of metal cations. Therefore, bacteria are commonly adsorbed on mineral surfaces in soils, sediments and groundwaters, mainly by hydrogen bonding and/or by electrostatic interactions (Young and Crawford 2004; Rong et al. 2008). This non-metabolic cell wall adsorption is significantly affected by pH, with larger amounts of bacteria adsorbed at lower pHs. The interaction of bacteria with minerals can cause both an increase in the extent of mineral dissolution and an inhibition of secondary mineral formation (Wightman and Fein 2004; Rong et al. 2008). The magnitude of this bacterial effect is proportional to the abundance of cells in a geologic system, and to the magnitude of the stability constants of the principal metal-bacterial surface complexes (Wightman and Fein 2004). Since minerals containing Fe^{3+} or Al^{3+} cations form highly stable surface complexes with bacteria, the solubility of these minerals should be strongly affected by bacterial surface adsorption (Fein et al. 1997).

Generally, bacterial adsorption to mineral surfaces is the first step towards the establishment of a biofilm. Cells in biofilms are embedded within an extracellular matrix composed of self-produced polymeric substances. Biofilm formation has been proposed to play an important role in altering the solubility of minerals (Welch et al. 2002) and also in contributing to P solubilization in soils (Jayasinghearachchi and Seneviratne 2006). One possible explanation for this phenomenon is that the production of both organic acids and exopolysaccharides (EPS) increases in mixed biofilms (Bandara et al. 2006). In keeping with these observations, the role of bacterial EPS in the microbially enhanced dissolution of P has been recently demonstrated (Yi et al. 2008). The increase of P solubilization brought about by EPS is attributed to its capacity for holding free P in the medium, therefore modifying the solubility equilibrium to accelerate the release of soluble P. On the other hand, Liu et al. (2006) showed that bacterial EPS strongly adsorb organic acids, which results in high concentrations of organic acids near the mineral.

10.4.2.4 Inorganic Acid Production and P Mobilization

The production of soluble P fertilizers is usually accomplished by sulfuric acid treatment of rock phosphate (RP). Therefore it is no surprise that acidophilic bacteria (e.g. *Acidithiobacillus* sp.), able to oxidize reduced sulfur compounds (such as pyrite) to sulfuric acid (H_2SO_4), may participate in the solubilization of P in soils. For instance, addition of *Acidithiobacillus thiooxidans* (formerly *Thiobacillus thiooxidans*) and low-grade phosphatic rock (PR) to an Oxisol caused a rapid drop in soil pH to levels below 4.0 and a concurrent increase in soluble-P level (Muchovej et al. 1989). The amount of P thus solubilized was sufficient for sustaining sorghum (*Sorghum bicolor*) growth at levels similar to those obtained using superphosphate. Similar results were obtained by Stamford et al. (2007) with the use of *A. ferrooxidans* and yam bean (*Pachyrhizus erosus*), in acid soils with low available P.

10.4.2.5 Bacterial Chelators and P Solubilization

As we will see in Sect. 10.5, organic acids both sequester cations and acidify the soil solution, thus causing solubilization of P. The chelating property of the organic anions is therefore as important as the proton effect. Indeed, it has been shown that the addition to soil of well-known chelators, such as EDTA, may enhance both mineral dissolution and P solubilization (Campbell and Eick 2002). Some bacterial metabolites, different from organic acid anions, may also contribute to P solubilization through chelation. Siderophores, for example, are biogenic chelators with a high affinity and specificity for iron, which can alter the solubility of iron oxides over a wide pH range (reviewed by Kraemer 2004). This can release P adsorbed on the surface of the Fe oxides. Recently, Hamdali et al. (2008) found that P solubilization from RP correlated well with the production of siderophores – but not with that of organic acids – by actinobacteria (*Streptomyces* sp. and *Micromonospora* sp.).

10.4.2.6 Reductive Dissolution of P-Containing Minerals

The reduction of hydrous ferric oxides under anaerobic conditions, characteristic of flooded soils, may release occluded P and ferrous iron (Fe^{2+}) to the soil solution (Stemmler and Berthelin 2003). This phenomenon is well documented in rice paddy fields. If soils are highly saturated with inorganic P, relatively large concentrations of soluble P can be released to the soil solution by reductive dissolution of minerals (Young and Ross 2001). Conversely, almost no soluble P will be mobilized to floodwater if the soil initial P content is low. The presence of redox-inactive Al oxides on the surface of ferric oxyhydroxides may negatively affect reductive dissolution rates of PO_4 and Fe^{2+} (Murray and Hesterberg 2006).

10.4.3 Organic P Mineralization

There is growing recognition of the importance of organic forms of P (P_o) for the development of sustainable agriculture practices, especially in tropical soils (Turner et al. 2006). A large proportion of soil P occurs in organic forms (phosphate esters, phosphonates, and anhydrides), which must be hydrolyzed by specific enzymes to release PO_4. Numerous soil micro-organisms are able to mineralize P_o by synthesizing phosphatases, and some are able to perform simultaneously inorganic P solubilization and P_o mineralization. For example, *Enterobacter agglomerans* and *Burkholderia cepacia* exhibited significant abilities to both solubilize hydroxyapatite and hydrolyze P_o (Kim et al. 1997, 1998; Rodriguez and Fraga 1999). Other bacterial strains with high P-solubilizing abilities have also been found to exhibit substantial acid or/and alkaline phosphatase activities (de Freitas et al. 1997), and it was found recently that a considerable proportion of soil micro-organisms harbor these combined properties (Oliveira et al. 2008).

10.5 Role of Organic Acids in P Mobilization from Acidic Soils

Both plants and soil micro-organisms exude or excrete low-molecular weight carboxylates (organic acid anions), able to efficiently chelate metal ions. Microbial production of organic acid P ligands may occur either constitutively or in response to P deficiency and is enhanced in the rhizosphere. Indeed, a large proportion of bacteria thriving in the rhizosphere may solubilize P by transforming sugars exuded through the roots into their respective sugar acids, whose amount and nature would depend on the type of sugars available (Deubel et al. 2000).

Two primary mechanisms have been proposed to explain the release of P from Fe-oxide surfaces in the presence of organic ligands and in the context of acidic soils: (1) ligand exchange and (2) ligand-enhanced dissolution of the Fe-oxides (reviewed in Johnson and Loeppert 2006). In ligand exchange processes, organic ligand exchanges for inorganic P at a mineral surface site, thus releasing P into the soil solution. During ligand-enhanced dissolution, the organic ligand is adsorbed first at a surface structural Fe site, and then the Fe-oxide surface is slowly dissolved, releasing adsorbed P to the soil solution. The relative effectiveness of specific organic acids in releasing P by either mechanism is related to the number and arrangement of organic acid carboxyl- and hydroxyl-groups (reviewed in Jones et al. 2003, and in Arcand and Schneider 2006). In general, the relative capacity to desorb P in soils varies in the following order: tricarboxylic- > dicarboxylic- > monocarboxylic-acid.

Some of the organic acids that have been shown to mobilize P from soils are citric, glutamic, succinic, lactic, oxalic, glyoxalic, maleic, fumaric, tartaric, and α-ketobutyric acids (Rodriguez and Fraga 1999; Khan et al. 2006). In addition, gluconic and 2-ketogluconic acids may be the most effective, particularly because of their extremely low pK_a(s) (~3.4 and ~2.6 respectively) (Goldstein 1995; Rodríguez Rodriguez and Fraga 1999), although their ability to solubilize P adsorbed to Fe- and/or Al-oxides has been debated (Whitelaw et al. 1999). These acids are produced through direct oxidation (or nonphosphorylating oxidation) of glucose, an alternative aldose utilization pathway which is expressed in a number of rhizobacteria (Goldstein 2007).

Citric acid is perhaps the best studied P-mobilizing agent in the context of acidic soils. P solubilization mediated by citric acid is not only due to acidification of the soil solution, but also to formation of Al and Fe complexes (reviewed by Jones 1998). For instance, citrate was among the most effective acids in releasing P from two Fe oxides, ferrihydrite and goethite (Johnson and Loeppert 2006), and from P-loaded synthetic goethite (Geelhoed et al. 1998). Input of citrate to acid soils not only results in release of soluble P (reviewed by Hocking 2001) but also reduces the sorption of newly applied fertilizer P (Jones and Darrah 1994). Addition of citric acid to soils may increase the amount of dissolved molybdate-reactive P (Drouillon and Merckx 2003; Hutchison and Hesterberg 2004). Citric acid mobilized and released more P from an upland clay loam Ultisol than did tartaric and oxalic acids (Wang et al. 2008).

A number of bacterial species are efficient producers of citric acid and therefore have been tested in P solubilization experiments. In one of these studies, Hoberg et al. (2005) demonstrated that *Pseudomonas fluorescens* released up to 60 mM citrate in the presence of P-loaded goethite.

In some cases, however, addition of organic acids (e.g. citric, oxalic, tartaric, gluconic, succinic, lactic, and acetic acid) to acid soils amended with RP failed in releasing P, even though the acid concentration was sufficient to decrease the pH to less than pH 4.0 (Srivastava et al. 2007). Indeed, besides the identity and the concentration of the organic acids, a number of other factors, such as Fe-oxide crystallinity, soil solution pH, and initial P content, may affect P release from natural soils (Johnson and Loeppert 2006; Hiradate et al 2007).

Organic acids may also affect P mobilization in soils indirectly, particularly by improving the ability of crops to obtain P from organic P compounds. For instance, organic acids can promote the growth of micro-organisms that mineralize organic forms of P (Richardson 1994). There is also evidence that organic acids can liberate adsorbed and complexed phytate, rendering it susceptible to breakdown by extracellular phytases (Jones 1998). In addition to their P-mobilizing abilities, organic acids also mediate detoxification of metals, particularly Al^{3+}, allowing plants to grow (Hocking 2001; Jones et al. 2003).

When considering the role of organic acids on P availability and uptake, it should be taken into account that research has often been conducted under laboratory conditions, which are vastly different from those that exist in vivo. Besides, organic acids are not only produced, but also consumed by soil micro-organisms (Jones et al. 2003). Organic acids are metabolized two to three times faster in the rhizosphere than in bulk soil, typically with 60% of the molecules present being mineralized and the remainder being incorporated into microbial biomass (Jones 1998). Nevertheless, organic acids may be protected from rapid biodegradation through an interaction with the soil solid phase (occurring by adsorption or fixation) (Jones et al 2003). Plants may also reduce the rate of organic acid microbial degradation by secreting phenolic compounds, mainly isoflavonoids, through their roots (Weisskopf et al. 2006).

10.6 Field Experiences with Phosphate-Solubilizing Bacteria

Massive application of PSB to increase the fertility of natural soils was carried out in the former Soviet Union during the 1950s (Mishustin and Naumova 1962). A large proportion of agricultural soils were inoculated with a fertilizer consisting of kaolin-impregnated *Bacillus megaterium* var. *phosphaticum* spores. The biofertilizer was called phosphobacterin. In general, the results were astonishing: addition of phosphobacterin to Soviet soils allowed crop yield increases of up to 70%! This was attributed to the mobilization of soil P as a result of microbial production of organic acids. In India, where almost 46% of all soils are classified as P-deficient, researchers also reported positive responses — although not as spectacular — to phosphobacterin applications (reviewed in Sundara et al. 2002). In contrast, experiments conducted in the United States during the 1960s did not confirm the value of phosphobacterin as a universal biofertilizer (Smith et al. 1961).

Significant areas of cultivated soils in Korea and China are P-deficient and have low crop productivity (Xie 1998). Therefore, scientists from these countries contributed numerous articles dealing with the use of PSB for agricultural purposes.

The enormous interest in this area is better reflected in the numerous patents issued to researchers, R&D institutions, and private companies. A number of PSB are even commercially produced as biofertilizers (Lucy et al. 2004; see Chap. 11). Application of PSB under field conditions proved beneficial to plant growth, yield, P uptake, and overall quality of various crops and legumes in different regions of the world (recently reviewed by Khan et al. 2006 and by Osorio Vega 2007). Field tests have been performed as single, dual- or multi-species inoculations alone or in combination with the application of PR. Although most experiments were conducted in non-acidic soils, various observations are relevant in the context of the present review and will be summarized here. For a more detailed account, the reader is referred to the excellent reviews from Rodriguez and Fraga (1999), Gyaneshwar et al. (2002), Khan et al. (2006) and Osorio Vega (2007).

10.6.1 Single-Species Inoculations

Numerous authors have reported on the beneficial effects of single-PSB species inoculation on growth and development of different crops, in many cases showing consistent increases in crop yield over several years. These published accounts were compiled by Lucy et al. (2004), and some will be presented here. Following a 3-year study, Sundara et al. (2002) concluded that application of *B. megaterium* var. *phosphaticum* to sugarcane cultures resulted in enhanced tillering, stalk population, and weight. The authors also reported increases in cane and sugar yields and an improvement in juice quality. The same study showed that a 25% reduction in the amount of P applied to sugarcane was possible when the P fertilizer was used in combination with PSB. Similarly, a 2-year study conducted under different field conditions showed that inoculation of sugar beet seeds with *Paenibacillus polymyxa* and *Pseudomonas putida* significantly increased leaf yield, when compared to the uninoculated controls (Çakmakçi et al. 2006). However, in many cases, inoculation of plants with PSB did not result in an increase of the crop parameters monitored (Gyaneshwar et al. 2002). Indeed, as recently pointed out by Goldstein and Krishnaraj (2007) and by Richardson (2007), when considering the hundreds of papers published on this subject, the number of failures equals the number of successful trials. The reasons explaining such discouraging results are discussed in Sect. 10.8 of this chapter.

A large number of legume-nodulating *Rhizobium* strains are able to solubilize inorganic phosphates (Chabot et al. 1996; Sridevi et al. 2007). Apart from their dual beneficial nutritional effect resulting from both P mobilization and N_2 fixation (Peix et al. 2001), rhizobia also establish synergistic interactions with arbuscular mycorrizal fungi (AMF) (Barea et al. 2002). Further advantages of rhizobial use as PGPR include their integration in well-established crop rotation systems and the ready availability of technologies for inoculation and inoculant production. Furthermore, since they have been used with legumes for many years without causing harm to the environment or to farmers (see Chaps. 8 and 11), rhizobia are perceived as environmentally friendly. In a pioneering work, Chabot et al. (1996) assayed P-solubilizing rhizobia in field trials, observing growth stimulations for lettuce (*Lactuca sativa*) and maize (*Zea mays*) similar in magnitude to those obtained with other PSM. Sorghum growth and P uptake were also significantly increased by rhizobial inoculation (Matiru and Dakora 2004). Kumar et al. (2001) showed that wheat inoculation with another N-fixing bacterium, *Azotobacter chroococcum*, increased P uptake and plant growth.

Unfortunately, few inoculation studies have been conducted in acid soils. In one such study, acid-resistant PSB were isolated from samples of various soil classes and cropping histories in the Himalayan regions of India by enrichment culture techniques (Pal 1998). The acid-tolerant PSB were further tested in field experiments conducted in a typical Inceptisol (pH 4.8), with and without added P sources. Seed inoculation with one of these strains, which exhibited the highest P-solubilization activity and acid tolerance, resulted in significant increases in grain and vegetative yield of finger millet (*Elosine coracana*), maize, amaranth (*Amaranthus hypochondriacus*), buckwheat (*Fagopyrium esculentum*), and French bean (*Phaseolus vulgaris*). Similarly, when tested in a pot experiment conducted in an Indonesian Ultisol (pH 4.9), a P-solubilizing *Pseudomonas* strain significantly improved sorghum growth, height, root P content, shoot dry weight, and root dry weight when compared to the uninoculated control (Widada et al. 2007). Recently, Fankem et al. (2008) reported that inoculation of either one of three PSB (*P. fluorescens* CB501, CD511 and CE509), isolated from acid soils in Cameroon and able to mobilize P from Al- and Fe-phosphates, increased grain yield and P uptake of maize when tested in the greenhouse (see Sect. 5.3.1.4).

10.6.2 Dual Inoculation Assays

Field effects of PSB may be enhanced upon their mixed inoculation with either other PSB, P-solubilizing fungi (PSF), NFB or AMF.

10.6.2.1 Co-Inoculation of PSB and NFB

Since N and P are the two major plant nutrients, combined inoculation of NFB and PSB may benefit the plant better than inoculation with either group of organisms alone (Gull et al. 2004; Khan et al. 2006). NFB and PSB are able to interact synergistically by providing nutrients, removing inhibitory products, or otherwise. In particular, it is well known that N_2-fixation is highly dependent on P- availability (Barea et al. 2005).

Kopler et al. (1988) found that inoculation of legumes with *Pseudomonas* spp. enhanced rhizobial-induced nodulation. Similar results were obtained by others in field assays conducted with different combinations of PSB, NFB and test crops (reviewed by Khan et al. 2006). Synergistic interactions on plant growth and yield have also been observed when co-inoculating PSB with other well-known NFB such as *Azospirillum* or *Azotobacter*. Furthermore, PSB exert a beneficial influence on the rhizosphere survival of *Azotobacter* (Ocampo et al. 1975). In some cases, results from dual inoculations involving two different PSB have been better than those obtained with each strain used separately (Kundu and Gaur 1980; Tiwari et al. 1989; Çakmakçi et al. 1999; Han et al. 2006).

10.6.2.2 Co-Inoculation of PSB and Nonmycorrhizal Fungi

Phosphate-solubilizing fungi (PSF) are among the best known and well-studied PSM (Whitelaw 2000; Bandara et al. 2006). As in the case of PSB, their P-solubilizing ability is related to the production of important amounts of organic acids. Not surprisingly, combined inoculation of PSF and NFB resulted in enhanced growth, nutrient uptake, and yield in several crops. For example, inoculation of mungbean with either *Bacillus subtilis* or *Bacillus circulans* and *Aspergillus niger* increased nodulation of plant roots by indigenous rhizobia, root and shoot biomass, as well as straw and grain yield (Gaind and Gaur 1991). Similarly, combined inoculation of either *B. megaterium* or *Pseudomonas striata* with *Aspergillus awamori* significantly increased productivity of potato (*Solanum tuberosum* L.) (Dubey and Billore 1992). Mixed populations of soil bacteria and fungi are currently prepared as commercial biofertilizers for improving P nutrition of plants (Richardson 2007).

10.6.2.3 Co-Inoculation of PSB and AMF

Arbuscular mycorrhizal fungi increase plant P uptake by: (1) increasing the mycorrhizal root absorptive area, (2) improving nutrient transfer efficiency and utilization of P within the host plant, and/or (3) enhancing the solubility of P in the rhizosphere through pH alteration of the surrounding soil (reviewed in Barea et al. 2005; see Chap. 9). Numerous reports of greenhouse and field trials have highlighted the beneficial effects of dual inoculations with PSB and AMF (reviewed in Barea et al. 2005; Khan et al. 2006; Osorio Vega 2007). Basically, a synergistic microbial interaction is established between AMF and PSB, thus improving P acquisition by the plant (Barea et al. 2005). For instance, mycorrhizal plants can release higher amounts of organic compounds into their rhizosphere than do non-mycorrhizal plants (Linderman 1988). PSB survive longer when associated to mycorrhizal roots rather than to non-mycorrhizal roots (Singh 1990; Toro et al. 1997; Barea et al. 2002), thus increasing the possibility of delivering P into the soil solution. Some AMF also produce and excrete organic acids, which contribute to the solubilization of mineral phosphates, particularly Fe-P (Bolan 1991).

The effect of AMF-PSB dual inoculations on the growth and nutrient uptake of sorghum was studied in pot experiments using an Indonesian Ultisol (pH 4.9) with a low P content (Widada et al. 2007). Inoculation of the AMF *Glomus manihotis* and the PSB *Pseudomonas* sp. increased plant dry weight 112 times with respect to that of the uninoculated plant. Plant dry weight and uptake of N, P, Fe and Zn increased more upon dual inoculation than with the PSB alone.

There is a certain degree of specificity between PSB, AMF, and crops. When AMF (*Glomus mosseae* or *Glomus fasciculatum*) were co-inoculated with different PSB isolated from an Oxisol, there was an increase in kudzu (*Pueraria phaseoloides*) growth, yield, and nutritional status with some bacteria, but not with others (Toro et al. 1996).

10.6.3 Multi-Species Inoculations

The higher plant P concentration obtained through co-inoculation of AMF and PSB might benefit resident or inoculated NFB and the functioning of their nitrogenases, leading to increased N fixation and positive feedback on root and mycorrhizal development (Barea et al. 2005). The effectiveness of multispecies inoculations was examined in a number of studies, with encouraging results (reviewed by Khan et al. 2006 and by Osorio Vega 2007). Again, few of these studies dealt with acidic soils.

10.6.4 Combined Use of PR and PSB

To enhance P nutrition of crops cultured on acidic soils, direct application of phosphatic rock (PR) has been recommended (Zapata and Roy 2004; Arcand and Schneider 2006). PRs are rich in calcium phosphate (Ca-P) complexes and, as such, are weakly soluble in acidic soils. PRs are cheaper than inorganic P fertilizer, create less environmental pollution as they require minimum processing, and their dissolution results in the slow release of P in the soil. However, the agricultural effectiveness of PR may differ with soil properties, climatic conditions, and the nature of the crop (Nahas 1996; Rajan et al. 1996). Because PR minerals are released slowly and their use as fertilizer often causes insignificant increases in crop yield (Zapata and Roy 2004), organic acid-secreting PSB, alone or in combination with AMF or PSF, have been used to improve PR agronomic value (Goenadi et al. 2000; Barea et al. 2002; Lin et al. 2002, Şahin et al. 2004). The combined use of PR and *Bacillus megaterium* var. *phosphaticum* for sugarcane cultures in India permitted a 50% decrease in superphosphate use without sacrificing yields (Sundara et al. 2002).

An acidophilic, sulfuric acid-producing *Acidithiobacillus* sp. has been inoculated together with elemental sulfur and PR. This resulted in increased total N and P in yam bean (*P. erosus*) when grown in a typical Brazilian Fragiudult soil (pH 5.8) with low available P (Stamford et al. 2007).

In spite of some successful trials, it is not clear if PSB can actually increase the effectiveness of PR under standard field conditions to such a magnitude that it can be used as an alternative fertilizer. For example, in a recent study, three well-characterized PSB (*Enterobacter asburiae*, *Bacillus coagulans* and *Citrobacter koseri*) failed to release P from an Indian Alfisol (pH 6.5) amended or not with PR (Srivastava et al. 2007).

A partially acidulated phosphatic rock (PAPR) is normally obtained by the partial chemical acidulation of PR to convert only a part of its P into water-soluble form. PSB have also been used to prepare PAPR (Rodriguez and Fraga 1999; Whitelaw 2000; Vassilev et al. 2001). This biologically-treated phosphatic rock fertilizer increased P nutrition of plants in acid soils (Zapata and Roy 2004). As compared to the chemical process, the PSB-mediated acidulation process presents the advantages of occuring at low temperatures and of being more selective for P extraction (Whitelaw 2000; Vassilev and Vassileva 2003).

10.7 How to Isolate Acid-Proficient Phosphate Solubilizing Bacteria?

For several decades, Pikovskaya (PVK) medium (Pikovskaya 1948) or Sperber medium (Sperber 1958a) were routinely used to screen PSB from soil samples. Both media contain some form of calcium phosphate, added as the sole P source, and the identification of PSB relies on observing a clear solubilization halo surrounding bacterial colonies. To improve the clarity and visibility of the results, bromophenol blue may be added to the medium (Gupta et al. 1994), yielding yellow-colored halos. In 1999, Nautiyal formulated a new chemically-defined growth medium (NBRIP medium) for rapid screening of PSM, in which each of the components was tested separately to increase the efficiency of P solubilization and, consequently, the sensitivity of the screening technique. Being chemically defined, NBRIP medium facilitates interpretation of the experimental outcome. However, the formation of a clear halo, which remained the only criterion for identification of PSM, still depended on a series of uncontrolled factors, such as the varying diffusion rates of different organic acids. Furthermore, the reliability of these halo-based techniques was questioned because many isolates not producing any visible halo in the plates were indeed able to solubilize inorganic phosphates in liquid media. Hence, a broth assay with bromophenol blue was developed, which resulted in more reliable outcomes (Mehta and Nautiyal 2001).

The media mentioned above contain slightly soluble P sources (calcium phosphate, tricalcium phosphate, hydroxyapatite, fluorapatite) and are designed for neutrophiles. Accordingly, few of the bacteria recovered on these media can effectively solubilize the more insoluble Fe- and Al phosphates, which are the main forms of P occurring in acidic, tropical soils (Gyaneshwar et al. 2002; He et al. 2003). Furthermore, with only a few exceptions, colonies grown on screening agar are mainly representatives of r-strategists, with a preferential ability to rapidly metabolize simple substrates (Brimecombe et al. 2001).

To isolate PSB useful as biofertilizers in acidic soils (here called acid-proficient PSB), the source of P in screening media was changed to more insoluble forms of P, typical of these soils. Such media contained $AlPO_4$, $FePO_4$ or zinc phosphate as the sole source of P (Illmer et al. 1995; Srivastava et al. 2007; Fankem et al. 2008).

Instead of pure mineral phosphates, test media used to isolate PSM or monitor their activity may contain a series of variable-charge minerals (artificially synthesized goethite, natural kaolinite, and/or montmorillonite) surface-loaded with inorganic P (He and Zhu 1998; Hoberg et al. 2005).

In addition to containing Al-P, Fe-P or other insoluble P-containing minerals, media used to isolate acid-proficient PSB should also reproduce the buffering properties of natural soils. Indeed, the buffering capacity of soils may diminish the efficiency of microbial acid production in P solubilization (Cunningham and Kuiack 1992). The addition of 100 mM Tris-HCl (pH 8.0) to a screening medium negatively affected the ability of two PSB to solubilize di-calcium phosphate and PR (Gyaneshwar et al. 1998).

The nature of the carbon source also influences the outcome of PSB isolation trials. Different carbon sources will favor various carbon utilization pathways, and thus influence the identity and amount of excreted organic acids.

Not only is the presence of a particular combination of sugars (glucose plus sucrose) necessary for solubilizing Al-P, but an increased concentration of total C (from 4 g l^{-1} to 16 g l^{-1}) allows this to happen more efficiently (Illmer et al. 1995). Furthermore, glucose concentration is a key factor for P solubilization in NBRIP liquid medium (Nautiyal 1999). Thus, the concentration of sugars added to different screening and/or test media may be considered one of the factors responsible for the isolation of PSB that *did not* perform well under field conditions (Richardson 2001). Indeed, most screening media contain unrealistically high concentrations of a single carbon source, glucose, which is only found in very low concentrations in most root exudates. In fact, rhizodepositions are composed of a mixture of sugars and other C sources, which can be further metabolized or transformed by rhizobacteria (Deubel et al. 2000). Consequently, PSB isolated using glucose as the sole C source may not be typical rhizosphere inhabitants or be capable of solubilizing P under rhizosphere conditions.

With a view to developing a screening method that would more realistically reflect nutrient levels in the rhizosphere, Harris et al. (2006) added lower amounts of glucose and sucrose (0.05%), plus exudates from wheat roots, to their medium (modified MIS medium). They were then able to isolate PSB that were not only good P solubilizers in laboratory tests, but resulted in increased grain yield and grain phosphorus content in test plants grown in non-sterile soil in the greenhouse.

Another important factor to be considered when screening PSB for biofertilization purposes is the pH of the medium, since this factor may drastically influence microbial utilization of C sources (Yao et al. 2000). Therefore, when isolating strains from acid soils, it appears advisable to utilize a medium with an acid, rather than a neutral, pH (Pal 1998).

The N source also influences the in vitro P-solubilizing ability of many PSB. In general, greater solubilization activities are observed in the presence of ammonium salts (NH_4^+), as compared to nitrate (NO_3^-) (Halder et al. 1992; Illmer et al. 1995). This has been attributed to the extrusion of protons to compensate for NH_4^+ uptake.

Culture-independent methods may be used both to detect and identify PSB. Since the direct oxidation of glucose has been proposed as the metabolic basis for a superior MPS^+ phenotype in Gram-negative bacteria, molecular probes targeting direct oxidation pathway genes may be used for detecting these bacterial populations in situ (Goldstein 2007). Indeed, this approach allowed the detection of populations of PSB in two alkaline desert soils (Goldstein et al. 1999). However, as was mentioned earlier, other bacterial-mediated mechanisms for mobilizing P in soils exist (see Sect. 10.4.2), which makes it difficult to define universal criteria for detection of all potentially beneficial PSB by molecular biology protocols.

In addition to optimizing the formulation of screening media, other factors should be considered in the selection of acid-proficient PSB. For example, as Sperber (1958a) first noticed, many isolates rapidly and irreversibly lose their ability to dissolve apatite on sub-culturing (Halder et al. 1990; Illmer and Schinner 1992). This phenomenon has not received much attention, although it may give rise to contradictory results. Such instability might arise from the segregation of genes located in plasmids, in the absence of a strong selection force under in vitro conditions. In line with this argument, genes related with the direct oxidation pathway of glucose are indeed localized in plasmids in certain bacterial species (Gupta et al 1997).

10.8 Why Do PSB Fail When Tested in the Field?

As mentioned above, comparisons of the effect of PSB-based biofertilizers in laboratory, greenhouse, and field studies lead frequently to very contrasting results. The factors involved in these inconsistencies, some of which were reviewed by Lucy et al. (2004), include the following:

1) *Lack of fundamental knowledge concerning the microbial mechanisms involved in P mobilization.* Early biofertilization trials were conducted with very limited information concerning the bacterial mechanisms involved in P solubilization from sparingly soluble sources. Even though this gap began to close during the 1990s, the basic solubilization mechanisms still remain incompletely understood (Illmer and Schinner 1995a; Rodriguez and Fraga 1999).
2) *Lack of adequate experimental design and analysis of results.* Illmer and Schinner (1995b) were the first to point out the frequent methodological mistake of comparing growth of plants in sterile soils with growth in non-sterile soils inoculated with PSM. Obviously, the extrapolation of these results

to outdoor experiments is impossible because of the non-sterile conditions and climatic variability in the field.

3) *Variable effectiveness of PSB as influenced by different soil types*. In many cases, the P-solubilization ability of PSB in natural soils is different from that observed under in vitro conditions. Indeed, most PSB have been isolated using neutral and unbuffered media, although it is well known that both the acidity and the buffering capacity of soils could limit microbially mediated P solubilization (Gyaneshwar et al. 1998). For example, inoculation with *E. asburiae* PSI3 was effective in enhancing plant growth in a Vertisol (pH 8.2) but not in an Alfisol (pH 6.6) (Srivastava et al. 2007). Therefore, PSB survival in a microcosm of the field environment should be examined during the initial stages of laboratory testing (Tang et al. 1995).
4) *Climatic variability*. Temperature fluctuations have been shown to significantly affect the P-solubilizing activity of micro-organisms (Dwivedi et al. 2004; Stamford et al. 2007).
5) *Antagonism and competition with other indigenous micro-organisms in the rhizosphere*. Many PSB have proven to be too vulnerable and unreliable to be used in agriculture (Illmer and Schinner 1995b). This has been attributed to their reduced ability to (a) survive and multiply, (b) colonize the rhizosphere, (c) compete for resources with native micro-organisms, and (d) avoid predation. All these factors may determine the rapid decline in the population size or density of exogenous PSM upon introduction into soils (Jacoud et al. 1998). Hence, it is highly recommended to select bacteria intended to be used as biofertilizers from naturally occurring, endemic populations, in order to take advantage of their ability to colonize a particular niche (Barea et al. 2005). It is also advisable to monitor the dynamics of rhizosphere colonization by the introduced bacteria, with a particular emphasis on their effects on native microbial populations (Castro-Sowinski et al. 2007).
6) *The frequent use of inappropriate screening media to identify and select PSB*. As discussed in Sect. 10.7, very often the media used for screening PSB favor the isolation of PSB with a better efficiency under laboratory conditions than in the soil.
7) *The pre-existent nutrient level of the soils and their moisture content*. The number, diversity, and metabolic activity of soil micro-organisms are influenced both by the identity and the availability of organic and inorganic nutrients (Welbaum et al. 2004). Field trials have shown that the effectiveness of PSB in promoting plant growth is highly dependent on the nutrient status of the soil (de Freitas and Germida 1990; Çakmakçi et al. 2006). Furthermore, in some field trials the effectiveness of PSB varied according to the amount of P previously added to the soil (Chabot et al. 1996).
8) *Bacteria-plant specificity*. Some PSB are only able to produce organic acids when grown in the presence of root exudates of the host plant (Hwangbo et al. 2003), which points to the existence of plant–PSB compatibility effects.
9) *Inoculum size*. Researchers have applied high PSB inoculant doses (reviewed by Lucy et al. 2004), even though the ability to colonize plant roots in high

numbers is not always necessary for a positive effect (Harris et al. 2006; Jacoud et al. 1998). In fact, excessively large numbers of bacteria may sometimes be detrimental to the germination and growth of certain seeds or plants (Chanway 1997). On the other hand, survival of the inoculant in large numbers in rhizospheric soil depends on the initial inoculum density (Jjemba and Alexander 1999).

10) *The nature of the carrier employed to apply bacteria to soils*. Even though no universal formulation for PSB inocula is presently available, a good carrier should have the capacity to deliver the right number of viable cells in appropriate physiological condition at the right time (Bashan 1998).

While keeping in mind the various factors mentioned above, it should also be stressed that, in order to lead to useful results, the field trials must be conducted under the full range of current and future conditions that will be experienced by the farmer (Hameeda et al. 2008).

10.9 Conclusions

Inoculation of crops with PSB may be a cost-effective alternative to inorganic P fertilizer application, and use of these bacteria may lead to improved crop yield and quality, particularly in tropical acidic soils with high P-fixation capacities. In the last two decades, significant contributions were made towards understanding the mechanisms underlying bacterially mediated P solubilization. The interactions taking place between rhizospheric micro-organisms, and between those micro-organisms and the plant, are also better known (Nehl and Knox 2006). Nevertheless, even though a few commercial products are already available and marketed in some countries, we are far from a general use of PSB. As Richardson (2007) stated recently, nearly 60 years after Gerretsen (1948) first demonstrated the potential of PSB as biofertilizers, not much has been delivered... besides promises. This long delay in establishing the use of PSB as a mature biotechnology is due to a combination of obstacles, some of which are biological (see above), while others are of a different nature.

For instance, a majority of farmers in developing countries are small-scale and resource-poor, and therefore rely primarily on low-input agriculture practices. Hence, farmers' access to bank loans with the aim of improving their agricultural techniques is extremely limited. This can be considered one major constraint to enhance crop productivity, even by means of traditional fertilization practices. Furthermore, even in countries where funds can be relatively easily obtained, the cost of using patented technologies and/or commercial biofertilizers (where available) might represent a major obstacle.

A second problem is logistic: how to bring these technologies within the reach of the small- and mid-scale farmers, who are usually settled in remote areas, far away from cities and ports? Also, how can artificial inoculation with PSB be promoted when the infrastructure required to store and transport biological products in large quantities into rural areas is not available?

A third obstacle, which is possibly the most difficult to overcome, is cultural in nature. Indeed, with the exception of rhizobia, the use of beneficial micro-organisms in many regions of the world is almost unknown. Consequently, when farmers are asked to replace well-known chemical fertilizers with bacterial inoculants, they are reluctant to do so. These hesitations result from fear of reducing soil fertility or because of the frequent association of microbes with human or animal diseases (Bashan 1998).

To overcome these constraints, it will be necessary to establish mechanisms of technology transfer, from developed countries to the developing world, from academic institutions to government agencies, and from private companies to the public sector. To promote this transfer, research centers with the capability of acquiring new technologies should be created in lesser developed countries. These centers should be able to adapt foreign technologies to local crops and to develop their own technologies (Herrera Estrella 1999). Moreover, national public programs aimed towards diffusing these new technologies among farmers should be launched, together with information and education campaigns. Altogether, this implies that the public sector should direct more resources towards agricultural research and technology diffusion.

Fortunately, there are encouraging signs coming from certain countries. For example, a year ago the Venezuelan government launched a project oriented towards mass-producing biofertilizers (PSB, NFB) and biological control agents to be delivered at low or no cost to farmers. In the particular case of PSB, two indigenous PSB, isolated from a Venezuelan savanna soil, are currently being produced in small-scale facilities throughout the country.

One of the most important messages that emerged from the State of Food and Agriculture report (FAO 2004) is that biotechnology — i.e. “the use of living (micro) organisms to make or modify a product for practical purposes” — is indeed capable of benefiting small, resource-poor farmers. However, as we have briefly discussed, biotechnology itself cannot overcome the problems that arise as a consequence of underdevelopment.

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