A STUDY OF PHOSPHATE SOLUBILIZING MICROORGANISMS IN THE LEGUMINOUS PLANTS

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A Study of Phosphate Solubilizing Microorganisms in the Leguminous plants

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By

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بسم الله الرحمن الرحيم

Dedication

I dedicate my this research work
To
My Loving Parents

DECLARATION

The material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

QURAT UL AIN

CERTIFICATE

This thesis, submitted by **Miss QURAT UL AIN** is accepted in its present form by the Faculty of Biological Sciences, Department of Microbiology, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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LIST OF ABBREVIATIONS

LFH	Laminar Flow Hood
RP	Rock Phosphate
P	Phosphorus
PKV Medium	Pikovskaya medium
TCP	Tricacium Phosphate
PSM	Phosphate Solubilizing Microbes
PSB	Phosphate Solubilizing Bacteria
PSF	Phosphate Solubilizing Fungi
Si	Solubility Index
Soln.	Solution
UV light	Ultra Violet light
wt.	Weight
CON	Control
TLC	Thin Layer Chromatography
PB	Pea Bacteria
PF	Pea Fungi
CPB	Chickpea Bacteria
CPF	Chickpea Fungi
LB	Lentil Bacteria
LF	Lentil Fungi

PGPR	Plant Growth Promoting Rhizobacteria
SP	Super Phosphate
SE	Standard Error
Spp.	Specie
VAM	Vesicular Arbuscular Mycorrhizae
Pi	Inorganic Phosphate

UNITS

CFU	Colony Forming Unit
cm	Centimeter
min	Minute
%	Percentage
ml	Millimeter
g	Grams
Ilb	Pounds (Pressure)
°C	Degree Centigrade

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QURAT UL AIN

ABSTRACT

The present study was conducted to characterize "Phosphate Solubilising icroorganisms" (PSM) and to evaluate the efficiency of PSMs to solubilize phosphorus in vitro onditions. Leguminous plants e.g pea, chickpea and lentils were selected for the isolation of PSMs. The microbial population, as well as the PSMs in the legume rhizosphere were enumerated by serial dilution plate method on Pikovskaya's (PKV) agar medium. The soil status of the legume plants was also checked. 4 phosphate solubilizing bacteria (PSB) were isolated from the leguminous plants and studied under stereomicroscope for their colonial morphology and were microscopically studied for their cell morphology and Gram staining. In case of fungi, over all 8 phosphate solubilizing fungi(PSF) were isolated and were identified microscopically. Colony diameter, halozone diameter and solubilization index were determined on solid PKV medium. Change of pH caused by 4PSB and 8PSF were also carried out in liquid PKV (broth) medium. These PSMs were further analyzed for the presence and absence of organic acids using thin layer chromatography (TLC) technique. The soil condition of the studied leguminous plants was alkaline. Microbial status of the rhizospheric soil revealed that soil had both phosphorus solubilizing and non-phosphorus solubilizing microorganisms though the enumeration results indicated that %age of PSM as compared to total microorganisms is very low. Gram staining revealed that PSB were Gram positive and mostly cocci. Most of the PSF belonged to division Ascomycota and Aspergillus genera. All the PSM including PSF and PSB dropped the pH of PKV broth medium and TLC results indicated the production of organic acids by PSMs.Out of the three studied legumes, PSM number was dominant in lentils from which 3 PSB and 5 PSF were isolated suggesting that phosphate solubilizing activity is more in lentils as compared to pea and chickpea. Results of the present study may provide a basis for the production of bioinoculants containing efficient PSM individually or in composite with the local deposits of phosphorus to fulfill phosphorus requirements of plants in phosphorus deficient soils. It is suggested for further research that a biofertilizer with the combination of PSM, N-fixers and VAM should be tried to get better yield of crops using useful microorganisms. Thus by such amendments the demand for expensive fertilizers can be reduced.

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

PHOSPHORUS "P"

Phosphorus was discovered by German alchemist Hennig Brand in 1669. In ecological terms, phosphorus is often a limiting nutrient in many environments, i.e. the availability of phosphorus governs the rate of growth of many organisms (Hammond, 1995).

It has long been recognized that at least ten elements are essential to the growth of higher order green plants. These were the major elements carbon, hydrogen, oxygen, nitrogen, sulphur, potassium, calcium, magnesium, phosphorus and iron (Ozanne, 1982). Next to Nitrogen, Phosphorus (P) is a vital nutrient for plants and microorganisms (Subba Rao, 1984; Barber, 1984; Brady, 1990), and one of the most important macronutrient of all living organisms (Illmer and Schinner, 1992). It constitutes 0.4% of dry weight of plant and 0.9% of animal tissue (Donahue et al., 1990). Natural sources of P are either organic or inorganic.The relative amount of two forms varies from soil to soil (Donahue et al., 1990; Coyne, 1999). The total amount of P (organic or inorganic) existing in the earth crust is of the order of 10^5 matric tons. But the amount and concentration of available P at any time is very low and seldom exceeds 0.1-1 ppm or 0.1-0.2 ppm in tropical soils (Paul and Clark, 1989; Brady, 1990). Mostly soils have pH between 4 and 8(Donahue et al., 1971; Barber, 1984) but extreme range is from 3.5-10 (Donahue et al., 1971). Iron and aluminium phosphates are usually found in acidic soils while at pH 7 calcium phosphates are dominant (Barber, 1984; Whitelaw, 2000). Phosphorus is mostly available at pH 6.5, if it is mineral phosphorus while organic phosphorus is mostly available at pH 5.5(Brady, 1990; Donahue et al., 1990; Jungk et al., 1993). The amount of phosphorus which is re-extractable by water, dilute acid or by carbonate solution usually available to plants is known as "labile P" and the remaining is designated as "fixed P" which is usually more than available P(Barber, 1984). Plant root take up or absorb P from labile soil solution pool in the form of H_2PO_4 or HPO_4 ions i.e. primary orthophosphates and secondary orthophosphate respectively (Beever and Burns, 1980; Parks et al., 1990; Yadev and Singh, 1991; Scheffer and Schachtschabel, 1992). As the plant root absorb P from the soil

solution it must be replenished. Replenishment of labile P pool takes place via mineralization of organic P and by desorption of P from surface of soil solids. This replenishment of adsorbed P is sometimes referred as buffering power of the soil(Brady, 984) but if the rate of P uptake at the root surface exceeds the replenishment, reducing the number of available P ions in soil, then P depletion zones are known to develop immediately around roots(Barber, 1984; Jungk, 1987).

The availability of inorganic P in ionic form is determined by soil pH because these factors i.e. mineral and soluble forms of Fe, Al, Mn , amount of organic matter decomposed and microbial activity in one way or the other depends upon soil pH (Nye and Tinker, 1977; Nath and Borach, 1983; Brady, 1990).In highly acidic solution, only $H_2PO_4^{-1}$ ions will be present and plants easily absorb these ions (Brady, 1990) and at basic pH, firstly $HPO_4^{-2}$and finally PO_4^{-2} are dominant and plants cannot absorb these. However, at pH 7 both HPO_4^{-2} and HPO_4^{-2} ions are found in soil solution. The most favorable pH range for maximum availability of P is 6-7 (Brady, 1990; Whitelaw, 2000).

OCCURRENCE

Due to phosphorus reactivity to air and many other oxygen containing substances, it is not found free in nature but it is widely distributed in many different minerals. Phosphate rock, which is partially made of Apatite (an impure Tricalcium Phosphate mineral), is an important commercial source of this element (Hammond, 1995).

Phosphorus content of soil varies from 0.02 to 0.5 % with an average of 0.05 % (Barber, 1984). Phosphorus exist in nature in variety of organic and inorganic forms. All phosphorus compounds found in sedimentary rocks, living organisms cultivated or non cultivated soils come from weathering of rocks. Due to which soils soluble phosphate is released. In the presence of calcium, phosphates are precipitated and sedimentary deposits rich in calcium phosphate are formed known as phosphorites. Phosphorus is found in forms of apatite(rock phosphate) in them generally as flourapatite $Ca_5(PO_4)_3F$, hydroxypatite $Ca_5(PO_4)_3OH$, hydroxyhapatite $Ca_{10}(PO_4)_6(OH)_2$ (Barber, 1984).

Phosphorus may be adsorbed on surface of iron and aluminium oxides and calcium carbonate (Barber, 1984). Soluble phosphorus or phosphorus ion is either primary

orthophosphate ($H_2PO_4^{-1}$) or secondary orthophosphate ion (HPO_4^{-2}) depending on pH system of soils. At pH 7.2 half phosphate would be present as $H_2PO_4^{-1}$and half as HPO_4^{-2} (Whitelaw, 2000)

PHOSPHORUS STATUS OF PAKISTANI SOIL

Almost all soils of the country are alkaline (pH=or > 8), calcareous to varying degrees, low in organic matter and also in available P (Rashid, 1994). In calcareous and alkaline soils an important factor that influences the availability of P is Ca or soluble Ca containing compounds. For example, if a Ca containing fertilizer (calcium super phosphate) is added to alkaline soil, HPO_4^{-2} will readily react with Ca to form less soluble P compounds and availability of P decreases i.e. calcium orthophosphate,

hydroxyapatite and flourapatite (Brady, 1999). The total quantity of soil P is 444.9-866 mg P Kg^{-1}that is much greater than that of available P which is only 3.05-27.1 mg P Kg^{-1} (Zia, 1990). Severe to moderate P deficiency has been recorded in more than 90% Pakistani soils. Moreover, the contribution from organic manures and crop residues is also limited. Hence P deficiency is being aggravated due to the continous mining with extensive cropping (Ahmad, 1992; Memon et al., 1992; NDFC, 1995).

IMPORTANCE FOR PLANT GROWTH

Phosphorus is the second key plant nutrient. Young plants absorb P rapidly if it is available. P is present in all plants in various concentrations according to the plant species, age and nature of plant tissues. The role played by this element is fundamental. It is essential for the development of meristematic tissues, in stimulation of early root growth and in hastening plant maturity (Donahue et a, 1990; Tisdale et al., 1993).In plants P increases the strength of cereal straw, stimulates root development, promotes flower formation and fruit production and especially essential for seed formation. Adequate P fertilization may improve the quality of certain fruits, forage, vegetables and grain crops and increases their resistance to diseases and adverse conditions such as extreme temperatures and lodging of cereals (Donahue et al., 1990; Tenebe et al., 1995).It

is, infact, an essential part of nucleoproteins in cell nuclei which controls cell division and growth and of DNA which carries the inheritance characteristics of living organisms. It plays an important role in many physiological processes of plant such as the utilization of sugar and starch, photosynthesis, and especially in the transfer of energy. Phosphate compounds through ATP, ADP and NADP act as "energy currency" in plants. In addition to this, P is an important constituent of a variety of biochemical compounds including nucleic acid, phospholipids, some proteins and enzymes (Donahue et al., 1990; Tisdale et al., 1993). For animals and people eating the plants, phosphorus is critical for growth of bones and teeth, which are mostly calcium phosphates (Donahue et al., 1990).

DEFICIENCY SYMPTOMS

Phosphorus content of soil may vary from 0.02-0.5% with an average of approximately 0.05%(Cathcart, 1980; Barber, 1984). Actually the concentration of available soil is much less than required by most of plants. Due to phosphorus deficiency in soil and its importance for plant growth expensive fertilizers are added to soil. To obtain higher yields farmers commonly apply more fertilizers than removed by crops, sometimes double or triple the deficiency of P in tropical soils is due to the extremely low level of total soil P. Unavailability of native P compounds and by the fixation of soluble P sources such as fertilizers and manures. Fixation of P takes place due to the reaction with Al, Ca, Fe and Mn compounds or adsorption on soil colloids and on clay particles (Brady, 1990; Jungk et al., 1993)

Phosphorus deficiency interferes with normal opening of stomata in certain plants. This results in 10% high leaf temperature during period of sunshine as compared to plants having adequate supply of phosphorus (Wallace and Deutsch, 1968).Lake of adequate P leads to the retarded growth of the plant. Visual symptoms appear in case of severe P deficiency of the plant i.e. accumulation of anthocyanin pigment, development of dead necrotic areas on the leaves, petioles and fruits. Abnormally dark green colour is developed in some species. Rusty brown lesions are also observed in potato tubers. Older leaves become dark brown as they die because of high mobility of P, deficiency symptoms appear first in older leaves. When plant matures, P translocates into the seeds

and fruits (Donahue et al., 1990; NDFC, 1995).

PHOSPHORUS FIXATION

Although P content in an average soil is 0.05%, only 0.1% of the total P present is available to the plants because of its chemical fixation and low solubility (Tilak et al., 2005). Phosphorus availability is low in acidic soils because of formation of iron and aluminium phosphate while in alkaline soil tricalcium phosphate [$Ca_3(PO_4)_3$] forms readily to reduce availability of soil phosphorus to plants (Donahue et al., 1971; Stevenson, 1986; Whitelaw, 2000). This process is called as phosphorus fixation. Holford (1983) suggested that precipitation by aluminium was a major cause of phosphorus fixation in soils having pH less than 4.5 but phosphorus is most available at pH 6.5 for mineral soils and at pH 5.5 for organic ones (Donahue et al., 1990). There is no efficient mechanism in soils to retain $H_2PO_4^{-2}$ or HPO_4^{-2} in large quantities as exchangeable anions (Donahue et al., 1990).

SOIL MICROORGANISMS

The fertility of soil depends not only on its chemical composition but also on qualitative and quantitative nature of microorganisms inhabiting it. Soils microorganisms are involved in a range of processes that affect P transformation and thus influence the subsequent availability of phosphate to plant roots (Richardson, 2001).The microorganisms inhabiting soil can be classified into bacteria, actinomycetes, fungi, algae and protozoa. Microorganisms are important component of soil. Soil processes such as decomposition, nutrient mobilization and mineralization, storage release of nutrients and water, nitrogen fixation and denitrification are mediated by soil bacteria and fungi (Alexander, 1971; Subba Rao, 1984). In the frame of agriculture, the microflora is of great significance because it has both beneficial and detrimental influence upon man's ability to feed himself (Motsara et al., 1995; Gaur, 1990; Whitelaw, 2000). There are specific soil microorganisms which bring about major transformation by different pathways centered upon carbon, nitrogen, phosphorus, sulfur, iron, manganese and other

minerals (Subba Rao, 1984; Kapoor and Mishra, 1989; Saha et al., 1995; Kulkarni and Nautyial, 1999; Whitelaw, 2000; Kulkarni and Nautyial, 2000).

PRESENCE OF PSM

The unmanaged excess caused by chemical fertilizer may be both an environment and economic problem because of excessive amounts of nitrates and phosphates in runoff from agricultural land can result in eutrofication of waterways. This situation has stimulated an interest in the use of alternative aspects of plant nutrients availability in soils which has a substantial pool of microorganisms having potentials to solubilize unavailable inorganic phosphate sources. Such organisms are known as phosphate solubilizing microorganisms (PSM). For over one hundred years, workers have recognized the ability of soil microorganisms to solubilize Pi from insoluble (i.e. nutritionally unavailable) organic and mineral phosphates (Whitelaw, 2000). To circumvent phosphorus deficiency, phosphate-solubilizing microorganisms (PSM) could play an important role in supplying phosphate to plants in a more environmentally-friendly and sustainable manner (Khan et al., 2007). The soil bacteria and fungi comprise the greatest percentages of phosphate solublizing microorganisms, known as PSM or Phosphate Solubilizing Bacteria (PSB) and Phosphate Solubilizing Fungi (PSF). These microorganisms are capable of solubilizing insoluble compounds and release phosphorus to soil solution (Fallah, 2006).Such organisms not only assimilates P but a large portion of soluble phosphate is released in quantities in excess of their own requirement (Gaur, 1990).

PSM includes different types of microorganisms such as bacteria and fungi that convert insoluble phosphatic compounds into soluble forms (Parks et al., 1990; Cunningham and Kuaick, 1992; Prerna-Akhaury et al., 1997; Raju and Reddy, 1999).The species of Pseudomonas, Bacillus, Micrococcus, Flavobacterium, Aspergillus, Penicillium, Fusarium, Sclerotium and others have been reported to be active in bioconversion (Gaur, 1990; Motsara et al., 1995).It has been reported that certain strains of rhizobium can also solubilize both organic and inorganic phosphates (Abd-Alla, 1994). Important genera of phosphate solubilizing bacteria are Bacillus and Pseudomonas (Illmer and Schinner,

1992; Motsara et al., 1995). Aspergillus and Penicillium are important P solubilizing fungal genera (Motsara et al., 1995).Strains from the genera Pseudomonas, Bacillus and Rhizobium are among the most powerful P solubilizers (Rodriguez and Fraga, 1999). Rajankar et al. (2007) observed that the fungi viz; Aspergillus spp., Penicillium spp. And Fusarium spp. have the more solubilizing ability of inorganic insoluble phosphate than bacteria , viz; B.subtilis, B.megatherium.Barroso and Nahas, (2005) reported soil isolate of the fungus Aspergillus niger showed high ability to solubilize both calcium and aluminum phosphates in culture medium.

OCCURRENCE AND DISTRIBUTION OF PSM IN PLANT RHIZOSPHERE

Plant root surfaces and soil in contact with roots comprise site of intense microbial activity and this environment under the influence of plant root is referred as Rhizosphere. Microorganisms are influenced in many ways by growing plants and the microbial processes are rapid in rhizosphere than in non-rhizosphere soil (Gaur, 1990).The rhizosphere is the region of soil that is immediately near to the root surface and that is affected by root exudates (Kennedy 1999); it was described for first time by Lorenz Hiltner 1904. There are different types of substances that diffuse from the roots and that stimulate the microbial activity, such as carbohydrates (sugars and oligo-saccharides), organic acids, vitamins, nucleotides, flavonoids, enzymes, hormones, and volatile compounds (Prescott et al.,1999). The result is a dense and active microbial population that interacts with the roots and within it. The rhizosphere effect on the soil microbial population can be measured comparing the population density (colonies forming units, CFU) between the rhizosphere soil (R) and the bulk soil (S), for which the"R/S ratio" is employed (Atlas and Bartha 1997). It has been observed by many investigations that a high proportion of P solubilizing microorganisms are concentrated in the rhizosphere of plants. As phosphate activities are found to be much higher in rhizosphere soil than in bulk soil (Seeling and Jungk, 1992). Inorganic P solubilizing microorganisms are also more in rhizosphere of plants than in bulk soil (Vesquez et al., 2000). It was reported that availability of various soil P fractions to different plant species may be different depending on the mechanisms plant roots can use under the given soil conditions (Jungk

et al., 1993).As observed with other soil microbes the number of PSM is more important in the rhizosphere than in non-rhizosphere soil (Kucey et al., 1989), and the number of phosphate solubilizing bacteria is more important than that of fungi (Kucey, 1983).

MORPHOLOGY OF PSM

PSM are characterized by the transparent zones of clearance (halozone) around their colonies (Nautiyal, 1999; Kumar and Narula, 1999) generally on Pikovskaya media (Pikovskaya, 1948). Mostly phosphorus solubilizing bacteria are 1.1 to 2.2 micrometer in cell size and are rod shaped and few are cocci and spirillum (Motsara et al., 1995). Stereomicroscopic studies of PSM colonies showed that PSM differ in their colony texture, elevation and shape etc(Urban, 1977).

Gram staining reaction shows that some PSB are either Gram positive or Gram negative (Urban, 1977; Pelczar et al., 1977; Pal, 1999).Important bacterial genera Bacillus and Pseudomonas are gram positive and gram negative respectively (Motsara et al., 1995) Aspergillus and Penicillium are two important phosphorus solubilizing fungal genera. These two can also be identified morphologically. Aspergillus has pitted conidiophores arising separately from substratum (Motsara et al., 1995) while Penicillium has penicillus structure(Urban, 1977). Similarly all PSM strains have their own morphology.

POSPHORUS SOLUBILIZATION BY PSM ON SOLID AND IN LIQUID CULTURES

Colony development on agar surface helps in identifying bacteria and fungi because individual species often form colonies of characteristic size and appearance. Sometimes it is possible to identify desired colony based on its overall appearance and to obtain its pure culture.The mechanism of P solubilization by PSM had been searched out on solid cultural media (Subba Rao, 1984; Illmer and Schinner, 1992; Gaur, 1990; Kumar and Narula, 1999; Whitelaw, 2000). Microbes grown on precipitated calcium phosphate in agar containing media, if capable of solubilizing calcium phosphate minerals produce halozones (Pikovskaya, 1948; Louw and Webley, 1959). Other forms of sparingly soluble P i.e. ferric or aluminium phosphate have also been included in precipitated phosphate

agar for the isolation of P solubilizing microorganisms (Banik and Dey, 1983; Martinez Curz et al., 1990). Rock phosphate suspended in solid agar plates has also been investigated for isolation of microorganisms capable of solubilizing rock phosphates (Singh et al., 1984; Surange et al., 1997; Toro et al., 1998). Illmer and Schinner (1992) screened 2 fungal strains having high abilities to solubilize inorganic phosphates from 600 colonies isolated from forest soils on solid medium and then tested their abilities for RP solubilization in unsterile soil. Phosphorus solubilization index has been evaluated through the measurement of colony and halozone diameter (Kumar and Narula, 1999).Many researchers have quantitatively investigated the ability of PSM to solubilize insoluble P in pure liquid culture mediums. A wide range of liquid media supplied with nutrients that are required for the growth of the PSM and with different P sources has been used in order to study the nutritional effects on the growth and physiological properties of PSM strains. The level of P solubilized by PSM and metabolites i.e. organic acids produced by PSM were also investigated in their liquid cultures (Halvorson et al., 1990; Halder et al., 1991; Illmer and Schinner, 1992; Gupta et al., 1994; He-ZhenLi et al., 1998; Narula et al., 2000; Whitelaw, 2000; Altomare,1999) It is reported that P solubilization was often higher when the initial insoluble P levels were high in liquid cultures and when volume of the liquid medium was adequate for dissolution (Illmer and Schinner, 1992; Cunningham and Kuiack, 1992). Banik and Dey (1982) reported low level of P solubilization by Aspergillus candidus and Aspergillus fumigatus despite the high initial insoluble P level. They suggested that the low volume of liquid medium might cause the limited dissolution of P.

PHYSIOLOGICAL ASPECTS OF PSM STUDIED IN LIQUID CULTURES

Despite the increasing number of authors whose attention is engaged by the study of phosphorus solubilizing microorganisms, only few data are available concerning the physiological properties of PSM (Illmer and Schinner, 1992). Some of the physiological properties of PSM that were investigated are nutritional requirements, oxygen demands and temperature effects. Glucose is the main carbon source for the growth of PSM but other carbon sources can also be utilized (Illmer and Schinner, 1992; Motsara et al.,

1995).

Many scientists had explained the importance of N sources for P solubilization by PSM (Asea et al., 1985; Cunningham and Kuack, 1992).It was observed that by increasing the oxygen input through higher shaking speed in culture of Penicillium and Pseudomonas strains, their P solubilization efficiency was increased (Einsele et al., 1985). Cunningham and Kuack (1992) concluded that optimum temperature for these 2 strains are 25 and 30°C respectively. It has been reported that production of citric acid by phosphorus solubilizing fungus, Penicillium bilaii, was promoted by shaking its culture (Illmer and Schinner, 1992; Motsara et al., 1995). Physiological properties of PSM were also influenced by stressed conditions. Occurrence of salt, pH and temperature tolerant phosphate solubilizing bacteria in alkaline soils had been investigated (Illmer and Schinner, 1992; Yahya and Al-Azavi, 1989). The isolated strains were invitro conditions and it was reported that the performance of PSM is severely influnced by environment factors especially under stressed conditions (Yahya and Al-Azavi, 1989; Gaind and Gaur, 1991; Johri et al., 1999; Nautyal et al., 2000).

MECHANISM OF PHOSPHORUS SOLUBILIZATION BY PSM

For over a century agricultural microbiologists and microbial ecologists have been interested in the ability of some bacteria to dissolve poorly soluble mineral phosphates such as tri-calcium phosphate or hydroxy-apatite (Goldstein, 1987, 1993). Many insoluble forms of calcium, iron and aluminum phosphate occur in soil, however, few studies are reported related to the solubilization of aluminum and iron phosphate (Barroso et al, 2006; Illmer and Schinner, 1992; Jones et al., 1991).Many research scientists have examined the reason that when phosphorus solubilizing microbes were applied to the insoluble P, a larger amount of unavailable P became available (Barber, 1984; Subba Rao, 1984). The role of organic acid produced by PSM in solubilizing insoluble P may be due to the lowering of pH, chelation of cations and by competing with P for adsorption sites in the soil. It has also been investigated that organic acids may also form soluble complexes with metal ions associated with insoluble P (Ca, Al, Fe) and thus P is released (Kepert et al., 1979; Omar, 1998).PSM have ability to bring insoluble phosphate sources

by producing organic acids such as formic, proponic, lactic, glycolic, fumaric and succinic acids (Rao, 1984), citric, oxalic, gluconic acid (Illmer et al., 1995). Moghimi and Tate (1978) have also added butyric acid, malic, malonic, adipic and 2 ketogluconic acid which either dissolve rock phosphate directly or chelate calcium ions to bring phosphorus into ions.Altomare (1999) proposed three possible mechanisms: acidification of the medium, production of chelating metabolites, and redox activity, isolated strain (T-22) was able to solubilize MnO_2, metallic zinc, and rock phosphate.

CHELATION

The PSM render insoluble phosphate into soluble form through the process of acidification, chelation and exchange reaction in soils (Son et al., 2006; Rodriguez et al., 2000; Berthelin et al., 1991; Banik., 1983). It is generally recognized that organic acid solubilizes RP through protonation and /or chelation reactions (Sagoe et al., 1998).Chelation involves the formation of two or more coordinate bonds between a molecule and a metal ion, thereby creating a ring like complex. Chelation by an organic acid ligand occurs through oxygen containing hydroxyl or carboxyl groups and takes place when five membered or less stable six membered ring is formed (Albert and Serjeant,1984; Abd-Alla, 1994; Altmare et al., 1999).The ability of low molecular weight organic acids to release P from ores or rocks, related to their ability to form stable metal complexes is well established (Mattey, 1992), related to their ability Many researchers have reported that organic acid were able to solubilize more P than inorganic ones at same pH. This difference is probably due to chelation. Kim et al (1997) reported that HCl able to solubilize less P from hydroxyapatite than citric and oxalic acids at same pH. They suggested that chelation by organic acids produced by microorganisms is involved. Whitelaw et al. (1999) observed that gluconic acid or Penicillium radicum inoculation alone was able to solubilize more P than HCl at same pH. Cunningham and Kuiack (1992) found increased solubilization of calcium biphosphate in the presence of citric acid compared to inorganic acid at the same pH.

DECREASE IN pH

Lowering of pH is considered to be responsible for phosphate solubilization. High phosphate solubilization is often associated with low pH in final culture solution. Trolldenier, (1992) provided evidence for solubilization of calcium phosphates due to acidification of rhizosphere. Whitelaw et al. (1999) found a positive correlation between activity of protons (H^+) and P solubilized from calcium hydrogen phosphate($CaHPO_4$), tricalcium phosphate $Ca_3(PO_4)_2$ and amorphous aluminium phosphate. Significant negative correlation was found with pH and solubilization of different calcium phosphate minerals by PSM (Thomas et al., 1985; Illmer and Schinner, 1992 and Venkateswarlu et al., 1994). But Nahas (1986) and Salih et al (1989) did not find negative correlation.

Narsian et al. (1993) reported that by an incubation period of 7 days pH, solubilization of $Ca_3(PO_4)_2$ by Aspergillus aculeatus reached a maximum coinciding with minimum pH on second day of incubation. After this time amount of phosphate solubilized decreased fluctuating in a manner which did not appear to be related to pH. Illmer and Schinner (1992), Goenadi and Saraswati (1993) and Whitelaw et al., (1999) also reported such behavior.

Fankem et al.(2006) reported that phosphate solubilization resulted from a combined effect of pH decrease of the media and organic acid production. Altomare, (1999) also reported the same idea that solubilization of metal oxides involves both chelation and reduction.Turan et al.(2006) reported that phosphorus solubilizing bacteria and fungi inoculation decreased solution pH and increased electrical conductivity, and Ca and P concentrations in solution culture. Chen et al.(2006) reported that P-solubilizing activity was associated with the release of organic acids and a drop in the pH of the medium. Song et al. (2008) reported that mineral phosphate solubilization was directly related to the pH drop.

OTHER MECHANISMS

Illmer and Schinner (1995) proposed other mechanism like protons from ammonium assimilation and from carbonic acid production can account for phosphate solubilization. According to Illmer et al. (1995) the production of organic acid is important mechanism

of aluminium phosphate solubilization but not the only one, other mechanisms may also be involved.

$$AlPO_4 + \quad + 2H^+ \_\_\_\_\_ > Al^{3+}.\ (aq) \quad + H_2PO_4^-$$

Asea et al. (1988) also reported presence of ammonium as responsible for phosphate solubilization. Kim et al. (1998) has also reported that P uptake and growth of plants could be due to production of carbon dioxide by respiration of soil microorganisms. Increased carbon dioxide concentration was highly correlated with solubilization of P by carbonic acid formed from carbon dioxide. Carbon dioxide when dissolves in water forms carbonic acid (H_2CO_3) which on dissociation releases proton. The produced proton can readily solubilize calcium apatite as shown and $H_2PO_4^-$ (primary orthophosphate ion) is absorbed by the plants.

$$Ca_5(PO_4)_3OH + 7H+ \_\_\_\_\_ > 5Ca^{2+} + 3H_2PO_4^- + H_2O$$

ORGANIC ACID PRODUCTION

Kucey et al. (1989) and Bar-yosef (1991) have shown that microbial solubilization of soil phosphates in liquid medium studies has often been due to the extraction of organic acids. In many studies the presence of organic acids i.e. oxalic acid, citric acid, lactic acid, gluconic acid etc in liquid culture filtrates were determined by paper chromatography or thin layer chromatography and by modern techniques such as High Performance Liquid Chromatography (HPLC), isotachophoresis and enzymatic methods have been used by others to allow more accurate identification of unknown organic acids.(Banik and Dey, 1982; 1983; Venkates Warula et al., 1984; Parks et al., 1990; Berthelin et al., 1991; Cunningham and Kuaik, 1992; Illmer and Schinner, 1992; 1995; Gupta et al., 1994; Singal et al., 1994; Illmer et al., 1995; Vassilev et al., 1995;1996; Mehta et al.,1996; Whitelaw et al., 1999; Maliha et al., 2004; Fankem et al., 2006). Kumar and Narula (1999) have used spectophotometric method for detection of indole acetic acid (IAA).Banik and Dey (1883) and Asea et al. (1988) detected organic acids in culture

solutions of PSM but did not show any correlation between the solubilization of P and amount of organic acids produced by PSM. Illmer et al. (1995) observed that organic acid production may be helpful but not the sole need for AlPO4 solubilization. It has been experimented that Pseudomonas spp. and Penicillium aurantiogriseum were very effective in solubilizing calcium phosphate without producing appreciable amounts of organic acids while Burgstaller et al. (1992) observed that Aspergillus and Penicillium solubilized P by producing large amount of organic acids. The production of citrate, oxalate and gluconate by Aspergillus niger has also been reported (Illmer et al., 1995). Bar-yosef et al. (1999) have detected gluconic and 2-ketoglutaric acids from Pseudomonas cepacia. . Vassileva,(1997) reported that citric acid-producing strain of Aspergillus niger, grown on olive cake-based medium, was able to solubilize rock phosphate. Chen et al (2006) reported that HPLC analysis detected eight different kinds of organic acids, namely: citric acid, gluconic acid, lactic acid, succinic acid, propionic acid and three unknown organic acids from the cultures of 36 isolates. Song et al (2008) reported that analysis of the culture medium by high pressure liquid chromatography identified gluconic acid as the main organic acid released.

ORGANIC ACIDS SOLUBILIZE MORE P THAN INORGANIC ACIDS

Inorganic anions also solubilize P but they are less effective than organic anions i.e. flourides, arsenates, borates, sulfates, chlorides and nitrates to some extent solubilized P from iron, aluminium and tricalcium phosphates (Danahue et al., 1971). Kim et al. (1997) reported that HCl was able to solubilize less P from hydroxyapatite than citric acid or oxalic acid at same pH (Whitelaw et al., 1999). It has been observed that inoculation of gluconic acid alone or Penicillium radicum alone was able to solubilize more P from $AlPO_4$ than HCl at same pH. Same results were obtained by inoculating unknown species of Penicillium, Pseudomonas (Illmer and Schinner, 1992) and also reported by the inoculation of Enterobacter agglomerans (Kim et al., 1997).Bolan et al. (1994) concluded that addition of organic acids caused the dissolution of soil components i.e. ferric acid and aluminium oxides and thereby decreased the adsorption by soil. Kpomblekou and Tabatabai (1994) also observed aliphatic acids with –OH or –COOH groups in position

suitable for the formation of complexes with metal cations are more effective than aliphatic and aromatic acids in releasing P from rock phosphates.

STUDIES OF PSM IN AGRICULTURE

Natural RP have been recognized as a valuable alternative source for P fertilizer, especially for acid soils. The economic value of the rocks increases considerably along with the increasing costs of SP production. Consequently, there is a growing interest in ways of manipulating such rock to obtain a more valuable product. Common efforts include the use of chemico-physical means, that is, partially acidulating RP (Hammond et al., 1986; Goenadi, 1990; Lewis et al., 1997; Rajan and Ghani, 1997), reacting with synthetic organic acids (Sagoe et al., 1998) and/or natural organic acids (Singh and Amberger, 1998, 1998,) and decreasing particle size (Babare et al., 1997).Importance of PSM inoculation can be noticed by its occurrence in many agricultural crop species i.e. cereals and legumes etc. (Alagawadi and Gaur, 1998; Gleddie, 1993; Chabot et al., 1993,1996; Kumrawat et al., 1997; Jain and Tiwari, 1997; Dubey et al., 1999).The dry matter contents grain yield and P uptake were increased by PSM inoculation in chickpea (Alagwadi and Gaur1988; Jain et al., 1999)Inoculation of soils with Penicillium billaii along with RP at the rate of 45 microgram of P per g of soil resulted increased plant dry matter production and P uptake by wheat and beans (Kucey, 1987) .Vassileva,(1999) reported that greater growth and P uptake of mycorrhizal and non-mycorrhizal plants were achieved when microbe-treated olive cake and rock phosphate were applied to soil compared with all other treatments.Rajankar et al. (2007) observed that the fungi viz; Aspergillus spp., Penicillium spp. and Fusarium spp. have the more solubilizing ability of inorganic insoluble phosphate than bacteria , viz; B.subtilis, B.megatherium. His observations suggested that application of biofertilizer prepared by above mentioned fungi should be helpful to reduce the salinity of the soil by neutralization phenomenon, because these microorganisms release the acid in very minute quantity in phosphate solubilization. Altomare (1999) reported the ability of a Trichoderma strain to solubilize insoluble or sparingly soluble minerals. Solubilization of metal oxides by Trichoderma involves both chelation and reduction. Both of these mechanisms also play a role in

biocontrol of plant pathogens, and they may be part of a multiple-component action exerted by T-22 to achieve effective biocontrol under a variety of environmental conditionsTuran et al. (2006) reported that phosphate solubilizing bacteria Bacillus (FS3) and Aspergillus (fungal strain FS9 and FS11) have great potential for use bio-fertilizer development in agriculture. The phosphorus solubilizing bacteria and fungi inoculation decreased solution pH and increased electrical conductivity, and Ca and P concentrations in solution culture.

Peix et al. (2001) reported that the strain SAOCV2 (Burkholderia cepacia) was able to mobilize P efficiently in the common bean. The N content in plants inoculated with the strain SAOCV2 was significantly higher than in uninoculated plants. His results suggested that the inoculation with strain SAOCV2 promotes the growth of common bean by several mechanisms, that include P mobilization, antagonism towards pathogenic species of Fusarium and, indirectly, by an increase in nodulation that may lead to an increase in N_2 fixation.

Goenadi et al. (2000) study was conducted to develop a simple, effective, and environmentally sound process to improve P availability of RP to crops by using a phosphate-solubilizing fungus (PSF), Aspergillus niger BCC F.194, isolated from tropical acid soils. The inoculation of the growth media with the PSF, *A.* niger resulted in the highest P solubility of the rock.Antarikanonda et al. (1991) isolated 102 PSMs from rhizosphere soil in Central Thialand.

They studied RP solubilization in 100 ml culture in one week. They found fungi more ctive in solubilizing phosphate than bacteria. Illmer et al (1995) found Aspergillus niger, Penicillium simplicissimum, P. aurantiogriseum and Peudomonas spp.to be very active in solubilizing hardly soluble aluminium phosphate. A. niger produced citric, oxalic and gluconic acids whereas other species did not produce organic acids in the detectable amount.Pal (1997) isolated phosphate solubilizing bacteria (PSB) from 60 soil samples of different land classes in Uttar Pradesh, India. Best strains of PSB from each land class ere compared for their phosphate solubilization and acid tolerance. Bacillus spp. Strain NoPAS-2, isolated from an acid soil (pH 4.8) had the highest P solubilizing capacity and highest acid tolerance.Omar (1998) tested 36 fungal species isolated from soil for their ability to solubilize rock phosphate (RP) in agar plates. Most of them were non-

solubilizers. A. niger and Penicillium citrinum had high activity. Both fungi caused a marked drop in pH of liquid culture media and solubilized considerable amounts of phosphate.Nautiyal et al. (2000) studied PSB strains NBRI 0603, NBRI 2601, NBRI 3246 and NBRI 4003, which exhibited diverse levels of phosphate activity under in vitro conditions in the presence of carbon and nitrogen sources. NBRI 0603 was most efficient strain in solubilizing P in the presence of 10% salt, pH 12 or 45 °C temperature as compared to other strains. They stated that organic acid production is an important mechanism in "P" solubilization but not the only one.El-Syed (1998) studied effect of combined inoculation of Rhizobium leguminosarum and PSB Pseudomonas striata and Paenibacillus polymyxa with and without added fertilizer on lentil yield and nutrient content under green house conditions. PSB increased available P content of soil. Combined inoculation of Rhizobium and PSB significantly increased nodulation, nitrogenase activity, dry matter content, grain yield, N and P uptake over the uninoculated control. Similar results were found by Dubey and Agarwal (1999) while studing effect of PSM as single and composite inoculant in rainfed soyabean.Pal (1998) conducted field experiments to study the effects of seed inoculation with PSB on Elusine coracana, Amaranthus hypochondriacus, Phaseolus vulgarus and Zea mays. Bacillus strain PAS-2 exhibited highest P solubilization.It also resulted in significant increase in grain and vegetative yield of all five crops. Kole and Hajra (1999) studied effect of Bacillus spp. (PSB) and Penicillium spp. (PSF) on availability and uptake of P by green gram. Inoculation increased available P_2O_5 (8-18%), dry matter yeild (23-34%), percentage P uptake (41-61%) by green gram in comparison with uninoculated control.Sarawgi et al. (1999) conducted field experiments to study effect of phosphate solubilizing bacterial culture inoculation and micro-nutrients on growth, nodulation and yield of chickpeas. Grain, straw yield and yield attributes of chickpeas were improved with increased P levels. All these characters were further improved with inoculation of PSB compared with P application alone. Maximum propotional impact of PSB and Rhizobium (34.1%) was observed with the application of 30Kg P_2O_5.In pea (Pisum sativum) increased growth was observed by dual inoculation with PSM along with application of 25.8Kg , 0.5Kg molybdenum and Rhizobium leguminosarum (Srivastava et al., 1998). Antoun. (1998) focused his interest on rhizobia, because these bacteria well

known for their beneficial symbiotic atmospheric nitrogen fixing symbiosis with legumes. The P-solubilization effect seems to be the most important mechanism of lant growth promotion in moderately-fertile soils, and was less effective in poor soils. Mutants altered in their P-solubilization activities (solubilizing significantly less P than the wild type) were used to determine the importance of this trait in strain R1 (Chabot et al., 1998). The results obtained, confirmed that growth promotion by P-solubilization is less effective in poor soils as observed under field conditions. Mehdi et al. (2006) reported that P-uptake efficiency was increased when P fertilizers were applied alongwith AM fungi and/or P-solubilizer rhizobial strains. Singh et al. (2005) reported that the inoculation of lentil seed with phosphate-solubilizing bacteria (PSB) also improved its seed and straw yield besides improving P use efficiency.Fankem et al. (2006) got isolates from oil palm tree root fragment and rhizospheric soils and their activity in mobilizing phosphates from insoluble sources was evaluated on agar plates and liquid culture media containing sparingly soluble phosphates. At the end of incubation time, phosphate solubilization resulted from a combined effect of pH decrease of the media and organic acid production. Among the ten isolates tested, three were identified as Pseudomonas flourescens and would be considered as potential biofertilizer. Further more each of the tested isolates were able to produce at least one of the most important organic acids such as citrate, malate and tartrate.Chen et al., (2005) reported the ability of a few soil microorganisms to convert insoluble forms of phosphorus to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields. The use of phosphate solubilizing bacteria as inoculants increases the P uptake by plants.Vikram and Hamzehzarghani (2008) studied ability of 16 isolates of Phosphate Solubilizing Bacteria (PSB) to promote growth parameters in greengram crop and were tested under green house conditions. The study consisted of 18 treatments which were replicated three times. Inoculation of greengram seeds with PSBV-14 recorded the highest nodule number, nodule dry weight, shoot dry matter and total dry matter in greengram plants 45 days after sowing. Similarly, treatment receiving the inoculation of PSBV-13 recorded the highest root length, root dry matter, P content and P uptake in root and shoot in greengram plants.Villegas and Fortin (2001) showed an interesting specific synergistic interaction between the P solubilizing bacterium Pseudomonas aeruginosa and the AM

fungus Glomus intraradices. Transformed mycorrhizal carrot roots or G. intraradices external mycelium and Pseudomonas aeruginosa solubilized more P from the sparingly soluble tricalcium phosphate when combined than when each alone. No synergistic effect was observed with the other two P-solubilizing bacteria tested (Pseudomonas putida and Serratia plymuthica) showing the specificity of this interaction.Rudresh et al. (2005) reported 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.Zaidi and Khan, (2005) reported that triple inoculation of AM fungus,(Glomus fasciculatum), phosphate solubilizing fungus (Aspergillus awamori), phosphate solubilizing bacterium (Bacillus subtilis) and nitrogen fixing (Bradyrhizobium sp. (vigna)) significantly increased dry matter yield , chlorophyll content in foliage and N and P uptake of green gram plants.

Induction of the systemic resistance against many pathogens, insect and nematodes (Ramamoorthy et al., 2001; Zehnder et al., 2001) is also a recent indirect mechanism of action of PGPR. All these traits that can be present in PGPR, illustrate how it is complex and difficult to associate the promotion of plant growth with P solubilization, and they explain in part the reason of obtaining better responses from plant inoculated with a mixture of PGPR. Furthermore, approximately two-thirds of all land plants form the arbuscular mycorrhizal (AM) type of association (Hodge, 2000). PGPR can promote mycorrhizal functioning. Antoun (trials) focused his interest on rhizobia, because these bacteria well known for their beneficial symbiotic atmospheric nitrogen fixing symbiosis with legumes, have an excellent potential to be used as PGPR with non legumes (Antoun et al., 1998). The P-solubilization effect seems to be the most important mechanism of plant growth promotion in moderately to fertile soils, and was less effective in poor soils. Mutants altered in their P-solubilization activities (solubilizing significantly less P than the wild type) were used to determine the importance of this trait in strain R1 (Chabot et al., 1998). The results obtained confirmed that growth promotion

by P-solubilization is less effective in poor soils as observed under field conditions.

PSM RESEARCH IN PAKISTAN

Khalil, (1995) isolated more than 50 PSM that brought 30-40% phosphate rock solubilization after 15 days incubation with PSM in culture. In other study phosphate availability from rock phosphate was increased in soil from 0.67 ppm in control to 17.78 ppm with PSM inoculation in 20 days.Khalil and Sultan, (2000) conducted two field experiments to evaluate survival of PSM under field condition. Results of first experiment indicated a gradual decrease in PSM population from 106 in the beginning of experiment to 104 by the end of the experiment. In the second experiment PSM inoculation with organic amendment increased plant shoot weight by 13% s compared to control.Alam, (2001) study was carried out to characterize PSM and their effect on plant growth and P uptake of crops (maize and wheat). Composite inoculation of PSM along with different sources revealed that TCP+PSM and RP+PSM increased growth and P uptake of plants.Rashid, (2001) suggested from the results of her study that the bioinoculants containing efficient PSM individually or in composite should be utilized with the local deposits of P to fulfill P requirements of plants in P deficient soils.

FAMILY LEGUMINOSAE

The fabaceae or leguminosae is a large and economically important family of flowering plants, which is commonly known as the legume family, pea family, bean family and pulse family. 'Leguminosae' is an older name and it refers to the typical fruit of these plants: the legume.It is found throughout the world, in many different environments and climates.It is the third largest family of flowering plants (after Orchidaceae and Asteraceae) with 730 genera and over 19,400 species, according to the Royal Botanical Gardens. The largest genera are Astragalus (more than 2,000 species), Acacia (more than 900 species), Indigofera (700 species), Crotalaria (600 species), Mimosa (500 species).Glycine max (soya bean), Phaseolus (bean), Pisum sativum (pea), Medicago sativa (alfalfa), and Arachis hypogae (peanut) are amongst the most well-known Fabaceae (http://en.wikipedia.org/wiki/Fabaceae).

FEW IMPORTANT PLANTS OR CROPS OF LEGUMINOSAE

LENTILS

Lentils are one of the oldest domesticated plant species that originated in Mediterranean region. Its cultivated species, Lens culinaris belongs to the genus Lens that is classified taxonomically in the order Rosales, sub-order Rosineae, family Leguminoseae and sub-family Papilionacea with special features of nitrogen fixation. It is used as a meat substitute due to high protein contents and quality, also used in gluten-free, diabetic, low salt, low calorie, low cholesterol and high fiber diets. It is an important winter pulse crop, grown on a total of 1.8 million hectares in the world, of which 60% is in the South Asia (Sultana, 2003)

CHICKPEA

Two types of chickpea are recognized, desi (colored, small seeded, angular and fibrous) and kabuli (beige, large seeded, rams-head shaped with lower fiber content) types (Malhotra et al., 1987). In India and Pakistan, chickpeas are consumed locally, and about 56% of the crop is retained by growers (Duke, 1981). Chickpea production increased from 1980 to 1990 by about a million tons (at 1.8 % annually), and there was a 5.6 % increase in yield over the decade (Oram and Agcaoili, 1994). During 2002-2004, the global chickpea production was 8.0 million tons from an area of 10.1 million ha, giving an average productivity of 786 kg ha^{-1} . During the past 20 years (1985-2004), the global chickpea area increased by 7%, yield by 24% and production by 33% chickpea has one of the highest nutritional compositions of any dry edible legume and does not contain any specific major anti-nutritional factors (icrisat,2008). The major chickpea growing countries are India, Pakistan, and Turkey in Asia, Ethiopia in Africa, California and Washington state in the U.S., Mexico and Australia (FAO, 1994)

PEA

Pisum sativum (pea) is an annual plant, with a lifecycle of one year. It is a cool season

rop grown in many parts of the world, planting can take place from winter through to early summer depending on location. For very early crops, a sandy loam is preferred; for large yields where earliness is not a factor, a well-drained clay loam or silt loam is preferred" (Duke, 1981). The average pea weighs between 0.1 and 0.36g. Peas are cultivated for the fresh green seeds, tender green pods, dried seeds and foliage (Duke, 1981). Green peas are eaten cooked as a vegetable, and are marketed fresh, canned, or frozen while ripe dried peas are used whole, split, or made into flour (Davies et al., 1985). Pea is the predominant export crop in world trade and represents about 40% of the total trade in pulses (Oram and Agcaoili, 1994). Total world dry pea production rose from 8.127 million metric tons in 1979-81 to 14.529 million metric tons in 1994 while acreage varied from 7.488 to 8.060 million hectares for the same years (FAO, 1994). Important production areas of the world include France, Russia, Ukraine, Denmark and United Kingdom in Europe; China and India in Asia; Canada and USA in North America; Chile in South America; Ethiopia in Africa, and Australia (FAO, 1994).

ECONOMIC IMPORTANCE OF LEGUMINOSAE

Their ability to fix atmospheric nitrogen reduces fertilizer costs for farmers and gardeners who grow legumes, and means that legumes can be used in a crop rotation to replenish soil that has been depleted of nitrogen. Legume seed and foliage has a comparatively higher protein content than non-legume material, due to the additional nitrogen that legumes receive through the process.Farmed legumes can belong to numerous classes including forage, grain, blooms, pharmaceutical/industrial, fallow/green manure and timber species, with most commercially farmed species filling two or more roles simultaneously.

Grain legumes are cultivated for their seeds, and are also called pulses. The seeds are used for human and animal consumption or for the production of oils for industrial uses. Grain legumes include both herbaceous plants like beans, lentils, lupins, peas and peanuts and trees such as carob and tamarind.There are of two broad typesof forage legumes. Some are sown in pasture and grazed by livestock. Other forage legumes are either broken down by livestock or regularly cut by humans to provide stock feed.Bloom

legume are farmed commercially for their blooms as well as being popular in gardens worldwide as ornamental trees and shrubs.

Industrial farmed legumes are cultivated for the production of indigo, gum arabic and the insecticide action of rotenone.

Fallow or green manure legume species are cultivated to be tilled back into the soil in order exploit the high nitrogen levels found in most legumes.

Various legume species are farmed for timber production worldwide (http://en.wikipedia.org/wiki/Fabaceae).

USE OF ARTIFICIAL FERTILIZER & DRAWBACKS OF ARTIFICIAL FERTILIZER

Since deficiency of Phosphorus is the most important chemical factor restricting plant growth. Chemical phosphatic fertilizer is widely applied to overcome this deficiency. Soluble forms of Phosphorus fertilizer used are easily precipitated as insoluble forms. This leads to excessive and repeated application of phosphorus fertilizer to cropland, which are non renewable sources of energy (Donahue et al., 1990).Global demand for fertilizers led to large increase in Phosphate (PO_4^{-3}) production in the second half of the 20th century. Phosphate rock, which contains the mineral apatite, an impure tri-calcium phosphate, is an important source of the element .Concentrated phosphoric acids, which may contain as much as 70 to 75% P_2O_5 content, have become of great importance to agriculture and farm production. World-wide demand for fertilizers has caused record phosphate production (Hammond, 1995)Only 20% of added phosphorus is used by a crop during the season phosphorus is applied (Donahue et al., 1990). A great portion of phosphorus from chemical fertilizers becomes insoluble turning into calcium or magnesium salts in calcareous soils and iron or aluminium salts in acid environments all of which are unavailable to plants (Fallah, 2006)Both biological and chemical processes are involved in P fixation but the chemical processes of P absorption and precipitation are more important in the retention of P fertilizers (Stevenson, 1986). To avoid this, fertilizers are placed in band about 5cm in one or both sides of seeds and also 5cm below it, to minimize the contact with the soil close enough to young roots. Even with these precautions, P fixation takes place (Hattori, 1973). Hence it is confirmed that most of the

added P undergoes the P fixation process (Seetharam et al., 1986; Donahue et al., 1990). Extensive use of P fertilizer causes Zn deficiency in applied season as excessive soluble P precipitates Zn as zinc phosphate both inside the plant and in the soil(Donahue et al., 1990). Chemical fertilizers have played a significant role in the green revolution, but unbalanced use of them,had led to reduction in soil fertility and to environmental degradation (Gyaneshwar et al., 2002). In addition unfavourable pH and high reactivity of aluminium and iron in soils decrease P availability as well as P-fertilizer efficiency (Borling et al., 2001; Hao et al.,2002)

BIOFERTILIZERS

Phosphorus is one of the major nutrients limiting plant growth. Most of the soil throughout the world are P deficient (Batjes, 1997) and therefore require P to replenish P demand by crop plants. To circumvent the P deficiency in soil, P fertilizers are applied. However after application, a considerable amount of P is rapidly transformed into less available forms by forming a complex with Al or Fe in acid soils (Norrish and Rosser, 1983) or calcium in calcareous soils (Lindsay et al., 1989) before plants root have had a chance to absorb it. Further, the use of rock phosphate as a phosphate fertilizer and its solubilization by microbes (Kang et al., 2002) through the production of organic acids (Maliha et al., 2004), have become a valid alternative to chemical fertilizers. Rock phosphate is widely distributed throughout the world, both geographically and geologically (Zapata and Roy, 2004). In conjugation with phosphate solubilizing microorganisms (PSM) rock phosphate provides a cheap source of P fertilizer for crop production. In this regard, several studies have conclusively shown that PSM solubilizes the fixed soil P and applied phosphates resulting in higher crop yields (Zaidi 1999; Gull et al., 2004; Rajankar et al., 2007; Turan et al., 2006; Fankem et al., 2006). The alternative approach is to use these PSM along with other beneficial rhizospheric microflora to enhance crop productivity. In this context, the simultaneous application of rhizobium and PS microorganisms (Parveen et al., 2002) PSM and arbuscular mycorrhizal (AM) fungi (Zaidi et al., 2003) has been shown to stimulate plant growth more than inoculation of each microorganism alone in certain situations when the soil is

P deficient.

Biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzymes, hydrogen cyanide, and siderophores or through competition for nutrients and space can improve significantly plant health and promote growth as evidenced by increases in seedling emergence, vigor and yield (Antoun and Kloepper, 2001). Microorganisms are involved in a range of processes that affect the transformation of soil phosphorus and are thus an integral component of the soil P cycle (Deubel and Merbach, 2005).

AIMS AND OBJECTIVES

Most soils of Pakistan are alkaline and low in available P. To overcome its deficiency chemical phosphatic fertilizers have been widely used. Most widely used phosphate fertilizer in in Pakistan is Diammonium Phosphate (DAP). During one year period, total DAP off take was increased by 40.1% in the year 1998-1999 to 1999-2000 (NFDC, 2000). Biofertilizer is a substitute of the artificial fertilizers that are not only costly but are not as effective and environment friendly as biofertilizers. PSM offers viable alternative to chemical phosphatic fertilizers. PSM research being carried out in other countries may not be applied directly to our own conditions because our field conditions and weather is different as compared to other countries. The research work using our own indigenous PSM species in comparison with the exotic one may open up more avenues for bringing up break through in the crops yield and production. The literature really lacks in having substantial domestic work.

The purpose of the present study is to gain acquaintance with legume's rhizospheric soil. This is primarily a survey work of the presence of PSM in the Pakistani soil of leguminous plants(having great economic importance). Aim is to know the species of the PSM that are present naturally in the rhizosphere of legumes, their frequency in the soil, their solubility index, their pH drop and acid production and in this way analyzing the microbial status that either fungi is dominant or bacteria in legumes with respect to the phosphate solubilization so that this survey could provide some information for further step i.e inoculum development that is the basic step for the biofertilizer production.

MATERIALS AND METHODS

MATERIALS AND METHODS

SAMPLING SITE

NARC was selected as a sampling site. The rhizosphere of plants i.e. Pea, Lentils and chickpea were taken as a research subject for the presence and absence of PSM. Five soil samples of each plant were taken.

COLLECTION OF SAMPLES

Soil samples were collected with the help of auger from the surface of the selected fields to the depth of 1-2.5 feet. Soil from the rhizosphere was taken because of the fact that PSM are situated more near the rhizospheric vicinity (Seeling and Jungk, 1992; Vesquez et al., 2000; Gaur, 1990).Sampling was done after the appearance of flowering. Distance for the soil collection was kept equal for the five samples of each plant so that the randomized results must be obtained. After putting them into the polythene bags they were properly labelled and dated. Samples were air dried, crushed and sieved through 2mm sieve. Then these samples were stored at 4 °C until PSM isolation.

SOIL TEXTURE

Soil of the selected plants and the whole fields of leguminous plants planted in NARC were having clay loam texture (NARC).

SOIL MOISTURE

Moisture content of each soil sample was calculated by percentage Gravimetric method (Pw).

$$Pw = \frac{\text{Weight of moist soil} - \text{Weight of oven dried soil}}{\text{Weight of oven dried soil}} \times 100$$

SOIL pH

Soil pH was measured in 1:1 soil water suspension with a pH meter using glass electrode

McLean (1982).

STERLIZATION OF THE GLASSWARE

All the glassware and the equipments required or used during isolation of the PSM were sterilized e.g. test tubes, petri plates, pipettes, tips,glass spreader, needles, loops and the flasks etc. These were sterilized in an autoclave at 121°C and 15 Ilb pressure for 20 minutes. Before sterilization the glassware was wrapped in the aluminium foil to prevent contamination.

PREPRATION OF PIKOVSKAYA'S MEDIA

Pikovskaya's media was prepared as explained in appendix-1, which is a special media prepared for PSM isolation (Pikovskaya, 1948).

AUTOCLAVING OF MEDIA

Pikovskaya's media consists of two solutions that were autoclaved separately, so that contamination of media do not occur.

MIXING OF SOLUTIONS

After autoclaving the solutions within half an hour the solution were mixed with each other in the Laminar hood so that the solidification and contamination of the media could not happen that can ruin the media.

POURING OF MEDIA

After the two solutions are mixed, the media prepared is then poured into the Petri plates. After solidification the plates are kept in the incubator for 24 hours for contamination checking. If media is sterilized no microbial colony appears in the Petri plates. If Petri plates are sterile, then they are ready for isolation of PSM.

SERIAL DILUTION

PSM were isolated from each sample by serial dilution. 1g of the soil sample was

dispersed in 10ml of autoclaved distilled water and was thoroughly shaken(Sharpley, 1960). 1ml of the above solution was again transferred to 9ml of sterile distilled water to form 10^{-2}dilution. Then again 1ml was transferred to 9ml of distilled (sterile) water to form 10^{-3} dilution. Similarly, 10^{-4}, 10^{-5}, 10^{-6}serials were made for each soil sample.

SPREADING OF SOIL SOLUTION ON PLATES

0.1ml from each dilution using micropipette (range; 0.1-1ml) was poured on Pikovskaya medium (Pikovskaya, 1948) in five replicates for microbial culturing and then spreaded with the help of sterilized glass spreader. They were labeled with the no. of soil sample and the dilution number. All this procedure was done in the LHF so that the other microbial spores are not dropped in the media that can contaminate the sample.

INCUBATION OF PLATES

Spreaded plates were incubated at 27-30°C for seven days. These plates were checked daily to find out the PSM presence on them.

MICROBIAL COUNTING

After the incubation time, the plates showing countable colonies (<300) were counted with the help of digital colony counter. Total number of fungal and bacterial colonies per 0.1ml were recorded with the help of digital colony counter in each replicate of PKV medium (Pikovskaya, 1948) poured Petri plates at 10^{-4} and 10^{-6} dilutions respectively. Total number of phosphorus solubilizing fungi and bacteria forming Halo zones around their colonies per 0.1ml were also recorded in the same manner. Total bacterial and fungal counts and phosphorus solubilizing fungal and bacterial counts per g of soil sample was calculated.

STATISTICAL ANALYSIS

Mean and standard error (± SE) of the enumeration counts (of each reading) was taken for pea, chickpea and lentil plants.

ISOLATION OF PSMs

PSM colonies making halozones were picked with the help of sterilized loop from mixed cultures and then streaked on fresh sterilized PKV agar plates to obtain pure PSM colonies. This was also done in the LHF so that the new plates are prevented from contamination. These plates were also labeled from where they were isolated.

MORPHOLOGICAL STUDY OF PSM

The morphological features of PSB colony were studied under stereomicroscope. Studied morphological features were color, shape, elevation, margin and texture of the bacterial colonies (Urban, 1977).After purification of PSF making halozone, pure cultures were transferred on fresh Czapeks (Cz) medium (composition of the czapek medium is given in appendix-2 for fungal identification. The shape and color of the fungal colonies were also noted.

MICROSCOPIC IDENTIFICATION

PSB were also morphologically identified by Gram's staining. Then on the basis of study of morphology and developed Gram's stain, bacteria were characterized.After the smear has been dried, heat-fixed, and cooled off, proceed as follows: Place slide on staining rack and cover specimen with crystal violet. Let stand for 1 minute. Wash briefly in tap water and shake off excess. Cover specimen with iodine solution and let stand for 1 minute. Wash with water and shake off excess. Tilt slide at 45° angle and decolorize with the acetone-alcohol solution until the purple color stops running. Wash immediately with water and shake off excess. Cover specimen with safranine and let stand for 30 seconds to 1 minute. Wash with water, shake off excess, and gently blot dry.

The smear is now ready to be read. (Use oil immersion lens.) Gram-positive organisms are easy to see because they stain a deep blue or blue-black. Gram-negative organisms stain a deep pink, but since the background material is also pink, minute and detailed inspection is necessary before reporting the results (NETC, 1986).For PSF, a small portion of each fungal colony along with agar was placed on a slide. A drop of trypan blue was added to material and immediately cover slip was placed over it, extra stain was

removed with the help of blotting paper. The slide was labelled and stored in slide box for further study. Morphological features of the phosphate solubilizing fungi were studied under microscope at x10 and x40 magnification.

PRESERVATION OF SAMPLE

PSM bacterial strains were streaked on fresh agar plates after every 10 days and preserved at 8°C for short term preservation as required for the determination of different research parameters. While fungal strains were preserved on the middle of the PKV agar media plates for short term duration. For long-term preservation, purified PSM colonies obtained after streaking were preserved in two ways, either in sterilized water in McConcky bottles or in PKV solid media containing slants and at room temperature 10ml sterile liquid paraffin was poured on to the slants showing growth of cultures. The paraffin layer was extended at least 2cm above its upper end of agar to prevent drying and then cultures were stored at 5°C (Malik, 1985).

CALCULATION OF SOLUBILITY INDEX (Si)

Sterlized PKV media was poured into sterilized Petri plates after solidification of the media, a pinpoint inoculation of bacterial or fungal strains is made on the plates under aseptic conditions. The plates were incubated at 27°C for seven days and observed for colony diameter and diameter of solubilization zone regularly during seven days. Then the ability of PSB and PSF to solubilize insoluble phosphate was studied by the determination of solubilization index (Si): the ratio of the total diameter (Colony + Halozone) and the colony diameter (Edi-Premono et al., 1996).

$$Si = \frac{\text{Colony Diameter + Halozone Diameter}}{\text{Colony Diameter}}$$

CHANGE IN pH IN PSM BROTH MEDIUM

As it is known that PSM produces acids .(Illmer et al., 1995; Vassilev et al., 1995;1996; Mehta et al.,1996; Whitelaw et al., 1999; Maliha et al., 2004; Fankem et al., 2006) so due to the production of the acids the pH of the media drops. So keeping this under consideration, the drop of pH in PSM broth medium is noted. Broth is prepared in the same manner as the Pikovskaya media, only agar is not added. Broth is poured in the sterilized flasks and PSF and PSB isolated are then added into the separate flasks (Each flask containing single PSF or PSM). All this is done in the LHF so that no other microflora is produced other than the inoculated PSMs. The pH of the broth is adjusted to 7.00 before pouring media into the flask so that we can compare the drop in pH with the control which is a broth flask without any PSM. Flasks are also labelled and were in two replicates. The flasks were placed in the incubator at 27°C.Then pH change in broth cultures was determined with the help of pH meter daily during seven days of incubation.

ORGANIC ACIDS DETECTION BY TLC

It is for long known that organic acids are produced by PSMs. So, organic acids produced by PSM strains in broth culture were detected by thin layer chromatography (TLC). Thin-layer chromatography (TLC) is a chromatographic technique that is useful for detection and separation of organic compounds. All forms of chromatography work on the same principle. They all have a stationary phase(a solid, or a liquid supported on a solid) and a mobile phase(a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates. Thin layer chromatography is done using a thin, uniform layer of silica gel or alumina coated onto a piece of glass, metal or rigid plastic. The silica gel (or the alumina) is the stationary phase. The stationary phase for thin layer chromatography also often contains a substance which fluoresces in UV light. The mobile phase is a suitable liquid solvent or mixture of solvents (Clark, 2007)Supernatant of samples, centrifuged at 1500 rpm for 15 minutes was taken. Then the supernatant of each sample was plotted onto the chromatographic silica plate along with the mixture of standard organic acids that works as referrence. For organic acids detection the polar solvent e.g pure 100% ethanol is used that is efficient in carrying the components of broth mixture (organic acids) of the PSM strains with it. Then the plate was placed in the TLC tank ,in which the solvent was

already present. When the solvent front gets close to the top of the plate, the plate is removed from the tank and the position of the solvent is marked with another line before it has a chance to evaporate. The plate was analyzed under UV light for the presence or absence of organic acids (Lee et al., 2001).

RESULTS

RESULTS

PHYSICO-CHEMICAL PROPERTIES OF SOIL

The physico-chemical analysis of the selected 15 soil samples of pea, chickpea and lentil (5 rhizospheric sample of each plant) are shown in the Table.1. Results of these analysis indicated that all the soil samples were generally alkaline with pH ranging from 7.77-8.06. All samples were Clay Loam in texture and their soil moisture ranged from 16.28-19.80.

Table 1: Physico-Chemical properties of 15 soil samples of pea(P), chickpea(CP) and lentil(L) selected for sampling

SITE NO.	LOCATIONS	SAMPLE CODE	SOIL TEXTURE	SOIL MOISTURE	pH
1	NARC	P1	Clay Loam	16.28	8.06
2	NARC	P2	Clay Loam	16.28	8.00
3	NARC	P3	Clay Loam	16.28	7.99
4	NARC	P4	Clay Loam	16.28	8.01
5	NARC	P5	Clay Loam	16.28	8.03
6	NARC	CP1	Clay Loam	19.8	7.99
7	NARC	CP2	Clay Loam	19.8	7.93
8	NARC	CP3	Clay Loam	19.8	7.89
9	NARC	CP4	Clay Loam	19.8	8.00
10	NARC	CP5	Clay Loam	19.8	7.90
11	NARC	L1	Clay Loam	16.8	7.79
12	NARC	L2	Clay Loam	16.8	7.77
13	NARC	L3	Clay Loam	16.8	7.81
14	NARC	L4	Clay Loam	16.8	7.78
15	NARC	L5	Clay Loam	16.8	7.80

P= Pea
CP= Chickpea
L=Lentil

MICROBIAL POPULATION OF SELECTED SOIL SAMPLES

Enumeration results of total fungal and bacterial population and fraction of phosphate solubilizing fungal and bacterial population of each plant i.e pea, chickpea and lentil are shown in Table. 2, 3 and 4 respectively. PSM were identified due to halozone (clear zone) formation around their colonies from non PSM. The highest count of PSB

($2.8x10^6g^{-1}$ of soil) was recorded in case of lentil while PSB were absent in case of pea.The PSF counts were highest in case of again lentil ($10.8x10^4g^{-1}$ of soil) and lowest ($0.8x10^4$ g^{-1} of soil) in chickpea.

Table. 2: Population of total fungi and bacteria of pea plant and population of hosphorus solubilizing fungi (PSF) and phosphorus solubilizing bacteria (PSB) of elected soil samples

Each value is the mean (±SE) of 5 replicates

SITE NO.	Total Fungi 1x 10^4cfu/ gram of soil	Total Bacteria $1x10^6$ cfu/gram of soil	PSM Fungi $1x10^4$ cfu/gram of soil	PSM Bacteria 1x 10^6 cfu/gram of soil
P1	24.8000 ± 0.59	17.0000 ± 0.28	2.0000 ± 0.0	NIL
P2	25.0000 ± 0.79	16.6000 ± 0.60	2.0000 ± 0.0	NIL
P3	24.0000 ± 1.07	17.6000 ± 0.72	1.8000 ± 0.33	NIL
P4	24.4000 ± 0.83	17.4000 ± 0.45	2.0000 ± 0.0	NIL
P5	24.6000 ± 0.82	17.8000 ± 0.59	1.8000 ± 0.33	NIL

Table. 3: Population of total fungi and bacteria of chickpea plant and population of phosphorus solubilizing fungi (PSF) and phosphorus solubilizing bacteria (PSB) of selected soil samples.

SITE NO.	Total Fungi $1x10^4$ cfu/ gram of soil	Total Bacteria $1x10^6$ cfu/gram of soil	PSM Fungi $1x10^4$ cfu/gram of soil	PSM Bacteria $1x10^6$ cfu/gram of soil
CP1	14.0000 ± 0.63	3.8000 ± 0.33	1.0000 ± 0.0	1.0000 ± 0.0
CP2	13.0000 ± 0.49	3.4000 ± 0.45	1.0000 ± 0.0	0.8000 ± 0.38
CP3	12.6000 ± 0.83	2.8000 ± 0.33	1.0000 ± 0.0	1.0000 ± 0.0
CP4	13.8000 ± 0.33	2.8000 ± 0.52	1.0000 ± 0.0	0.8000 ± 0.38
CP5	13.6000 ± 0.72	3.4000 ± 0.45	1.0000 ± 0.0	1.0000 ± 0.0

Each value is the mean (±SE) of 5 replicates

Table. 4: Population of total fungi and bacteria of lentil plant and population of phosphorus solubilizing fungi (PSF) and phosphorus solubilizing bacteria (PSB) of selected soil samples.

SITE NO.	Total Fungi 1x 10^4 cfu/gram of soil	Total Bacteria 1x 10^6 cfu/gram of soil	PSM Fungi 1x 10^4 cfu/gram of soil	PSM Bacteria 1x 10^6 cfu/gram of soil
L1	18.2000 ± 0.52	10.8000 ± 0.44	4.6000 ± 0.22	2.8000 ± 0.18
L2	19.6000 ± 0.45	10.4000 ± 0.45	4.6000 ± 0.22	2.4000 ± 0.22
L3	18.4000 ± 0.92	10.4000 ± 0.53	4.4000 ± 0.36	2.6000 ± 0.22
L4	17.6000 ± 0.67	10.4000 ± 0.45	4.4000 ± 0.36	2.6000 ± 0.22
L5	18.6000 ± 0.87	10.2000 ± 0.59	4.4000 ± 0.36	2.6000 ± 0.22

Each value is the mean (± SE) of 5 replicates

MORPHOLOGICAL FEATURES OF PHOSPHORUS SOLUBILIZING BACTERIA (PSB)

Total 4 PSB were isolated from the plants i.e pea, chickpea and lentil. 3 PSB were isolated from lentils and 1PSB from chickpea while PSB were absent in case of pea. These PSB were further studied for their morphological features e.g colony shape, elevation, texture, color and margins as shown in the Table.5a. All these features were analyzed in stereo microscope. Bacterial strains and their shapes were identified by Gram staining. All PSB were Gram positive and three out of four PSB were cocci in shape , one was bacillus (Table. 5b.)

Table 5a: Colony morphology of PSB with the help of stereo microscope.

COLONY	COLOR	TEXTURE	SHAPE	ELEVATION	MARGIN
LB1	DirtyYellow (blackish)	Granular	Irregular	Raised	Curled
LB2	Yellow	Smooth	Circular	Flat	Entire
LB3	Maronish Brown	Smooth	Irregular	Flat	Serrate
CPB1	White	Smooth	Irregular	Flat	Lobate

LB= Lentil Bacteria
CPB= Chickpea Bacteria

Table 5b: Gram's staining and cell morphology of PSB.

STRAIN NO.	GRAM STAINING	SHAPE
LB1	Gram Positive	Cocci
LB2	Gram Positive	Cocci
LB3	Gram Positive	Bacillus
CPB1	Gram Positive	Cocci

IDENTIFICATION OF PHOSPHORUS SOLUBILIZING FUNGI (PSF)

The isolated phosphate solubilizing fungi (PSF) were isolated from the mixed microbial culture and were identified on Czepack's medium and then under microscope preparation showed that majority of the isolates PF1, LF2, LF4 and CPF1 belonged to the Aspergillus genus while PF2 belonged to Penicillium genus, LF3 from Mucor, LF5 from Geotrichum

genus. All these PSF isolates showed different colors as shown in the Table.6

Table 6: Identification of fungal strains (PSF) isolated from rhizospheric soil of pea, chickpea and lentil.

STRAIN NO.	COLONY COLOR	classification	MICROSCOPIC IDENTIFICATION
PF1	Black	Ascomycota	Aspergillus niger
PF2	Green	Ascomycota	Penicillium spp.
LF1	Yellow	Ascomycota	Epicoccum spp.
LF2	Golden yellow	Ascomycota	Aspergillus cydowi
LF3	Black	Zygomycota	Mucor spp.
LF4	Orangish green	Ascomycota	Aspergillus versicolor
LF5	Pink	Ascomycota	Geotrichum candidum
CPF1	Black	Ascomycota	Aspergillus niger

PF= Pea Fungus
LF= Lentil Fungus
CPF= Chickpea Fungus

COMPARISON OF PSM NUMBER IN THREE LEGUME PLANTS

If we compare the number and presence of the PSMs in the soil rhizosphere of the three legume plants i.e pea, chickpea and lentils it is obvious that lentils are dominated by PSMs as shown in Table.7.

Table 7: Comparison of PSM number isolated from pea, chickpea and lentil

No. of PSM isolated from pea plant	No. of PSM isolated from Chickpea plant	No. of PSM isolated from Lentils
2 PSF (PF1 and PF2) PSB absent	1 PSF (CPF1) 1 PSB (CPB1)	5 PSF(LF1, LF2, LF3, LF4 and LF5) 3 PSB (LB1, LB2 and LB3)

BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF PSM

PSM are known to produce a clear zone around their colony which is the identification character of them as shown in the figures 1,2,3,4,5,6,7,8,9,10,11,12,13 and 14 in which the PSMs are in mixed and in pure cultures. Every colony has its own unique appearance.

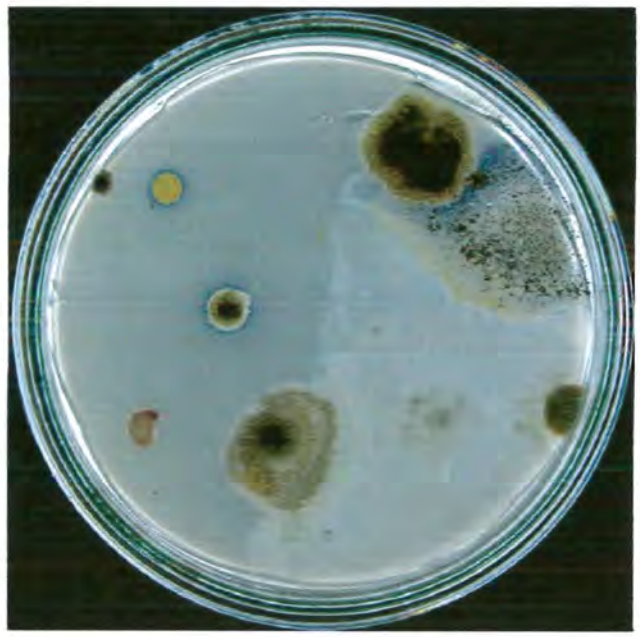

Fig.1:Halozone produced by solubilization of suspended tricalcium phosphate on Pikovskaya's agar medium by PSB (LB1 and LB2) and PSF (LF3).

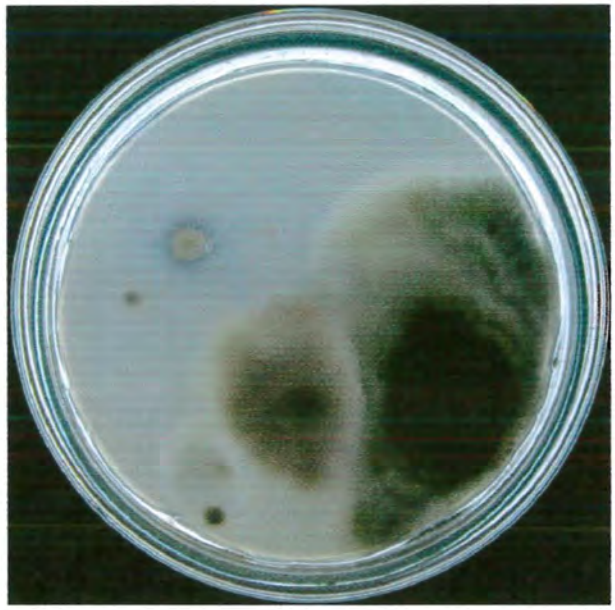

Fig.2: PSB (CPB1) solubilizing P and producing halozone in mixed culture

Fig.3: PSF (LF4) solubilizing P and producing halozone in mixed culture

Fig.4: P solubilization and halozone production by PSF in the mixed culture

Fig.5: P solubilization and halozone production by PSM in mixed culture

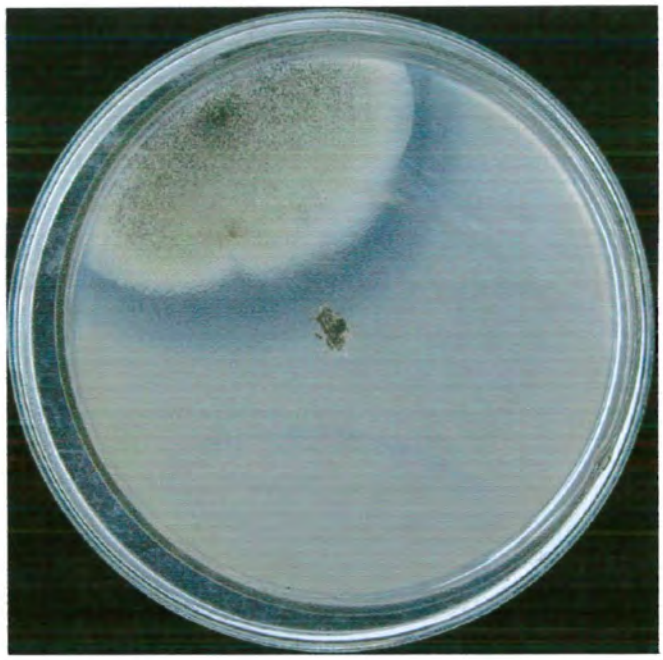

Fig.6: PSF (CPF1) solubilizing P and producing halozone

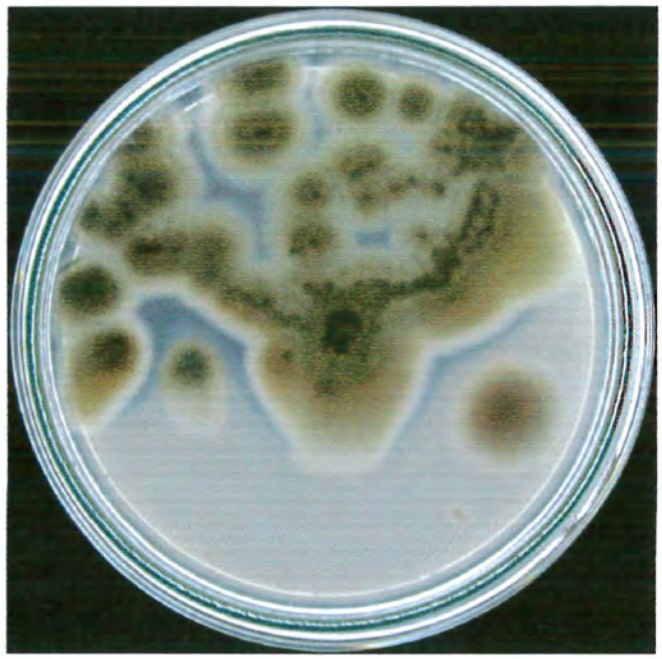

Fig.7: PSF (LF4) solubilizing P and producing halozone

Fig.8: PSF (CPF1) solubilizing P and producing halozone

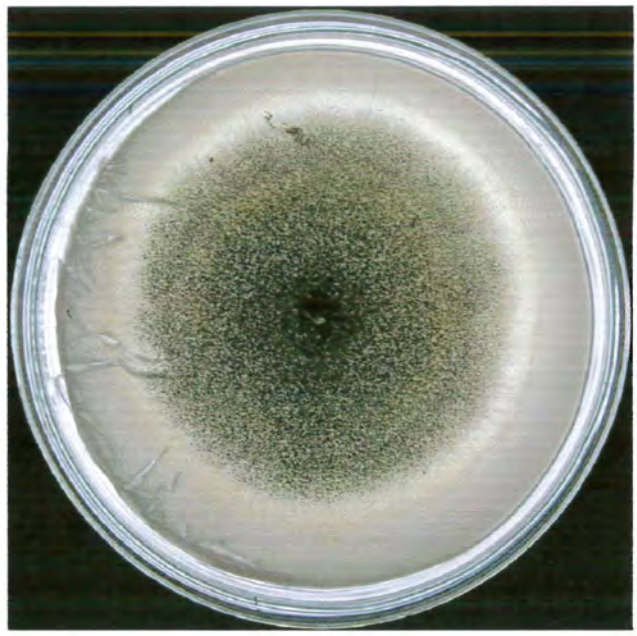

Fig.9 : PSF (LF3) solubilizing P and producing halozone

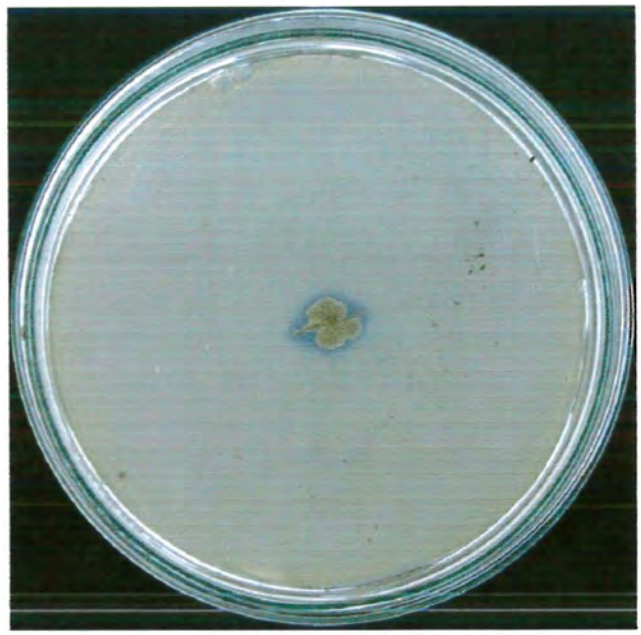

Fig.10: PSB (CPB1) solubilizing P and producing halozone

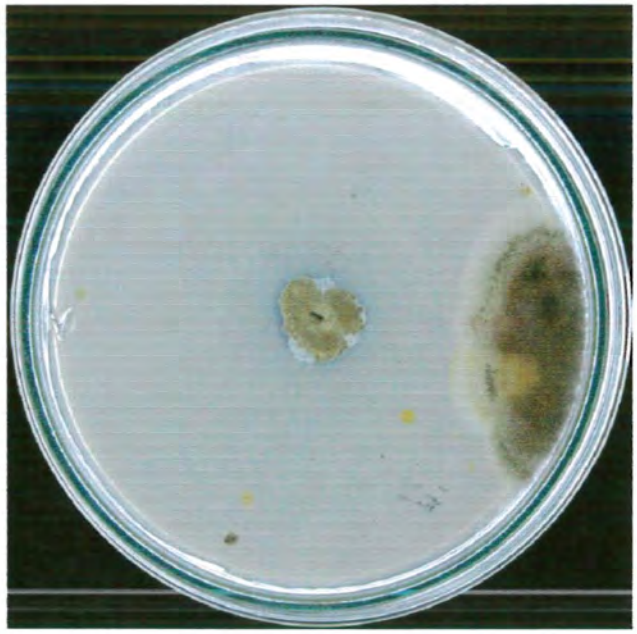

Fig.11: PSB (LB1) solubilizing P and producing halozone

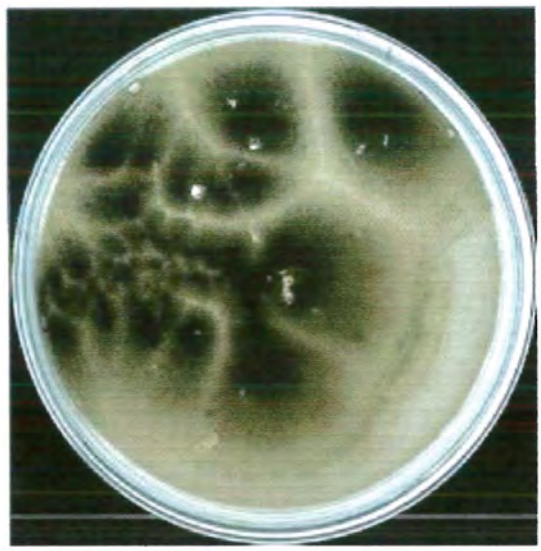

Fig.12: PSF (PF2) solubilizing P and producing halozone

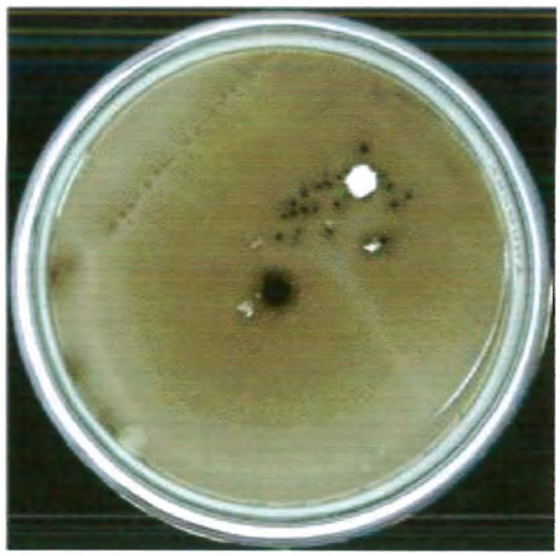

Fig.13 : PSF (LF2) solubilizing P and producing halozone

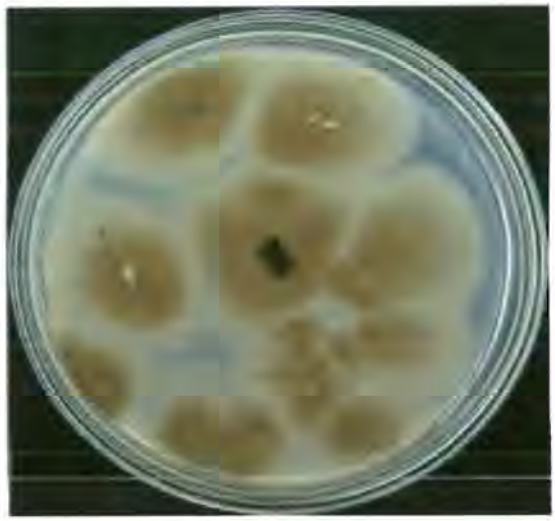

Fig.14: PSF (LF1) solubilizing P and producing halozone

Colony Diameter, Halozone Diameter and Solubilization Index

Colony diameter (c.d.), colony + halozone (z.d.) diameter and solubilization index (Si) of the isolated PSM's including PSF and PSB from the 3 legume plants were measured during seven days of incubation. It was observed that there was a steady increase in the colony, and halozone diameter during the incubation period. It was observed that fungal strains showed greater colony and halozone diameter while bacteria were showing little increase so, the colony diameter range of fungal strains was larger than the range of bacterial strains. The largest colony diameter was of LF1 (6.7) and lowest of LF5 (1.55) in case of phosphate solubilizing fungi as shown in the Table.12 (in appendix). PSB colony diameter ranged from 1.10(LB1) to 0.45 (LB3) as shown in the Table 13 (in appendix). The highest solubility index in case of PSF was of PF1 and the solubilization index was 2.56 (table 8) while in case of PSB the highest solubility index value was in case of LB1 which was 2.36 and lowest was in case of LB3 which was 2.11 (table 9).The change or increase in colony diameter, colony + halozone diameter and the solubilization index is shown in the Figures15a, 15b, 16a, 16b, 17a and 17b.

Table 8: Solubility index (Si) of the PSF strains.

PSF STRAINS	SOLUBILITY INDEX (Si)
PF1	2.56
PF2	2.14
CPF1	2.55
LF1	2.21
LF2	2.18
LF3	2.52
LF4	2.37
LF5	2.19

Tabe 9: Solubility index (Si) of PSB strains

PSB STRAINS	SOLUBILITY INDEX (Si)
LB1	2.36
LB2	2.30
LB3	2.11
CPB1	2.35

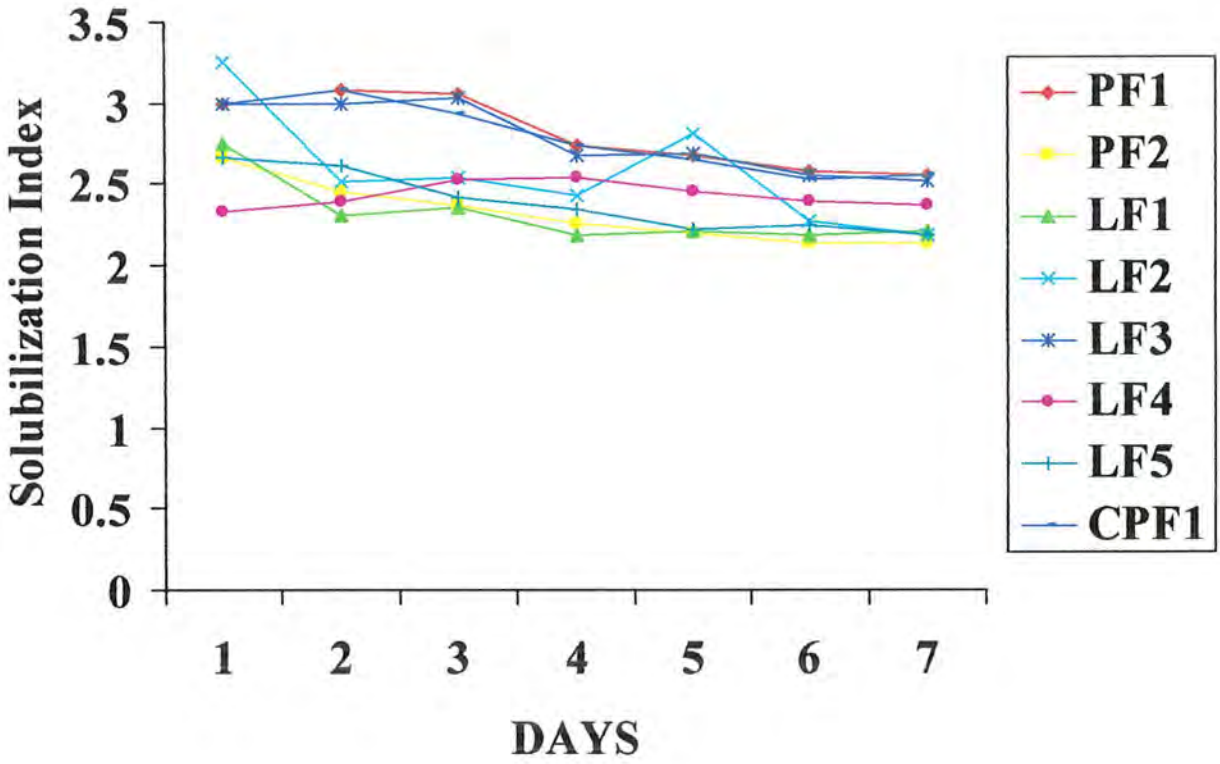

Fig.15a : Solubilization index of PSF strains during seven days
Each value is the mean of two replicates

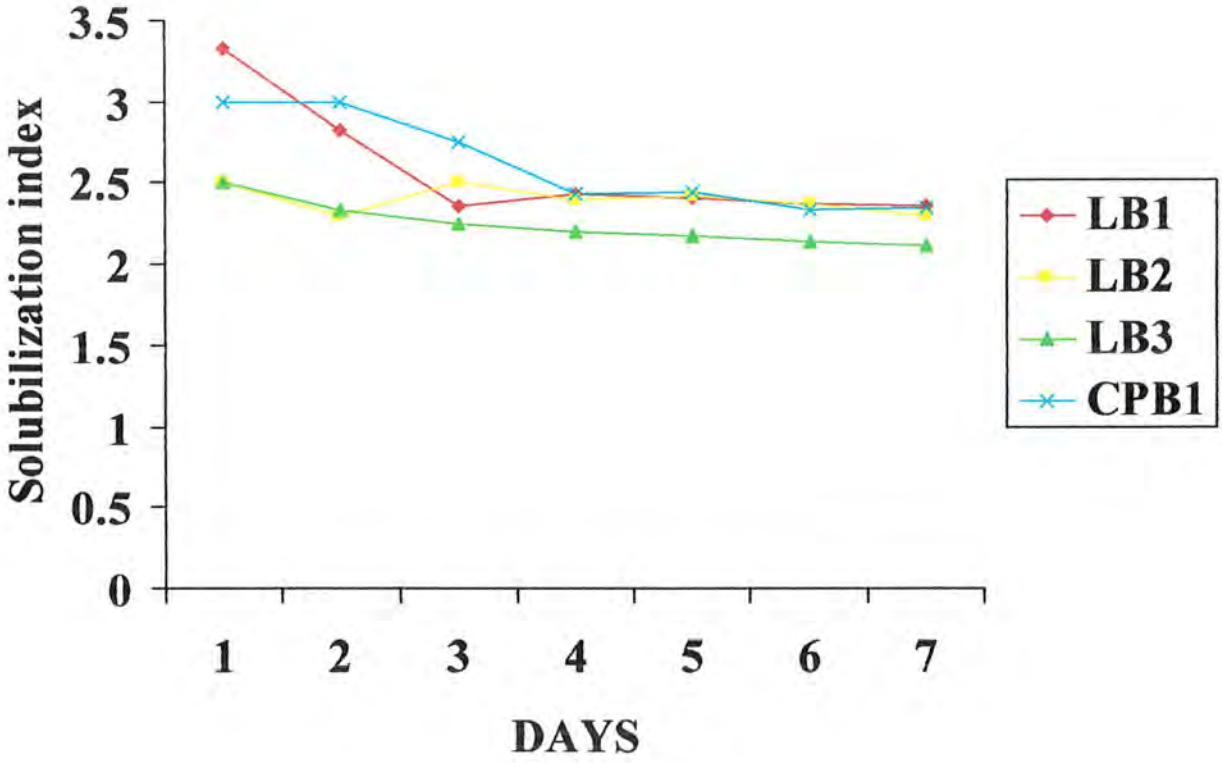

Fig.15b: Solubilization index of PSB strains during seven days
Each value is the mean of two replicates

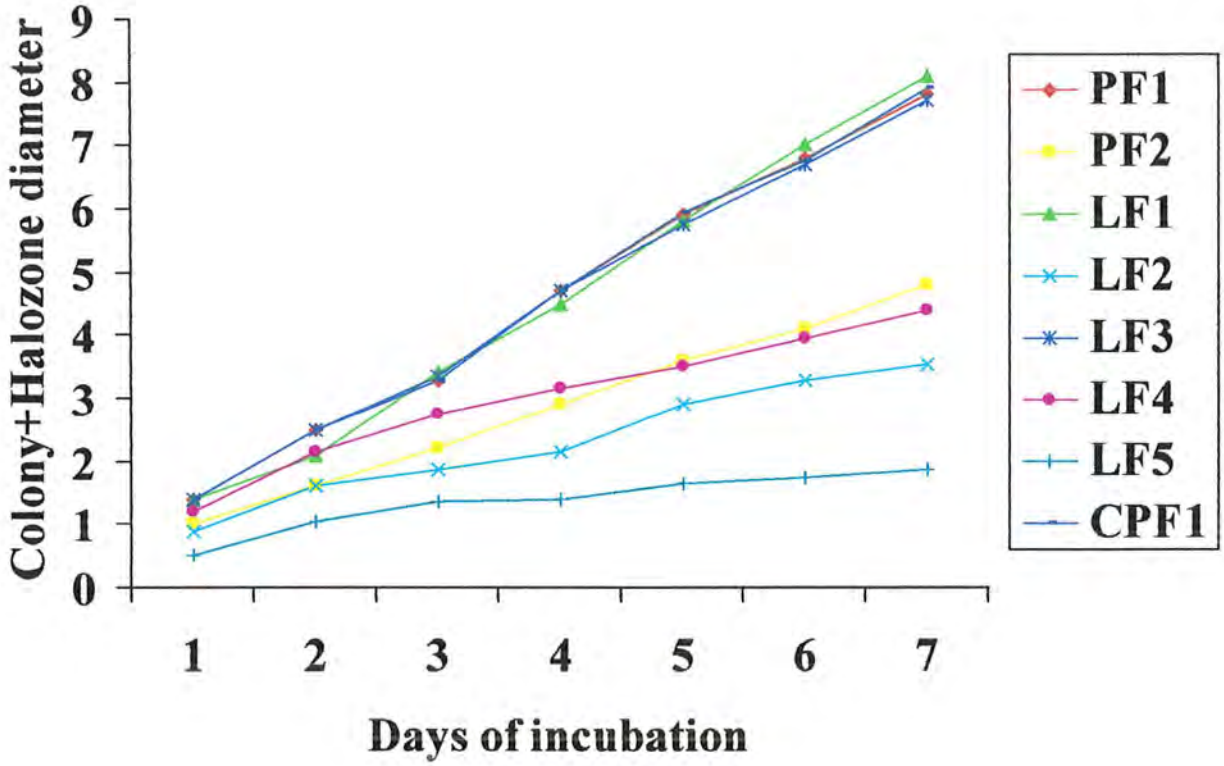

Fig.16a: Increase in colony+halozone diameter by PSB on solid Pikovskaya'smedium during seven days.
Each value is the mean of two replicates

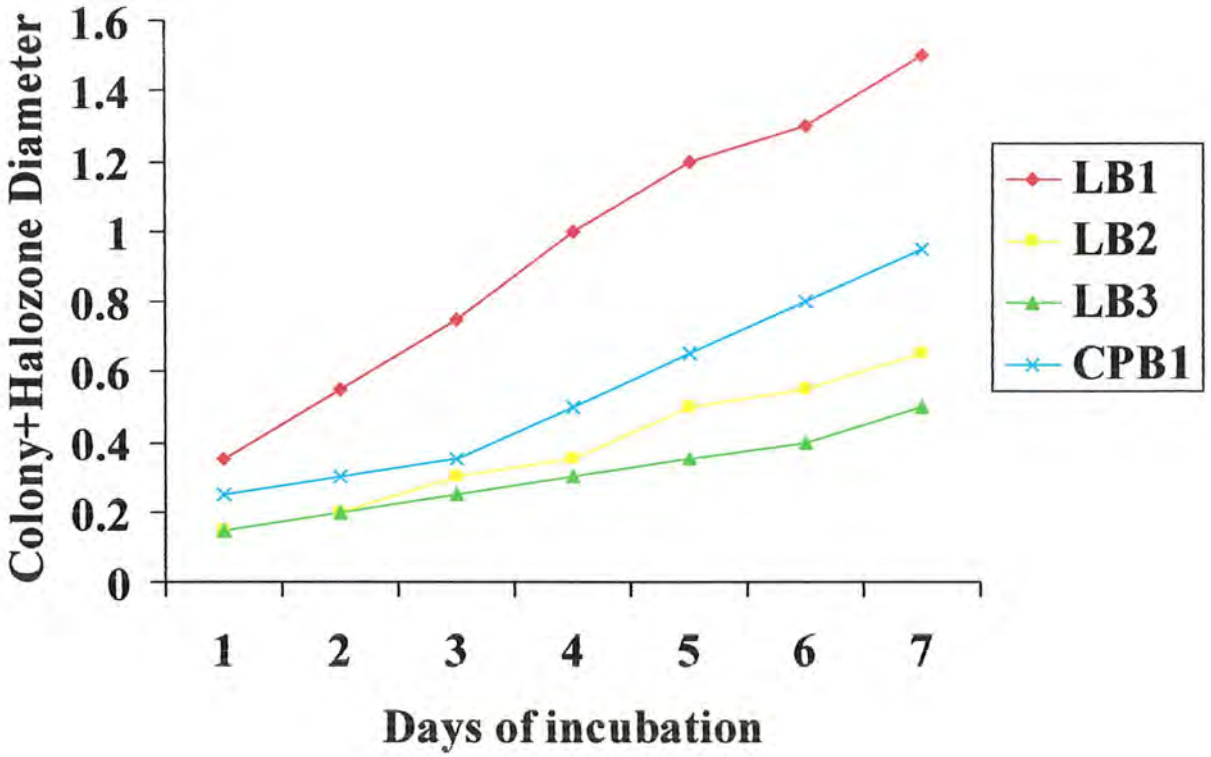

Fig.16b: Increase in colony+halozone diameter by PSB on solid Pikovskaya's medium during seven days.
Each value is the mean of two replicates

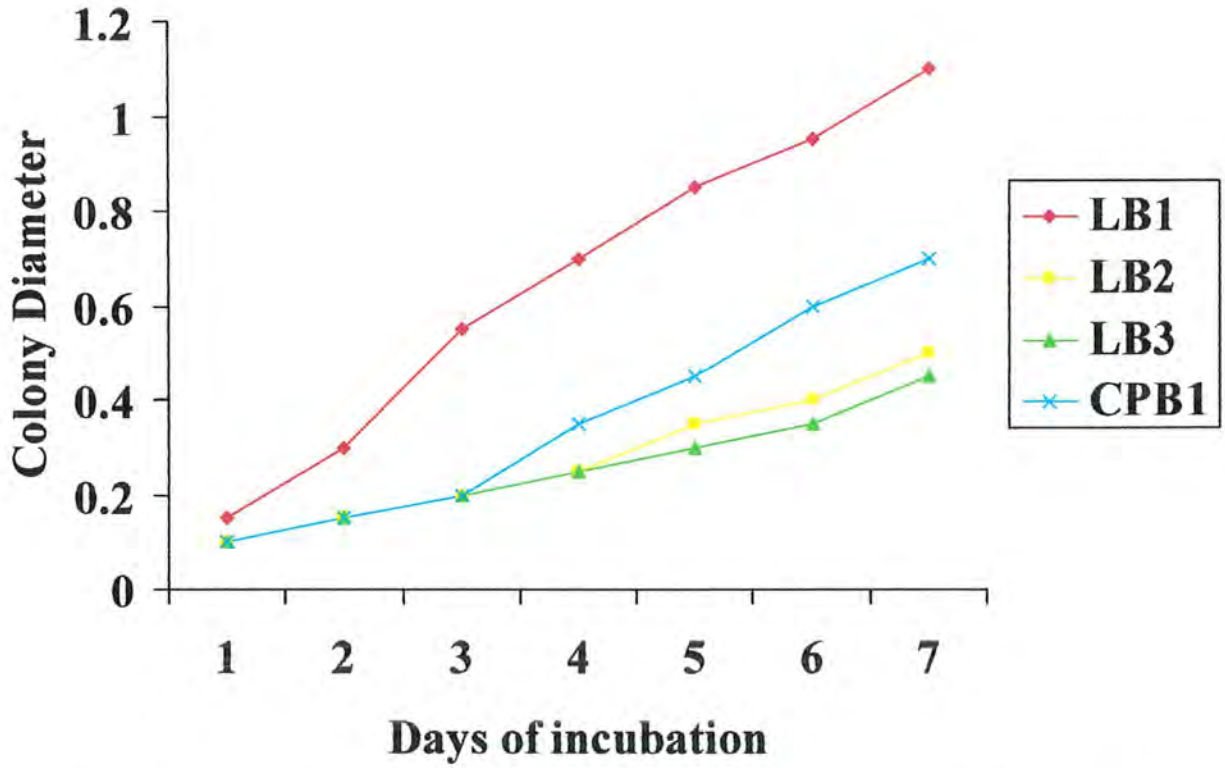

Fig.17a: Increase in colony diameter of PSB on solid Pikovskaya's medium during seven days of incubation
Each value is the mean of two replicates

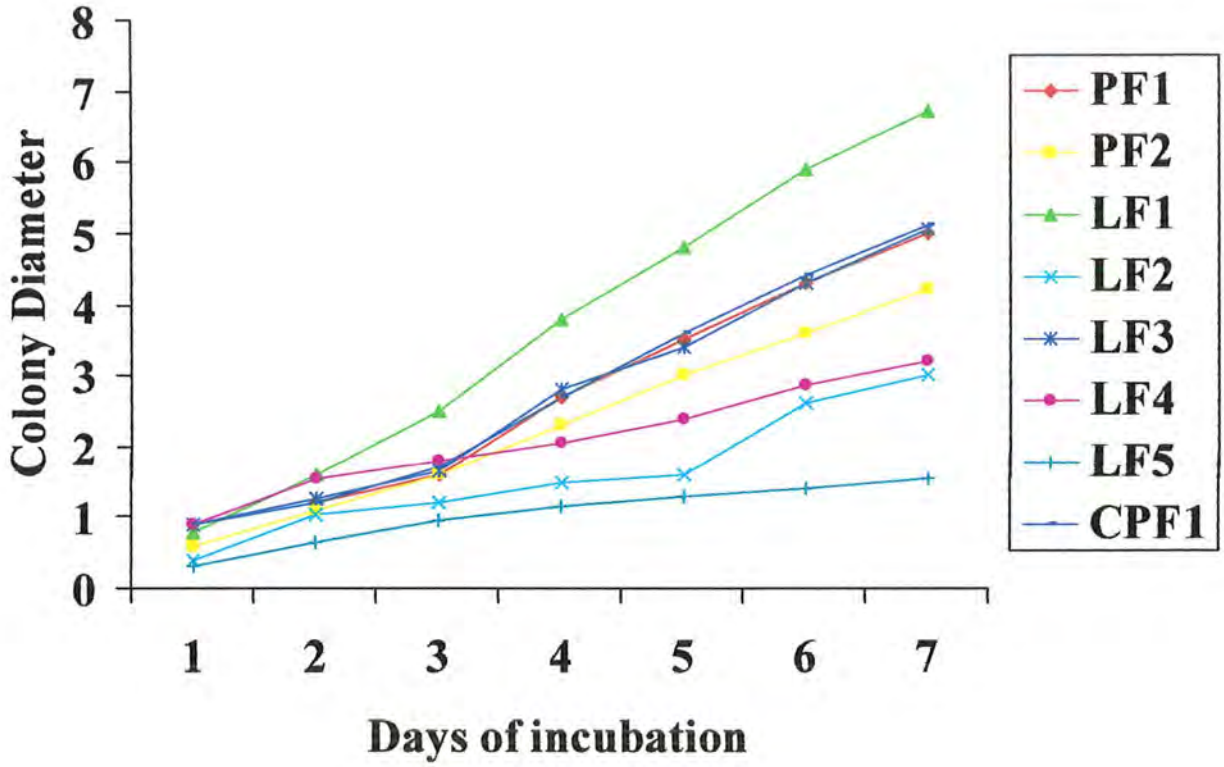

Fig.17b: Increase in colony diameter of PSF strains on solid Pikovskaya's medium during seven days
Each value is the mean of two replicates

pH DROP IN LIQUID CULTURES

PSM isolates were studied for their characteristic that they lower the pH of broth edium. Results showed that most of the phosphate solubilizing microorganisms lowered the pH of Pikovskaya broth medium as compared to control. Some PSM showing larger halozones did not lower the pH significantly. Fluctuations in pH drop were also noted during seven days of incubation as shown in the table.14 and 15 (in appendix) for PSF and PSB respectively. Lowest pH recorded in case of PSF was of CPF1 which was 4.44 while there was a little pH drop in case of LF5 which was 6.30 as shown in the Table.10. In PSB the pH ranged from 5.60 (LB2) to 6.50 (CPB1) as shown in the Table.11. Fungi were found to be more active in lowering the pH of the broth cultures as compared to bacteria as shown in the Fig.18a and 18b.

Table 10: pH Drop of PSF strains

PSF STRAINS	pH DROP
PF1	4.45
PF2	6.01
CPF1	4.44
LF1	5.61
LF2	5.38
LF3	4.45
LF4	5.25
LF5	6.30

Table 11: pH drop of PSB strains

PSB STRAINS	pH DROP
LB1	5.94
LB2	5.60
LB3	6.48
CPB1	6.50

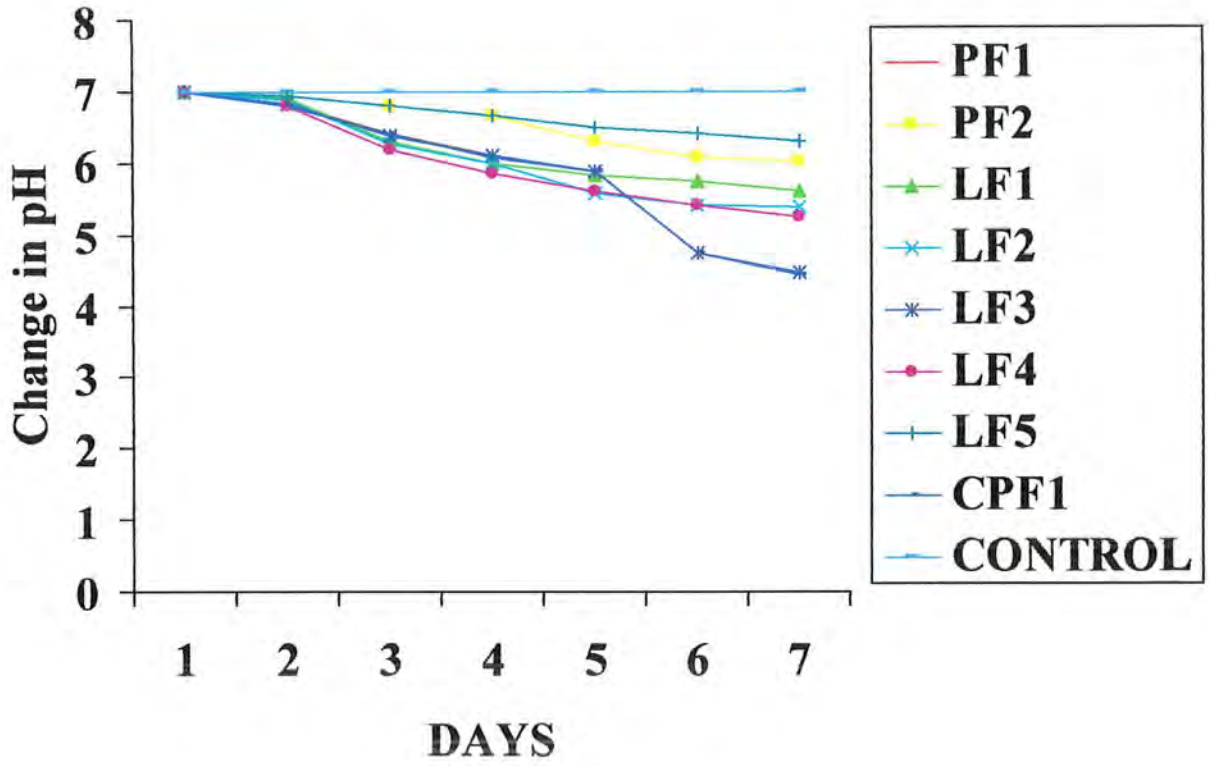

Fig.18a:Drop of pH of Pikovskaya's broth medium by phosphate solubilizing fungi (PSF)
Each value is the mean of two replicates

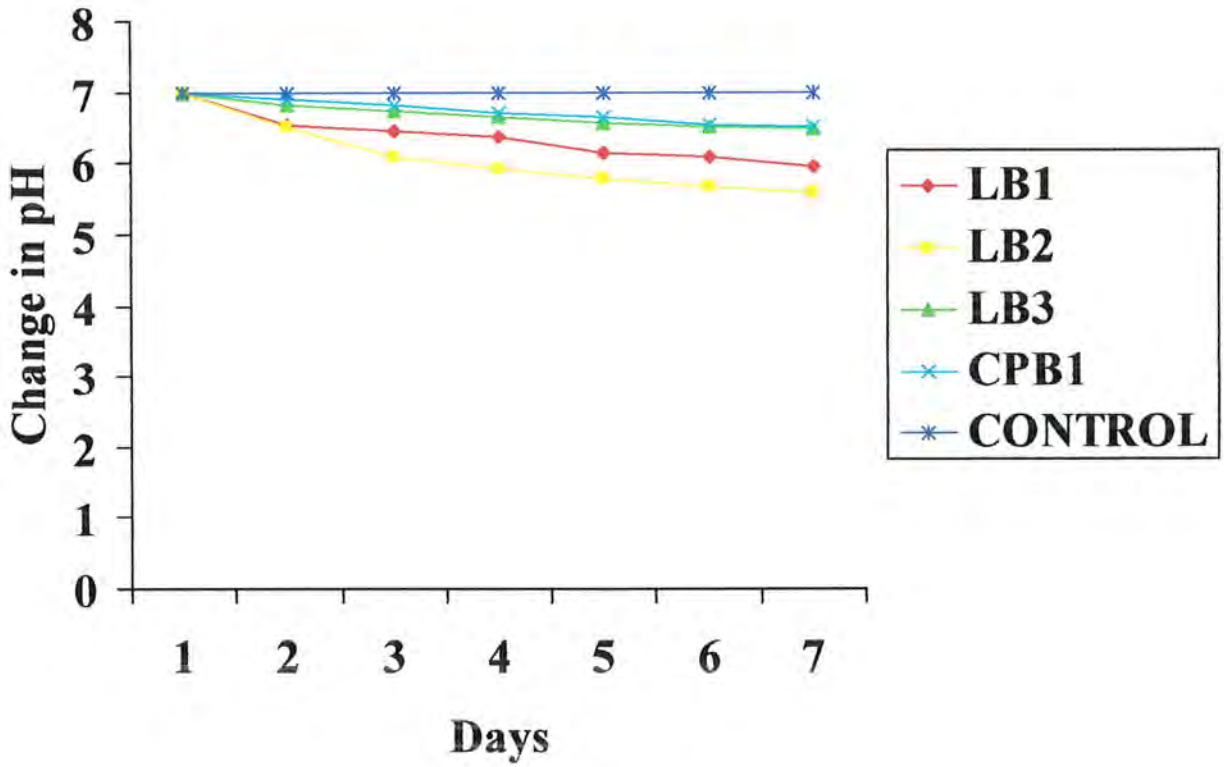

Fig.18b : Drop of pH of Pikovskaya's broth medium by phosphate solubilizing bacteria (PSB)
Each value is the mean of two replicates

THIN LAYER CHROMATOGRAPHY

The strains of PSB and PSF of the selected legume plants were checked for the presence and absence of organic acids by Thin Layer Chromatography. The chromatographic plate was analyzed under UV light. The chromatograph irradiated by UV light clearly shows the bands and very diminished spots produced by all the strains (PSB and PSF) of the plants as well as the mixture that was plotted along with the sample strains to work as the referrence. The mixture was of organic acids i.e Citric acid, oxalic acid, malic acid, acetic acid and lactic acid. While the control plotted on TLC showed no movement.The bands and spots provides an idea of the presence of organic acids produced in the broth medium of the PSB and PSF sample. Figures 19, 20 and 21 shows the bands that are produced by the PSM strains isolated from pea, chickpea and lentil plant.

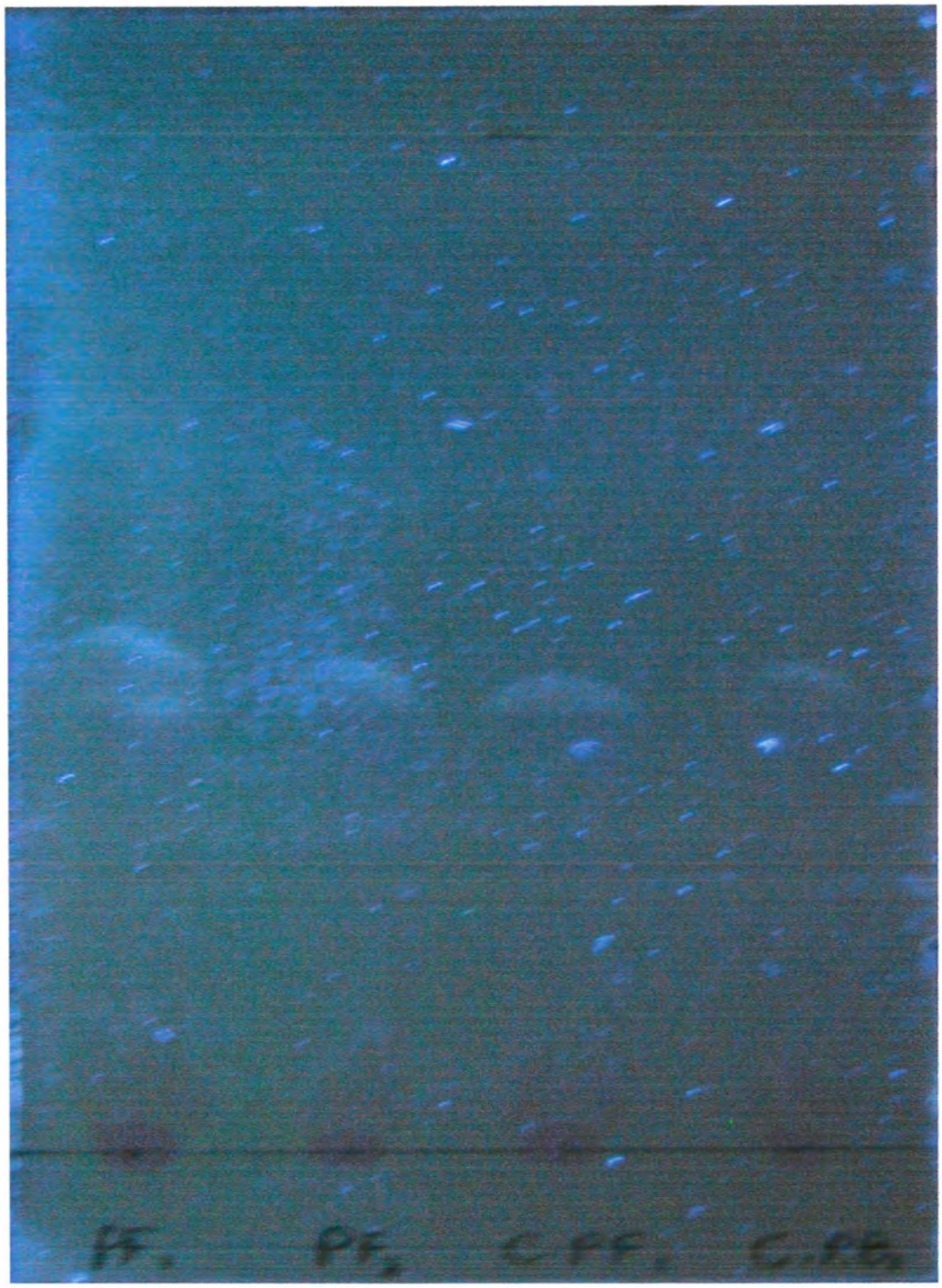

Fig. 19: TLC plate of strains PF1, PF2, CPF1 and CPB1 showing bands Produced by organic acids movement.

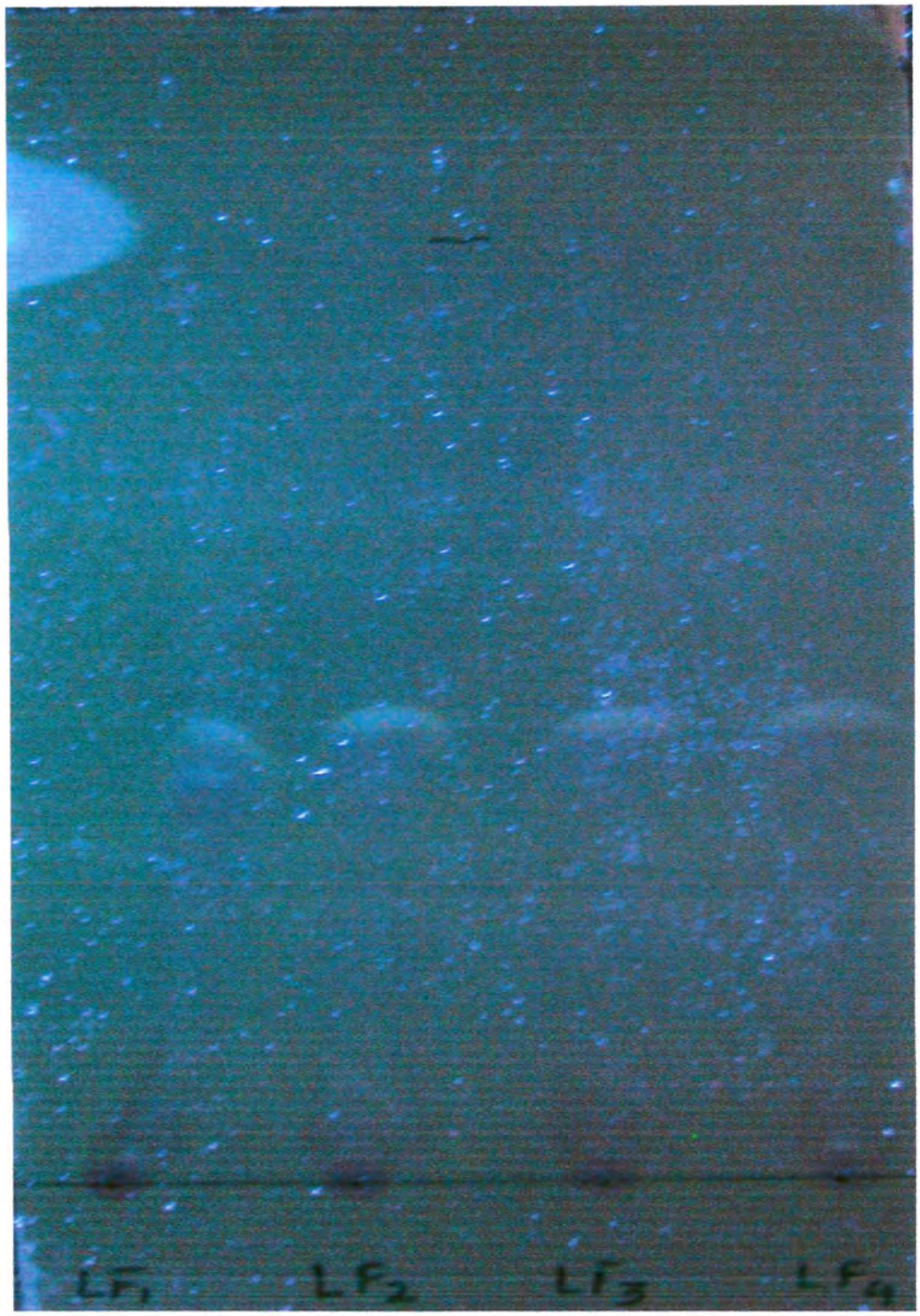

Fig. 20: TLC plate of strains LF1, LF2, LF3 and LF4 showing bands bands produced by organic acids movement.

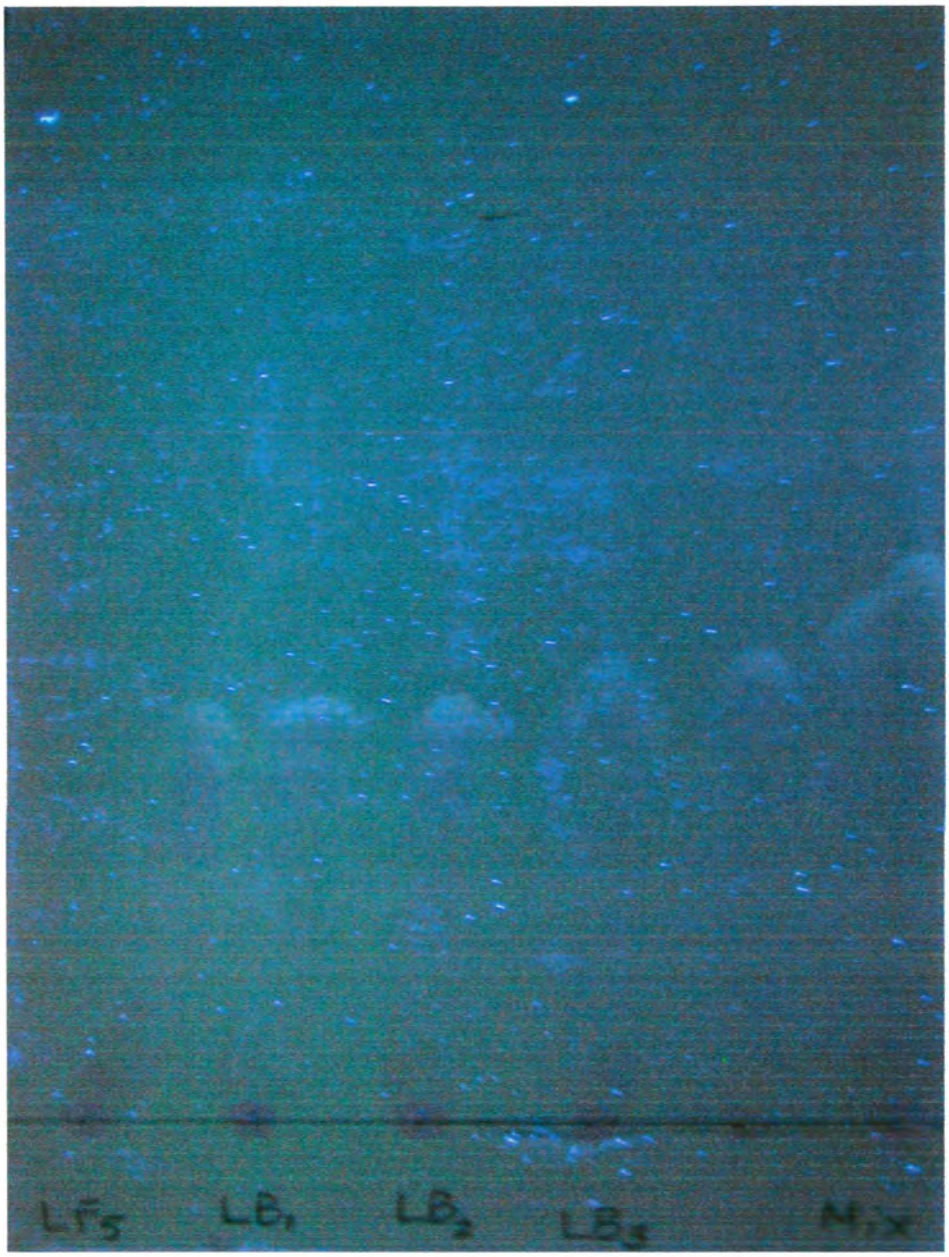

Fig. 21: TLC plate of the strains LF5, LB1, LB2,LB3 and Mixture (of the organic acids used as reference) showing bands produced by organic acids movement.

DISCUSSIONS

DISCUSSIONS

The physical and chemical soil properties of the selected legume plants i.e soil moisture and soil pH varied to some extent from plant to plant (Pea, chickpea and lentil) during present study. These soil properties indicate that all the fields were alkalinewithout chemical fertilization but the soil texture was the same or uniform e.g clay loam. Brady, (1990) stated that phosphorus fixation tends to be more pronounced in fine textured soils than in coarse textured ones. Average phosphorus fixation is 71% in clayey, 62% in clay loam, 56% in loam and 29% in loamy sand soils. Phosphorus availability is low in acidic soils because of formation of iron and aluminium phosphate while in alkaline soil tricalcium phosphate [$Ca_3(PO_4)_3$] forms readily to reduce availability of soil phosphorus to plants (Donahue et al., 1971; Stevenson, 1986; Whitelaw, 2000). This process is called as phosphorus fixation. So alkalinity suggests the unavailability of the phosphorus to the plant. There was great variation for the microbial distribution in rhizospheric soil of the three legume plants during present study. This observation is similar to the Brady, (1990) who concluded that physical and chemical properties of soil influence the distribution of the microbial population in the soil. Nahas et al. (1994) also reported that physical and chemical properties of soils influence microbial population of soils. During present study the phosphate solubilizing microorganisms were recognized by the halozone formation as it is known that PSM are characterized by the transparent zones of clearance (halozone) around their colonies (Nautiyal, 1999; Kumar and Narula, 1999) generally on Pikovskaya media (Pikovskaya, 1948). PSM have important role in the solubilization of insoluble phosphorus sources (Singh and Kapoor, 1999; Roy et al., 1999; Richardson, 2001; Fallah, 2006; Khan et al., 2007). Enumeration studies showed that PSM constitute very minute % of the total microbial population associated with rhizosphere of different legume plants. PSB and PSF constituted different % as compared to one another. This difference may be due to different soil conditions. As different plants have differential capabilities to exude chemicals, they regulate microbial population of rhizosphere (Alexander, 1977).

Considering the types of microorganisms, from the rhizospere of pea plant two fungal strains were isolated i.e Aspergillus niger (PF1) and Penicillium spp. (PF2) while bacterial strains were absent. From chickpea plant, one fungal strain was isolated i.e Aspergillus niger (CPF1) and one bacterial strain (CPB1). Whereas from lentils five

fungal strains were isolated i.e Epicoccum spp.(LF1), Aspergillus cydowi (LF2), Mucor (LF3), Aspergillus versicolor(LF4) and Geotrichum candidum(LF5). 3 bacterial strains e.g LB1, LB2 and LB3 were also isolated from the rhizosphere of lentils. This difference in the number and distribution was may be due to the same effect that plants having differential capabilities to exude chemicals e.g there are different types of substances that diffuse from the roots and that stimulate the microbial activity, such as carbohydrates (sugars and oligo-saccharides), organic acids, vitamins, nucleotides, flavonoids, enzymes, hormones, and volatile compounds (Prescott et al., 1999).

Analysis of results reveal that though having different species, Aspergillus was the common genus in the rhizosphere of the three plants studied. Barroso and Nahas, (2005) reported soil isolate of the fungus Aspergillus niger showed high ability to solubilize both calcium and aluminum phosphates in culture medium. Aspergillus and Penicillium are important P solubilizing fungal genera (Motsara et al., 1995). So the results were in accordance with Cruz et al. (1990), Narsian et al. (1995,1993) and Rao, (1999) as well that also suggest that Aspergillus and Penicillium are the most common genera.PSB showed different morphological characters and Gram staining on Pikovskaya's agar medium. Similar results were observed by Urban, (1977), Pal, (1998) and Kumar and Narula, (1999) on different media. In case of PSB it was observed that all are Gram positive and cocci were dominant as out of four PSB strain only one was Bacillus. While Motsara et al. (1995) suggests that mostly phosphorus solubilizing bacteria are 1.1 to 2.2 micrometer in cell size and are rod shaped and few are cocci and spirillum.Results from the present study of the legume plants suggest that the fungal strains are dominant over bacteria in their presence as well as in their solubilization. This observation is in accordance with Rajankar et al. (2007) who observed that the fungi have more solubilizing ability of inorganic insoluble phosphate than bacteria.So as a whole, lentils were supposed to be dominant in phosphorus solubilization due to the presence of higher number of fungal as well as bacterial strains having phosphorus solubilizing capability. But considering plants other than legumes in the present study, it was observed that higher number of bacterial and fungal strains were isolated by the researchers e.g Rashid, (2001) and Alam, (2001) in the rice, maize and wheat. This difference may be due to the fact that in leguminous plants the nitrogen fixing microorganisms are present that are

called rhizobium and these are capable of solubilizing phosphorus as well so due to their presence the PSMs are restricted. Also there might be a negative interaction (amensalism) between the two kinds of microorganisms i.e rhizobium and PSMs. By the production of toxins and antibiotics they regulate each other presence or there might be a competition for space, food etc between these microorganisms (Alexander, 1977).

Studies on agar plates revealed that phosphate solubilizing microorganisms formed clear zones by solubilizing suspended tricalcim phosphate. Halozone measurements ranged from 1.85-8.1 cm in case of fungi and 0.50-1.50 cm in case of bacteria of the legume plants while the solubilization index ranged from 2.14-2.56 cm in case of fungal strains and 2.11-2.36 cm in case of bacterial strains of the legumes. Similar results were found by Kucey, (1983, 1987), Edi-Premono et al. (1996) and Kumar and Narula, (1999). Most of the PSM strains lost their ability to form halozone on Pikovskaya's agar medium on subculturing. This result is in accordance to Kucey, (1983, 1989), Alam, (2001), Rashid, (2001) and Illmer and Schinner, (1992). LB2, PF2, LB3 and LF5 have shown such character.In all the legume plants studied during present study, phosphate solubilizing fungi formed larger halozones as compared to bacteria. These results are in accordance to Chabot et al. (1993), Alam, (2001), Rashid, (2001) and Nahas, (1996).pH studies showed a drop of pH from 7-4.44(CPF1) in the fungal strains of the legume plants and 7-5.60 (LB2) in case of legume's bacterial strain. Similar results were observed by Alam, (2001), Rashid, (2001), Bar-Yosef, (1999), Illmer and Schinner, (1995) and Khalil and Sultan, (2000). So the pH change infact, the drop in the pH clearly indicates the production of the acids that have lowered the pH and changed the alkalinity of the broth into acidity similar results were found by different researchers e.g Song et al. (2008) and Turan et al. (2006).Danahue et al. (1971), Kim et al. (1997), Whitelaw et al. (1999), Illmer and Schinner, (1992) and Kim et al. (1997) reported that phosphate solubilization is more efficient by the production of organic acids as compared to inorganic acids. So keeping these views under consideration, organic acid production was detected by TLC for the legume plants i.e pea, chickpea and lentil. The purpose was to find out whether all the three plants are able to produce acids or not.As the acidity was found out in the broth, Thin Layer Chromatography was done to find out the acid production. As it is known that organic acids are produced more commonly in the broth medium of PSMs so the TLC of

the PSF and PSB strains for the production of organic acids was done that demonstrated the presence of organic acids though the acids type and quantity was not found out. The purpose of the TLC was to detect the presence of organic acids only. The plate irradiated by UV light provided the idea of the presence of organic acids. This result of production of organic acids is in accordance to the results of the previous findings of Gupta et al. (1994), Singal et al. (1994), Illmer et al. (1995), Vassilev et al. (1995, 1996) Mehta et al. (1996), Whitelaw et al. (1999), Maliha et al. (2004) and Fankem et al. (2006) that not only detected the presence but also the type of organic acids produced. The results suggests that in legume plants the production of organic acids by PSB and PSF is the reason of phosphate solubilization though other reasons may also be responsible but organic acid production is one of the reasons. .As Illmer et al. (1995) observed that organic acid production may be helpful but not the sole need for phosphate solubilization.The results clearly provides the view that the basic principle of the phosphate solubilization by PSMs i.e halozone formation, pH drop, production of acids is similar in different plants studied.Thus the findings of the present study can be used to know the indeginous phosphate solubilizing microorganisms of the legumes studied e.g pea, chickpea and lentils. This would in turn help to produce inoculum that can be used practically in increasing the growth of the plants which in turn will provide us more grain yield by using PSM. Enhanced phosphorus uptake by PSM can replace synthetic fertilizers used commercially which are very expensive. Still more research is needed in this field such as exact relationship between PSM and Nitrogen fixers and effect of PSM along with mycorrhiza and nitrogen fixing microorganisms on growth of plants may also be studied.

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APPENDICES

APPENDIX - 1

CHEMICAL COMPOSITION OF PIKOVSKAYA'S MEDIUM

Glucose	10g
TriCalcium Phosphate	2.5g
Ammonium Sulphate ($(NH4)_2SO_4$)	0.5g
Sodium Chloride (NaCl)	0.2g
Magnesium Sulphate ($MgSO_4 .7H_2O$)	0.1g
Pottasium Chloride (KCl)	0.2g
Yeast Extract	0.5g
Manganeese Sulphate ($MnSO_4$)	trace
$FeSO_4 . 7H_2O$	trace
Agar	15g
Distilled Water	1000ml
pH	Adjusted to 7.0 ± 0.2

All above mentioned chemicals are added into 1000ml distilled water except TriCalcium Phosphate whose suspension is made separately

PREPRATION OF TRICALCIUM PHOSPHATE SUSPENSION

Stock suspension of 2.5 percent tricalcium Phosphate is prepared in 0.5 percent Gum Arabic solution and pH is adjusted to 8.4 and is sterlized.

APPENDIX - 2

CZAPEK AGAR CZ MEDIA

Stock solution A :

Sodium Nitrate	40g
Potassium Chloride	10g
Magnesium Sulphate	10g
Ferrous Sulphate	0.2g

Dissolve in 1 litre distilled water and store in refrigerator

Stock solution B :

Dissolve 20g of K_2HPO_4 in 1 litre distilled water and store in refrigerator to each mixed litre of medium then add 1ml of both a & b.

a) Zinc sulphate 1g in 100ml

b) Copper sulphate 0.5g in 100ml water

Autoclave for 20 minutes at 121°C at 15 Ilb

Table. 12:Colony(cm), Colony+Halozone(cm) and Solubilization Index of PSF of three legume plants determined on Pikovskaya's solid media.

Day 1	PF1	PF2	LF1	LF2	LF3	LF4	LF5	CPF1
c.d	0.9	0.6	0.8	0.4	0.9	0.9	0.30	0.9
z.d	1.4	1.00	1.4	0.9	1.4	1.20	0.50	1.4
Si	3.00	2.66	2.75	3.25	3.00	2.33	2.67	3.00
Day 2								
c.d	1.2	1.1	1.6	1.05	1.25	1.55	0.65	1.2
z.d	2.5	1.6	2.1	1.60	2.50	2.15	1.05	2.5
Si	3.08	2.45	2.31	2.52	3.00	2.39	2.61	3.08
Day 3								
c.d	1.6	1.6	2.5	1.20	1.65	1.80	0.95	1.7
z.d	3.3	2.2	3.4	1.85	3.35	2.75	1.35	3.30
Si	3.06	2.37	2.36	2.54	3.03	2.53	2.42	2.94
Day 4								
c.d	2.7	2.3	3.8	1.50	2.80	2.05	1.15	2.70
z.d	4.7	2.9	4.5	2.15	4.70	3.15	1.40	4.70
Si	2.74	2.26	2.18	2.43	2.68	2.54	2.35	2.74
Day 5								
c.d	3.5	3.00	4.8	1.60	3.40	2.40	1.30	3.6
z.d	5.9	3.6	5.8	2.90	5.75	3.50	1.65	5.95
Si	2.68	2.2	2.21	2.81	2.69	2.46	2.22	2.65
Day 6								
c.d	4.30	3.6	5.9	2.60	4.30	2.85	1.40	4.40
z.d	6.8	4.1	7.00	3.30	6.70	3.95	1.75	6.75
Si	2.58	2.14	2.19	2.27	2.56	2.40	2.25	2.53
Day 7								
c.d	5.00	4.2	6.7	3.00	5.05	3.2	1.55	5.10
z.d	7.8	4.8	8.1	3.55	7.7	4.40	1.85	7.90
Si	2.56	2.14	2.21	2.18	2.52	2.37	2.19	2.55

Si = Solubility index
c.d = Colony Diameter
z.d = Colony + Halozone Diameter

Table. 13: Colony(cm), Colony+Halozone(cm) and Solubilization Index of PSB of three legume plants determined on Pikovskaya's solid media during seven days of incubation.

Day1	LB1	LB2	LB3	CPB1
c.d	0.15	0.1	0.1	0.10
z.d	0.35	0.15	0.15	0.25
Si	3.33	2.5	2.5	3.00
Day 2				
c.d	0.30	0.15	0.15	0.15
z.d	0.55	0.20	0.20	0.30
Si	2.83	2.3	2.33	3.00
Day 3				
c.d	0.55	0.20	0.20	0.2
z.d	0.75	0.30	0.25	0.35
Si	2.36	2.50	2.25	2.75
Day 4				
c.d	0.70	0.25	0.25	0.35
z.d	1.00	0.35	0.30	0.50
Si	2.43	2.40	2.20	2.43
Day5				
c.d	0.85	0.35	0.30	0.45
z.d	1.20	0.50	0.35	0.65
Si	2.41	2.42	2.17	2.44
Day 6				
c.d	0.95	0.40	0.35	0.60
z.d	1.30	0.55	0.40	0.80
Si	2.37	2.37	2.14	2.33
Day 7				
c.d	1.10	0.50	0.45	0.70
z.d	1.50	0.65	0.50	0.95
Si	2.36	2.30	2.11	2.35

Si = Solubility index
c.d = Colony Diameter
z.d = Colony + Halozone Diameter

Table 14: Change in pH caused by PSF in broth culture.

PSF CULTURE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
PF1	7.00	6.82	6.40	6.10	5.87	4.73	4.45
PF2	7.00	6.93	6.80	6.65	6.30	6.09	6.01
CPF1	7.00	6.81	6.41	6.09	5.87	4.74	4.44
LF1	7.00	6.90	6.30	6.00	5.83	5.75	5.61
LF2	7.00	6.89	6.26	5.99	5.58	5.42	5.38
LF3	7.00	6.83	6.39	6.11	5.87	4.73	4.45
LF4	7.00	6.80	6.20	5.85	5.63	5.43	5.25
LF5	7.00	6.95	6.80	6.65	6.50	6.41	6.30

Each value is the mean of two replicates

Table 15: Change in pH caused by PSF in broth culture.

PSB CULTURES	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
LB1	7.00	6.53	6.45	6.37	6.16	6.10	5.94
LB2	7.00	6.50	6.10	5.93	5.79	5.68	5.60
LB3	7.00	6.82	6.73	6.65	6.58	6.52	6.48
CPB1	7.00	6.90	6.81	6.70	6.65	6.55	6.50

Each value is the mean of two replicates