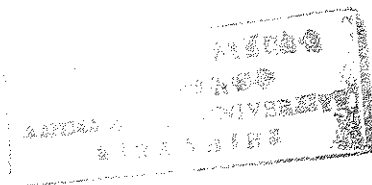


**ADDIS ABABA UNIVERSITY  
SCHOOL OF GRADUATE STUDIES**

**ISOLATION AND CHARACTERIZATION OF PHOSPHATE  
SOLUBILIZING BACTERIA FROM SOME ETHIOPIAN SOILS  
AND THEIR EFFECT ON THE GROWTH OF FABA BEAN  
(*Vicia faba* L.)**

**A thesis submitted  
School of Graduate studies  
of Addis Ababa University**

**In Partial Fulfillment of  
the Degree of Master of Science in Biology**

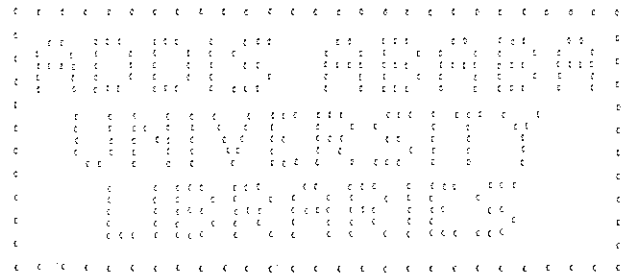


**WASSIE HAILE**

**APRIL, 1999**

**Dedicated To:**

My mother, W/o Achamyesh Haile  
and  
My uncle, Ato Getahun Haile



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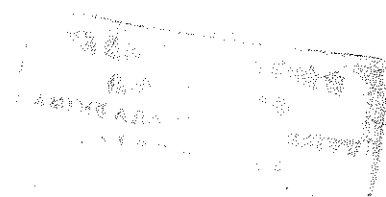
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## List of abbreviations

CEC	Cation exchange capacity
g	gram
masl	meters above sea level
me	milli-equivalent
mg	milli-gram
ml	milli-liter
N <sub>tot</sub>	Total nitrogen
OB	Old bone
OM	Organic matter
P <sub>ava</sub>	Available phosphorus
PPM	Parts per million
P <sub>tot</sub>	Total phosphorus
PR	Rock phosphate
PSB	Phosphate solubilizing bacteria
PSM	Phosphate solubilizing microorganisms
TCP	Tricalcium phosphate
TSP	Triple super phosphate

## ABSTRACT

Three phosphate solubilizing bacteria coded as Jim41, Gim10 and Hag12 were isolated from Jimma, Gimbi and Hageremariam soils, respectively. They were characterized to generic level and found to belong to *pseudomonas spp.* Tricalcium phosphate (TCP), rock phosphate (PR) and old bone (OB) solubilization efficiency of these isolates and one reference commercial strain (*Pseudomonas striata*), introduced from India was studied in the laboratory at 5, 10, 15 and 20 days of incubation. At the end of the specified incubation date, samples were taken and filtered to remove insoluble material. The filtrate was then centrifuged and analysed for soluble P using colorimeter. The result revealed that all PSB isolates solubilized significantly ( $P \leq 0.01$ ) greater amount of tricalcium phosphate and rock phosphate over the uninoculated control. The highest amount of solubilization was achieved with Jim41 followed by Gim10, Hag12 and *Pseudomonas striata* in that order. Isolate Gim10, Hag12 and *P. striata* solubilized significantly ( $P \leq 0.01$ ) greater amount of old bone than the control and isolate Jim41. This suggests that isolate Jim41 cannot be used as PSB inoculant where old bone is required as insoluble p fertilizer.

Greenhouse pot experiments with regard to the effect of inoculation of PSB isolates on the growth of faba bean were conducted. The treatments included two PSB isolates coded as Jim41 and Gim10, and two P-sources (rock phosphate and triple super phosphate) in various combinations, with and without *Rhizobium*. The result showed that following inoculation, the two PSB inoculants established well in the rhizosphere of faba bean and displayed the highest population during 7<sup>th</sup> week. Inoculation of PSB isolates along with the application of rock phosphate significantly ( $P \leq 0.01$ ) improved the dry matter yield of faba bean compared to the control with and without inoculation of *Rhizobium*. Isolate Jim41 and Gim10 inoculated with

rock phosphate were found to increase the dry matter yield of faba bean by 34 and 39% over the uninoculated control without inoculation of *Rhizobium*, and by 33 and 31% with the inoculation of *Rhizobium* respectively. The dry matter yield increment obtained from inoculation of both PSB isolates along with PR was comparable to that obtained with the application of triple super phosphate (TSP). This implies that there may be a possibility to use the less expensive PR together with PSB than the more expensive TSP. Analysis of plant tissue P content revealed that the PSB inoculants improved the P-uptake of faba bean where PR was applied as p-sources. Inoculation of PSB has also improved the nitrogen accumulation of faba bean. In the light of the present finding many of the conclusions are subject to verification. Thus, future research directions are discussed.

## 1. Introduction

Agriculture is the backbone of Ethiopian economy in which 85 % of the population depend for its livelihood. Moreover, it accounts for 52 % of GDP and for 90 % of the total foreign exchange earnings (Yohannes, 1994). The Country has a total land area of 112.3 million hectares 52 % of, which is suitable for cultivation of crops. However, only 16.4 million hectares of it is under cultivation now (Getachew *et al.*, 1998).

Despite the huge working force involved in agriculture and the enormous potentials the country has, it is unable to produce enough food to feed its population and achieve self-sufficiency in food. Poor soil fertility status especially deficiency of nitrogen and phosphorus among others account for this failure (Andrews and Jones, 1978, Yohannes, 1994).

Phosphorus, which is the most important nutrients required by plants for their growth next only to nitrogen, is deficient in most Ethiopian soils limiting crop production (Tekalinge and Haque, 1987). According to Desta (1982) 70-75 % of Ethiopian soils are deficient in Phosphorus. The deficiency may arise due to fixation by aluminum and iron (Brady, 1990). Phosphorus fixation is found to be common in the acidic soils of South, South Western, Western regions and highland Vertisols (Sahlmedhin and Ahmed, 1983; Tekaligne *et al.*, 1988). Even worse, the use of P-fertilizers to rectify the deficiency are complexed with metals and become an unavailable to crops in acidic and alkaline soils (Brady, 1990).

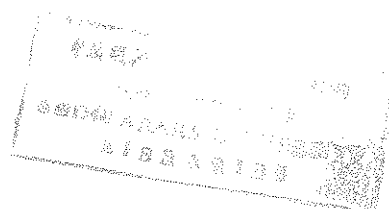
In order to prevent P-fixation and improve the fertilizer use efficiency of crops, various methods are developed. These methods include liming, application of organic matter and inoculation of seeds or soil with phosphate solubilizing microorganisms (Tisdale *et al.*, 1985; Brady, 1990; Diez *et al* 1992).

Phosphate solubilizing microorganisms (PSM) have a unique ability to use phosphorus contained in insoluble P-compounds and release soluble P-in excess of their requirement which could be taken up by plants (Alexander, 1977; Rao, 1982). In addition to increasing P-uptake, phosphate solubilizing microorganisms benefit plants by producing growth promoting hormones like indole acetic acid, gibberellic acids etc., inducing the proliferation of other useful micro organisms in the rhizosphere and by antagonizing plant pathogenic microorganisms (Mishustin and Naumova, 1962; Biswas *et al.*, 1993).

Consequently, the use of PSM as inoculant of seed or soil has long been practiced in the USSR and East European countries (Tisdale *et al.*, 1985). Nowadays, inoculation of seeds or soil during planting is becoming a common practice in many countries especially in India, and appreciable increase in the yield of crops like wheat, rice maize, beans etc. was reported (SubaRao, 1993).

Faba bean (*Vicia faba* L) is the predominant high land pulse crop grown in Ethiopia. It is a cheap source of protein for the majority of the population (Westphal, 1974; Asfaw *et al.*, 1994). Moreover, it helps to improve soil fertility through its nitrogen fixing ability in symbiotic association with *Rhizobium*.

Despite its importance, the yield of faba bean per unit area in the country is very low (Alem *et al.*, 1990). According to Central Statistics Authority (1997) the national average yield of faba bean is 10.4q/ha, whereas in the neighboring Sudan 18q / ha is obtained (Ahmed, 1996) which is almost double of that produced in Ethiopia. Deficiency of phosphorus is one of the factors that adversely affect the yield and nitrogen fixing ability of faba bean (Sahlemedhin and Desta, 1988).



Currently, the phosphate fertilizer used to grow crops in Ethiopia is imported from abroad and its ever-increasing cost makes it unaffordable to subsistence farmers. Even if it is affordable, the fertilizer use efficiency of crops is very low. According to Birwas et al., (1994) only 15-25 % of the applied P fertilizers is utilized by crops in a season. All these necessitate the development of alternative or supplementary means of improving p-nutrition for enhanced crop yield.

Natural rock phosphate deposits and huge accumulation of bones from abattoirs are available in the country. The exploitation of such cheap sources of phosphorus by the action of PSM may reduce our dependence on imported fertilizers to appreciable extent. Further more application of PSM alone without insoluble phosphate can increase phosphate availability by preventing P-fixation of already existing phosphorus in the acidic and alkaline soils. Once effective PSM strains are obtained it is easy to produce them commercially and supply to farmers at very low price.

However, only limited research work has been done so far in Ethiopia on the various aspect of PSM not the least their production on commercial scale for inoculation purposes. The present work was therefore, carried out to isolate phosphate solubilizing bacteria (PSB) from some Ethiopian soils and see their relative efficiency in increasing the phosphorus uptake of faba bean (*Vicia faba*. L.).

## **2. Objectives**

### **2.1 General Objective**

- To study the effect of phosphate solubilizing bacteria isolated from some Ethiopian soils on the growth and phosphorus uptake of faba bean.

### **2.2 Specific Objectives**

- To isolate phosphate solubilizing bacteria from some Ethiopian soils.
- To characterize these isolates.
- To test insoluble phosphate solubilization efficiency of PSB isolates.
- To evaluate the effect of these isolates on the dry matter yield and p-uptake of faba bean.

### 3. Literature Review

#### 3.1 The Importance of Faba bean in Ethiopian Agriculture

Faba bean (*Vicia faba* L.) is originated in Mediterranean and West Asian countries and has been domesticated in Turkey and Iran (Janick *et al.*, 1981). It reached Ethiopia soon after its domestication around 5<sup>th</sup> B.C. Secondary centers of diversity must have been established in Afghanistan and Ethiopia, that are mountainous countries where Faba bean is well adapted (Bond, 1976).

Faba bean is the major food legume grown in Ethiopia. According to Central Statistics Authority (1997), it accounts for 50% of total legumes produced. It grows in areas with altitude ranging 1800-3000 masl. The crop serve as the most important source of protein for the majority of the population and reasonable amount of its grains are exported annually to generate foreign exchange earnings. Moreover, it helps to improve soil fertility due its nitrogen fixing ability in symbiotic association with *Rhizobium*.

Despite the importance of faba bean, its yield per unit area is very low compared to that obtained in other countries (Alem *et al.*, 1990). According to Central Statistics Authority (1997) the national average yield of faba bean is 10.4q/ha whereas in the neighboring Sudan an average yield of 18q/ha is obtained (Ahmed, 1996) which is almost double of that produced in Ethiopia. Some of the factors which account for this low yield are insect pests, diseases, weeds, and poor soil fertility (Anagaw and Asnakew, 1994, Asfaw *et al.*, 1994).

Deficiency of phosphorus is one of the factors that determines soil fertility and hence, affects the yield and nitrogen fixing ability of faba bean. In most experiments conducted with P-fertilizers in Ethiopia, it is found that faba

bean responds well to the application of soluble p-fertilizers. This suggests that most soils of faba bean growing areas are deficient in phosphorus (Sahlemedhin and Desta, 1988; Angaw and Asnakew, 1994).

### 3.2 The role of Phosphorus in Plants

Phosphorus is one of the most important nutrients required by plants for their growth and development. With the exception of nitrogen, there is no other element as critical as is phosphorus. It is constituent of nucleic acid, proteins, phospholipids and coenzymes such as NAD and ATP.

In soils, phosphorus occurs in both organic and inorganic forms (Khasawneh *et al*, 1980). But plants take it up almost exclusively in in-organic forms principally as  $H_2PO_4$  for they take this up more easily than  $HPO_4^{-2}$  form (FAO, 1984). The specific role of phosphorus as plant nutrient is manifested in its useful effect on cell division and fat albumen formation, flowering and fruiting including seed formation and crop maturation. It also enhances root development and strengthening of straw of cereal crops and help to prevent lodging and improve the quality of crops especially forage and vegetable crops (FAO, 1984; Brady, 1990). Despite its importance phosphorus is deficient in most soil to fulfil the need of plants (Tisdale *et al.*, 1985, Brady, 1990). Deficiency of phosphorus results in stunting of growth, delay in maturity, dark coloration and purple streaks of plants (FAO, 1984).

The deficiency arises due to the fact that, compared to other major nutrients the amount of native phosphorus in soils is very low or it exists in unavailable form to be taken up by plants (Brady, 1990). Besides, soluble phosphate fertilizer that is applied to correct deficiency and thereby increase crop yield, but it is usually fixed in the soil and become unavailable to plants or crops (Velayuthan, 1980). Such P-fixation occurs in

highly weathered acidic soils dominated by  $\text{Al}^{3+}$  and  $\text{Fe}^{3+}$  ions (Brady, 1990). Similar p-fixation could occur in arid and semi arid soils containing high amount of  $\text{Ca}^{+2}$  (Diez *et al.*, 1992). The situation is very severe in the tropical soil as they contain appreciable amount of those factors responsible for P-fixation (Larsen, 1967). According to Andrew and Jones (1978), most soils in the tropics are in advanced stage of weathering with high content of iron and aluminum oxide which results in high P-fixation.

Phosphorus deficiency is the most important problem of Ethiopian soils (Desta, 1982). The deficiency is very severe in acidic soils of southern, south western and western regions. In these areas  $\text{Al}^{3+}$  and  $\text{Fe}^{3+}$  are totally incriminated for P-fixation (Sahlemedhin and Ahmed, 1983). Tekaligne and Haque (1987) studied the phosphorus status of some Ethiopian soils and reported that sorption of P is significantly correlated with exchangeable and extractable forms of iron and aluminum as well as pH and organic matter content. However, the role of  $\text{Al}^{3+}$  was found to be more important in P-fixation than  $\text{Fe}^{3+}$ . Desta (1982) studied the phosphorus content of highland soils and reported that more than 70-75% of soils are characterized by P-deficiency. Birhanu (1985) reported that 70% of vertisols have available P below 5 PPM, which is very low to support good plant growth. According to Tekaligne *et al.*, (1988), the P-fixation in vertisols of Ethiopia is related more to calcium which is a predominant cation in all profiles than  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$ .

### 3.3 Phosphate solubilization and phosphate solubilizing microorganisms

Phosphate solubilizing microorganisms are found in nature and are capable of solubilizing phosphate contained in sparingly soluble phosphate compounds (Alexander, 1977). These microorganisms endowed with solubilizing ability release soluble P-in excess of their requirement which could be taken up by plants (Rao, 1982).

The earliest report on phosphate solubilizing microorganisms come from the work of Stalstrom (1903) cited in Asfaw (1988). He mixed rock phosphate, bone meal, tricalcium phosphate and calcium carbonate in agar plate and found that certain microorganisms form clear zones around their colonies due to dissolution of complex phosphates. However, it was Pikovskaya (1948) who made systematic studies on tricalcium phosphate solubilization and recommended a medium for isolation, enumeration and maintenance of phosphate solubilizing microorganisms.

Representative species of microorganisms endowed with phosphate solubilizing ability occur in fungi, bacteria and actinomycetes (Gaur, 1988). The population of PSM is distributed more in the rhizosphere than the nearby soils (Paul and SundraRao, 1971) and the metabolic activity of PSM varies depending on the position from where they are

isolated. For instance, Katznelson and Bose (1959) studied the metabolic activity and phosphate dissolving capacity of bacteria isolated from wheat rhizoplane, rhizosphere and non rhizosphere soils. Their finding revealed that, those bacteria isolates from rhizoplane are more active in oxidizing glucose and alanine than those isolated from rhizosphere and non rhizosphere soils.

The solubilization capacity of PSM varies from strain to strain and with the nature of insoluble phosphorus compounds (Gaur, 1988). Agnihotri (1970) studied phosphate solubilizing potential of the 18 fungi species commonly found in forest seedbeds invitro, using tricalcium phosphate, hydroxyapatite and flouroapatite as phosphate source. He found that among 18 fungi tested, 14 solubilize tricalium phosphate. Five of his fungal isolates namely *Asperigillus flavus*, *Fusarium oxysporium*, *Sclerocium rolfsii*, *Cylindrocladium* spp and *Penicillium* spp. showed a good ability of

solubilization of all sources of phosphates tested. He further noticed that in the presence of insoluble phosphates, pathogenic fungi (i. e *Phythium* and *Rhizoctonia* spp.) grow poorly and failed to sporulate, whereas antagonist of these fungi such as *Pencillium*, *Aspergillus* and *Trichoderma* spp. sporulate. This phenomenon can be exploited to protect seedlings from damping off diseases.

Banik and Dey (1982) studied the efficiency of PSM isolated from alluvial soils in India, using  $\text{Ca}_3(\text{P}_04)_2$  agar plate and found that two fungal strains *Aspergillus fumigatus* and *Aspergillus candidus*, two bacterial strains *Bacillus firmus* B7600 and *Bacillus firmus* 137651 and one actinomycete strain AC56 (*Streptomyces* sp.) are efficient solubilizers. Will and Sylvia (1990) conducted a laboratory experiment to determine the ability of *Klebsiella pneumoniae*, *Azospirillum lipoferum* and *Alcaligenus denitrificans* to solubilize  $\text{CaHPO}_4$ , with visible clear zones around colonies within 96 hrs. Among the species tested, *Klebsiella pneumoniae* showed rapid growth and wider clear zones around 4 mm.

The solubilizing ability of PSM on different types of inorganic phosphate is not similar. Badiya and Gaur (1974) tested 14 bacterial isolates that has been found to effectively solubilize tricalcium phosphate. Out of the test PSB isolate, only 5-10% were found to solubilize rock phosphate (Gaur, 1988).

The solubilization effect is generally due to the production of organic acids in the medium in which the microorganisms grow (Sperber, 1957; SubaRao, 1993). The none volatile acids liberated in varying quantities in the medium are citric, glutamic, succinic, lactic, glyoxilic malic, fumaric, tartaric and alpha-ketobutyric acids. The solubilizing action of these organic acids have been attributed to their chelating property which enables them to form stable complex with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Al}^{3+}$  and release soluble

phosphate (Rao, 1982). There are a number of factors affecting phosphate solubilization by PSM. These factors include carbon source, nitrogen source, rock phosphate concentration, particle size of rock phosphate, pH, temperature, aeration and incubation period (Gaur, 1988).

### 3.4 Phosphomicroorganisms as Inoculant

Application of industrially manufactured phosphate fertilizers is becoming increasingly expensive for sustained crop production. Especially in developing countries, farmers could not afford the high price of industrially manufactured fertilizers since they are poor. Besides, even if they can afford to apply the fertilizers, there is poor fertilizer use efficiency by the crop. According to Biswas *et al.*, (1994), only 15-25% of applied P-fertilizers are utilized by crops due to immediate P-fixation in the soil. Therefore, the use of alternate methods, which improve P-nutrition and increases the productivity of crops, is important. Such methods include application of organic matter, liming and inoculation with PSM (Tisdale *et al.*, 1985; Diez *et al.*, 1992).

PSM have been shown to solubilize phosphate contained in insoluble form in the soil (Gaur,1988, SabaRao,1993). This ability of PSM can be manipulated to use low grade rock phosphate as direct application fertilizers and prevent the fixation of soluble P-fertilizers thereby improving the fertilizers use efficiency (Algawadi and Kulkarni,1993). PSM, besides increasing the phosphorus up take of plants, they are known to produce growth promoting hormones and prevent pathogenic microorganisms.

An increase in the yield of maize, rice, sorghum, barely, chickpea, soybean, groundnut, sugar beet, cabbage and tomato to the extent of 10-20 % due to inoculation with PSM was reported by Algawadi and kulkarni (1993). Twenty five percent increase in seed yield of black gram was obtained due

to inoculation with PSB (Tomar *et al.*, 1993). Asfaw (1993) reported a 20% increase in the yield of tef inoculated with phosphate solubilizing fungi. Datta *et al.* (1982) inoculated two varieties of rice, Java and IR-8 with phytohormone producing PSB (*Bacillus firmus*) planted in Negaland, India and found appreciable increase in the yield of both varieties. According to Kundu and Gaur (1980) inoculation of cotton with *Pseudomonas striata* and *Aspergillus awmori* increased the straw yield by 70% over the control. A number of trials conducted in India revealed that application of 50kg P<sub>2</sub>O<sub>5</sub> / ha as rock phosphate and inoculation with PSB give yield equivalent to that obtained with application of 50 Kg/ha as super phosphate containing 46% P<sub>2</sub>O<sub>5</sub> (Algawadi and Kulkarni,1993). However, inoculation with PSM does not always bring increase in the P-uptake and yield (Biswas *et al.*, 1994 ).

Inoculation is expected to improve plant growth if the bacteria establish well in the rhizosphere. Soil fertility, organic matter content of soil, and the interaction between inoculant strain and that of rhizosphere microorganisms are some of the factors affecting the establishment of inoculum in the rhizosphere. According to Biswas *et al.*, (1994), positive response to phosphobacteria is obtained only in soil low in available P and high organic matter content.

### **3.5 Interaction between phosphate solublizing microorganisms**

#### **(PSM) and nitrogen fixing bacteria.**

Phosphorus among other is one of the most important factors affecting effective biological nitrogen fixation (Alexander, 1977; Munn,1977). Beneficial effect on biological nitrogen fixation due to phosphorus application was reported on various leguminous crops (Robson *et al.*, 1981). A similar positive effect has been observed on biological nitrogen fixation by inoculating leguminous crops with phosphate solubilizing microorganisms (Kundu and Gaur, 1984).

Asfaw (1988) studied the associative effect of *R. japonicum* and PSB on phosphate solubilizing activity by inoculating sterilized soil either with *P. striata*, or *Bacillus polymaxa* or their respective mutants M<sub>20</sub> and M<sub>142</sub> singly or in combination with *Rhizobium japonicum*. The result revealed that available P<sub>2</sub>O<sub>5</sub> content at 30 days reach a maximum of 41.7ppm and 40ppm in *Bacillus polymaxa* and its mutant M<sub>142</sub>, respectively in combination with *Rhizobium japonicum*. He further tested the associative effect of *R. japonicum* and PSB on nodulation and nitrogen fixation of soybean ( *Glycine max. L* ), and obtained much higher nodulation and nitrogen fixation when *R. japonicum* was combined along with PSB over the uninoculated control.

Pant *et al.*, (1995) reported a significant increase in the number of nodules, nodule dry weight and fodder yield of soybean coinoculated with PSB, *P. striata* and nitrogen fixing bacteria, *Bradyrhizobium japonicum*. Inoculation effect of nitrogen fixing *Azotobacter chroococcum*, and two phosphate solubilizing microorganism, *P. striata* and *A. awamori* on yield and nutrient up take in rice was studied and the result showed that, these organisms appreciably increase the yield and uptake of nutrients when rice is inoculated with the mixture of the three organisms (Kundu and Gaur, 1984).

Phosphate solubilizing microorganisms have also been shown to interact with another useful microorganism known as arbuscular mycorrhizal fungi (AM) (Barea *et al.*, 1975). AM fungi forms a symbiotic association with a wide variety of plants and enable the later to acquire more nutrients through its extraradial hyphae, confers them tolerance to water stress, protect them from metal ion toxicities and diseases (Garret, 1981; Harely and Smith, 1983). Piccini and Azcon (1987) conducted an experiment to see the effects of dual and single inoculation of alfalfa plants with PSM and VAM fungi in the presence of Bayovar rock phosphate when sand-vermiculite was used as

a culture medium. The result indicated that dual inoculation with PSB and A-mycorrhiza significantly increased alfalfa growth. These authors also found that root weight and percentage of root colonization by AM endophytes were improved by dual inoculation. Sreenivasa and Krishinara (1992) studied the interaction between two AM fungi, namely *Glomus fasciculatum* and *Glomus macrocarpum*; and phosphate solublizing bacteria *Pseudomonas striata* and found that plant dry biomass and up take of P, Zn and Fe are appreciably increased by dual inoculation of *Glomus macrocarpum* and *pseudomonas striata*.

## 4. Materials and Methods

### 4.1 Soil sample collection for isolation of phosphate solubilizing bacteria (PSB)

Soil samples at a depth of 0-30cm were collected from red soils of Jimma, Gimbi and Hageremariam. From each of these areas 50 samples a total of 150 samples were collected in plastic bags.

### 4.2 Isolation of phosphate solubilizing bacteria

Phosphate solubilizing bacteria were isolated following the method developed by Pikovskaya (1948) cited in Asfaw (1988).

Ingredients: Glucose	5g
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	5g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5g
Yeast extract	0.5g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2g
NaCl	0.1g
MnSO <sub>4</sub>	Trace
FeSO <sub>4</sub>	Trace
Agar	15g
Distilled water	1000ml
pH	7.0

The medium was autoclaved at 121<sup>0</sup>C and pressure 15psi for 15 minutes. Fifteen ml of the molten agar medium was poured in a petridishes and allowed to solidify.

Ten grams of soil from each sample was suspended in 90ml of sterilized water to make 1:10 dilution. These were shaken on platform shaker for 30 minutes to break clogs. Then series of dilutions viz. 10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup> 10<sup>-5</sup> and 10<sup>-6</sup> were made. From dilutions of 10<sup>-5</sup> and 10<sup>-6</sup>, 0.1ml of suspensions

were transferred on a petridishes containing Pikovskaya's medium. The suspensions were spread uniformly on a petridishes using glass rod spreader and incubated for 5 days at  $30\pm 2^{\circ}\text{C}$ . Colonies showing clear zones around them were picked and subcultured for purification. The purified cultures were maintained on Pikovskaya's agar slant for subsequent experiments.

#### 4.3 Characterization of PSB Isolates

The PSB isolates were characterized to their respective genera using the following tests.

**4.3.1 Cultural characteristics:** Colonies were characterized on the basis of elevation, margin and size.

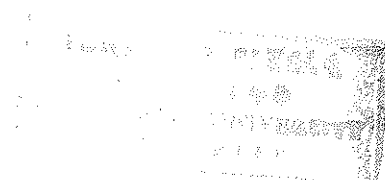
**4.3.2 Gram reaction:** twenty four hour young cultures of PSB isolates were gram stained and their reaction was recorded.

**4.3.3 Microscopic observation:** simple staining of the PSB isolates was performed and stained smears were examined under the microscope to observe cell shape (rods, cocci etc.) and cellular arrangement (single, pairs, chains, etc.).

**4.3.4 Spore staining:** forty eight hours old cultures of PSB isolates were spore stained following procedure the described in Aneja (1993).

**4.3.5 Flagella Staining:** Flagella staining was performed using the method described in Aneja (1993). The presence, absence, number and position of flagella on the isolates were recorded.

**4.3.6 Motility test:** Isolates were inoculated by stabbing into motility agar medium and incubated for 3-5 day at  $30\pm 2^{\circ}\text{C}$ . Diffused growth throughout the test tube is an indication of motility. While growth only along the stab is an indication of non-motility(Collins and Lyne, 1976).



### 4.3.7 Biochemical Test

**4.3.7.1 Fluorescent pigment production:** King' s medium B was used for this experiment (Korobko, 1988).

Ingredients: Peptone	20g
K <sub>2</sub> H <sub>2</sub> SO <sub>4</sub>	1.5g
MgSO <sub>4</sub> .7H <sub>2</sub> O	1.5g
Glycerin	10ml
Agar	17g
Distilled water	1000ml
pH	7.2

Fifteen ml of the medium was distributed in a petriplate and autoclaved at 121°C and pressure of 15 psi for 15min. Then each isolate was inoculated into the cold medium and incubated for up to 5 days. Then the diffusing pigment was observed using UV- light adjusted at 230nm.

**4.3.7.2 Catalase production:** The colonies were flooded with 2 to 3 drops of 3% H<sub>2</sub>O<sub>2</sub> solution. Formation of bubbles indicates a positive reaction (Collins and Lyne,1976).

**4.3.7.3 Cytochrome oxidase Production:** A small piece of whatman filter paper was soaked with 1% aqueous solution of tetramethyl - p - phenyl diamine dihydrochloride. And a loopful of 24hours old culture was scrapped and rubbed on the filter paper. The appearance of a blue color in 10 to 20 seconds was considered as a positive test for oxidase (Collins and Lyne, 1976).

4.3.7. 4 Oxidation -fermentation test: The test was performed following the method described in Aneja (1993).

Ingredients:	peptone	2.0g
	NaCl	5.0g
	K <sub>2</sub> HPO <sub>4</sub>	0.2.g
	Bromothymol blue(0.2%)	0.08g
	Agar	2.50g
	Distilled water	1000ml
	PH	7.1

Three to four ml of the basal medium was distributed into each test tube and sterilized by autoclaving at 121<sup>0</sup>C and pressure of 15psi for 15 minutes. After sterilization, 1ml of 10%filter sterilized glucose solution was added to give a final 1% concentration. Two tubes, were inoculated by stabbing with same culture and one of the tubes was sealed by adding sterile paraffin oil. Then inoculated tubes were incubated at 30±2<sup>0</sup>C for 3 to 4 days. Acid production in the open tube only and in both open and sealed tubes is an indication of oxidative and fermentative utilization of glucose respectively.

4.3.7.5 Methyl red and Vogues proskauer test(MRVP test): The test was conducted as described in Aneja (1993).

Ingredients of MRVP medium :-	Peptone	7g
	Glucose	5g
	K <sub>2</sub> SO <sub>4</sub>	5g
	Distilled water	1000ml
	pH	7.0
Ingredients of methyl red indicator:-	Methyl red	0.1g
	Ethyl alcohol	300ml
	Distilled water	100 ml

## Reagents

VPI ; - 0.6g of alpha naphthol was dissolved in 100ml of 96 % ethanol

VPII;- 40g of KOH was dissolved in 100ml of water.

Five ml of MRVP broth was distributed in test tubes and autoclaved at 121°C and pressure of 15psi for 15 minutes. Two MRVP tubes were inoculated with 24hr old cultures and incubated at  $30 \pm 2^{\circ}$  C for 2-3 days. Then 5 drops of methyl red indicator was added to one of the tubes inoculated with each culture. The development red color indicates a positive reaction.

Twelve drops of VP reagent I and 2-3 drops of VP reagent II were added to the other test tube. The formation of pink color is a characteristic of acetoin production.

**4.3.7.6. Indole production test:** - Test tubes containing 1% tryptone broth were inoculated with each PSB culture and incubated for 48hrs. After that 1ml of Kovac's (dimethylamino benzaldehyde) reagent was then added and shaken for 10-15 min. The test tubes were allowed to stand. The development of a cherry (red) color in the top layer was an indicates a positive result for indole production (Aneja, 1993).

### **4.3.7.7 Nitrate reduction test:-**

Reagents:- Solution 1:- 0.8g of sulphanilic acid was dissolved in 100ml 5 acitic acid with gentle heating. Solution 2:-0.5g of alpha-naphthylamine was dissolved in acetic acid with gentle heating.

A heavy suspension of the inoculum of isolates was added in 1ml of peptone water containing 0.2mg of 2%  $\text{KNO}_3$  and heated to 37°C for 15 min. in water bath. Then 3 drops of solution 1 was added and immediately

followed by the addition of 3 drops of solution 2. The mixture was then shaken and allowed to stand for 2 min. The appearance of pink color at this stage is considered as a positive reaction (that is reduction of nitrate to nitrite). When no color change occurred, zinc dust was sprinkled in to these tubes. The development of red color confirms a negative reaction (Collins and Lynne, 1976).

#### 4.3.7.8 Utilization of Trehalose, *Meso*-Inositol and Lactose as sole carbon source.

The test was performed as recommended by Korobko (1988).

Ingredients:	$N_4H_2PO_4$	5g,
	$K_2HPO_4$	0.5g,
	$MgSO_4.7H_2O$	0.2g,
	NaCl	0.5g,
	Yeast extract	1g
	Agar	12g
	Bromocrestol Purple	1.5%
	Alcohol solution	7ml
	Carbon	5g
	Distilled water	1000ml

The medium was distributed at 7ml volume in to test tubes, autoclaved and inoculated with 24hrs of each isolate. Inoculated tubes were incubated at  $30\pm 2^\circ C$  for 3 to 5 days and examined for acid production. A yellow color is an indication of acid production, as a result of utilization of the carbon source.

#### 4.4 Tricalcium phosphate (TCP), Rock phosphate (PR) and Old Bone meal (OB) solubilization efficiency test

The following PSB isolates were used in the test; Jim41, Gim10, Hag12 and *Pseudomonas striata* (introduced from India). Fifty ml of Pikovskaya's broth (with out phosphorus source) was dispensed in 250ml Erlenmeyer flasks and to these 250mg of tricalcium phosphate, 333.33mg of Morocco rock phosphate and 200mg of old bone

were separately added to each flask. The above quantities give equivalent amount of phosphate ions. The flasks were sterilized at 121°C and pressure 15psi for 15 minutes.

This autoclaved and cooled medium was inoculated with 0.1ml of 24 hrs active culture (containing  $10^7$  cells/ml) suspension of each PSB isolates. The flasks were kept on a rotary shaker for 10 hrs per day until the day of sampling. Sixteen replicate flasks were used for each PSB isolate of which four flasks were removed at 5, 10, and 15 and 20 days and analyzed for phosphorus and pH changes.

Soluble phosphorus was quantitatively determined following the method developed by Koenig and Johnson (1942) cited in SubaRoa (1993). From culture broth, insoluble materials were removed by filtering through whatman filter paper No.1 and the filtrate was centrifuged at 13000 rpm for 15 minutes.

Ten ml of the neat filtrate was taken and to which 2.5 ml of Barton's reagent (its ingredients is shown below) was added and the volume was adjusted to 50 ml with distilled water. After 10 minutes, color intensity was read in a colorimeter adjusted at 430 nm wavelength.

## Ingredients and preparation of Barton's reagent:

Solution A: Twenty five gram of ammonium molybdate was dissolved in 400ml water.

Solution B: One and point five gram of ammonium metavanadate was dissolved in boiling water. After cooling, 25ml concentrated nitric acid was added. Afterwards A and B solutions were mixed and adjusted to a liter.

A standard curve was prepared by dissolving 0.2195  $\text{KH}_2\text{PO}_4$  in distilled water and the solution was made up to 1lt (1ml=50ppm of P). Further dilution of 10ml in 250 ml was made so as to give 1ml = 2ppm of P. Aliquots of 2, 3, 4, 5, 6, 8, 10, 15, and 20 ml of 2ppm stock solutions were transferred into 50ml volumetric flasks. To these 2.5 ml of Barton's reagent was added and the volume was made up to 50ml with distilled water. After 10 minutes the color developed was read in a colorimeter. A standard graph was then prepared from which P values for experimental samples were calculated.

### 4.5 Pot experiment

#### 4.5.1 Soil

The soil for pot experiment was brought from Sheno, North Shewa, 70 km from Addis Ababa. The soil was milled and passed through 4 mm-sized mesh. The physical and chemical characteristics of the soil is shown in Table 1. Farmyard manure at a rate of 1ton/ha (0.25g/Kg of soil) was incorporated to maintain the organic matter content at 2% level.

Table 1. Physical and chemical characteristics of the soil used in the pot experiment.

PH	Texture	Om %	N <sub>tot</sub>	<u>Meq/100gofsoil</u>					<u>PPM</u>				
				Na	K	Ca	Mg	Pay	Fe	Mn	Zn	P <sub>tot</sub>	
				<u>CEC</u>									
6.84	clay	1	0.08	0.4	0.9	35	18	56	6.69	7.2	49.3	0.64	7.2

Then it was sterilized at 121<sup>0</sup>C and pressure of 15psi for 1 hour intermittently for two days and two kilograms of sterilized soil was filled in plastic pots of size 4 X 4cm.

#### 4.5.2 Treatments

Two phosphate solubilizing bacterial isolates Jim41 and Gim10 based on their efficiency test performed in the laboratory were used for this experiment. Besides, two phosphorus sources, triple super phosphates at rate of 75kg/ha (17.25mg P<sub>2</sub>O<sub>5</sub>/kg of soil) and Morocco rock phosphate at a rate of 200 kg/ha (17.2mg P<sub>2</sub>O<sub>5</sub>/kg of soil) were included in the treatment. The various combinations of treatments are shown below. The trial was performed in two sets. The first set was conducted without *Rhizobium* inoculation and the second set with *Rhizobium*.

<u>Treatments</u>	<u>Set-I (with out <i>Rhizobium</i>)</u>	<u>Set-II(with <i>Rhizobium</i>)</u>
	<u>Code</u>	<u>code</u>
1. Non fertilized, Non inoculated control	NoIo	NoIoR
2. Isolate Jim41	Jim41	Jim41R
3. Isolate Gim10	Gim10	Gim10R
4. Triple super phosphate	TSP	TSPR
5. Rock phosphate	PR	PRR
6. Isolate Jim41 + Rock phosphate	Jim41+PR	Jim41+PRR
7. Isolate gim10 + Rock phosphate	Gim10+RP	Gim10 + PRR

The experiment was laid out in a completely randomized design with four replications.

#### 4.5.3. Inoculum Preparation

The two phosphate solublizing bacterial isolates Jim41 and Gim10 were grown on Pikovskaya's broth at  $30 \pm 2^{\circ}\text{C}$  on a rotary shaker for 5 days, until the population reached  $10^8$  cells/ml of broth. An efficient carrier based *Rhizobium* strain (*Rhizobium leguminosarum*) containing  $10^9$  cells/g of peat was used for the second set of experiment.

#### 4.5.4. Seed sterilisation, inoculation and sowing

Fababean seeds of variety CS20DK which had been obtained from Holleta Research Center were surface sterilized with 1% HgCl<sub>2</sub> for 3 minutes and subsequently they were dipped in to 70% alcohol for three minutes and washed with deionized sterilized water several times.

For treatments with *Rhizobium*, seeds were inoculated using two step methods as recommended by Somasegran and Haben (1994). Seeds were first coated with 40% gum Arabic (sticker). Then a carrier based inoculum of *Rhizobium* containing 10<sup>9</sup> cells/g of peat was inoculated at a rate of 7g per kilogram of seeds.

Just before sowing, seed were treated with either of Jim41 and Gim10 isolate at a rate of 10<sup>8</sup> / seed as per the treatment. Four seeds per pot were planted and later thinned to three. Plants were watered with deionized water at field capacity.

#### 4.5.5. Data collection

##### 4.5.5.1. Enumeration of PSB

In order to see the establishment of PSB, following inocuation, soil samples from the rhizosphere at 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> weeks were taken and the bacteria were counted using dilution plating method.

##### 4.5.5.2 Dry matter yield Determination

After two months of growth, stems were cut at the soil line with sterile razor blade. Then the shoots were placed inside labeled paper bags and oven dried to constant weight at 70°C for two days to determine dry weight.

#### 4.5.5.3 Nodule number and weight determination

The roots were carefully removed, adhering soils were removed with running water on the sieve. Nodules were collected, counted and dry weights were determined.

#### 4.5.5.4 Determination of total Nitrogen content of plants samples

Total nitrogen was analysed according to the manual of NSL (1994). Dried plant samples from each pot were milled into powder using electrical mill so as to Pass through 0.5mm sieve. 0.3g powder was taken from each sample and was subjected Kjeldahl digestion for determination of total nitrogen in the sample. macro Kjeldahl Procedure for determination of total nitrogen involves three processes: Digestion, distillation and titration. During digestion organic nitrogen is converted to ammonia-nitrogen with the help of potassium sulphate which raises the temperature when copper sulphate is used as a catalyst. Then the amount of ammonia liberated is estimated by distilling the digest with sodium hydroxide. The ammonia liberated is trapped by boric acid and titrated with HCl.

##### 4.5.5.4.1 Reagent preparation

A) Sulphuric acid - Selenium mixture: 3.5g of selenium powder was dissolved in one litter of sulphuric acid by heating to about 330<sup>0</sup>C, while covering with watch glass until the color changes to light yellow.

B) Digestion mixture: 7.2 g of salicylic acid was dissolved in 100ml of sulphuric acid-selenium mixture.

C) Mixed indicator: 0.5g of bromocresol green plus 0.1g of methyl red was dissolved in 100ml 95% Ethanol and the pH was adjusted to 4.5.

D) Receiver solution: A 4% boric acid receiver solution was prepared by dissolving 40g boric acid in 600ml of very hot distilled water. The solution

was then mixed and cooled to room temperature. This was followed by the addition of 10ml of bromocresol green solution (100mg in 100ml methanol) and 7ml of methyl red solution (100mg in 100ml Methanol). The whole mixture was then diluted to 1l with hot distilled water and gently mixed. Twenty-five ml of this solution was transferred to a 250ml Erlenmeyer flask to which 100ml distilled water was added. Based on the color of this mixture, two to three drops of 0.1M HCl or 0.1M NaOH was added to the stock solution. The transfer of 25ml portion followed this and mixing it with water until color change appeared. This procedure was repeated successively until the 25ml boric acid and 100ml water mixture assumed a neutral grey color. This color was used as a reference end point for titration of ammonia with acid in the determination of ammonia in the samples.

#### 4.5.5.4.2 Kjeldahl Digestion procedure

0.3g of finely grinded plant sample were transferred to a digestion tube to which 2.5ml of the digestion mixture (B) was added. The mixture was carefully swirled to moisten the plant material and allowed to stand for at least 2hrs. The tubes were then placed in a heating block and heated at 100<sup>0</sup>C for at least 2hrs after which time they were removed cooled and 3ml 30% H<sub>2</sub>O<sub>2</sub> solution was successively added by mixing after each addition. The tubes were again placed on the preheated block and heated at 330<sup>0</sup>C until the digest turned to colorless or light yellow. After removing the tubes from the block, it was cooled at room temperature and 48.3ml of distilled water was added to the digest and mixed. This mixture was allowed to stand over night. Total nitrogen in the plant samples was determined by distillation of the aliquots (50ml from the digest with 40% sodium hydroxide, which was received in 25ml of 4% boric acid (in a kjeltec 1002 distilling unit). It was then titrated with 0.1N HCl to the end point of mixed acid indicators. A blank tube with out sample was treated similarly as control tubes.

Total nitrogen in the sample was calculated using the formula:

$$\%N = \frac{(T-B) \times N \times 14.007 \times 100}{\text{Weight of the sample in mg}}$$

Where, T= titration volume of the sample

B= titration volume for the blank

N= normality of the acid

#### **4.5.5.5 Analysis of phosphorus contents of plant samples**

The phosphorus content of plant samples was determined using methavanadate method(NSL, 1994). The plant samples were calcinated in the furnace at 450°C and digested with strong acid to liberate the organic phosphorus. The phosphorus in the solution was determined colorimetrically by using molybdate and metavanadate for color development.

##### **4.5.5.5.1 Reagent preparation**

A) Solution I: 20g ammonium molybdate was dissolved in 25ml distilled water in 1l capacity volumetric flask.

B) Solution II: 1.25g of ammonium metavanadate was dissolved in 300ml distilled water by heating. Then 425ml of concentrated perchloric acid was added.

Solution II was then added to solution I and the volume was adjusted to a liter with distilled water.

##### **4.5.5.5.2 Standard phosphorus solution preparation**

A) 500ppm P solution: 3g of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) were oven dried at 105°C for 2 hours. After cooling in a desecrator 2.197g  $\text{KH}_2\text{PO}_4$  was measured and dissolved in 1lt of distilled water.

B) 100ppm P solution: 50ml of the 500ppm P solution was taken in 250ml volumetric flask and diluted to 250ml with distilled water.

C) 10ppm P solution: 50ml of the 100ppm P solution was taken in 500ml volumetric flask and diluted to 500ml with distilled water.

D) Phosphorus working solution standards of 0, 1, 2,3, 4 and 5ppm P: 0, 5, 10,15, 20 and 25 ml of the 10ppm P solution were pipeted in 50ml

volumetric flasks.

**4.5.5.5.3 Procedure:** 5ml sample from digests was pippered into 50ml volumetric flasks. Then 10ml of the molybdate and vanadate solutions was added to the samples and the standards and the volume were brought up to 50ml with distilled water. After 10 minutes the color developed was read on spectrophotometer at 460nm wavelength.

#### **4.5.5.5.4 Calculations**

Standard curve was prepared, with absorbance on x-axis and concentrations on y-axis. From which P-values in the samples were calculated.

$$\text{PPM} = \frac{C.V1.V2}{S.A}$$

Where C = P concentration in sample digest read from the curve in PPM.

V1 = Volume of the digest.

V2 = Volume of the digestion

S = Weight of the material calcinated in grams

A = Aliquots (5ml)

PPM = Concentration. 1000

## **4.6 Statistical Analysis**

Data on soluble P, dry matter yield, plant total nitrogen and plant phosphorus contents were subjected to two way analysis of variance using MSTAT4C computer software. Means were separated using Least Significance Differene (LSD) and Duncun's Multiple Range Test (DMRT) at 0.5 and 1% level of significance.

## 5. Results

### 5.1 Characterization of Phosphate Solubilizing bacteria (PSB) Isolates

A total of three phosphate solubilizing bacterial coded as Jim41, Gim10 and Hag12 were isolated from soil samples collected from Jimma, Gimbi and Hageremariam respectively. These isolates were grown on Pikovskaya's agar plate and formed clear zones around their colonies. Isolate Jim41 formed clear zone measuring a diameter of 4mm, whereas isolate Gim10 and Hag12 formed a clear zones measuring a diameter of 3 and 2.5mm, respectively after 96hrs of growth.

Some of the cultural, morphological and biochemical characteristics of these isolates are shown in Table 2. By comparing these characteristics to those described in Bergey's manual of Systematic Bacteriology(Krieg and Holt, 1984), attempts were made to classify them into their respective genera. Accordingly they were all found to belong to *Pseudomonas spp.*

However, the PSB isolates were found to be different because of marked differences in some of the characteristics tested. For instance isolate Jim41 produced fluorescent pigment, whereas the other two did not have any pigment. Nitrate reduction was observed in only isolate Hag12. The test organisms showed discrepancies in the utilization of meso-Inositol, Trehalose and lactose as sole carbon source.

Table 2. Some Cultural, Morphological and Biochemical Characteristics of PSB Isolates

Characteristic	Isolates		
	Jim41	Gim10	Hag12
Gram reaction	-	-	-
Shape of cells	Rod	Rod	Rod
Color	Greenish yellow	Yellow	White
Spore	-	-	-
Motility	Mo	Mo	Mo
Flagella	Po	Po	Po
Fluorescent pigment	+	-	-
Catalase	+	+	+
Oxidase	+	+	+
OF-test	O	O	O
MR	-	-	-
VP	-	-	-
Indole	-	-	-
Nitrate reduction	-	-	+
Growth at 41°C	-	+	+
Utilization: Inositol	-	+	-
Trehalose	-	+	+
Lactose	-	-	+

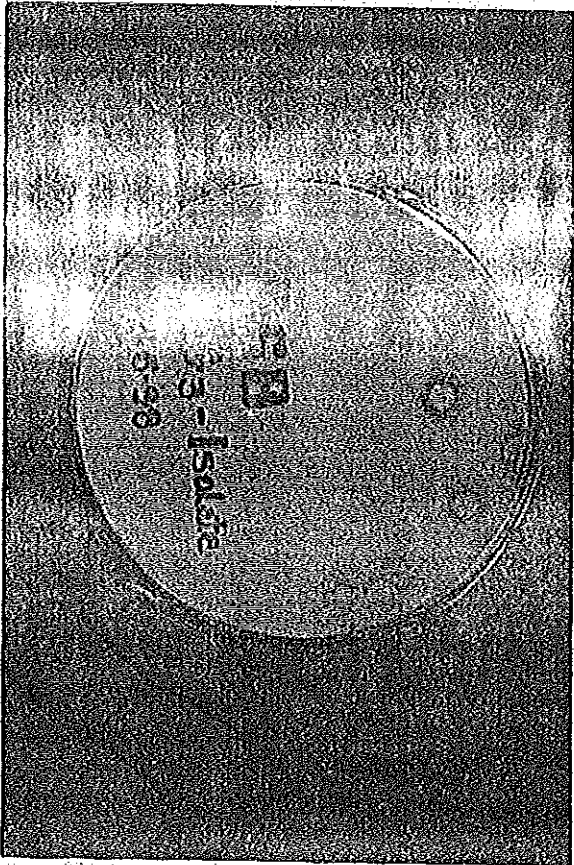
- = Negative for the test

O = Oxidative

Po = Polar

+ = Positive for the test

Mo = Motile



Picture 1  
PSB Isolate H&G 12

## 5.2 Tricalcium phosphate solubilization efficiency of PSB isolates

The results of tricalcium phosphate solubilization by different isolates and the associated pH changes in the medium are shown in Table 3. All bacterial isolates used in the test solubilized significantly ( $P \leq 0.01$ ) greater amount of tricalcium phosphate over the uninoculated control. The highest amount of solubilization was recorded by bacterial strain coded as Jim41 followed by Gim10, Hag12 and *Pseudomonas striata*. The result showed that PSB isolated from Ethiopian soils were found to be more efficient in solubilizing TCP than *P. striata* introduced from India.

There was a progressive increase in TCP solubilization up to 15 days of incubation. But the increase thereafter was less marked. Increase in solubilization was followed by a corresponding decrease in the pH of medium inoculated with PSB isolates.

## 5.3. Rock phosphate (PR) solubilization efficiently of PSB isolates

Table 4 shows the amount of rock phosphate (PR) solubilized by the isolates. All isolates tested solubilized significantly ( $P \leq 0.01$ ) greater amount of rock phosphate over the uninoculated control. The highest amount of soluble P per 50ml of medium was released in flasks inoculated with Jim 41 isolate followed by Gim10, Hag12 and *P striata*.

The solubilization increased up to 20 days of incubation where the maximum amount of soluble P was released. There was a drastic drop in the pH of the medium inoculated with all PSB isolates, whereas in the uninoculated flask, an increase in pH was recorded.

Table 3. Tricalcium phosphate (TCP) Solubilization efficiency of PSB isolates.

PSB isolates	5 days		10 days		15 days		20 days	
	pH	mg of p/50ml	PH	mg of p/50ml	pH	mg of p/50ml	pH	mg of p/50ml
Jim41	5.20	14.07	4.50	15.50	4.0	21.00	4.0	20.88
Gim10	5.60	11.43	5.00	13.00	4.6	17.00	4.3	18.05
HAG12	5.80	9.63	5.58	11.50	4.9	14.88	4.8	15.25
<i>P. striata</i>	6.10	8.80	5.78	10.00	5.3	12.75	5.0	13.25
Control	6.67	1.95	6.68	1.90	6.5	2.05	6.8	2.30
LSD*( 0.01)	-	2.99	-	2.07	-	3.25	-	4.07

\* Least significant difference at 1% level of significance

Table 4. Rock Phosphate (PR) solubilization efficiency of PSB Isolates

PSB isolates	5 days		10 days		15 days		20 days	
	pH	mg of p/50ml	pH	Mg of p/50ml	pH	mg of p/50ml	pH	mg of p/50ml
Jim 41	4.8	5.05	3.7	5.50	3.25	6.02	3.20	6.66
Gim10	5.0	4.77	4.5	5.00	3.90	5.42	3.50	6.04
HAG12	5.3	4.25	4.7	4.12	4.10	5.02	3.50	5.48
<i>P. striata</i>	5.3	4.07	4.9	4.43	4.50	4.53	4.60	5.06
Control	7.3	0.70	7.2	0.55	7.40	0.53	7.20	0.62
LSD (0.01)	-	0.73	-	0.72	-	0.39	-	0.40

#### 5.4. Old bone (OB) solubilization efficiency of PSB isolates

The amount of phosphate solubilized from old bone and the corresponding pH decrease of the medium by PSB isolates is shown in Table 5. Out of the four PSB isolates tested, three of them namely, Gim10, Hag12 and *P striata* solubilized significantly ( $P \leq 0.01$ ) greater amount of old bone over the uninoculated control. More or less these isolates were equally efficient in solubilizing old bone. There was no significant difference in the amount of old bone solubilized between PSB isolate Jim41 and uninoculated control.

The maximum solubilization was achieved at 20 day of incubation. There was a drastic drop in the pH of inoculated flasks with all PSB isolates except with Jim41 isolate where there was a slight increase above neutral pH.

#### 5.5 Establishment of PSB inoculants in the rhizosphere of faba bean

The result in Table 6 shows the population of PSB isolates, Jim41 and Gim10 in the rhizosphere of faba bean at different intervals during their growth inoculated singly or in combination with rock phosphate. The population of both bacteria increased with time and was found to be highest at 7<sup>th</sup> week with and without *Rhizobium* inoculation.

Table 5. Old bone solubilization efficiency of PSB isolates

PSB isolates	5 days		10 days		15 days		20 days	
	pH	mg of p/50ml	pH	Mg of p/50ml	pH	mg of p/50ml	pH	mg of p/50ml
Jim 41	7.40	3.00	7.20	3.11	7.01	3.20	7.40	3.30
Gim10	3.70	25.08	3.60	28.63	3.30	29.25	2.28	32.50
HAG12	3.90	23.02	3.65	25.30	3.48	27.70	3.10	29.95
<i>P. striata</i>	4.00	22.90	3.73	24.50	3.50	27.25	3.25	29.60
Control	6.90	2.28	6.90	2.78	6.85	3.05	6.90	2.95
LSD (0.01)	-	3.55	-	5.69	-	4.21	-	5.17

Table 6. Population of Phosphate Solubilizing Bacteria in the Rhizosphere of Fababean

	Without <i>Rhizobium</i>			With <i>Rhizobium</i>		
Time (Weeks)						
Treatment	3	5	7	3	5	7
N <sub>0</sub> I <sub>0</sub>	-	-	-	-	-	-
Jim 41	1.13x10 <sup>4</sup>	9.8x10 <sup>6</sup>	2.10x10 <sup>7</sup>	1.2x10 <sup>4</sup>	8.5x10 <sup>6</sup>	2.21x10 <sup>7</sup>
Gim10	2.5 x 10 <sup>4</sup>	1.07x10 <sup>7</sup>	3.27x10 <sup>7</sup>	3x10 <sup>4</sup>	9.3x10 <sup>6</sup>	2.50x10 <sup>7</sup>
TSP	-	-	-	-	-	-
RP	-	-	-	-	-	-
Jim 41 + PR	1.7x10 <sup>4</sup>	1.34x10 <sup>7</sup>	1.81x10 <sup>7</sup>	2.3x10 <sup>4</sup>	1.05x10 <sup>7</sup>	1.76x10 <sup>7</sup>
Gim10 + PR	3.3.8x10 <sup>4</sup>	1.98x10 <sup>7</sup>	2.63x10 <sup>7</sup>	3.2x10 <sup>4</sup>	1.11x10 <sup>7</sup>	290x10 <sup>7</sup>

## 5.6 The Effect of Inoculation of Phosphate Solubilizing Bacterial on the Dry matter Yield of Faba bean

The effect of inoculation of phosphate solubilizing bacteria on the dry matter yield of Faba bean without and with *Rhizobium* is presented in Figure 1. In both cases, there was a highly significant ( $P \leq 0.01$ ) difference in the mean dry matter yield in response to treatments.

Inoculation with phosphate solubilizing bacterial isolate Jim41 and Gim10 along with the application of rock phosphate significantly increased the dry matter yield compared to treatments that received rock phosphate or PSB inoculant alone and control irrespective of *Rhizobium* inoculation. As far as treatments without *Rhizobium* are concerned, Jim41 and Gim10 together with rock increased the dry matter yield of Faba bean by 34 and 39% respectively over the control. *Rhizobium* treatments of the same experimental set up increased the dry matter yield by 33 and 31% respectively. Neither inoculation with either of PSB isolates alone nor the application rock phosphate significantly improved the dry matter yield compared to control. The two PSB isolates along with rock phosphate produced a dry matter yield, which was comparable to that obtained by the application of triple super phosphate, and this was true for both sets of experiments. Under the conditions the two PSB isolates, Jim41 and Gim10 were of equal magnitude in their effect on the dry matter yield of faba beans.

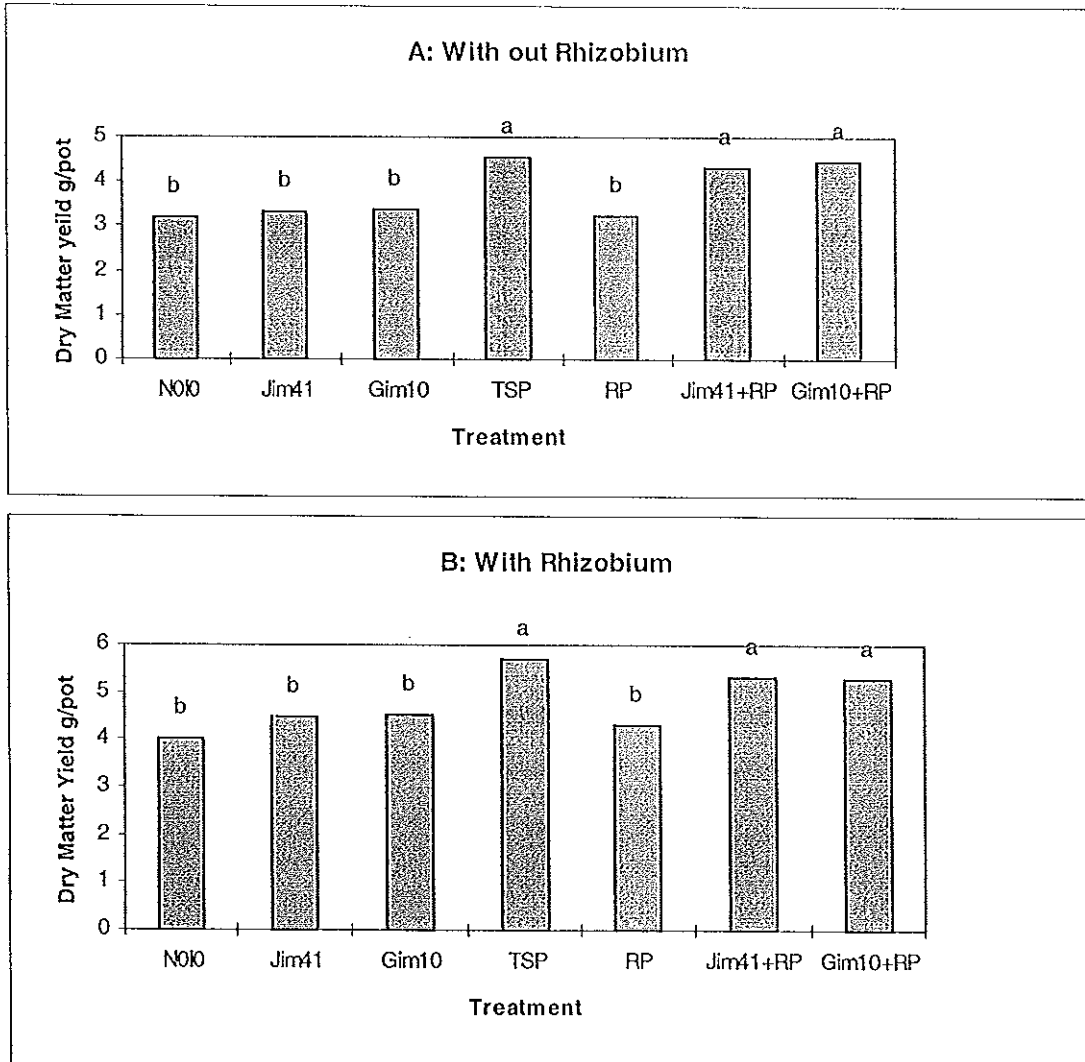
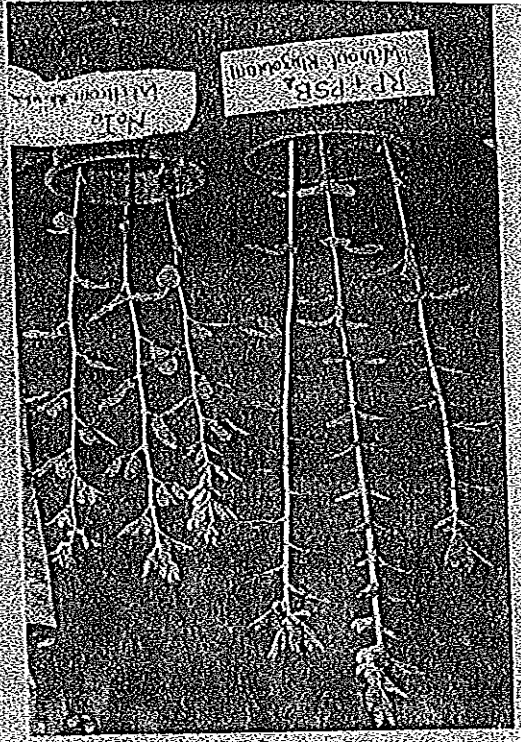


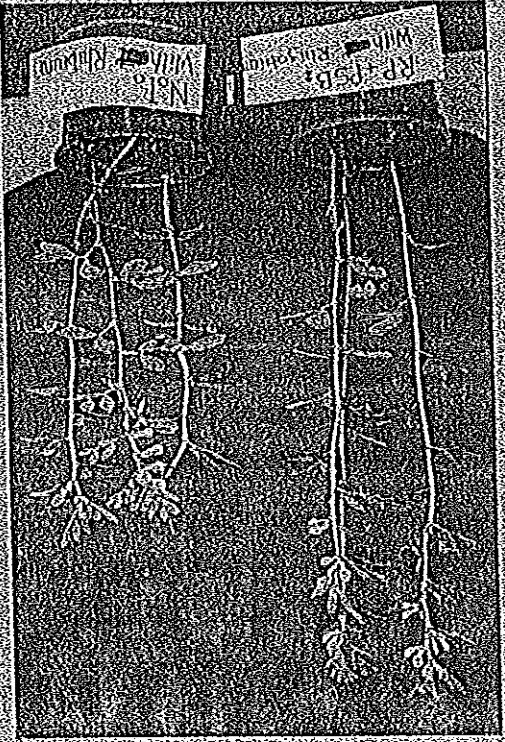
Figure 1. The effect of phosphate solubilizing bacteria on the dry matter yield of fababean (A) without Rhizobium and (B) with Rhizobium inoculation.

777  
777  
777  
777  
777

Picture 2



Picture 3



### 5.7 The effect of inoculation of phosphate solubilizing bacteria on the P-uptake of faba bean

The concentration of P in the dry matter as influenced by inoculation of PSB without and with *Rhizobial* inoculation is shown in Table 7. Inoculation with PSB along with the application of rock phosphate significantly ( $p < 0.05$ ) increased the P-uptake of plants compared to the control and treatments that received rock phosphate or PSB alone.

**Table 7. The effect of phosphate solubilizing bacteria on the P-uptake of faba bean**

Treatments	Concentration of P mg/g	
	Without Rhizobium	With Rhizobium
N <sub>0</sub> I <sub>0</sub>	1.31b*	1.21c
Jim41	1.34b	1.23c
Gim10	1.32b	1.24c
TSP	2.03a	1.70a
PR	1.31b	1.18c
Jim41+PR	2.14a	1.51b
Gim10+PR	1.99a	1.47b

\* Means followed by the same letter are not significantly different from each other according to duncun's multiple range test (DMRT)

However, there was no significant difference in P-uptake among treatments that received either TSP or PSB together with rock phosphate.

**5.8 The effect of inoculation of PSB isolates on the nitrogen nutrition of faba bean.**

The percent nitrogen content, nodule dry weight and number are presented in Table 8. Those treatments that received triple super phosphate or PSB inoculants along with the application of rock phosphate produced the highest ( $p \leq 0.01$ ) percent nitrogen accumulation.

**Table 8. The effect of PSB on the nitrogen content of fababean (Set-II)**

Treatments	N content in %	Nodule dry weight in g/pot	Nodule number
N <sub>0</sub> I <sub>0</sub>	1.82c*	0.060	61
Jim41	2.23c	0.076	99
Gim10	2.48c	0.081	124
TSP	3.41a	0.091	101
PR	2.00c	0.080	103
Jim41+PR	3.21ab	0.090	120
Gim10+PR	3.36a	0.100	90

\* Means followed by the same letter are not statistically different from each other according to DNRT

Even though, it was not statistically significant, there was a difference in the percent N-fixed between plants inoculated with PSB and those uninoculated and unfertilized ones. The corresponding nodule dry weight and number were in consistent with percent N-accumulated.

## 6. Discussion

### 6.1 Phosphate Solubilizing Bacterial isolates from some Ethiopian soils.

Phosphate solubilizing microorganisms occur in nature and have the ability to render insoluble phosphate compounds soluble. Such microorganisms could easily be grown and isolated in a medium containing tricalcium, ferric, aluminum and rock phosphate and old bone (Alexander, 1977; SubaRao, 1993).

In the present study three phosphate solubilizing bacteria coded as Jim41, Gim10, and Hag12 were isolated from red soil samples collected from Jimma, Gimbi and Hageremariam respectively. They were all found to belong to the genus *Pseudomonas*. This result adds to the earlier report that most efficient and frequently encountered phosphate solubilizing bacteria are belonging to the genera of either *Bacillus* or *Pseudomonas* (Gaur, 1987; Sundram, 1994).

### 6.2. Tricalcium phosphate, rock phosphate and old bone solubilization efficiency of PSB Isolates

All PSB isolates including *psuedomonas striata* introduced from India solubilized significantly greater amount of trialcium and rock phosphates over the uninoculated control (Table 3 & 4). The presence of small amount of soluble phosphorus in the uninoculated control flask is believed to be due to the release of  $PO_4$  ions during

autoclaving. This is in agreement to that reported by Agnihotri (1970). There was a progressive increase in the solubilization of tricalcium phosphate by PSB isolates up to 15 days of incubation. There after the increase was less marked. The result is in conformity with the finding of Gaur (1987). With rock phosphate, the solubilization

continued up to 20 days of incubation. In a similar studies, Asfaw (1988) reported that maximum solubilization of rock phosphate was achieved at 20 days of incubation.

As the solubilization of tricalcium and rock phosphate increase, there is a corresponding decrease in the pH of inoculated medium (Agnihotri, 1970; Paul and SundraRao, 1971; Guar, 1987). Paul and SundraRao (1971) studied the relation between tricalcium phosphate solubilization ability of 12 PSB isolates and the pH of the medium. Their result revealed that there is a perfect inverse correlation with amount of TCP solubilized and pH of the medium inoculated with PSB cultures. According to these authors, among twelve PSB tested; the highest amount of tricalcium phosphate solubilization was achieved by *Bacillus brevis* with the lowest pH of 4.4. A similar decrease in the pH of inoculated medium was observed in the present investigation.

The decrease in the pH of the inoculated medium was due to the release of organic acids. Such acids include formic, acetic, propionic, lactic, succinic etc.. (Rao, 1982, SubaRao, 1993). These acids lower the pH and bring about dissolution of bound forms of phosphate. Some of the hydroxyacids may chelate with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{3+}$ ,  $\text{Al}^{3+}$ ,  $\text{Fe}^{3+}$  etc. ions and resulting in effective solubilization (Sperber, 1957).

The type, effectively and quantity of acids released during solubilization of insoluble P-compounds vary with the type of organism and type of insoluble P-compounds. For instance, Gaur (1987) studied extracellular organic acids released by *pseudomonas striata*, *Schwanniomyces acidetalis*, *Aspergillus awmori* and *Penicillium digitatum* and found that *Pseudomonas striata* produced malic, glyoxalic, succinic, and fumaric, citric, tartaric and  $\alpha$  - ketogluconic acids. In the culture filtrate of *Schwanniomyces accidentalis* five organic acids (succinic, fumaric, citric and  $\alpha$  Ketobutyric acids) were identified. Where as *Aspergillus awmori* and *peniucillium dittantum*

synthesized succinic, citric and tartaric acids only. Louw and Webley (1959) reported that most bacteria dissolvers of resistant natural phosphates and silicates produce high amount of alph-ketogluconic acid.

In the present study, the drop in the pH of inoculated medium containing rock phosphate was more drastic than inoculated medium containing tricalcium phosphate. The result is in agreement to that reported by Gaur (1972). This could be due to the high insolubility of rock phosphate compared to tricalcium phosphate.

Thus the same PSB isolate when inoculated in a medium with rock phosphate releases more acid (a consequence of which is a drop in pH) than when does with tricalcium phosphate.

In the case of old bone solubilization study, it was found that Jim41 the most efficient PSB isolate in solubilizing tricalcium phosphate and rock phosphate was the least in solublizing old bone. This suggests that the mechanism employed by PSB isolates in solubilizing old bone is different from that employed in solubilizing TCP and PR. The solubilization of old bone is probably due to the production of enzymes such as acid phosphatases produced by PSB isolates. This could be substantiated by the finding of Guar (1972) who reported that the solubilization of calcium phytate and lecithin by microorganisms was due to the production of enzymes such as phytase and lecithinase.

The maximum amount of soluble phosphorus was brought about from old bone followed by tricalcium phosphate, but the least from rock phosphate irrespective of PSB isolates. This is in line with the finding of Guar (1972).

### **6.3. Establishment of Phosphate solubilizing bacterial inoculants in the rhizosphere of faba bean.**

Inoculation does not always bring an improvement in the growth of plants because there are a number of factors affecting the establishment of inoculated bacteria (Biswas *et al.*, 1994). Thus, the desired improvement in plant growth is expected only when the inoculated bacteria establish well and reach sufficient level of population size.

In the present study, it was found that the number of both PSB inoculants Jim41 and Gim10 increased progressively and was highest at the 7<sup>th</sup> week. Similarly, Kundu and Gaur (1980) found the highest population of phosphobacteria at 7<sup>th</sup> week of inoculation. In general, the number of phosphobacteria in this experiment was sufficiently high to bring the required improvement in the growth of fababean (Kundu and Gaur, 1984).

### **6.4. The effect of inoculation of phosphate solubilizing bacteria on the dry matter yield of fababean.**

Inoculation of PSB isolates together with rock phosphate was found to increase the dry matter yield of Fababean appreciably as compared to the control with and without *Rhizobium* (Figure 1 A & B). The importance of PSB inoculation in augmenting the yield of several crops have been reported by various authors (Datta *et al.*, 1982; Datta *et al.*, 1992; Tomar *et al.*, 1996). For instance a 70% increase in the straw yield of rice over the control was obtained due to inoculation with PSB (Kundu and Guar, 1984). A 25% increase in seed yield of blackgram was obtained due to inoculation with phosphate solubilizing bacteria (Tomar *et al.*, 1993).

The dry matter yield increase due to PSB inoculation together with rock phosphate was highest in the presence of *Rhizobium* than in its

absence. This implies that the yield of crops could further be improved by co-inoculation of nitrogen fixing and phosphate solubilizing bacteria. This is in agreement with Asfaw (1988) who reported that combined inoculation of PSB and *Rhizobium japonicum* showed a beneficial associative effect on soybean over their respective single inoculations.

PSB inoculation together with rock phosphate gave dry matter yield comparable to that obtained with the application of triple super phosphate. This finding is in line with that of Algawadi and Kulkarni (1993) who have found that application of rock phosphate at a rate of 50 kg P<sub>2</sub>O<sub>5</sub>/ha to soil and inoculation with PSB gave equivalent grain yield to that obtained with application of 50 kg/ha as super phosphate containing 46% P<sub>2</sub>O<sub>5</sub>. But inoculation with PSB alone did not significantly increase the dry matter yield compared to the control. However, there was a slight improvement in response to inoculation and this increase may be due to the production of growth promoting hormones. In this regard several works revealed that PSB inoculation alone could improve plant growth not only by increasing phosphorus uptake but also by producing plant promoting hormones (Mishustin and Naumova, 1962; Kundu and Gaur, 1984).

In the laboratory study, isolates Jim41 was more efficient in solubilizing tricalcium and rock phosphate than isolate Gim10. But they were found to be equally important in their effect on the growth in pot experiment. This is because conditions in the laboratory are quite different from those in soils thus PSB isolate efficient in laboratory may not remain efficient when inoculated into soil (Biswas *et al*; 1994).

### **6.5 The effect of inoculation of phosphate solubilizing bacteria on P-uptake of faba bean.**

In the present study the two PSB inoculants along with rock phosphate significantly increased the P-uptake of the faba bean (Table 7). The corresponding increase in the dry matter yield of the crop (Figure 1 A&B) should have been due to increased P-uptake. In this regard Datta and Banik (1997) studied the effect of phosphobacterium (*Bacillus firmus*) plus Missouri rock phosphate on the grain and P-uptake of different rice varieties. Their result revealed that bacterial inoculation along with Missouri rock phosphate significantly improved the grain and P-uptake of all rice varieties. In a similar studies Piccini and Azcon(1987) reported that PSB inoculation enhanced the yield and P-uptake of alfalfa plants.

However, when the two PSB inoculated alone, they did not improve the P-uptake. This is probably due to the low amount native P existing in unavailable form in the soil used in this experiment (Table1). That is to say, whatever P found in the soil used in the pot experiment was present in the available form.

### **6.6 The effect of inoculation of PSB on the nitrogen nutrition of faba bean.**

Inoculation of PSB along with rock phosphate improved the nitrogen fixation (Table 8). This is due to increased availability of P by PSB inoculants. Even though, it was not significant, those plants inoculated with PSB isolate alone had higher percent N than uninoculated and unfertilized control plants. This shows that PSB inoculants promote nitrogen fixation ability of *Rhizobium* spp. This is in agreement with (Kundu and Gaur, 1984; Piccini and Azcon, 1987), who independently reported that inoculation of PSB together with rock phosphate improves the nodulation and nitrogen fixing ability of leguminous crops.

## 7. Conclusions and recommendations

### 7.1 Conclusions

In the present work three Phosphate solubilizing bacteria coded as Jim41, Gim10 and Hag12 were isolated from some Ethiopian soils. They were characterized under the genus *Pseudomonas*.

It was found that all the test organisms solubilized significantly greater amount of tricalcium phosphate and rock phosphate. However, with old bone, two isolates (Gim10 and Hag12) and *P.striata* solubilized appreciable amount with equal efficiency. Whereas, isolate Jim41 which was the most efficient strain in solubilizing TCP and PR was found to be poor in solubilizing old bone. This implies that isolate cannot be used as inoculant of seed or soil, where old bone is needed as insoluble P fertilizer. The maximum amount of soluble P was released by PSB isolates from old bone, followed by tricalcium phosphate and rock phosphate.

Following inoculation in the pot experiment, the two PSB isolates, Jim41 and Gim10 successfully established themselves in the soil.

Inoculation of PSB along with application of rock phosphate increased the dry matter yield and P-uptake of faba bean appreciably. The increment was comparable to that obtained with the application of triple super phosphate. This shows that there is a possibility to replace triple super phosphate fertilizer with PSB together with cheaper rock phosphate.

Neither inoculation with PSB nor rock phosphate alone increased P-uptake of faba bean. From this it could be concluded that the PSB inoculants solubilized the rock phosphate so as to make it available to the crop. Inoculation with PSB has also improved the nitrogen accumulation of faba bean.

## 7.2 Recommendations

Based on the above conclusions the following can be recommended.

- Further isolation and screening program should be launched to get more efficient PSB isolates.
- Field inoculation experiment shall be conducted in different locations with different crops to see whether the PSB inoculants persist in benefiting crops.
- The interaction of PSB inoculants with other useful microorganisms such as nitrogen fixing bacteria and AM fungi in promoting plant growth should be investigated.
- Appropriate carrier material should be developed for efficient PSB isolates
- Effective PSB isolates shall be produced on commercial scale as inoculant and distributed to the farming community.

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