4. Phosphate Solubilizers

4.1. Isolation of Microbial Strains

A considerable number of bacterial species are able to exert a beneficial effect upon plant growth. Mostly they are associated with the plant rhizosphere, so they are called as rhizobacteria. This group of bacteria has been termed plant growth promoting rhizobacteria, and among them are strains from genera such as *Alcaligenes, Acinetobacter, Arthrobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Paenibacillus, Pseudomonas, Rhizobium, and Serratia*. They are used as biofertilizers or control agents for agriculture improvement, and there are numerous researchers for the area with the agricultural environment conservation.

Phosphorus is second only to nitrogen in mineral nutrients most commonly limiting the growth of crops. Phosphorus is an essential element for plant development and growth making up about 0.2 % of plant dry weight. Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As the results, the amount available to plants is usually a small proportion of this total.

Several scientists have reported the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate.

4.1.1. Mechanisms of phosphate solubilization

The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases play a major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings.

The production of organic acids by phosphate solubilizing bacteria has been well documented. Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Also, 2-ketogluconic acid is another organic acid identified in strains with phosphate solubilizing ability (Table 1).

Table 1. Microbial strains producing organic acid

Organic acid	Strains
Gluconic acid	*Pseudomonas* sp., *Erwinia herbicola, Pseudomonas cepacia, Burkholderia cepacia*
2-Ketogluconic acid	*Rhizobium leguminosarum, Rhizobium meliloti, Bacillus firmus*

Strains of *Bacillus* were found to produce mixtures of lactic, isovaleric, isobutyric and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers. Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodriguez et al. 1999. Biotechnology Advances 17: 319-339).

Chelating substances and inorganic acids such as sulphideric, nitric, and carbonic acid are considered as other mechanisms for phosphate solubilization. However the effectiveness and their contribution to P release in soils seems to be less than organic acid production.

4.1.2. Isolation of mineral phosphate solubilizer

Detection and estimation of the phosphate solublization ability of microorganisms have been possible using plate screening methods. Phosphate solubilizers produce clearing zones around the microbial colonies in media. Insoluble mineral phosphates such as tricalcium phosphate or hydroxyapatite are contained in the media.

Also the bromophenol blue method that produce yellow halos following pH drop through the release of organic acids is more reproducible and has greater correlation in comparison with the simple halo method. However, clearing zones on agar plate method is generally used (Fig. 1). Pikovskays's medium (Table 2) is a general medium for selection of phosphate solubilizer.

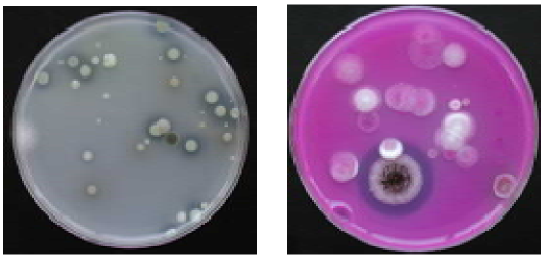

Fig. 1: Phosphate solubilizer forming clear zone.
Bacteria (left), Fungi (right)

Table 2. Composition of medium for phosphate solubilizers
(Pikovskays's medium)

Components	Amounts (g l^{-1})
Glucose	*10*
$Ca_3(PO_4)_2$	*5*
$(NH_4)_2SO_4$	*0.5*
NaCl	*0.2*
$MgSO_4$.7H2O	*0.1*
KCl	*0.2*
Yeast extract	*0.5*
$MnSO_4$. H_2O	*0.002*
$FeSO_4.7H_2O$	*0.002*
pH	*7.0*

4.2. Inoculant Production

Microorganisms with potential as plant growth promoters have been used to produce inoculants. Potential materials that are able to support good growth and survival of bacteria are needed in inoculant production. Many materials have been evaluated, including different types of coals, bentonite, corn oil, mineral soils, peat, peat moss, vermiculite, and perlite. Peat is commonly used material for inoculant carrier. Finely ground peat is most commonly used in conventional inoculant production. However, peat is not always available, because some peat may inhibit growth of some *Rhizobium* strains. There have been reports on the difficulty of obtaining autoclaved or gamma-irradiated peat as carriers. The high temperature during steam sterilization or the high dosage needed for irradiation might generate toxic substances for bacteria. However, perlite can be easily sterilized with no risk of producing toxic substances, because it is a volcanic stone, composed of a little-hydrated aluminium silicate.

The agronomic use of agro-wastes as substrates causes changes in the soil affecting its physico-chemical characteristics and microbial activity in the rhizosphere. The breakdown of such materials to simple sugars provides energy sources for heterotrophic microorganisms such as P-solubilizing and nitrogen fixing bacteria. Normally, the growth and metabolic activity of soil microorganisms are limited by the availability of nutrients. From this reasons, several kinds of agro wastes such as rice straw compost will be good carrier materials for the inoculants and improver of soil condition as following case study. However, the peat moss with phosphate solubilizer showed good survival of inoculants and effects on crops.

4.2.1. Cultivation

○ Media: proper media for inoculant (nutrient broth, yeast extract broth etc.)

○ Incubation condition: temperature, light, incubation period

In case of *Bacillus* sp.
-Nutrient broth for 3days at 27±1℃ on rotary shaker at 150 rpm.

4.2.2. Collection

○ After incubation period, collect microbial cells by centrifuge

○ Wash cells with distilled water or diluted saline solution

In case of *Bacillus* sp.
-Centrifuge 10 minutes at 4000-5000 rpm (Fig.2)
-Wash cultures with sterilized tap water
-Re-centrifuge washed cultures 10 minutes at 4000-5000 rpm


Fig. 2: Cultures centrifuged

4.2.3. Formulation of inoculants using carrier materials

If it is necessary, mix cells using carrier materials such as peat, vermiculite, perlite etc.

4.3. Inoculant Application

4.3.1. Inoculation method of phosphate solubilizer

Generally biofertilizers in powder form are applied as for organic matters onto the soil. This type is very convenient for users on the management of biofertilizer. Some biofertilizers are costly products for farmers, so their use would be restricted on the specific condition of agronomy.

Microorganisms are generally supplied by producers of biofertilizer, so it would only necessary that the users or farmers follow the application method prepared by manufacturers. However, the popular application method is regarded as next procedure (Fig. 3).

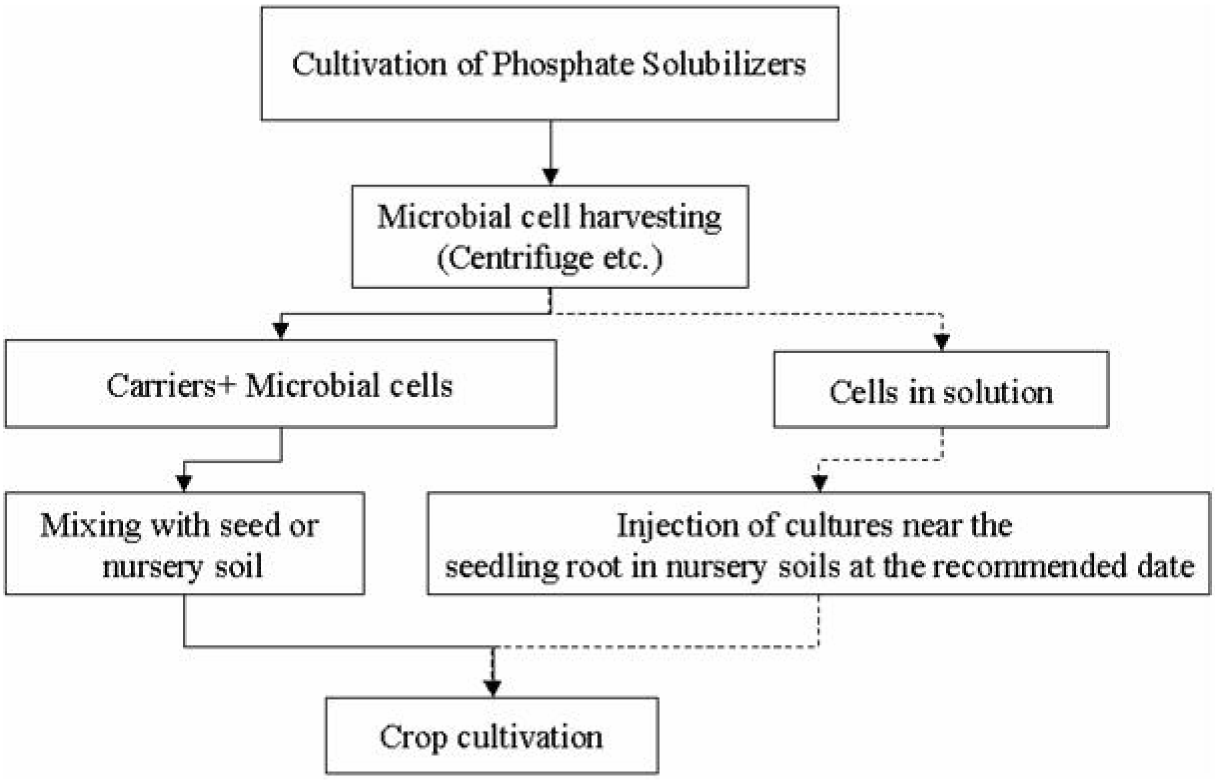

Fig. 3: Inoculation method of phosphate solubilizers.

4.3.2. Improvement of phosphate solubilizers

An alternative approach for the use of phosphate solubilizing bacteria as microbial inoculants is the use of mixed cultures or co-inoculation with other microorganisms. This evidence points to the advantage of the mixed inoculations of PGPR strains comprising phosphate solubilizing bacteria.

The effect of a combined inoculation of *Rhizobium*, a phosphate solubilizing *Bacillus megaterium* sub sp. *phospaticum* strain-PB and a biocontrol fungus *Trichoderma* spp. On growth, nutrient uptake and yield of chickpea were studied under glasshouse and field conditions. Combined inoculation of these three organisms showed increased germination, nutrient uptake, plant height, number of branches, nodulation, pea yield, and total biomass of chickpea compared to either individual inoculations or an uninoculated control (Rudresh et. al, 2004.).

On the other hand, it has been postulated that some phosphate solubilizing bacteria behave as mycorrhizal helper bacteria. It is likely that the phosphate solubilized by the bacteria could be more efficiently taken up by the plant through a mycorrhizal pipeline between roots and surrounding soil that allows nutrient translocation from soil to plant. Considerable evidence supports the specific role of phosphate solubilization in the enhancement of plant growth by phosphate solubilizing microorganisms. However, not all laboratory or field trials have offered positive results. Therefore, the efficiency of the inoculation varies with the soil type, specific cultivars, and other parameters.

4.3.3. Case study of improvement of soil condition for phosphate solubilizers on the fifty-years long-term experiment in rice paddy soils in Korea

A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere soil. Also the fungal genera with this capacity are *Penicillium* and *Aspergillu* (Suh, et al., 1995; Whitelaw et al., 1999). A continued exploration of the natural biodiversity of soil microorganisms and the optimization and manipulation of microbial interactions in the rhizosphere of crops represents a prerequisite step to develop more efficient microbial inoculants. Also, it is desirable to find methods to manage indigenous microbes in soil for sustainable agriculture. The answer may be obtained from diverse field tests managed for improvement of soil condition. A case study for phosphate solubilizers has been conducted on a fifty-year long-term experiment in rice paddy soils in Korea.

4.3.3.1. Changes in physical properties of soils

An investigation was made on the effect of different soil amelioration on the physical and chemical properties of soil, the yield or quality of rice, and the activity of soil microbes in the plot where NPK, lime, and compost had been applied for fifty years.

The physical properties were most significantly affected by the application of compost, which reduced the bulk density and hardness of soil and increased the liquid and gas phase of soil and cation exchange capacity of soil (Table 3).

Table 3. Effect of materials on physical properties of paddy soils

Treatment	Bulk density ($g\ cm^{-3}$)	Phase distribution (%)			CEC ($Cmol^{+}kg^{-1}$)	Hardness (mm)
		Solid	Liquid	Gas		
Control	1.39	52.5	29.8	17.7	7.8	13.8
NPK	1.25	47.1	26.4	26.5	9.0	14.0
NPK+C	1.19	44.7	28.3	26.9	11.2	13.0
NPK+L	1.23	46.4	24.7	28.9	8.8	14.0
NPK+S	1.20	45.2	24.0	30.8	9.0	14.8
NPK+C+L+S	1.16	43.6	28.3	28.1	10.8	12.8

L; Lime, C; Compost, S; Silicate

4.3.3.2. Changes in chemical properties of soils

The application of compost tended to increase organic matter, available phosphorus and silicate, exchangeable cations in the soil (Table 4).

Table 4. Effect of materials on chemical properties of paddy soils

Treatment	pH (1:5)	OM ($g\ kg^{-1}$)	$Av.P_2O_5$ ($m\ kg^{-1}$)	Ex. ($cmol^{+}kg^{-1}$)			$Av.SiO_2$ ($mg\ kg^{-1}$)
				K	Ca	Mg	
Control	6.0	18	40	0.10	5.0	0.9	89
NPK	5.7	21	194	0.12	4.7	0.9	44
NPK+C	5.7	32	241	0.11	5.7	1.1	47
NPK+L	6.6	23	166	0.12	5.9	1.5	51
NPK+S	6.6	20	167	0.09	7.7	1.2	256
NPK+C+L+S	7.0	33	270	0.12	10.0	1.6	386

L; Lime, C; Compost, S; Silicate

4.3.3.3. Changes in microbial diversity of soils

Diversity is an important concept in ecology, often in environmental monitoring and conservation management. But diversity is an intuitively obvious concept that is difficult to define and quantify. There are two parts to diversity: the number of species and the evenness of the distribution. A sample with more species is more diverse: while an evenly distributed community is more diverse than an unevenly distributed community with the same number of species. However, microbial communities are severely affected by agricultural managements such as tillage, and organic materials and chemicals for soil amendment.

A pattern was revealed from the long-term experiment showing different profiles of the ratio of functional species to bacteria. The ratios of fluorescent bacteria, Bacillus and Gram-negative bacteria to total aerobic bacteria in CLAPK (compost + lime + fertilizer) and CAPK (compost + fertilizer) plots were higher than other treatments. This result represents effect of amendments on soil microbial community (data not shown). Nevertheless microbial diversity was simplified, the functional groups were triggered by the application of substances to soil.

Apart from fertilization and enzymatic decomposition of organic compounds, microbial P-mobilization would be the only possible way to increase plant-available phosphorus. Many species of bacteria are able to solubilize phosphates in vitro and some of them can mobilize P in plants.

The phosphate solubilizing activity is significantly affected by material input. High positive correlation was observed between the phosphate solubilizing bacteria and organic matter contents in soils. The treatment of the agricultural materials is presented in Table 5. The available phosphorus contents ranged from 40 to 105 mg. FLC contained highest amount of available phosphorus than control. FLC showed high ratio of PB/TB between treatments. This means that lime and compost used as soil improver had positive effects on phosphate solubilizers.

Table 5. Effect of materials on available phosphorus and PB/TB ratio in paddy soils

Treatment	PH (1:5)	Av.P_2O_5 (mg kg^{-1})	PB/TB ratio (%)
Control	6.0	40	0.6
F	5.6	73	2.2
FL	6.3	73	5.0
FC	5.6	87	6.3
FLC	6.1	105	16.8

F; fertilizer (plot of ammonium sulfate and/or urea), FL; fertilizer + lime, FC; fertilizer + compost, FLC; fertilizer + lime + compost, PB/TB; phosphate bacteria/total aerobic bacteria

Modern agricultural practices largely rely on high inputs of mineral fertilizers to achieve high yield. These practices are now being reevaluated and are coming under increased scrutiny as our awareness of environmental conservation from the impact of chemical overuse. This research is very necessary to improve soil condition on environmentally friendly agriculture that is being practiced at present.

Also we should imagine that improvement of soil condition includes activation of functional microbes related to specific materials input. As shown in Table 6, diversity of phosphate solubilizers in soils was highly controlled by the agricultural management. Only three types of treatment are shown in the table. But as was commented earlier, compost directly affected microbial community in soils.

Table 6. Effects of amendment on phosphate solubilizer in soils

Treatment	Species of isolate	Number of isolate	
Chemical Fertilizer	*Bacillus simplex*	1	4
	Bacillus megaterium	2	
	Pseudomonas chlororaphis	1	
Lime + fertilizer	*Bacillus pumilis*	2	5
	Pseudomonas chlororaphis	1	
	Pseudomonas coronafaciens	1	
	Arthrobacter globiformis	1	
Compost + fertilizer	*Bacillus pumilis*	3	13
	Bacillus licheniformis	2	
	Pseudomonas fluorescens	4	
	Bacillus marinus	1	
	Bacillus subtilis	1	
	Bacillus lentimorbus	1	
	Pseudomonas chlororaphis	1	

4.3.3.4. Effects of organic matters on nutrient absorption of rice plants

Organic matter results in several benefits such as better soil structure that provides a more suitable medium for plant growth, supplies nutrients for the plant and also help to build up target microorganisms. The combined use of organic matter with inoculant in the build up of high bacterial populations that improve plant growth.

Effect of materials on amount of nutrient absorbed by rice is presented in Table 7. It is possible to suggest that the only driving force to dissolve phosphate is microbial activity, especially phosphate solubilizing bacteria, from the results shown in the table.

Table 7. Effect of materials on amount of nutrient absorbed by rice cultivated in paddy soils (unit: $kg\,ha^{-1}$)

Treatment	T-N	P_2O_5	K_2O	CaO	MgO
Control	46.4(50)	33.2(60)	76.4(63)	22.9	12.0
NPK	93.4(100)	54.9(100)	120.4(100)	38.0	23.4
NPK+C	107.5(115)	64.9(118)	161.9(134)	41.9	28.6
NPK+L	89.7(96)	52.5(96)	124.3(103)	36.7	22.9
NPK+S	94.0(104)	53.8(98)	137.1(114)	37.7	25.0
NPK+C+L+S	106.3(114)	63.1(115)	164.0(136)	35.4	26.0

L; Lime, C; Compost, S; Silicate

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