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SCREENING AND CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM THE RHIZOSPHERE SOIL OF *ERYTHRINA BRUCEI*

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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ABSTRACT

Screening and Characterization of Phosphate Solubilizing Bacteria from the Rhizosphere Soil of *Erythrina brucei*

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The study was undertaken to investigate the native phosphate solubilizing bacteria (PSB) isolated from *Erythrina brucei* rhizosphere in National Botanical Research Institute's phosphate growth medium, and assess their multiple beneficial traits. The population of PSB was estimated to be 5.7×10^6 cfu per gram of soil. Nine persistent PSB isolates based on the formation of visible halos were screened for further studies. The halo size was ranged from 2.67 (EbPSB 2) to 8.67mm (EbPSB 7). These isolates also showed a wide range of variations in tolerance level to pH (4.5-11), salt (2.5-10%) and temperature (4-41^{0}c), and most isolates were sensitive to streptomycin and Kanamycin (5-50µg/ml). On the basis of phenotypic characterization, these isolates were tentatively identified as *Pseudomonas* and *Bacillus*. Some of the isolates also showed production of IAA (EbPSB isolate 1, 3, 6 and 7) and antagonism against *Fusarium oxysporum* (EbPSB isolate 1 to 4 and EbPSB 7). Isolates were also tested for their quantitative P-solubilization efficiency on insoluble-P sources. The results revealed that the PSB isolates significantly ($P< 0.01$) solubilized a higher amount of P over uninoculated control. The highest amount was recorded from TCP by EbPSB 7 (41.37mg/100mL) with a concomitant reduction of pH to 3.25 followed by Ferric phosphate and Egyptian rock phosphate and bone meal, respectively. Role of PSB isolates on enhancing germination capacity and seedling vigor of chickpea seed was also performed *in vitro*. EbPSB isolate 6, 7 and 8 resulted in a 100% germination and vigor index of 1260, 830 and 745, respectively. Four isolates showing better germination and seedling performance were further tested for growth promoting efficiency of chickpea plant under greenhouse. These isolates showed a significant positive effect on plant height and dry weight of shoot and root compared to uninoculated pots. EbPSB 6 showed superiority in root (14.7cm/pant) and shoot length (13.8 cm/plant), while EbPSB 7 and EbPSB 3 showed better dry weight in shoot (0.53g/plant) and root (0.401g/plant). In general, the study showed that PSB are diverse in their ability to solubilize P and improve germination and plant growth, suggesting that the most potent PSB isolates, especially EbPSB 6 and EbPSB 7, are worthy of further field testing and of great potential application in improving the yield of these grain legumes.

Keywords: Antagonistic activity, *Fusarium oxysporum*, IAA, NBRIP, Phosphorous

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LIST OF ACRONYMS

%(I)&(D)	Percentage Increase and Decrease
BPB	Bromophenol Blue
EbPSB	*Erythrina brucei* Phosphate Solubilizing Bacterial isolate
ErP	Egyptian Rock Phosphate
FeP	Ferric Phosphate
IARP	Intrinsic Antibiotic Resistance Profile
LB	Luria Bertani
MR-VP	Methyl Red-Voges Proskauer
NBRIP	Nutrient Botanical Research Institute's Phosphate Growth Medium
O/F	Oxidation-Fermentation
PDA	Potato Dextrose Agar
PGI	Percentage Growth Inhibition
P_i	Inorganic Phosphorous
P_o	Organic Phosphorous
PSB	Phosphate Solubilizing Bacteria
PSF	Phosphate Solubilizing Fungi
PSM	Phosphate Solubilizing Microorganism
PVK	Pikovskaya Medium
RCBD	Randomized Complete Block Design
SE	Solubilization Efficiency
SIM	Sulfur Indole Motility
TCP	Tri Calcium Phosphate

CHAPTER ONE
INTRODUCTION

Phosphorus (P) is the major plant growth limiting nutrients despite being abundant in soil in both inorganic and organic forms. This is mainly due to *Pi fixation* that results in the adsorption of P_i on soil solid surfaces and/or precipitation of P_i by Aluminum and Iron ions (Osorio, 2008). To circumvent the problem of 'P' deficiency, phosphate-based fertilizers amounted to 40 million metric tons of P per year were consumed globally for agricultural production and productivity (Vance *et al*., 2003). However, its application encounters low utilization efficiency (25%) of applied phosphatic fertilizer by plants due to chemical fixation (Sandeep *et al*., 2008). This can no longer be achieved by current farming practices alone, which heavily rely on the use of chemical fertilizers; limit its adoption in many developing countries (Osorio, 2008).

In soil, an extensive range of bacteria, fungi and actinomycetes are able to release soluble P from various forms of insoluble phosphate compounds. These microorganisms are termed as Phosphate Solubilizing Microorganisms (PSMs) (Chen *et al*., 2006). The population varies from soil to soil and ranges from less than 10^2 colony forming units (cfu) to $3x10^6$ cfu per gram of soil. (Peix *et al*., 2001). An extensive range of microbes belonging to the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Enterobacter*, *Micrococcus*, *Agrobacterium*, *Aspergillus*, *Penicillium*, *Streptomyces*, *Nocardia* and *Trichoderma* are able to solubilize various forms of precipitated P (Rodriguez and Fraga, 1999).

PSM can grow in media containing insoluble tricalcium phosphate (Pikovskaya, 1948; Nautiyal, 1999), for they release high amounts of P_i in the soil through the production of organic acids, H^+ excreted and chelation. However, P solubilization is a complex phenomenon, which depends on many factors such as nutrition, physiology and growth conditions of the culture (Cunningham and Kuiack, 1992). For the last few decades, there has been long standing interest to manipulate soil microorganisms to improve P nutrition of plants. Seed or soil inoculation with PSM is known to improve solubilization of fixed soil P and applied P resulting in higher crop yields and thus maintain soil health and quality (Deubel and Merbach, 2005).

Most soils in Ethiopian especially Vertisols (heavy black clay soils) are deficient in P when assayed by chemical methods (Tekalign Mamo and Haque, 1987). It is also established that more than 70% of Ethiopian agricultural soils are characterized by P deficiency (Desta Beyene, 1982), which

is very severe in acidic soils of southern, southwestern and western regions. In these areas Al^{3+} and Fe^{3+} are totally incriminated with P fixation (Tekalign Mamo *et al*., 1988). Given the downside and limited access of most farmers to phosphate fertilizers in Ethiopia it is necessary to screen and incorporate into cropping systems some efficient strains of PSMs that can supply P to plants in a more environmentally-friendly and sustainable manner. To this effect, previous writers have attempted to isolate and screen effective PSM from rhizosphere soils of different plants, like faba bean (Wassie Haile, 1999; Assefa Keneni *et al*., 2010) and lentil (Yirgalem Kumelachew, 2009).

In Ethiopia, there are some 12.7 million hectares of Vertisols of which 7.6 million are distributed in the central highlands. They are potentially among the most productive soils, where N and P are the two most important element which are relatively low in Vertisols (Tekalign Mamo *et al*., 1988). Some of the Vertisols are sown to water-tolerant crops such as teff, and chickpea only towards the end of the main rains. This latter practice does not exploit the high productive potential of these soils (Bull, 1988). However, many of these soils are underutilized, about 75% of the highlands Vertisols are uncropped, and crop production is highly constrained because of their very poor internal drainage system causing seasonal waterlogging and part of the arable land is usually affected by erosion unless it is well protected by physical or biological measures (Tilahun Amede *et al*., 2004). Every effort should be exerted to conserve the soil and water of the farmland. Thus, for sustained agriculture on Vertisols, crops with deep-rooted system and adequate drought resistance should be incorporated. In addition, building up organic matter in these Vertisols, will contribute to better workability and soil water conservation (Jutzi *et al*., 1988).

Chickpea is a hardy, deep-rooted, dryland crop and one of the major annual grain legume or 'pulse' crop in Ethiopia. The deep-tap root system enhances the use water from greater depths than other pulse crops and has greater drought tolerance when stored subsoil water is available. Chickpea is an excellent source of protein, fiber, complex carbohydrates, vitamins, and minerals, and has the capacity to fix N. Ethiopia is the sixth largest producer of chickpea worldwide and the largest producer of chickpea in Africa accounting for about 46% of the continent's chickpea production (Menale Kassie *et al*., 2009). It is also one of the newly emerging export commodities being promoted for expansion in Ethiopia. Despite its crucial role in poverty reduction and food security in Ethiopia, chickpea seed production is limited, and chemical fertilizers are the most important input required for its cultivation. In P-deficient soils, chickpea responds poorly to N fertilization without P fertilization (*i.e.* P deficiency most limits nitrogen fixation) (Kosgey, 1994).

The potential of legume tree cropping for sustained cereal yields by incorporating the prunings from hedges of perennials has been proved for the tropical lowlands. In the Ethiopian cool tropics, green fodder is scarce during the dry season and the inclusion of tree legumes in the mixed crop-livestock systems might help alleviate the fodder scarcity as well as increase crop yields by the application of prunings. Agroforestry trees might also recover leached nutrients, fix atmospheric nitrogen and, hence, add additional nutrients to the ecosystem (Anthofer *et al*., 1998).

Erythrina brucei Schweinf. emend. Gillett (Papilionaceae) is an endemic leguminous tree that grow in different agro-ecological zones of Ethiopia (Thulin, 1989). It is a multi-purpose tree legume with desirable agroforestry attributes such as rapid establishment, tolerance to sunlight, presence of spreading canopy, high rate of litter production and rapid litter decomposition and vigorous regrowth after cutting. This tree legume also improve the nutrition of livestock since it has relatively high leaf-nutrient concentration and fairly rich in protein. Thus, it have a bright chance of being integrated into the existing farming system, which in turn limits the proliferation and spreading of pests, and soil erosion which is appeared to be a concern on Vertisols (Legesse Negash, 2002). Teklu Erkossa *et al*. (1999) studied its potential for gully stabilization on a Vertisol. The tree often occurs on farmlands in association with some important annual and perennial crops: barley, enset, maize, sorghum and coffee (Legesse Negash, 2002). *E. brucei* is among the best nitrogen fixing trees tested, and can initiate root nodules when treated with bacterial strains of the genus *Bradyrhizobia* (Fassil Assefa, 1993). Shasho Megersa and Fassil Assefa (2011) studied on the rhizosphere effect of microorganisms and mycorrhizae on growth and biological nitrogen fixation of *Erythrina brucei*; however, the study lacks a well-thought and extensive study of PSB and their PGP properties of these isolates. Hence, the present study initiated to selectively isolate and characterize PSB from the rhizosphere soil of *Erythrina brucei* and evaluate their effect on improving the germination and plant growth of chickpea under *in vitro* and *in vivo* conditions. Attempt was also made to establish the relationship of PSB isolates, solubilizing efficiency, pH and P-solubilization, and determine IAA production and antagonistic activity against *Fusarium oxysporum*.

CHAPTER TWO
OBJECTIVE OF THE STUDY

2.1. GENERAL OBJECTIVE

- To investigate the native phosphate solubilizing bacteria isolated from rhizosphere soil of *Erythrina Brucei* and evaluate their effect on germination and plant growth promotion of chickpea (*Cicer arietinum* L.)

2.2. SPECIFIC OBJECTIVES

- To isolate and evaluate the relative density of phosphate solubilizing rhizobacteria from rhizosphere soil of *Erythrina brucei*.
- To characterize the PSB isolates on the basis of their morphological, biochemical and physiological properties.
- To evaluate the phosphate solubilizing efficiency of PSB isolates on solid and liquid medium with various inorganic phosphate source.
- To optimize phosphate solubilization of the most potent PSB isolate on different carbon (C) and nitrogen (N) sources.
- *In vitro* evaluation PSB isolates on the basis of IAA production and their antagonistic activity towards *Fusarium oxysporum* by dual inoculation technique**.**
- To evaluate the effect of inoculation of selected PSB isolates on different germination parameters *in vitro* and plant growth of chickpea (*Cicer arietinum* L.) under greenhouse conditions.

CHAPTER THREE
LITERATURE REVIEW

3.1. THE LIVING SOIL

When you look at the soil, it may seem like lifeless clay and rocks, but in fact the soil is very much alive. Soil is a structured, heterogeneous and discontinuous system, generally poor in nutrients and energy sources, with microorganisms living in discrete microhabitats. It is also fundamental and irreplaceable, since it governs plant productivity of terrestrial ecosystems and maintains biogeochemical cycles (Anderson *et al*., 1995). Here we focus on soil that is in active, current communion with living plant roots.

The living population inhabiting soil includes macrofauna, mesofauna, microfauna and microflora. Among the microflora: bacteria, fungi, actinomycetes, protozoa, and algae are the most common ones (Egamberdiyeva, 2006). Only 1 to 10% of soil microbiota can be cultured, so there is still much to learn about soil as an environment for microbial life. Bacteria are by far the most numerically abundant soil microbes (Maunuksela, 2001). Even if the available space is extensive in soil, the biological space, the space occupied by living microbes, represents a small proportion, generally < 5% (Ingham *et al*., 1985). Another peculiarity of soil is the presence of 'hot spots', zones of increased microbial/biological activity. In soils, the rhizosphere is probably the greatest of hot spots (Roesti, 2005).

3.2. THE RHIZOSPHERE

Below the soil surface, the rhizosphere is the crossroads of the soil habitat, a hub of biological, chemical, and physical activity surrounding the plant roots. This makes the rhizosphere a complex, dynamic and interactive microenvironment (Hawkes *et al*., 2007).

3.2.1. Definition of Rhizosphere

The term "*rhizosphere*" was introduced by German scientist Lorenz Hiltner in 1904 to designate a narrow zone of soil subjected to influence of living roots (Brimecombe *et al*., 2007). The extent may vary with soil type, plant species and age of plant, but generally extends from the root surface out into the soil for up to a few millimeters (Ali, 1997). The rhizosphere is the area where plants *via* their root systems obtain the necessary nutrients and excrete/diffuse some of the byproducts of their physiological activities. It is a zone of tremendous microbial growth (Starkey, 1958). Rhizosphere microbiota thus provides a critical link between plant and soil environments (Brimecombe *et al*., 2007). Indeed, a myriad of physiological activities occur at the rhizosphere level (Feiro, 1978). The

rhizosphere region includes: (i) ectorhizosphere (the area immediately surrounding root), (ii) rhizoplane (the root surface), and (iii) endorhizosphere (the area within the root) (Abat, 2006).

3.2.2. Characteristics of Rhizosphere Soil

The rhizosphere develops around each root as it grows, changing the chemical, physical, and biological environment that can differ substantially from that of nearby soils. Quantitatively and qualitatively, nutrient availability, water potential, and redox state distinguish this habitat from bulk soil and consequently constrain distribution, abundance, composition, trophic interactions and activity of the diverse rhizosphere biota (Hawkes *et al.*, 2007). A comprehensive review of the rhizosphere physical, chemical, and biological changes (Figure 1) and their association with nutrient availability is being discussed herewith in the following paragraphs.

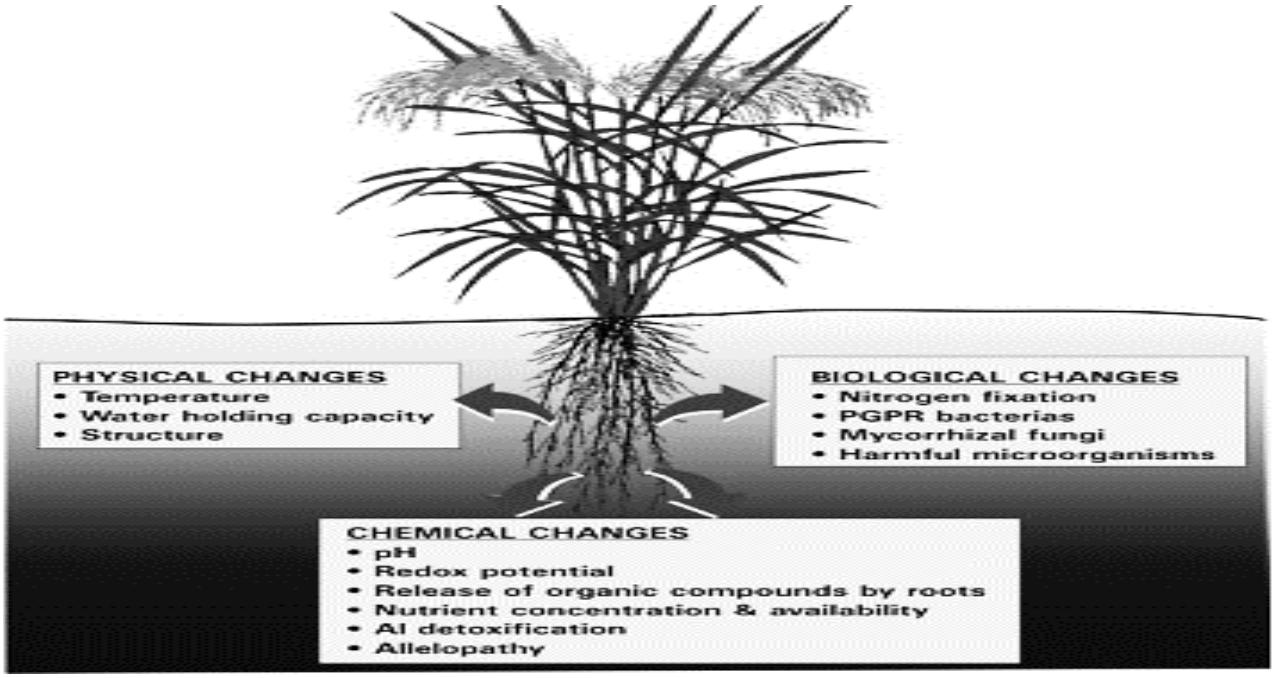


Figure 1: *Physical, chemical, and biological changes in rhizosphere* (Source: Fageria and Stone, 2006)

3.2.2.1. Physical properties

Physical properties are characteristics, processes, or reactions of a soil caused by physical forces, which depend on the amount, size, shape, arrangement and mineral composition of its particles. Physical properties most strongly influencing nutrient uptake include temperature, water availability, and soil structure. These changes are brought about by addition of organic matter by root residue and microorganisms (Fageria and Stone, 2006). The soil temperature affects physical, chemical, and biological processes in rhizosphere and nutrient availability. When soil T^0 is higher than 35^0C and lower than 10^0C, most of the biological processes are adversely affected. The optimum T^0 for maximum production of root material ranges from 20^0C to 30^0C (Brimecombe *et al.*, 2001, 2007).

Plant roots add a substantial amount of organic matter to the rhizosphere that improves soil physical properties such as bulk density, porosity, and pore size distribution in favor of higher infiltration rate and water-holding capacity (McRae and Mehuys, 1985). Plant roots and associated microbes also improve the soil structure and stability *via* root exudates, such as mucilage and sugars (Lombi *et al*., 2001). The size, arrangement, and stability of soil aggregates have a wide influence on soil physical and chemical processes, root growth, and consequently nutrient uptake (Amezketa, 1999).

3.2.2.2. Chemical properties

Several chemical changes take place in rhizosphere due to plant root and soil environmental interactions. Among these: pH, oxidation potential, rhizodeposition, nutrient concentrations, and root exudates are prominent. These changes significantly influence nutrient solubility and their uptake by plants (Fageria and Stone, 2006). Plant roots are known to change the pH of the rhizosphere by extruding protons *via* H^+-ATPase in epidermal cells (Schmidt *et al*., 2003). Changes in soil pH influence nutrient solubility and, hence, nutrients availability to plants. At lower pH (<5.5), availability of micronutrients is higher, and decreases with increasing pH (Fageria and Stone, 2006). Root activity alters rhizosphere redox potential through respiratory oxygen consumption and ion uptake (Bloom *et al*., 1992). Plant roots exude a large and complex assortment of organic and inorganic compounds into nearby soil (rhizodeposition) (Jaeger *et al*., 1999). These compounds bring several chemical changes in root environment, affecting microbial population and availability of nutrients (Neumann and Romheld, 2001). The quantity and quality vary with plant species, genotype, age, physiological status, etc (Jaeger *et al*., 1999).

3.2.2.3. Biotic Interactions

A variety of biotic interactions occur in the rhizosphere that can affect the diversity and composition of microbial community. An important biological change associated with the rhizosphere is root colonization by beneficial and harmful microbes. The plant-beneficial microbes include nitrogen fixing bacteria, plant-growth-promoting rhizobacteria (PGPR), and biocontrol agents (Brimecombe *et al*., 2007). It is typically more common in rhizosphere than in bulk soil, and its importance in determining microbial community composition exceed or equal to that of abiotic factors (Fageria and Stone, 2006). When root passes *via* soil and activates the indigenous microbial community, competition or coordination for resources/space may result, which determine the resulting rhizosphere community. During this process, bacteria interact with each other, and with roots, *via* the release and detection of chemical signal (Perry *et al*., 2007). Since 1990s, interest has particularly developed in quorum sensing

in rhizosphere, in which symbiotic, pathogenic, or plant-associated soil bacteria perceive threshold concentration of signal, inducing a change in gene expression and thus behavior. The signal nature can be specific to one organism or more general (Brelles-Marino and Bedmar, 2001).

3.2.3. Competition in the Rhizosphere

The ability to compete for nutrients and suitable niches within indigenous microbial population in rhizosphere is an important trait for effective survival and propagation of any soil microbes (Walsh *et al.,* 2001). Unless an organism compete favorably and effectively scavenge and utilize the available nutrients, it will not constitute a significant proportion of the rhizosphere-rhizoplane population. Nutrient competition varies at different rhizospheres depending on available sources of carbon, nitrogen, sulfur, phosphate, and micronutrients (O'Sullivan and O'Gara, 1992).

Successful colonization of rhizosphere by microbial inoculants depends on rhizosphere competence. Thus, bacterial strain need to possess particular traits such as chemotaxis towards root exudates, compound mediating attachment and a capacity to metabolize root exudates (Walsh *et al*., 2001). Since, *Pseudomonas* possesses root competence traits they are used as models of rhizobacteria (Sorensen *et al*., 2001). They constitutes up to 10% of the culturable rhizospheric microflora. Some Pseudomonad isolates act as biocontrol agent against soil pathogen, *Pythium ultimum*, by metabolizing constituents of seed exudates to produce inhibitory compounds (Glick and Bashan, 1997). Competitive exclusion of deleterious organisms by fluorcent pseudomonads (O'Sullivan and O'Gara, 1992) and *Azospirillum brasilense* (Bashan and de-Bashan, 2002) have been also observed.

3.3. Rhizosphere Microbial Communities and Rhizosphere Effect

The rhizosphere region is a highly favorable habitat for the proliferation, activity and metabolism of numerous microbes. Thus, studying organisms in rhizosphere, and more generally in soil, is not a straightforward task. Physically removing microbes from soil is also nontrivial, particularly from intact rhizosphere soil (Roesti, 2005). However, the recent development and popularity of molecular techniques, like small subunit 16S ribosomal DNA gene (16S rDNA), has allowed us to identify and characterize complex microbial communities belowground (Doi *et al.,* 2007).

The rhizosphere microflora can be enumerated intensively by microscopic, cultural and biochemical techniques. Microscopic techniques reveal the types of organisms present and their physical association with the outer root surface. The cultural technique most commonly followed by "serial dilution and plate count method" reveal the quantitative and qualitative population of microflora. At

the same time, a cultural method shows the selective enhancement of certain categories of bacteria. The biochemical techniques used are designed to measure a specific change brought about by the plant or by the microflora (My Agriculture Information bank, 2009).

Plant species can be important in determining the structure of rhizosphere microbial communities. Within plant species, microbial communities can be affected by plant genotype, nutrient status, pathogen and mycorrhizal infection. Within root systems, microbial communities can even differ among root zones and distances from the root surface (Marilley and Aragno, 1999). The rhizosphere is also characterized by large environmental fluctuations (see section 3.2.2), which may promote high microbial diversity in rhizosphere. Rhizosphere microbial communities can significantly influence phytopathogenes development (Egamberdiyeva, 2006), nutrient acquisition (Artursson *et al*., 2006), biotransformation of hazardous organic compounds (Anderson *et al*., 1995), and ecological fitness of plants (Barea *et al*., 2005). The rhizosphere harbors very high numbers and activities of organisms that can reach 10^{10}-10^{12} per gram of soil as compared to often $<10^{8}$ in bulk soil (Reyes *et al*., 2006).

The term 'rhizosphere-effect' indicates the overall influence of plant roots on soil microbes. It is often expressed as the ratio of number of microorganisms in rhizosphere to non rhizosphere soil, the R/S ratio; commonly range from 5-20, but occasionally as high as 100 and above (Anderson *et al*., 1995).Several factors such as soil type, moisture, pH, temperature, plant age and relative humidity are known to influence rhizosphere effect (Manoharachary and Mukerji, 2006)

Bacteria: The greater rhizosphere effect is observed with bacteria (values ranging from 10-20 or more) than with actinomycetes and fungi. Bacteria that are adapted to rhizospheric living conditions, rhizobacteria, can affect the plant development either negatively (phytopathogenes) or positively (PGPR) (Roesti, 2005). The bacterial population is enormous ranging from 10^{8}-10^{9} per gram of rhizosphere soil (Jaeger *et al*., 1999), but the most common genera are as follows: *Pseudomonas, Bacillus, Agrobacterium, Arthrobacter, Alcaligenes, Azotobacter, Clostridium* and *Micrococcus*. They cover about 4-10% of the total root area occurring profusely on the root hair and rarely in the root tips. Gram-negative, rod shaped, non-sporulating bacteria *(Pseudomonas)* which respond to root exudates are predominant, while Gram-positive, rods, cocci and aerobic spore forming *(Bacillus, Clostridium)* are comparatively rare in the rhizosphere (Rodríguez and Fraga, 1999). The proliferation of rhizobacteria have been reported to takes place in zone of root elongation (Jaeger *et al*., 1999), indicating that this is an area of maximum root exudation.

Fungi: In contrast to their effects on bacteria, plant roots do not alter/enhance the total count of fungi in rhizosphere. However, rhizosphere effect is selective and significant on specific genera *(Fusarium, Verticillium, Aspergillus* and *Penicillium).* The R:S ratio of fungal population is narrow in most plants, usually not exceeding 10. The soil serial dilution and plating technique used for enumeration of fungi may often give erratic results as most of the spore formers produce abundant colonies in culture media giving a wrong picture/estimate (Krasilnikov, 1968).

Actinomycetes, Protozoa and Algae: It is generally understood that the actinomycetes are less stimulated in rhizosphere than bacteria. Only negligible changes in rhizosphere effect are noted with regard to protozoa and algae. When antagonistic actinomycetes increase in number they suppress bacteria. Among the actinomycete, the phosphate solubilizers *(Nocardia, Streptomyces)* have a dominant role to play. As a rule they are not significantly influenced by their proximity to the plant roots and their R:S ratios rarely exceed 2 to 3:1 (Krasilnikov, 1968).

3.4. PHOSPHORUS IN THE SOIL SYSTEM AND ITS AVAILABILITY TO PLANTS

3.4.1. Phosphorous

Phosphorus (P) was chemically identified in 1699 by German alchemist Henning Brandt in search of the legendary '*Philosopher's Stone*' which would supposedly turn metal into gold. Whilst he found no such magical stone, Brandt discovered the pure form of P, which glowed in the dark (Emsley, 2000). The word phosphorus is derived from the Greek "phos" meaning *light* and "phorus" meaning *bringing* (Johnston and Steen, 2000).

Although P is widely distributed, the elemental form of P does not occur by itself in nature. Rather, it is a very stable and often assumed to lack a gaseous species for atmospheric transport, and does not readily leach (Morton, 2003). The reason for the stability of P in soils is that they are extremely reactive with other compounds, to form phosphates (Minnesota Department of Agriculture, 2004). Phosphorous is essential to all known life forms, next to N and classed as an essential macronutrient taken up by plant roots for growth and development (Grover, 2003).

3.4.2. Essential Role of Phosphorous (P)

Phosphorus, the master key element, is known to be associated with a plethora of vital functions and responsible for several characteristics of plant growth and metabolism (Mahdi *et al*., 2011). Phosphorus is an integral part of cellular activities and a number of important compounds present in plant or animal cells (Bagyaraj *et al*., 2000). Phosphate is a vital component of Adenosine Tri-

Phosphate (ATP) and Adenosine Di-Phosphate (ADP). They are high-energy P compounds that control most processes in plants including photosynthesis, respiration, protein and nucleic acid synthesis, and nutrient transport. Phosphorous is a vital component of nucleic acid structure (DNA and RNA) of all living things (Cordell, 2010). P is also one of the primary structural components of biological membranes, phospholipids, which surround plant cells (Johnston and Steen, 2000). So, cellular machinery is difficult to be imagined without P being involved (Mahdi *et al*., 2011).

The role of P in food production is arguably the most significant to society given a) around 90% of P used globally is for food production and b) there is no substitute for P in food production and food is a fundamental basis for human societies (Cordell, 2010). In addition to aforementioned roles, the agronomic literature is full of examples where adequate supply of P in the early stages of plant growth promotes many physiological functions including: (1) promote early root formation, flower formation and seed production, (2) promote a more uniform and earlier crop maturity, (3) improve crop quality and nitrogen fixing capacity of legumes and (4) increased stalk and stem strength, and also resistance to plant diseases. Due to this and other reasons, P becomes a necessary and beneficial input for crop production (Armstrong, 1999; Cordell, 2010). It is clear, therefore, that inadequate supply of P to crops causes P deficiency leading to reduction in number and surface area of leaf, photosynthetic capacity, and a decline of plant growth (Grant *et al*., 2001). A deficiency of P also affects the quality of fruit and the formation of seeds (Johnston and Steen, 2000), delayed maturity, reduced quality of forage, vegetable, and grain crops, and decreased disease resistance (Armstrong, 1999).

3.4.3. Forms and Availability of Phosphorous in Soil

The total soil P concentration usually ranges from 100 and 3000mg P kg^{-1} of soil (Frossard *et al*., 2000), and soil solution in agricultural soils, which is the main source of P for plant roots, contains between 0.01 and 3.0mg P L^{-1} or around 0.1% of the total P (Kuhad *et al*., 2011). Thus, most of the P is often present in unavailable forms to growing plants (Schachtman *et al*., 1998). Mineral forms of P constitute the biggest reservoirs, represented primarily by rocks and deposits formed during geological age. The principal characteristic of these minerals is their insolubility (Achal, 2005). Inorganic soil phosphorus (P_i) consist of apatite, complexes of Fe, Al (in acidic conditions) or Ca phosphates (in neutral to alkaline soils), and P absorbed on clay particles. A second major component of soil P is organic matter (P_o), present largely in the forms of inositol phosphate, accounting for up to 50% of the total organic P. Other organic P found in the form of phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters (Mahdi *et al*., 2011). The proportion of total soil

P present in organic forms ranges from 30 to 65% (Frossard *et al*., 2000). In soils, P may exist in many different forms. In practical terms, however, P in soils can be thought of existing in 3 "pools":

The **solution P** pool is very small and usually contains only a fraction of a pound of P per acre. The solution P pool consists of usually in orthophosphate form (plant available P), but small amounts of organic P (unavailable P) may exist as well (Achal, 2005). Phosphorus in the soil solution of most agricultural soils ranges from <0.01 to 1 ppm (Mullins, 2009). The solution P pool is important because it is the pool from which plants take up P. A growing crop would quickly deplete the P in soluble P pool if it was not being continuously replenished (Busman *et al*., 2002).

The **active P** pool is P in solid phase attached to soil particles and which is relatively easily released to soil solution. The amount of P in active P pool is generally between 10-300 pounds per acre (Minnesota Department of Agriculture, 2004). As plants take up P, the concentration of P in solution is decreased and some phosphate from the active P pool is released to the solution pool. The ability of the active P pool to replenish the solution P pool in a soil is what makes a soil fertile. The active P pool contains P_i that reacted with Ca/Al, and organic P that is easily mineralized (Busman *et al*., 2002).

The **fixed P** pool contains inorganic phosphate compounds that are very insoluble and organic compounds that are resistant to mineralization by soil microorganism. The amount of P in the fixed P pool can range from 300 to 3000 pounds per acre (Minnesota Department of Agriculture, 2004). Phosphate in this pool may remain in soils for years without being made available to plants and may have very little impact on fertility of soil (Busman *et al*., 2002).

Many factors influence content of soil P, among these: (i) plant and chemical nature of its parent material; (ii) degree of weathering; and (iii) climatic conditions are the major ones. In addition, erosion, crop removal and P fertilization also affect it (Bagyaraj *et al*., 2000).

3.4.4. Plant-Available Phosphorus in the Soil System

Despite of being so rich, the concentration of soluble/bioavailable P is usually very low in soils due to the phenomenon of chemical fixation and immobilization of phosphate (Tate, 1984). The term available P is often used to express the amount of soil P in solution that can be extracted by roots and utilized by plant for growth and development. Since it is one of the most unavailable and inaccessible nutrient, establishing a plant's requirement for P, and the ability of the soil to supply this requirement, is crucial in managing cropping systems (Holfold, 1997).

Determining the amount of P available for plant growth is accomplished by having soil tested. In laboratory, soil samples are subjected to various methods of P extraction. The amount extracted over a specified length of time is considered plant available P and include both soluble and active pools of P. Soil test results are reported in parts per million. For soils with pH of 7.4 or less, Bray extraction method is used, while for pH higher than 7.4 Olsen is used (Frank *et al*., 1998)

Concentrations of P in plant tissues typically range from 0.1 to 0.5% on a dry weight basis and most crops utilize between 20 to 90 pounds of P_2O_5 each year (Mullins, 2009). Plants take up P almost entirely as the negatively charged primary orthophosphate anion ($H_2PO_4^-$) and smaller amounts of the 2^0 orthophosphate anion (HPO_4^{2-}), which occur in soil solutions at very low concentrations (0.1-10 µM) (Raghothama, 1999; Hinsinger, 2001). This is an active, energy dependent process where uptake occurs against an electrochemical gradient and is mediated by H^+ co-transport (Frossard *et al*., 1995). The ratio of these two ions in soil solution is pH dependent where, acidic soils favor $H_2PO_4^-$, and alkaline favor HPO_4^{2-}. Maximum availability of soil P occurs between pH of 6.5-7.0 (Diaz *et al*., 2011). Uptake of these phosphates by plants from soil solution causes an imbalance in the equilibrium between the different pools of P, which lead the formation of a concentration gradient of P_i between the solid phase and the soil solution around the roots. This gradient regulates the rate of P diffusion toward the plant root. However, the rate of P_i diffusion is quite slow (10^{-12}-$10^{-15} m^2 s^{-1}$) which limits the P_i supply and creates a depletion zone of P_i around the roots of approximately of 1–2 mm, potentially leading to P deficiency in plants (Schachtman *et al*., 1998).

In many plants, a prolonged reduction in the availability of P_i reduces their yield potential. However, in response to persistently low levels of P_i in the rhizosphere, plants have evolved highly specialized physiological and biochemical mechanisms to cope with low P availability (Raghothama, 1999).

Generally, three broad categories of P efficiency mechanisms exist in plants to increase availability and uptake of P under deficiency conditions: (i) alteration of the geometry or architecture of the root system, (ii) secretion or exudation of chemical compounds into the rhizosphere, and (iii) association with rhizosphere microorganisms (Rengel and Marschner, 2005). The mechanisms they have developed act both to increase acquisition of P from the soil and to improve its use internally (Raghothama, 1999). The third mechanism is being discussed in detail herewith in the next section with related to PSM.

Table 1: *Multiple responses of plants to phosphate deficiency* (Source: Raghothama, 1999)

Morphological responses
Increased root:shoot ratio; changes in root morphology and architecture; increased root hair proliferation; root hair elongation; accumulation of anthocyanin pigments; proteoid root formation; increased association with mycorrhizal fungi
Physiological
Enhanced Pi uptake; reduced Pi efflux; increased Pi use efficiency; mobilization of Pi from the vacuole to cytoplasm; increased translocation of phosphorus within plants; retention of more Pi in roots; secretion of organic acids; protons and chelaters; secretion of phosphatases and RNases; altered respiration; carbon metabolism; photosynthesis; nitrogen fixation; and aromatic enzyme pathways
Biochemical
Activation of enzymes; enhanced production of phosphatases; RNases and organic acids, changes in protein phosphorylation; activation of glycolytic bypass pathway
Molecular
Activation of genes (RNases, phosphatases, phosphate transporters, Ca-ATPase, vegetative storage proteins, β-glucosidase, PEPCase, novel genes such as *TPSII*, *Mt 4*)

The P availability for plant growth depends not only on the total amount of P but also on its solubility, which in turn dictated by chemical reactions and biological interaction (Muraleedharan *et al*., 2010). In addition, different parameters such as growth stage, plant species, soil pH, concentration of Ca^{2+}, Fe^{3+} and Al^{3+}, amount of organic matter, soil moisture, texture and root exudates can affect P concentration in plants (Hinsinger, 2001; Diaz *et al*., 2011).

3.5. PHOSPHATE SOLUBILIZATION BY SOIL MICROORGANISMS

3.5.1. Introduction

Worldwide, 5.7 billion hectares contain too little available P for sustaining optimal crop production. Suboptimal levels of P can lead to a 5-15% loss in the yield of plants (Hinsinger, 2001). To circumvent P-deficiency, synthetic fertilizers are applied. The repeated and injudicious applications, however, lead to (i) the loss of fertility, (ii) disturbance in microbial diversity and their metabolic activities, and (iii) reduced yield of agronomic crops. Moreover, after application, almost 75-90% of applied P fertilizer is precipitated into less available forms (Sundara *et al.*, 2002), creating a demand for suitable alternatives to mobilize this important bioelement.

Given the negative impacts and increasing costs, the search for environmentally friendly and economically feasible strategy has started. Thus, major scientific advances in the field of Microbiology and Biotechnology provide a biological alternative to the use of chemical fertilizers, and for improving

crop production in low or P deficient soil (Dobbelaere *et al*., 2003). Such strategies enable the efficient use of PSM as an alternative and/or supplementary to the use of P fertilizers in agriculture (Mehrvarz *et al*., 2008). The latter sections aims to identify the different types of PSM, the types of P they can utilize, the mechanisms employed, and the potential for managing them in agricultural context.

3.5.2. Phosphate Solubilizing Microorganisms (PSMs)

Evidence of naturally occurring rhizosphere PSM dates back as early as 1903 (Kucey *et al*., 1989), and the trial to improve P availability to crops *via* microbial means was first demonstrated in Russia (Sundara *et al*., 2002). PSMs refer to a group of microbes that as components of P cycle, not only assimilate P but also cause a large portion of soluble P to be released in excess of their requirement from insoluble P sources (Kumar, 2003), which include bone meal, rock phosphate, tricalcium phosphate, etc (Gangasuresh *et al*., 2010). It was estimated that PSMs may constitute 20 to 40% of the culturable population of soil microbes and a significant proportion can be isolated from rhizosphere (Kucey, 1983). Therefore, their use in agricultural practice would not only offset the high cost of phosphatic fertilizers but also mobilize insoluble P in fertilizer and soils (Rodriguez and Fraga, 1999).

Several scientists have reported the ability of different rhizosphere microbes that have the potential to enhance the rate of P_o or P_i cycling by solubilizing insoluble organic and mineral bound P (Kucey, 1983; Khan *et al*., 2009), and have therefore been used to enhance the solubilization of reprecipitated soil P for crop improvement (Sharma *et al*., 2007). Although there is good evidence for PS by microbes in pure culture, it is difficult to validate in plant-microorganism systems (Bashan and Holgin, 1997).

Among the different soil fungal communities, *Penicillium* and *Aspergillus* are identified to be the most active phosphate solubilizing fungi (PSF) (El-Azouni, 2008*).* JumpStart®, the first P-solubilizing inoculant on the market, contains the fungus *P. bilaiae*, as the active ingredient (Takeda and Knight, 2006). In addition, *P. radicum* and *P. aurantiogriseum* solubilized of TCP, Fe-P, and Al-P (Illmer *et al*., 1995). Agnihotri (1970) reported the solubilization of fluorapatite, hydroxyapatite and TCP by *Aspergillus niger*, *A. flavus*, *Fusarium oxysporum*. Arbuscular mycorrhizae fungi are also known to enhance P nutrition of plants especially in P-deficient soils by scavenging the available P due to the large surface area of their hyphae and by their high-affinity P uptake mechanisms (Kuhad *et al*., 2011).

In addition to PSF, P-solubilizing bacteria (PSB) are also present in soil and plant rhizospheres. The population is higher in rhizosphere than non-rhizosphere (Katznelson *et al*., 1962). PSB strain belonging to genera *Pseudomonas, Bacillus, Enterobacter, Serratia, Rhizobium, Azospirillum* and

Burkholderia are the most important ones. They have the ability to solubilize P_i compounds such as TCP, RP, and hydroxyl apatite (Mahdi *et al*., 2011). Rodriguez and Fraga (1999) compared 13 bacteria of different genera for their solubilizing abilities on different insoluble P substrates and indicated that *Pseudomonas, Bacillus* and *Rhizobium* species were among the most powerful P solubilizers, while TCP and hydroxyl apatite more degradable substrates. In addition, *Actinomycete*, *Micromonospora*, *Nocardia* and *Streptomyces* have also been reported to solubilize P (Kuhad *et al*., 2011).

In general, PSF exhibit superior PS activity than their bacterial counterpart on both solid and liquid culture (Kucey, 1983). This may be due to the presence of fungal hyphae that was found to be attached to P-mineral particles in liquid culture. Furthermore, fungi hyphae are able to reach greater distances more easily than bacteria. The PS ability in bacteria is generally reduced/lost upon repeated sub culturing but no such loss has been observed in PSF (Kucey 1983). However, since bacteria tend to be more robust and more amenable for large volume high density culture and high-volume production, the current research emphasized on the biotechnological potential of bacterial isolates.

3.5.3. Occurrence and Distribution of Phosphate Solubilizing Bacteria

Phosphate solubilizing bacteria are ubiquitous but they are reportedly varied in density and mineral PS ability, generally isolated from rhizosphere soil using serial plate dilution method or by enrichment culture technique (Zaidi *et al*., 2009). PSB can be found in cocci (0.5µm), bacilli (0.5-0.3µm) or spiral (1-100µm) shapes. Bacilli are common, whereas spirilli are very rare (Khan *et al*., 2009). As observed by different researchers, the numbers of PSB are concentrated in rhizosphere, and they are known to be metabolically more active than those isolated from non rhizosphere. PSB constitute 1-50% and fungi 0.5-0.1% of the total respective population (Kuhad *et al*., 2011).

An extensive range of aerobic and anaerobic soil bacteria including actinomycetes are able to solubilize various forms of insoluble P_i compounds (Gulati *et al*., 2008). The prominent genera involved in mineral PS are *Pseudomonas, Bacillus, Rhizobium, and Enterobacter* along with *Burkholderia, Agrobacterium, Microccocus, Aerobacter, Erwinia, Serratia, Flavobacterium,* and *Xanthomonas* sp. (Kuhad *et al*., 2011; Whitelaw, 2000). P-solubilizing ability of PSB is affected by many physiological factors such as C and N source in medium, mineral source, pH, aeration, incubation temperature and incubation period (Vassilev and Vassileva, 2003). The PSB population also depends on different soil properties (physical and chemical properties, organic matter, and P content) and cultural activities (Kim et *al*., 1998).

3.6. ASSESSING PHOSPHATE SOLUBILIZATION BY MICROORGANISMS

3.6.1. Assessing Techniques

There are two main techniques that have been used for detection and estimation of the phosphate solubilization ability of microorganisms. One uses a precipitated phosphate agar plate assay and the other uses a liquid media/culture broth. The agar plate screening method are used widely in the initial selection for PSMs (Jia Xie, 2008). Microorganisms capable of solubilizing P minerals are grown on an agar medium with insoluble phosphates (like TCP) as the only P source and produce a visible clear zone around their colonies (Pikovskaya, 1948). To improve the clarity of clear zone, dyes such as Bromophenol blue (BPB) and alizarin red S is often used in the agar media (Cunningham and Kuiack, 1992). It is used to screen large numbers of isolates quickly and simultaneously (Jia Xie, 2008).

Despite the popularity, the reliability of this halo-based technique is questioned as many isolates which did not produce any visible halo on plates could solubilize insoluble P_i in liquid medium (Nautiyal, 1999). Thus, the existing plate assay fails when halo zone is inconspicuous or absent. This may be due to the varying diffusion rates of organic acids secreted by organism (Whitelaw, 2000). In contrast to indirect measurement of PS by plate assay, the direct measurement of PS in broth assay is more accurate and reliable (Nautiyal, 1999). The liquid culture technique measures the released P from the initial insoluble P substrate used. The rate of PS is typically estimated by subtracting the final culture solution-P from un-inoculated control (Rodriguez and Fraga, 1999). Unfortunately, the liquid media method is labor intensive and time consuming (Jia Xie, 2008).

3.6.2. Novel Microbiological Media for PSM Screening

Phosphate solubilizing microorganisms are routinely screened by a plate assay method using Pikovskaya (PVK) agar medium. It was first suggested by Pikovskaya in 1948 to contain (g/L): glucose, 10g; $Ca_3(PO_4)_2$, 5g; $(NH_4)_2SO_4$, 0.5g; NaCl, 0.2g; $MgSO_4.7H_2O$, 0.1g; KCl, 0.2g; yeast extract, 0.5g; $MnSO_4.H_2O$, 0.002g; and $FeSO_4.7H_2O$, 0.002g. This medium has been widely used for isolation, enumeration and maintenance of PSMs (Kumar, 2003). To improve the clarity and visibility of the halo, a modified PVK medium using BPB was suggested. But, this too has not improved the PVK plate assay (Nautiyal, 1999). This and other reasons led to the development of a suitable medium for screening PSMs and to establish a procedure for identification of the most efficient P-solubilizers.

Therefore, a novel-defined microbiological growth medium, National Botanical Research Institute's phosphate growth medium (NBRIP), was formulated for screening PSMs. It was developed based on

PVK in 1999 by Nautiyal. It contains (g/L): glucose, 10g; $Ca_3(PO_4)_2$, 5g; $MgCl_2.6H_2O$, 5g; $MgSO_4.7H_2O$, 0.25g; KCl, 0.2g and $(NH_4)_2SO_4$, 0.01g. NBRIP was comparable to PVK agar medium; however, in broth assay, NBRIP medium consistently resulted in 3-fold increase in PS. The rate of PS was increased with increased concentrations of glucose. Also, glucose, xylose and sucrose are reported to be good C sources for PSF, while for bacteria glucose, galactose and sucrose were effective (Kuhad *et al*., 2011). P-solubilization ability was improved with $(NH_4)_2SO_4$ at a lower concentration and was 27.1% more effective than KNO_3 as observed by Nautiyal (1999). Moreover, quantities of TCP and hydroxyapatite solubilized were much greater than RP, Al and Fe phosphate (Kuhad *et al*., 2011).

Thus, NBRIP can be used as a defined medium because it excludes the use of yeast extract. Secondly, NBRIP is more efficient in a broth assay compared to PVK (Nautiyal *et al*., 2000). However, screening a large number of isolates for quantitative P-estimation requires quite a lot of time, labor, and chemicals, Mehta and Nautiyal (2001) modified NBRIP medium using BPB, a blue-colored dye that decolorizes owing to a drop in pH, as an indicator will help to qualitatively evaluate the level of PS quickly and reliably based upon visual observations. Accordingly their invention provides a novel synergistic formulation containing 0.01-0.1 mg/ml BPB was designated, "NBRI-BPB" which comprises (g/L): glucose, 10; $Ca_3(PO_4)_2$, 5; $MgCl_2.6H_2O$, 5; $MgSO_4.7H_2O$, 0.25; KCl, 0.2; $(NH_4)_2SO_4$, 0.1 and BPB, 0.025 g. Therefore, it is suggested that NBRI-BPB should serve as an excellent formulation for the easy and fast screening and grouping of a large number of phosphate solubilizers (Mehta and Nautiyal, 2001; Nautiyal *et al*., 2003).

3.7. Mechanisms of Phosphate Solubilization

At the molecular genetics level, the precise mechanism used by different PSMs still remains mostly unidentified. Nevertheless, several theories have been proposed to explain the mechanism of microbial P-solubilization (Rodriguez and Fraga, 1999). Important among them are: (i) Acid production theory, and (ii) proton and enzyme theory.

3.7.1. Acid Production Theory

According to this theory, the process of PS is due to the production of organic acids which is accompanied by the acidification of the medium. A drop in pH from 7.0 to 4.0 was recorded by many workers (Panhwar *et al*., 2009). Organic acids produced by PSM are determined by paper chromatography or thin layer chromatography and by modern techniques such as high pressure liquid chromatography and enzymatic methods (Illmer *et al*., 1995). The release of organic acids such as

gluconic, 2-ketogluconic, citric, lactic, succinic, oxalic and acetic acid by PSB as a primary mechanism of inorganic PS, has been well documented (Rodriguez and Fraga, 1999; Rashid *et al*., 2004; Kuhad *et al*., 2011). Similarities or differences may be explained on the basis of the type and quantity of acid produced, which depends on the PSM strain, media composition, growth conditions and several other factors (Alam *et al*., 2002). Besides the acid strength, the type and position of the ligand determine isolates effectiveness on solubilization process (Kuhad *et al*., 2011).

In case of Gram negative bacteria, gluconic acid seems to be the most frequent and principal organic acid produced by PSB such as *Pseudomonas cepacia* (Goldstein *et al*., 1993), *Azospirillum brasilense* (Rodriguez *et al*., 2004) and *Gluconacetobacter diazotrophicus* (Crespo *et al*., 2011). Cunningham and Kuiack (1992) reported that citric acid was major organic acid produced by *P. bilji* when nitrogen source was nitrate. Rashid *et al*. (2004) also found oxalic and citric acids to be the major acids produced by all the tested PSM strains. According to Roger *et al*. (1993), gluconic acid and 2-ketogluconic acid were the predominant organic acids released by strains of *Pseudomonas*. Moreover, citrate released by strains of *Serratia* sp. has been shown to effectively mobilize iron phosphate (Perez *et al*., 2007). Although many researchers support organic acid theory, the amount of soluble P is difficult to correlate with the organic acid produced (Illmer *et al*., 1995). Two most probable explanations for this are:

3.7.2. Proton and Enzyme Theory

Proton extrusion mechanism is a probable mechanism of PS in some microbes, which is due to the release of H^+ accompanying respiration and/or NH_4^+ assimilation (Illmer *et al*., 1995). Thus, more solubilization occurs when ammonium was used as sole N source in the media (Narsian and Patel, 2000). In addition, mineralization of most P_o compounds in soils is carried out by means of phosphatase enzymes (Grover, 2003). Phosphatases are collective name for extracellular enzymes that cleave phosphate from P_o compounds **(**e.g. phospholipids),liberating P_i (Kumar, 2003**).**These observations indicate that PS is a complex phenomenon which depends on factors such as nutritional, physiological and growth conditions of the cultures (Cunningham and Kuiack 1992).

Besides these two mechanisms the production of chelating substances (Cunningham and Kuiack, 1992), H_2S, CO_2 (Kumar, 2003) mineral acids, siderophores (Khan *et al*., 2009) biologically active substances like IAA, gibberellins and cytokinins (Kucey *et al.*, 1989) are also correlated with PS. Chelation involves the formation of two or more coordinated bonds between a molecule (the "ligand") and a metal ion resulting in a ring structure complex (Whitelaw, 2000)

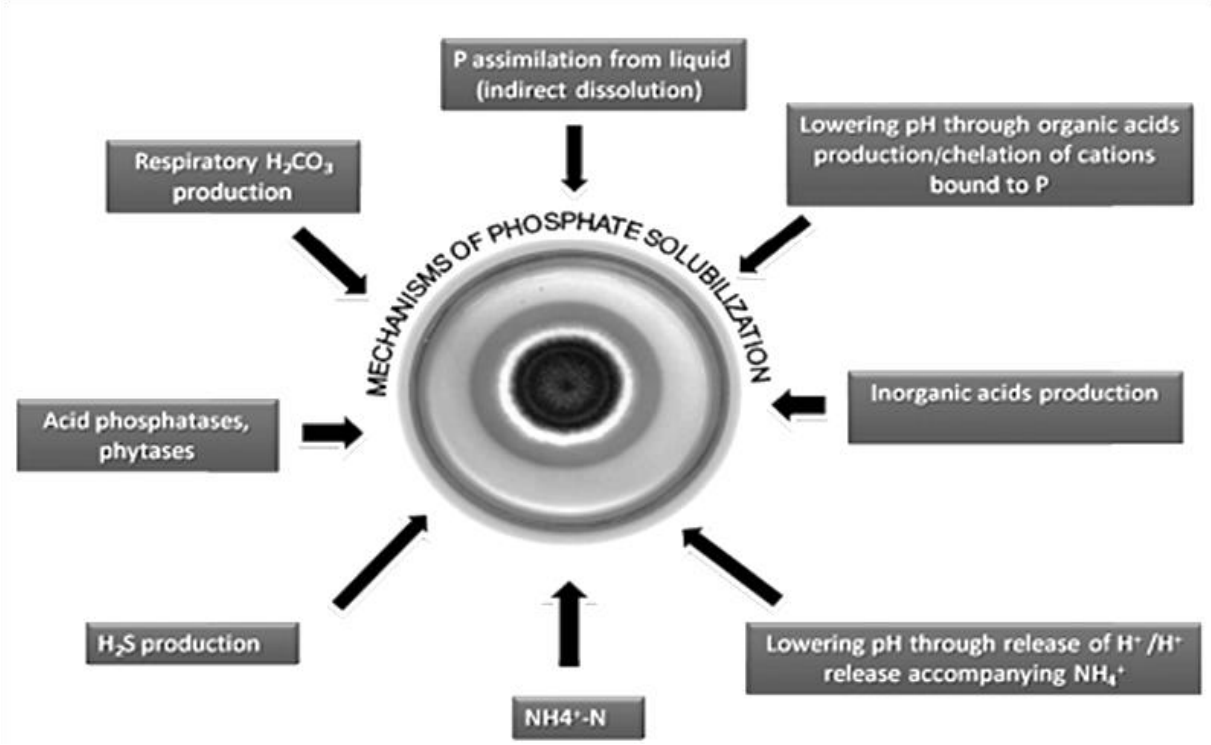


Figure 2: *Mechanism of phosphate solubilization by PSM* (Source: Khan *et al*., 2012)

3.8. The Role of Phosphate Solubilizing Bacteria on Crop Production

The substantial number of Free-living bacterial species, mostly those associated with the rhizosphere, may exert a beneficial effect upon plant growth. This group of bacteria has been termed "plant growth promoting rhizobacteria" or PGPR (Kloepper *et al*., 1980). Generally, about 2–5% of rhizosphere bacteria are PGPR (Antoun and Prevost, 2005). The beneficial effects of these rhizobacteria on plant growth can be direct or indirect. Examples of direct plant growth promotion include (a) biofertilization, (b) stimulation of root growth, (c) rhizoremediation, and (d) plant stress control. Mechanisms by which rhizobacteria can promote plant growth indirectly include antibiosis, induction of systemic resistance, and competition for nutrients and niches (Lugtenberg and Kamilova, 2009). The use of those bacteria as biofertilizers or biocontrol agents in agriculture has been a focus of research for a number of years.

Biofertilizer is still an unclear term but it is most commonly referred to the use of soil microorganisms to increase the availability/uptake of mineral nutrients and/or growth stimulus for target plants. The main sources of biofertilizers are bacteria, fungi, and cyanobacteria. The principle behind this strategy is that microorganisms have various abilities that could be exploited for better farming practices. Among PGPR, PSB are considered as promising biofertilizers for agricultural improvements (Hayat *et al*., 2010), since converting insoluble soil phosphorous to a form accessible to plants is an important trait for PSB (Mahdi *et al*., 2010).

Commercial biofertilizers claiming to undergo PS using mixed bacterial cultures have been developed. Examples of these are: 'Phylazonit-M', a product containing *Bacillus megaterium; Azotobacter Chroococcum,* which allows an increase in N and P supply to plants; and the product known as 'KYUSEI EM' (Rodríguez and Fraga, 1999). RSCL BIO-P is also a commercial biofertilizer containing PSB *viz*., *B. megaterium,* which can be used to increases the content and quality of protein, yield by 10% and helps to reduce 25-30% of recommended dose of P fertilizer (Rajshree Sugars & Chemicals Ltd.). The use of microbial products has certain advantages over conventional chemicals: (*i*) they are considered safer and cheaper; (*ii*) they do not accumulate in food chain; (*iii*) self-replication of microbes circumvents the need for repeated application; (*iv*) the target organism seldom develop resistance; and (*v*) biofertilizing agents are eco-friendly and pose no damage to environment (Gaur, 2010). Therefore, it should be recommended that an integrated approach of applying biofertilizers as a supplement to chemical fertilizers for maximizing not only the yield but also agrosystem stability is the best way of integrated nutrient supply in agriculture (Mahdi *et al*., 2010).

According to Yadav *et al*. (2011), PSB treatments with combination of chemical fertilizer were found effective to increase seed yield as compared to without combination. This is supported by many researchers (Mishra *et al*., 2010). Single and dual inoculation along with P fertilizer was 30-40% better than P fertilizer alone for improving grain yield of wheat, and dual inoculation without P fertilizer improved grain yield up to 20 % against sole P fertilization (Afzal and Bano, 2008). Furthermore, PSB when used with other PGPR, such as *Rhizobium* (Gangasuresh *et al*., 2010), *Azotobacter* (Faccini *et al*., 2007) or VAM (Toro *et al.,* 1997), act synergistically to enhance crop yields (Kuhad *et al*., 2011). PSB enhanced seedling length of *Cicer arietinum* (Sharma *et al.*, 2007), while co-inoculation with PGPR reduced P application by 50% without affecting corn yield (Yazdani *et al.,* 2009). *In vitro* studies by Diriba Muleta (2007) also revealed *Bacillus, Pseudomonas, Erwinia, Ochrobactrum,* and *Serratia* sp. to have a potent inhibitory effect against emerging coffee pathogens of *Fusarium* sp.

Despite the benefits, the commercialization of biofertilizers on a global scale has been limited due to variable and inconsistent field results (Bashan and Holgin, 1997). Inconsistency in efficiency of inoculation has been attributed to adverse conditions such as interaction of microorganisms, physical and chemical conditions of soil, poor ability of PGPR strain to colonize roots, environmental factors (high T^0 and low rainfall), specific cultivar (Kuhad *et al*., 2011). So, in order to achieve successful plant-microbe-soil interactions with biofertilizers, both the genotype of organisms and environmental conditions must be assessed (Dobbelaere *et al*., 2003).

CHAPTER FOUR
MATERIALS AND METHODS

The present study was undertaken at the Department of Microbial, Cellular and Molecular Biology, College of Natural Science, Addis Ababa University. In this study attempts were made to selectively isolate phosphate solubilizing bacteria from rhizosphere soil of *E. brucei* plants. The selected isolates were characterized and tentatively identified up to genus level based on cultural, morphological and biochemical properties. The efficient P-solubilizers were further evaluated for their ability to quantitated P release, produce IAA and determine their antagonistic activity against *Fusarium oxysporum*. Isolates showing a combination of the above beneficial characters were selected and tested for their effect on germination *in vitro* and plant growth promotion of chickpea plant under greenhouse.

4.1. RHIZOSPHERE SOIL COLLECTION

The soil samples were collected from ten randomly selected *Erythrina brucei* plants grown in different compounds in and around Addis Ababa in 2011. The composite rhizosphere soil samples from each plant were collected in a separate alcohol sterilized polythene bags, and immediately transported to the Applied Microbiology laboratory. The soil samples were air-dried, crushed to pass through a 2mm sieve, and were thoroughly mixed and stored at 4^0C for further work.

4.2. ISOLATION AND ENUMERATION OF PHOSPHATE SOLUBILIZING BACTERIA

Viable total counts of PSB were enumerated by the standard serial dilution plate count method as colony forming units using NBRIP (Table 4.1) as described by Nautiyal (1999), containing 20µg/mL cyclohexamide to inhibit fungal colonies **(**Prevost and Antoun, 2008).

Table 2: *Composition of NBRIP medium (g/L)*

Glucose	10g
Ca_3PO_4	5g
$MgCl_2.6H_2O$	5g
$MgSO_4.7H_20$	0.25g
KCl	0.2g
$(NH_4)_2SO_4$	0.1g
Agar	15g
pH 7.0	

All ingredients were added to an Erlenmeyer flask containing a stir bar, to which 1 liter of distilled water was added. The pH of the media were adjusted initially to 7.0 and sterilized by an autoclave for 15 min at 121^0C and 15psi. NBRIP plates were made by dispensing approximately 20mL of medium into each sterile petriplate.

Ten gram of each soil sample was aseptically weighed and transferred into 90mL of 0.85% NaCl solution contained in 250mL Erlenmeyer flasks, and mixed thoroughly for 30 minutes using an orbital shaker at 200 rpm. The samples were allowed to settle for 10 minutes. Aliquots 1mL of the liquid suspension was periodically transferred with sterile pipettes to 9mL of 0.85% NaCl in test tubes to serially dilute it up to 10^{-7}. Finally, from the last three dilute solution (10^{-5}, $10^{-6}$and 10^{-7}); 0.1mL of each sample was spread plated on the NBRIP medium in triplicate. The plates were inverted and incubated at 30^0C for 7 days to periodically observe for colony formation.

The number of bacterial colonies showing clear zone of P-solubilization on the medium was counted, and expressed as colony forming unit per gram of soil. The CFU gram^{-1} was calculated as follows:

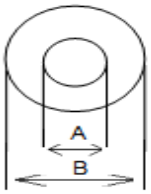
$$\text{CFU/g} = \frac{\text{Mean Number of colonies}}{\text{Volume plated (mL)} \times \text{Dilution factor}}$$

4.3. Designation of PSB Isolates

Single colony showing clear zones was picked and restreaked onto fresh NBRIP solid medium to obtain pure colonies, and preserved on NBRIP agar slants at 4^0C for future works. The PSB colonies were properly labeled with specific numbers as follows: *EbPSB 1*, *EbPSB 2*, *EbPSB 3*, etc; where, Eb stands for *Erythrina brucei,* and PSB- phosphate solubilizing bacteria.

4.4. Qualitative measurement of Phosphate Solubilization

Solubilization efficiencies (SE) were determined by spotting 10µLof overnight-grown cultures (~10^6cfu/mL) on NBRIP plates using sterile micropipette in triplicate (Perez *et al*., 2007). The plates were incubated at 30^0C for 7 days. The halo and colony diameter for each isolates were measured by using metric scale, and the halo size was calculated by subtracting colony diameter from solubilization diameter. SE was also calculated by the following formula (Sharma *et al*., 2007):



$$SE = \frac{\text{Solubilization diameter}}{\text{Growth diameter}}$$

A- Growth diameter (colony)
B- Solubilization diameter (colony+halo zone)

The PS activity of the isolates was undertaken on a liquid media following the protocol of Mehta and Nautiyal (2001). In brief, the isolates were grown in NBRIP medium containing a pH indicator of Bromophenol Blue for 3 days at 30^0C with continuous agitation. At the end of incubation, OD_{600nm} shift of the culture supernatants of each isolates were calculated by subtracting the final OD_{600nm} values from the initial values (Perez *et al*., 2007).

4.5. PHENOTYPIC CHARACTERIZATION OF PHOSPHATE SOLUBILIZING RHIZOBACTERIA

4.5.1. Cultural and Morphological Characteristics

For identification of PSB strains on the basis of colony and cell morphology, PSB isolates were grown overnight in NBRIP broth and 1mL cell suspension adjusted to 10^6cfu/mL was spread plated on the same medium. The plates were incubated at 30^0C for three days. The cultural characteristics of the isolates were recorded based on colony color, shape, elevation and pigmentation. Likewise, gram staining and endospore stain were also performed according to Aneja (2003).

4.5.2. Biochemical Characteristics

4.5.2.1. Catalase test

The catalase test was performed by adding three drops of 3% hydrogen peroxide to an 18-24 hour culture on a glass slide (Harisha, 2007). The culture was observed for the immediate appearance of bubble. The occurrence of gas bubbles showed positive for catalase reaction.

4.5.2.2. Cytochrome oxidase test

The oxidase test was based on the production of the enzyme indophenol oxidase by organisms containing cytochrome C. The test was done by adding oxidase reagent (1% tetra methyl-p-phenyldiamine dihydrochloride) on a piece of Whatman filter paper (Aslanzadeh, 2006). A loopful of 18-24 hour culture was then scrapped and rubbed on it. The appearance of a blue/dark purple color showed positive for oxidase test.

4.5.2.3. Gelatin hydrolysis

The gelatin liquefaction ability of the bacterial isolates was examined on Gelatin medium consisting (g/L) of beef extract 3g, peptone 5g and gelatin, 120g at pH 6.8, in test tubes (Aneja, 2003). The PSB isolates were stab inoculated in triplicates with a sterile needle, and incubated at 30^0C for three days. After the 3rd day of incubation, the isolates in test tubes were kept at 4^0C for 30 minutes to evaluate for gelatin liquefaction by gently tipping. A medium that readily flows as the tube is gently tipped was taken as positive for gelatin liquefaction.

4.5.2.4. Starch hydrolysis

The ability of the isolates to utilize starch was detected on triplicate plates of starch agar consisting (g/L) of beef extract, soluble starch 10g and agar 12g at pH 7.5, inoculated with the test isolate and incubated at 30^0C for three days according to Atlas (2010). After 3 days of incubation, the plates were flooded with Gram's iodine, which react with starch to produce purple-blue color. A clear zone around bacterial growth after 15 to 30 minutes of addition indicates starch hydrolysis.

4.5.2.5. Sulfur Indole Motility (SIM) test

A 5mL SIM agar tube was stab inoculated with PSB isolates and incubated at 37^0C for 48 hours. SIM medium (g/L) contains: Peptone, 30g; Beef extract, 3g; $Fe(SO_4)_2.(NH_4)_2 \cdot 6H_2O$, 0.2g; $Na_2S_2O_3$, 0.025g and Agar, 3g at pH 7.3 (Harisha, 2007). After 2 days of incubation, the SIM culture tubes were examined for H_2S production and motility before the addition of 5 drops of Kovac's reagent, prepared by dissolving 5g dimethylamine-benzaldehyde in 75mL amyl alcohol and slowly adding 25mL concentrated HCl, for indole production test. The development of red color at the top of the tubes confirms indole production.

4.5.2.6. Methyl red-Voges proskauer (MR-VP) test

A 5mL MR-VP broth (Oxoid CM 43) was inoculated with isolates and incubated at 37^0C for 48 hours. For Methyl Red test, 2.5 mL of the broth culture was transferred to a fresh tube and 3-5 drops of methyl red indicator was added. To prepare MR indicator, 0.01g of methyl red was dissolved in 25mL of 95% ethyl alcohol and 25mL of distilled water. Positive and negative result was indicated by the presence of red or yellow-orange color, respectively. For VP test, the other half of the broth was mixed with 18 drops (0.5mL) of Barritt's reagent A followed by equal amounts of reagent B. Barritt's reagent consists of two solutions, *i.e.*, solutions A and B. Solution A was prepared by dissolving 3g of α-naphthol in 50mL of 95% ethyl alcohol, whereas, solution B was prepared by dissolving 8g of KOH in 50mL of distilled water. A positive reaction was indicated by the development of pink color that develops within 15 minute. No color change indicates negative VP (Harisha, 2007).

4.5.2.7. Oxidation-Fermentation test

The purpose was to determine whether the PSB isolates metabolize glucose oxidatively, or by fermentation. The medium used for O/F test are presented in Table 3 (Atlas, 2010). Three milliliter of the basal medium was distributed into each tube and sterilized by autoclaving. 0.3mL of 10% filter sterilized glucose solution was aseptically added to the medium to make a final concentration of 1%.

Table 3: *Composition of O/F test medium*

The compositions of the basal medium in g/L:	
NaCl	5g
Peptone	2g
K_2HPO_4	0.3g
Agar	3g
Bromthymol blue	0.03g
Composition of carbohydrate solution per 100mL:	
Glucose	10g
pH 7.1±0.2	

Each isolate was aseptically inoculated into two tubes O/F medium (*i.e.* one opened and the other sealed by sterile paraffin) with a sterile needle. The tubes were incubated at 30^0C for 3-5 days. Acidic production in both tubes, changing from green to yellow (fermentative); acid production in open tube only (yellow) is an indication of oxidative utilization of glucose. A green color in both tubes indicates that the isolate is unable to utilize the carbohydrate tested, *i.e.* inert (Alexander and Strete, 2001).

4.5.2.8. Citrate utilization test

The citrate utilization test was undertaken on Koser citrate broth (Oxoid CM 65). Using aseptic technique, each PSB isolate was inoculated and incubated for 2 days at 30^0C. The change of the medium to a blue color represents a positive citrate test (Aneja, 2003).

4.5.3. Physiological Characteristics

For each physiological test, an inoculation of a loopful of 48hrs old broth culture was streaked on NBRIP medium. The inoculated plates were incubated at 30^0C for 7 days unless stated otherwise. All tests were carried out on triplicate. Ultimately, the growth of each PSB isolate was determined qualitatively and recorded as (±) for slight growth, (+) for good growth and (-) for no growth.

4.5.3.1. Determination of PSB growth on different pH, salt, and temperature gradients

For determination of the PSB isolates under salt, pH, and temperature stressed conditions NBRIP agar medium was used. The selected isolates were screened for their salt tolerance efficacy level by plating them on to NBRIP plates amended with different concentrations of NaCl (2.5%, 5%, 7.5% and 10% w/v). For pH induced P-solubilization, the pH of NBRIP medium was adjusted to seven different levels (pH 4.5, 5, 6, 8, 9, 10, 11) by 1N HCl or 1M NaOH. Plates with salt and pH stress induced NBRIP medium was inoculated with PSB isolates and incubated at 30ºC for 7 days. The growth of each isolate at different incubation temperatures (4^0C, 37^0C, 41^0C) was also evaluated by inoculating the PSB isolates on NBRIP plates (Nautiyal *et al*., 2000).

4.5.3.2. Determination of intrinsic antibiotic resistance profile (IARP)

The resistance of the selected PSB isolates to different antibiotics was evaluated by streaking each isolate on NBRIP containing freshly prepared filter sterilized antibiotics, at concentration of 5, 10 and 50µg/mL. The stock solution of each antibiotic was first prepared as described by Lupwayi and Haque (1994). The antibiotics used to develop IARP of PSB isolates were Chloroamphinicol, Erythromycin, Kanamycin, Nalidixic acid, Neomycin and Streptomycin. Antibiotics containing NBRIP agar medium were streak inoculated with a loopful of test PSB isolates and incubated at 30^0C for 5 days. Appearance of growth of the isolates shows resistance reaction whereas inhibition indicates sensitivity.

4.6. SCREENING OF PSB ISOLATES FOR INDOLE ACETIC ACID PRODUCTION

All the test PSB isolates were screened for IAA production (Bric *et al*., 1991). Briefly, test bacteria were inoculated in Luria Bertani (LB) broth (containing g L^{-1}: Tryptone-10, NaCl-10, yeast extract-5, pH 7.5) supplemented with L-Tryptophan (5mM) incubated at 30^0C for 48 hour. Cells were removed from the culture medium by centrifugation at 8,000 rpm for 10 minute. Two milliliters of the supernatant was mixed vigorously with two drops orthophosphoric acid and 4mL of Salkowaski's reagent (50mL, 35% perchloric acid; 1mL 0.5% FeCl). Development of a pink color indicates IAA production.

The intensity of pink color developed was read at 530nm in a UV-spectrophotometer (Sahasrabudhe, 2011). From a standard curve prepared with known concentrations of IAA, the quantity of IAA in culture filtrate was determined and expressed as PPM of the medium. Pure IAA was used for preparing standards of 0, 12.5, 25, 50 and 100µg mL^{-1}.

4.7. SCREENING FOR ANTAGONISTIC ACTIVITY IN PSB ISOLATES

The selected PSB isolate were screened for their antagonistic activity towards the test organism, *Fusarium oxysporum* f. sp. *ciceri*, by dual inoculation technique in 9cm sterile petridish containing fifteen milliliter of PDA (*Oxoid CM 139*) (Wang *et al*., 2009). To test the inhibitory effects of the rhizobacterial isolates against the pathogen, a mycelial disc of the test fungus (5mm in diameter) obtained from the peripheral region of 5 day-old cultures of *F. oxysporum*, was placed in the center of a fresh PDA plate. Then, bacterial isolates from 2 day-old cultures were streaked across both sides of the mycelium disc approximately 3cm away from the disc center. The paired PDA plates were then incubated at 30^0C for 7 days.

At the end of the incubation period, radial growth was measured (Figure 3). The percentage growth inhibition (PGI) of tested pathogens in presence of selected PSB isolates was calculated by the reduction percentage of mycelium expansion compared to control plates without bacteria following the formula of Whipps (1987):

$$PGI = \frac{(R1 - R2)}{R1} \times 100$$

Where R_1 represents the radius of the colony of the test fungus in the direction with no bacterial colony and R_2 is the radius of the fungal colony in the direction of the antagonist. All *in vitro* antagonism assays were made in duplicates.

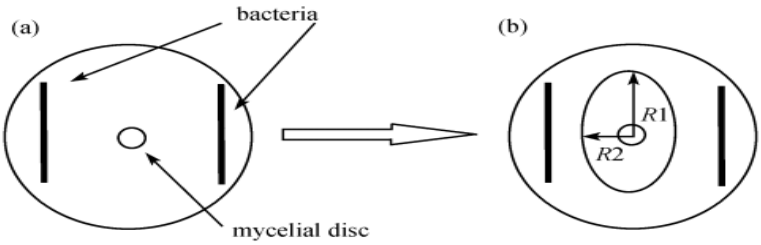


Figure 3: *Description of the in vitro antagonism assay* (Source: Wang *et al*., 2009) (a) One mycelial plug of *F. oxysporum* placed at center of the plate, and PSB isolates streaked across each side of mycelial plug, (b) The PGI of the fungus calculated by the formula of Whipps (1987).

4.8. Quantitative Estimation of Phosphate Solubilization in Broth Culture

4.8.1. Phosphate Solubilization under Various Insoluble Phosphate sources

Insoluble phosphates such as, Tricalcium phosphate, Ferric (III) phosphate, Egyptian rock phosphate and Bone meal sources were used for the quantitative estimation of P-solubilization (PS). Quantitative measurement of PS of each isolate was carried out on hundred milliliter of NBRIP broth without having any P source dispensed in to a 250mL Erlenmeyer flask. Different insoluble P sources were dried separately and added at the rate of 500mg/100mL (Nautiyal, 1999). One mL of 24 hr bacterial culture suspension was added to each flask aseptically and incubated on a rotary shaker at 200 rpm. Autoclaved, uninoculated medium served as control. Samples were withdrawn every 3 days for a total of 12 days of incubation. The samples were centrifuged at 4,000 rpm for 20 minutes and each supernatant was filtered through Whatman No. 1 filter paper. The filtrate was used to measure the pH and soluble P by following chlorostannous reduced molybdophosphoric acid blue method in H_2SO_4 modified from Jackson (1958).

To a 5 mL aliquot of the clear filtrate, 2 mL of sulfomolybdic acid solution and 1.0 mL of freshly prepared chlorostannous acid reagent were added, and thoroughly mixed. After mixing, the volume was made up to 50mL with distilled water. Absorbance of the resultant blue color solution was read in a Spectrophotometer at 700nm. Simultaneously, a standard calibration curve was prepared from which the amount of phosphorus solubilized from experimental samples were calculated. Finally, the sample's absorbance was checked against the calibration curve and determines the correct concentration of P, which is given by the formula:

$$\text{mg/100mL P} = \frac{\text{[P] calculated from the curve}}{\text{Initial volume used (mL)}} \times 50 \text{ mL}$$

4.8.2. Effects of Nutrients on Phosphate Solubilization

The effects of different sources of carbon and nitrogen on phosphate solubilization were studied sequentially in batch cultures in NBRIP medium. The different C sources (Glucose, Fructose and Starch) at 1% concentration and N sources ($(NH_4)_2SO_4$, KNO_3 and NH_4NO_3) at 0.05% (Seshadri *et al*., 2004) were amended to evaluate the performance, so a categorical experimental design was applied to select the best combination of carbon and nitrogen sources that can optimize P solubilization activity of the PSB isolate. Thereafter, pH change of the medium was recorded and estimation of P-solubilization was done quantitatively by the same method described above.

4.9. Germination Bioassay and Green House Evaluation of PSB Isolates

4.9.1. Effect of Isolates on Seed Germination

To evaluate the effect of rhizobacteria on seed germination and seedling vigour, chickpea seeds (*Cicer arietinum* L. Ararti variety) obtained from Ethiopian Agricultural Research Organization (EARO), Debrezeit were used. The surface of the seeds was sterilized with 3% sodium hypochlorite solution for 3 minutes and subsequently they were dipped into 95% ethanol for 10 second, after which they were washed with sterile distilled water 6-7 times (Sharma *et al*., 2007). The inoculants for treating seeds were prepared by suspending cells from 48 hours old nutrient broth. Seeds were dipped for 5 hours in rhizobacterial culture suspension. For the control, seeds dipped in sterile distilled water were used (Aipova *et al*., 2010). In accordance with aseptic regulations, both treated and untreated seeds were placed onto dampened filter paper in petridishes and incubated at 30^0C for 5 days.

After 5 days of incubation, germinating parameters were measured, *viz*, the number of germinated seeds, radicle and plumule length and finally the percentage increase in radicle and plumule length due to treatment was compared. In addition, Vigour index was computed by multiplying the total length of seedling (mean root length + mean shoot length) with germination percentage (Sharma *et al*., 2007). The experiment was performed in duplicates.

4.9.2. Nursery Plantation Experiment

Four PSB isolates (EbPSB 3, EbPSB 6, EbPSB 7 and EbPSB 8) were selected as representative to evaluate their growth promoting efficiency on chickpea. The representative isolates were selected on the basis of different combination of parameters *viz*., physiological tolerance, maximum P-solubilization, IAA production, antagonistic activity and their effect on chickpea seed germination and seedling vigour index improvement. Chickpea was selected due to its significance as grain

commodities, and source of food. A nursery experiment was conducted under glasshouse conditions located on the College of Natural Science using sterile sand culture (Hydroponics). These test cultures were amended in pots along with tricalcium phosphate and evaluated on chick pea, in terms of plant height, root dry weight, shoot dry weight as per control treatments.

4.9.2.1. Pot preparation

The sand used for greenhouse trial was sieved through a 2mm diameter sieve, carefully rinsed in 23% sulfuric acid overnight and washed thoroughly with distilled water. The air dried sand was autoclaved and approx. 500g was filled into sterilized plastic pots (9×9×10cm). Finally, the pots were saturated with sterile distilled water prior to sowing seeds.

4.9.2.2. Potting

The seeds of chickpea (*Cicer arietinum* L.) cultivar having uniform size and color were sterilized as before and transferred by using sterile forceps into sterile sand in pots at a depth of 3-5 cm. Three seeds were planted into each pot, which were later thinned down to one. All pots were irrigated once a week after sowing.

4.9.2.3. Inoculation

Inoculation of plants with PSB isolates were performed after one week. Starter cultures of the selected test isolates were grown in test tubes containing 10mL Nutrient broth for two days. All the sowed seeds received one milliliter of a 48 hour old microbial culture (10^9cfu/mL) by pipetting onto the base of the emerging seedlings of chick pea. Uninoculated control pots were also set up.

4.9.2.4. Treatments and experimental design

The pots were arranged in Randomized Complete Block Design (RCBD) in three blocks or replications with 7 treatments. The experimental design was performed as follows - treatment 1: uninoculated potted sand with addition of soluble phosphate (K_2HPO_4); treatment 2: uninoculated potted sand with addition of $Ca_3(PO_4)_2$; treatment 3: pots inoculated with EbPSB 3 having TCP; treatment 4: pots inoculated with EbPSB 6 having TCP; treatment 5: pots inoculated with EbPSB 7 having TCP, and treatment 4: pots inoculated with EbPSB 8 having TCP. TCP was supplemented at the same concentration of KH_2PO_4 as in normal Hoagland's solution (Katiyar and Goel, 2003).

A modified Hoagland nutrient solution (Hoagland and Arnon, 1950) devoid of P was applied to each pot (20mL) once a week, and sterilized water was added whenever necessary to maintain soil moisture levels near field capacity. Plants were grown in a greenhouse for 50 days.

Table 4: *Composition of a modified Hoagland nutrient solution for growing plant* [1]

Compounds	Molecular weight (g/mol)	Conc. of stock solution		Volume of stock solution per 500mL of final solution (mL)
		mM	g/100mL	
Macronutrients[2]				
$Ca(NO_3)_2.4H_2O$	236.16	1000	23.6	2.5
KNO_3	101.10	1000	10.1	2.5
$MgSO_4.7H_2O$	246.49	1000	24.6	1
KH_2PO_4	136.09	1000	13.6	0.5
Micronutrients[3]				
H_3BO_3	61.84	46	0.29	0.5
$MnCl_2.4H_2O$	197.91	9.15	0.18	
$ZnSO_4.7H_2O$	289.55	0.76	0.022	
$CuSO_4.5H_2O$	249.71	0.32	0.008	
$Na_2MoO_4.2H_2O$	241.95	0.1	0.0025	
FeEDTA	367.05	18.25	0.67	0.5

[1.] After Hoagland and Arnon (1950), modified, devoid of P-source.
[2.] Prepare stock solutions of individual macronutrient separately.
[3.] A combined stock solution contained all micronutrients except Fe.

After 50 days of growth, chickpea plants were harvested by cutting at the soil line with a razor blade and growth parameters were recorded. Shoot and root length were measured in centimeter. Then plants were dried in an oven at 65^0C for 3 days till constant weight was obtained. Later, the weight of dry matter was recorded in gram (Yadav *et al*., 2010).

4.10. DATA ANALYSIS

Experimental designs were performed with replication. Means were compared using Duncan's multiple range tests at 0.01 and 0.05 levels of significance. In all the cases, the quantitative data obtained were subjected to analysis of variance using IBM SPSS 19.0 statistical version computer software.

CHAPTER FIVE
RESULT

5.1. IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIAL ISOLATES

The number of rhizobacteria showing zone of solubilization on NBRIP medium as estimated by colony forming units per gram of soil was found to be $8.7\pm0.84 \times 10^6$. From the total of fifteen selected PSB isolates, nine persistent and efficient colonies were screened for further investigation. All the selected PSB isolates were identified to genus level based on their morphological and biochemical characters and the results are presented in Table 5.

The different PSB isolates showed similarities and differences in their cultural characteristics (Table 5). All of them were characterized by round colony margin, opaque texture and displayed white color, except EbPSB 1 and EbPSB 7 that appeared to be light yellow**,** and with raised colony elevation, except EbPSB 2 that appeared flat. All isolates imparted green pigmentation, except the colorless isolates EbPSB 3 and EbPSB 6.As far as cellular morphology is concerned, all of them were motile gram negative, except EbPSB 9 that was gram positive and, all but EbPSB 2 were rod shaped bacteria, and were not spore formers except EbPSB 9.

The biochemical reactions of the isolates showed that all of them were catalase and oxidase positive (Table 5). All except EbPSB 5, EbPSB 6 and EbPSB 8 liquefied gelatin, and only EbPSB 6 and EbPSB 9 were found to hydrolyze starch. No isolate was involved in indole and H_2S production, and failed to respond to MR test. The only isolate that was neither oxidative nor fermentative was EbPSB 9. All of them were found positive for citrate utilization.

Table 5: *Morphological and biochemical characterization of phosphate solubilizing rhizobacteria*

CHARACTERS	PHOSPHATE SOLUBILIZING RHIZOBACTERIAL ISOLATES								
	EbPSB 1	**EbPSB 2**	**EbPSB 3**	**EbPSB 4**	**EbPSB 5**	**EbPSB 6**	**EbPSB 7**	**EbPSB 8**	**EbPSB 9**
Cultural Characteristics									
Color	*Light yellow*	*White*	*White*	*White*	*White*	*White*	*Light Yellow*	*White*	*White*
Colony Shape	*Round*	*Round*	*Round*	*Round*	*Round*	*Round*	*Round*	*Round*	*Round*
Elevation	*Raised*	*Flat*	*Raised*	*Raised*	*Raised*	*Raised*	*Raised*	*Raised*	*Raised*
Optical feature	*Opaque*	*Opaque*	*Opaque*	*Opaque*	*Opaque*	*Opaque*	*Opaque*	*Opaque*	*Opaque*
Pigmentation	*Green*	*Green*	-	*Green*	*Green*	-	*Green*	*Green*	-
Microscopic Examination									
Gram stain	-	-	-	-	-	-	-	-	+
Cell shape	*Bacilli*	*Cocci*	*Bacilli*	*Bacilli*	*Bacilli*	*Bacilli*	*Bacilli*	*Bacilli*	*Bacilli*
Motility	+	+	+	+	+	+	+	+	+
Endospore stain	-	-	-	-	-	-	-	-	+
Biochemical Reactions									
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	-	-	+	-	+
Starch hydrolysis	-	-	-	-	-	+	-	-	+
Indole production	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-
MR test	-	-	-	-	-	-	-	-	-
VP test	-	-	-	+	-	+	-	-	+
O/F test	***O***	***O***	***O***	***O***	***O***	***F***	***O***	***O***	***I***
Citrate utilization test	+	+	+	+	+	+	+	+	+
Genus	***Pseudomonas***	***Pseudomonas***	***Pseudomonas***	***Pseudomonas***	***Pseudomonas***	***Pseudomonas***	***Pseudomonas***	***Pseudomonas***	***Bacillus***

Note: *+= Positive for the test, -= Negative for the test O= Oxidative, F= Fermentative, I= Inert*

5.2. ECOPHYSIOLOGICAL CHARACTERISTICS OF ISOLATES

5.2.1. pH, Salinity and Temperature Tolerance

The isolates showed differences in their tolerance to pH, salt and temperature (Table 6).

Table 6: *Tolerance of isolates to pH, salinity and temperatures on NBRIP medium*

	EbPSB 1	EbPSB 2	EbPSB 3	EbPSB 4	EbPSB 5	EbPSB 6	EbPSB 7	EbPSB 8	EbPSB 9	No of (+/±) Isolates
pH tolerance										
4.5	-	±	±	-	-	±	±	-	±	**5**
5	±	±	±	-	±	+	+	+	±	**8**
6	+	+	+	+	+	+	+	+	+	**9**
8	+	+	+	+	+	+	+	+	+	**9**
9	-	+	±	+	-	+	+	+	±	**7**
10	-	-	±	±	-	±	±	-	±	**5**
11	-	-	-	-	-	±	-	-	-	**1**
Salt tolerance										
2.5%	±	±	+	±	+	+	+	+	+	**9**
5%	-	-	+	-	±	+	+	+	+	**6**
7.5 %	-	-	-	-	-	±	±	-	-	**2**
10%	-	-	-	-	-	-	-	-	-	**0**
Temperature tolerance										
4^0C	-	-	-	-	-	-	-	-	-	**0**
37^0C	+	+	+	+	+	+	+	+	+	**9**
41^0C	±	±	±	±	±	±	+	±	+	**9**
	6	**8**	**10**	**7**	**7**	**12**	**11**	**8**	**10**	

Note: **(-)** *= No growth,* **(+)** *= Good growth,* **(±)** *= Limited growth*

All isolates were tolerant to pH 6 and 8 with optimum growth, whereas five isolates, 8 isolates, seven isolates, five isolates and one isolate were found to be tolerant to pH 4.5, pH 5, pH 6, pH 9, pH 10 and pH 11, respectively. Likewise, the most pH tolerant isolate was EbPSB 6 that grew on all tested pH, whereas EbPSB isolates 3, 7 and 9 failed to grow at pH 11. All the PSB isolates tolerate the minimum tested 2.5% NaCl, but all failed to grow on 10% salt concentration. On the other hand, six isolates and two isolates were found to be resistant to 5% and 7.5% NaCl concentration, respectively. The most salt tolerant isolates were EbPSB 6 and EbPSB 7 that grew on 7.5% NaCl. When temperature stressed PS was considered, all isolates were found to grow at higher temperatures of 37^0C and 41^0C, but failed to grow at 4^0c (Table 6).

5.2.2. Intrinsic Antibiotic Resistance Profile (IARP)

The level of antibiotic resistance of the isolates showed variation to different antibiotics and concentrations (Table 7).

Table 7: *Levels of antibiotics resistance by different PSB isolates*

Isolates / Antibiotics		EbPSB 1	EbPSB 2	EbPSB 3	EbPSB 4	EbPSB 5	EbPSB 6	EbPSB 7	EbPSB 8	EbPSB 9	No of (+/±) Isolates
Chl	5 µg/mL	+	+	+	+	+	+	+	+	+	9
	10 µg/mL	±	+	+	+	±	+	+	±	+	9
	50 µg/mL	±	±	+	±	-	±	-	-	-	5
Ery	5µg/mL	+	+	+	±	+	+	+	+	±	9
	10µg/mL	±	±	+	±	+	+	+	+	-	8
	50µg/mL	±	±	±	±	±	+	+	±	-	8
Kan	5 µg/mL	+	+	+	+	±	+	±	±	-	8
	10 µg/mL	+	+	+	+	-	±	-	-	-	5
	50 µg/mL	±	±	±	±	-	-	-	-	-	4
Nal	5 µg/mL	+	+	+	+	+	+	+	±	+	9
	10 µg/mL	±	±	±	+	+	+	±	±	±	9
	50 µg/mL	±	+	±	+	±	+	±	-	-	7
Neo	5 µg/mL	+	+	+	+	+	+	+	+	+	9
	10 µg/mL	+	+	±	±	±	±	+	±	+	9
	50 µg/mL	+	+	±	±	±	-	+	±	-	7
Str	5 µg/mL	+	+	+	±	+	-	+	+	-	7
	10 µg/mL	±	+	±	±	±	-	±	-	-	6
	50 µg/mL	±	-	±	-	-	-	±	-	-	3
		18	*17*	*18*	*17*	*14*	*13*	*15*	*12*	*7*	

Note: **(-)** = *No growth,* **(+)** = *Good growth,* **(±)** = *Limited growth*

All the isolates were tolerant to 5 and 10µg/mL of chloramphenicol, nalidixic acid and neomycin. All except EbPSB 9 tolerate erythromycin at all concentration. The majority of the isolates were sensitive to 10 and 50 µg/mL Kanamycin and streptomycin. However, the majority of the isolates were sensitive to 50µg/mL Kanamycin and streptomycin. Among the isolates, the most resistant ones that grew on all tested antibiotics were EbPSB 1 and 3 followed by EbPSB 2 and 4 that tolerated all but 50µg/mL of streptomycin. EbPSB isolate 7, 5 and 6 were relatively tolerant compared to the rest of the isolates in that they grew on all tested antibiotics, except 50µg/mL chloramphenicol, 10 and 50µg/mL kanamycin, and 50µg/mL streptomycin. The most sensitive isolate was EbPSB 9 that failed to grow on kanamycin, streptomycin and erythromycin at concentration of 10 and 50µg/mL.

5.3. Qualitative Screening of Phosphate Solubilizing Bacteria

The rate of growth and PS of the bacterial isolates are tabulated in the Table 8. The data is presented as mean value of colony diameter, solubilization zone, halo size and SE (%) of different PSB isolates. The highest solubilization diameter of 12.33mm was recorded from isolate EbPSB 7 followed by 12.00cm measured from isolate EbPSB 6. The lowest solubilization diameter of 9.33mm was displayed by isolate 2. The growth diameter of the isolates did not corroborate with solubilization diameter in that EbPSB 2 and 9 showed slight and significantly higher growth diameter than several isolates.

Table 8: *Plate based assay for phosphorus solubilization by PSB isolates in NBRIP medium*

PSB Isolates	Solubilization Diameter (mm)	Growth Diameter (mm)	Halo Size (mm)	Solubilization Efficiency (%)
EbPSB 1	9.67±0.67^{d}	5.33±0.33^{bcd}	4.0±0.58^{cde}	177±14.53^{cd}
EbPSB 2	9.33±0.33^{d}	6.33±0.33^{ab}	2.67±0.33^{e}	143±7.0^{d}
EbPSB 3	10.0±0.58^{cd}	5.33±0.33^{bcd}	4.33±0.33^{cd}	181±1.0^{cd}
EbPSB 4	10.67±0.67^{bcd}	6.00±0.0^{abc}	4.0±0.0^{cde}	167±0.0^{cd}
EbPSB 5	11.33±0.67^{abc}	5.67±0.58^{abc}	5.33±0.33^{bc}	194±5.67^{bc}
EbPSB 6	12.0±0.0^{ab}	4.33±0.33^{de}	7.67±0.33^{a}	280±20.0^{a}
EbPSB 7	12.33±0.33^{a}	4.00±0.0^{e}	8.67±0.33^{a}	308±8.33^{a}
EbPSB 8	11.33±0.33^{abc}	5.0±0.58^{cde}	6.33±0.88^{b}	234±34.53^{b}
EbPSB 9	10.33±0.33^{cd}	6.67±0.33^{a}	3.67±0.33^{de}	156±6.96^{cd}

Note: *Values are mean of triplicates and expressed as mean ± SE. Means within column followed by the same letters are not significantly different (P< 0.05).*

Differences in halo size was also exhibited by the isolates, where EbPSB 7 and 6 showed significantly (P< 0.05) higher values (8.67 and 7.67mm, respectively) from other isolates, followed by insignificantly different values (mm) recorded by EbPSB 8 (6.33), EbPSB 5 (5.33) and EbPSB 3 (4.33). EbPSB isolate 7 and 6 displayed the highest solubilization efficiency (%) of 308 and 280, respectively. The lowest solubilization was recorded from EbPSB 2 (143), which was twice as low as the SE of the most effective isolates (Table 8).

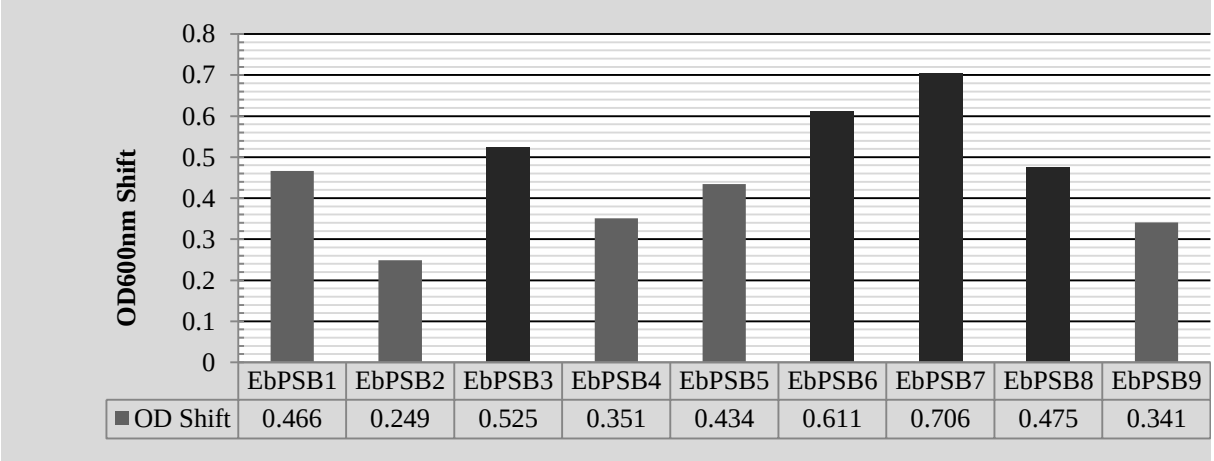


Figure 4: *Qualitative analysis of phosphate solubilization activity in NBRI-BPB medium.*

The OD_{600} shift of the culture supernatant after incubation in NBRI-BPB medium for three days. Some isolates including the control showed no significant change in the absorbance of the supernatant as well as color change, while others exhibited OD_{600} shifts of more than 0.249 units. The highest OD_{600} shift (0.706) was recorded by EbPSB 7 followed by EbPSB 6 (0.611), EbPSB 3 (0.525) and EbPSB 8 (0.475). While, the lowest OD_{600} shift values were attributed to EbPSB 2 and 9.The data showed clear differences in the acidification (*i.e.* color change) of the medium. The most dramatic color change is, however, associated with NBRI-BPB broth inoculated with EbPSB 7.

5.5. *IN VITRO* IAA PRODUCTION AND ANTAGONISTIC ACTIVITY OF PSB ISOLATES

The results of the *in vitro* screening revealed that only four PSB isolates were found to produce IAA, and five out of 9 PSB isolates significantly reduced the mycelial growth of *F. oxysporum* (Table 9).

Table 9: *Production of IAA and antagonism by PSB isolates*

PSB Isolates	IAA Production (µg mL^{-1})	*In vitro* Antagonism Assay		
		R_1 (cm)	R_2 (cm)	Percentage Growth Inhibition (PGI: %)
EbPSB 1	13.33	4.30^{a}	1.3^{b}	69.75^{a}
EbPSB 2	-	3.15^{b}	1.6^{a}	49.20^{b}
EbPSB 3	22.33	4.25^{a}	1.4^{b}	67.03^{a}
EbPSB 4	-	2.75^{c}	1.4^{b}	48.94^{b}
EbPSB 5	-	-	-	-
EbPSB 6	26.00	-	-	-
EbPSB 7	20.67	2.85^{bc}	1.3^{b}	54.37^{b}
EbPSB 8	-	-	-	-
EbPSB 9	-	-	-	-

***Note**: Values are expressed as a mean of two replicates.*
Values which share same letter are insignificantly different.

The production of IAA (µg mL^{-1}) was highest in EbPSB 6 (26.0µg mL^{-1}), followed by EbPSB 3 (22.33µg mL^{-1}), EbPSB 7 (20.67µg mL^{-1}) and EbPSB 1 (13.33µg mL^{-1}) as described in Table 8**. T**he percent inhibition of pathogen growth (PGI) by the isolates tested varied between 48.94 and 69.75%. The most effective antagonistic PSB isolates were EbPSB 1 (69.75%) and EbPSB 3 (67.03%) that were significantly different ($P<0.05$) from the rest of the isolates. On the contrary, the lowest PGI values of 48.94 and 49.2 were recorded by EbPSB 4 and 2, respectively.

5.5. QUANTITATIVE ESTIMATION OF PS ACTIVITIES UNDER VARIOUS INSOLUBLE P SOURCES

Inoculation of the selected PSB isolates (*i.e.* EbPSB 3, EbPSB 6, EbPSB 7 and EbPSB 8) into NBRIP liquid medium supplemented with either TCP, $FePO_4$, Egyptian rock phosphate or Bone meal resulted in a gradual increase in the concentration of the released soluble P, and a subsequent decrease in pH value of the supernatant.

5.5.1. Tricalcium Phosphate Solubilization

The results of tricalcium phosphate solubilization by the selected isolates and the associated pH changes in 100mL NBRIP medium are shown in Table 5.

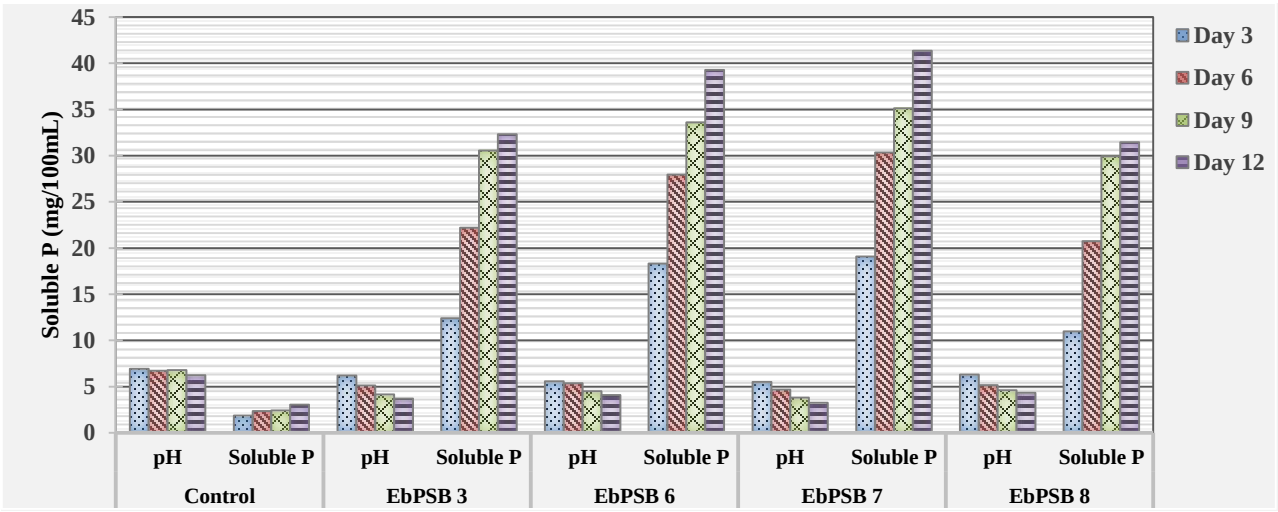


Figure 5: *Tricalcium phosphate solubilization efficiency (mg/100mL) of PSB isolates*

The result showed that all the PSB isolates solubilized significantly higher amount of tricalcium phosphate over uninoculated one ($P<0.01$). The highest amount of TCP solubilization (41.37mg/100mL) was recorded for EbPSB 7 followed by EbPSB 6 (39.29mg/100mL), EbPSB 3 (32.32mg/100mL) and EbPSB 8 (31.44mg/100mL) on 12th day of incubation. A fall in pH (initially 7.0) of the culture filtrate was observed, and the mean difference was significant at $P< 0.05$ over control. In case of EbPSB 7 and EbPSB 3, the pH was greatly reduced to 3.25 and 3.69 (12th day), respectively. When the effect of pH on TCP solubilization was studied, a negative correlation was obtained ($r= -0.961$) and it was significant at the 0.01 level (Figure 5).

5.5.2. Ferric Phosphate Solubilization

The results for ferric P solubilization efficiency of selected PSB isolates are shown in Figure 6.

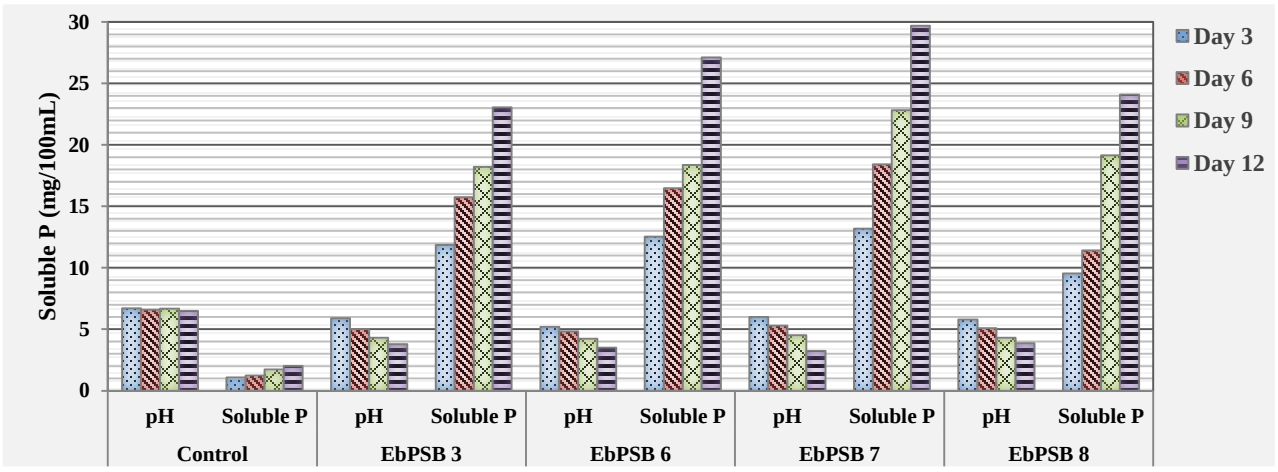


Figure 6: *Ferric phosphate solubilization efficiency (mg/100mL) of PSB isolates*

The amount of P released (mg/100mL) and pH change by different isolates of PSB on $FePO_4$ as a function of time was highly significant over control at P< 0.01 and P< 0.05, respectively. EbPSB 7 (29.69mg) was comparatively the most efficient solubilizer compared to all the other isolates followed by EbPSB 6 (27.12mg), EbPSB 8 (24.09mg) and EbPSB 3 (23.05mg). A drop in the pH of the medium from 7.0 to 3.25 (EbPSB 7), 3.50 (EbPSB 6), 3.78 (EbPSB 3), and 3.86 (EbPSB 8) was also observed. There was inverse relationship between soluble P and decrease in pH (r= -0.955).

5.5.3. Egyptian Rock Phosphate Solubilization

All the tested isolates solubilized significantly (P<0.01) higher amount of Egyptian rock phosphate over uninoculated (Figure 7).

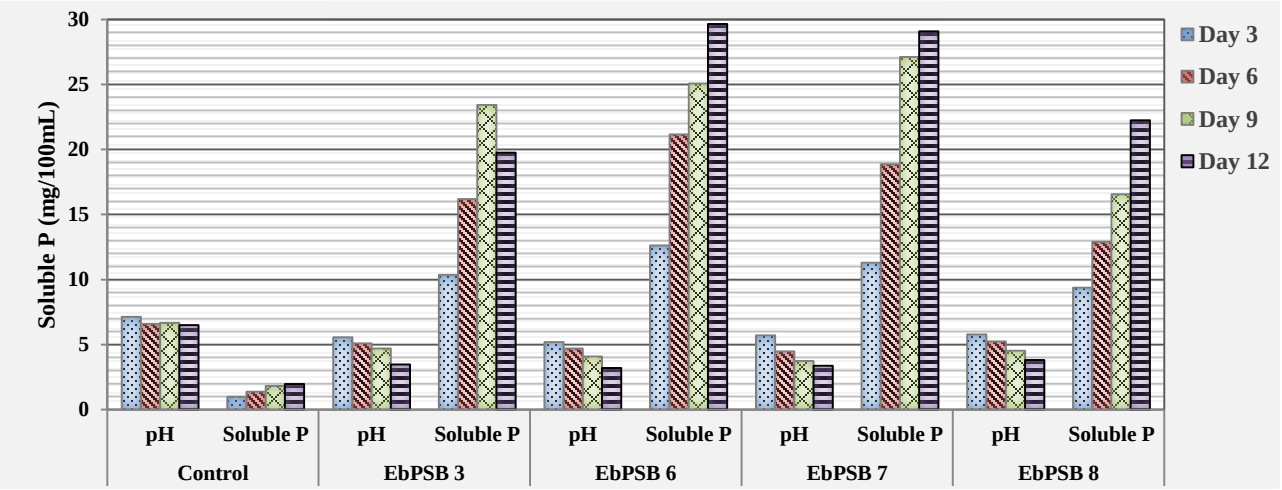


Figure 7: *Egyptian rock phosphate solubilization efficiency (mg/100mL) of PSB isolates*

The solubilization increased steadily up to 12 days of incubation except in EbPSB 3. The highest (29.64mg/100mL) amount of soluble P was released in the flask inoculated with EbPSB 6 followed by EbPSB 7 (29.08mg), EbPSB 8 (22.23mg) on 12^{th}, and EbPSB 3 (23.41mg) on 9^{th} day of incubation. . P solubilization activity of EbPSB 3 decreased after 9 days of incubation to 19.76mg. A progressive decrease in pH value of the culture filtrate was observed and found to be highly significant (P< 0.01) compared to the uninoculated medium. Pearson correlation analysis showed a significant (P< 0.01) inverse relation between soluble P and pH (r= -0.957).

5.5.4. Bone Meal Solubilization

The amount of soluble P (mg/100mL) released from bone meal and the corresponding pH decrease of the medium by the PSB isolate is shown in Figure 8.

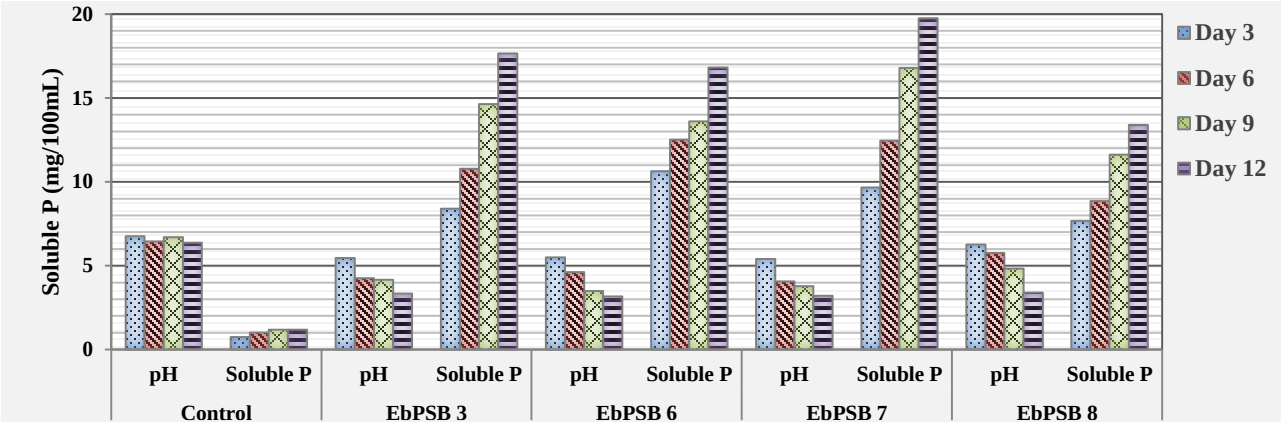


Figure 8: *Bone meal solubilization efficiency (mg/100mL) of PSB isolates*

There was a significant amount of phosphate solubilization (P< 0.01) and pH change (P< 0.05) by the selected PSB isolates over the uninoculated control. However, the maximum soluble P concentration produced in the presence of bone meal was 19.76, 17.66, 16.82 and 13.39mg/100mL for EbPSB 7, EbPSB 3, EbPSB 6 and EbPSB 8, respectively. A drastic drop of pH to 3.17 (EbPSB 6), 3.21 (EbPSB 6), 3.35 (EbPSB 8) and 3.4 (EbPSB 3) was recorded on day 12. There was a significant (P<0.01) negative correlation between soluble P and pH (r= -0.931).

Table 10: *Maximum phosphate released (mg/100mL) by PSB isolates per day of incubation*

Isolate	Maximum P release per day (mg P release/day)			
	TCP	FeP	ErP	BM
EbPSB 3	3.52(3rdday)	3.59 (3rd day)	3.12 (3rd day)	2.55 (3rd day)
EbPSB 6	5.49 (3rd day)	3.81 (3rd day)	3.87 (3rd day)	3.29 (3rd day)
EbPSB 7	5.75 (3rd day)	4.03 (3rd day)	3.42 (3rd day)	2.97 (3rd day)
EbPSB 8	3.06 (6th day)	2.82 (3rd day)	2.79 (3rd day)	2.31 (3rd day)

The effectiveness of each isolate in releasing P from different phosphate sources per day of incubation were compared (Table 10). In almost all P sources used the highest solubilization rate (mg P release/day) were recorded in the first three days of incubation, except in TCP inoculated with EbPSB 8 (on day 6). Maximum P release per day was achieved by EbPSB 7 when inoculated in TCP (5.75) and FeP (4.03) broth, however, EbPSB 6 was superior in ErP (3.87) and BM (3.29) compared to the other isolates. EbPSB 3 showed maximum rate of solubilization in FeP (3.59) followed by TCP (3.52), ErP (3.12) and BM (2.55). The lowest solubilization rate per day of incubation in all P sources used was recorded by EbPSB 8.

5.6. CARBON AND NITROGEN INFLUENCE ON PHOSPHOROUS SOLUBILIZATION

P-solubilization activity was evaluated by selecting the most effective isolate (EbPSB 7) in the presence of three carbon and three nitrogen sources sequentially in batch cultures in NBRIP medium. The results showed diverse levels of PS activity in the presence of the various carbon and nitrogen sources (Table 11).

Table 11: *Categorical factors to determine the best combination of carbon and nitrogen source on phosphate solubilization by EbPSB 7*

Nitrogen Source	Carbon Source						Mean ± SE	
	Glucose		Fructose		Starch			
	pH	*Soluble P (mg/100mL)*	*pH*	*Soluble P (mg/100mL)*	*pH*	*Soluble P (mg/100mL)*	***pH***	***Soluble P (mg/100mL)***
$(NH_4)_2SO_4$	3.85	33.65	4.19	21.05	5.07	13.56	**4.37^{a}±0.364**	**22.75^{a}±5.862**
KNO_3	4.45	19.06	4.84	13.86	5.53	10.54	**4.94^{a}±0.316**	**14.49^{a}±2.479**
NH_4NO_3	4.27	23.78	4.14	14.72	5.18	10.64	**4.53^{a}±0.327**	**16.38^{a}±3.883**
Mean ±SE	**4.19^{b} ±0.178**	**25.49^{a} ±4.298**	**4.39^{b} ±0.225**	**16.54^{ab} ±2.267**	**5.26^{a} ±0.139**	**11.58^{b} ±0.990**		

As indicated in Table 11, the isolate was able to solubilize insoluble P with all combination of carbon and nitrogen sources. For any given carbon source, changes in N source had no significant effects on pH or phosphate solubilization, while for any given nitrogen source, changes in C source had a significant ($P<0.05$) effect on PS. The C-source which resulted in the most extensive solubilization of TCP by EbPSB 7 was glucose followed by fructose > starch, and for N it was ammonium sulphate followed by NH_4NO_3> KNO_3. The optimal amount of P (33.65mg/100mL) was solubilized in media containing glucose as C and ammonium sulphate as N source. Whereas starch and KNO_3 based media showed the lowest PS value (10.54mg/100mL), followed by starch and NH_4NO_3 (10.64mg/100mL) as the C and N sources respectively. The acidification of the culture media varied with different C and N sources but not found significant.

5.7. CHICKPEA SEED GERMINATION AND PLANT GROWTH

5.7.1. Effects on Seed Germination

Results on the effect of seed germination and seedling growth due to treatment by the isolated strains are given in Table 12.

Table 12: *Effect of PSB on germination and seedling length of Cicer arietinum seeds*

PSB used	Germination %	Radicle Length		Plumule Length		Vigor Index
		cm	%(I)&(D)	cm	%(I)&(D)	
Control	83.33	2.90 ± 0.8^{b}	-	1.3 ± 0.4^{b}	-	385.014 ± 110^{bcd}
EbPSB 1	91.67	3.55 ± 1.8^{b}	18.31(I)	1.75 ± 0.7^{ab}	25.71(I)	397.500 ± 180^{bcd}
EbPSB 2	66.67	2.50 ± 1.7^{b}	-16(D)	1.80 ± 1.3^{ab}	27.78(I)	286.681 ± 200^{cd}
EbPSB 3	91.67	4.95 ± 0.4^{ab}	41.41(I)	2.35 ± 0.6^{ab}	44.68(I)	669.191 ± 82.5^{bcd}
EbPSB 4	66.67	4.65 ± 0.5^{ab}	37.63(I)	1.90 ± 0.4^{ab}	31.58(I)	491.250 ± 3.8^{bcd}
EbPSB 5	83.33	3.00 ± 1.1^{b}	3.33(I)	1.25 ± 0.3^{b}	-4(D)	354.153 ± 112.5^{cd}
EbPSB 6	100	8.90 ± 1.3^{a}	67.42(I)	3.70 ± 0.4^{a}	64.86(I)	1260.00 ± 90^{a}
EbPSB 7	100	6.05 ± 2.6^{ab}	52.07(I)	2.25 ± 0.1^{ab}	42.22(I)	830.000 ± 260^{b}
EbPSB 8	100	4.75 ± 0.6^{ab}	38.95(I)	2.70 ± 0.4^{ab}	51.85(I)	745.000 ± 15^{bc}
EbPSB 9	58.33	2.40 ± 0.5^{b}	-20.83(D)	1.10 ± 0.2^{b}	-18.18(D)	262.500 ± 52.5^{d}

***Note**: Values are expressed as a mean of two replicates ±SE. **%(I) & (D)**= Percentage Increase and Decrease. Means followed by the same letter are not significantly different (P< 0.05).*

Seed treatment with PSB isolates brought a general increase in various germination parameters as observed in all except EbPSB 9. However, the rate of enhancement varied with bacterial strains. The highest germination percentage (100%) was recorded from the seeds treated with EbPSB 6, 7 and 8 followed by EbPSB 1 and EbPSB 3 (91.67%). The lowest germination rate of 58.3% was recorded for EbPSB 9 followed by 66.67% germination from seeds treated with EbPSB 2 and 4. The different treatments also showed a significant variations in radicle (2.4-8.9cm) and plumule (1.1-3.7cm) length. Inoculation with EbPSB 6 showed a maximum increase in both radicle and plumule length to a point of 8.9cm (67.42%) and 3.7cm (64.86%), respectively. Meanwhile the vigor index reaches up to 1260 by isolate EbPSB 6 that is significantly superior over the other isolates followed by insignificantly different values recorded by EbPSB isolate 7 (830), 8 (745) and 3 (669).

5.7.2. Greenhouse Test on Growth Promotion

It was evident that growth of chickpea plants was not only enhanced in presence of available phosphorus (KH_2PO_4) in comparison to plants growing in P-deficient soil, but it was also significantly stimulated by TCP along with PSB inoculation (Table 13).

Table 13: *Effect of PSB on growth promotion of chickpea plant*

Treatment	Shoot length plant^{-1} (cm)	Root length plant^{-1} (cm)	Shoot dry weight plant^{-1} (g)	Root dry weight Plant^{-1} (g)*
$\mathbf{I_0P_{KH2PO4}}$	17.00±1.39^{a}	12.70±1.04^{a}	0.426±0.050^{a}	0.382±0.026^{a}
$\mathbf{I_0P_{TCP}}$	10.87±0.29^{bc}	12.40±1.79^{a}	0.159±0.048^{b}	0.080±0.014^{b}
$\mathbf{I_3P_{TCP}}$	13.43±0.38^{ab}	13.33±0.33^{a}	0.432±0.062^{a}	0.401±0.050^{a}
$\mathbf{I_6P_{TCP}}$	14.70±1.89^{a}	13.80±1.56^{a}	0.403±0.084^{a}	0.389±0.046^{a}
$\mathbf{I_7P_{TCP}}$	13.57±0.78^{ab}	13.23±0.43^{a}	0.530±0.089^{a}	0.393±0.051^{a}
$\mathbf{I_8P_{TCP}}$	13.70±1.32^{ab}	12.73±0.49^{a}	0.502±0.123^{a}	0.377±0.056^{a}

***Note**: Values are expressed as a mean of triplicates ± SE. $\mathbf{I_3, I_6, I_7, I_8}$ represents the inoculation treatments with isolates EbPSB 3, EbPSB 6, EbPSB 7, EbPSB 8,respectively, while $\mathbf{I_0}$ represents uninoculated plant. $\mathbf{P_{KH2PO4}, P_{TCP}}$ representing applied phosphorous sources (KH_2PO_4 and $Ca_3(PO_4)_2$). Mean values followed by same letter are not significantly different (P< 0.05). * Significant at 1%.*

The shoot length was increased with PSB treated plants over uninoculated control, but not found significant. The highest shoot length 17cm plant^{-1} was recorded in fertilized control (I_0P_{KH2PO4}) followed by statistically at par values due to isolates EbPSB 6 (14.7cm). Isolates EbPSB 8, EbPSB 7 and EbPSB 3 showed significantly higher shoot length over control (13.7cm, 13.6cm and 13.4cm, respectively). Similarly, EbPSB 6 treated plant showed the highest root length (13.8cm/plant) followed by EbPSB 3 and EbPSB 7 (13.3cm and 13.2cm, respectively) in comparison to control and other isolates. Plant height in the fertilized control (12.7cm) was on par with other inoculated treatments; consequently, there was no significant difference amongst the inoculants.

A significant increase in shoot dry matter (P< 0.05) of chickpea plant was observed in response to PSB isolates. The highest shoot dry matter (0.530g plant^{-1}) was recorded by isolate EbPSB 7 followed by statistically at par values due to EbPSB 8 (0.502g) and EbPSB 3 (0.432g). Isolates EbPSB 6 (0.403g) and fertilized control (0.517g) showed statistically significant increase of shoot dry weight over negative control (I_0P_{TCP}). Root dry weight was significantly increased (P< 0.01) in chickpea inoculated with selected PSB isolates, and the highest was recorded by I_3P_{TCP} (0.401g/plant) and I_7P_{TCP} (0.393g) followed by I_6P_{TCP} (0.389g/plant), I_8P_{TCP} (0.377gm/plant) and I_0P_{KH2PO4} (0.382gm).

CHAPTER SIX
DISCUSSION

The results of the present investigation revealed the presence of bacterial isolates involved in solubilization of insoluble phosphate in rhizosphere soils of *Erythrina brucei*. The population of these bacteria was estimated to be $8.7 \pm 0.84 \times 10^6$cfu g^{-1} of soil. Similarly, Banik and Dey (1982), Assefa Keneni *et al*. (2010) and Krishnaveni (2010) estimated PSB population to be $1.6x10^6$ cfu/g (alluvial soil), $>5x10^6$ cfu/g (yield sustained faba bean) and $1\text{-}2x10^6$ cfu/g (black and red soils), respectively. But, the PSB population in this study was slightly higher than the ones reported in Alberta soils ($1.4x10^4$ - $11.5x10^5$ cfu/g) by Kucey (1983) and in tropical soils ($2x10^1$ - $13x10^5$ cells/g) by Venkateswarlu *et al.* (1984). Such variations in PSB populations might be attributed to different soil factors such as, nutrient, pH, moisture content, organic matter and enzyme activities, and plant species (Ponmurugan and Gopi, 2006).

In the primary screening step, fifteen PSB isolates were selected from NBRIP agar plate, of which six of them showed a great reduction and/or subsequent loss of their PS activity (no visible halo) upon repeated sub culturing (app. 5) (data not shown). Similarly, Kucey (1983) and Diriba Muleta (2007) have reported that a high percentage of the bacterial isolates lose their abilities when sub-cultured. The nine persistent PSB isolates were tested for their morphological, biochemical, eco-physiological and agronomical characters. Colonies of all the isolates except EbPSB 9 were gram negative rods, round, motile, non-spore forming, catalase and oxidase positive, indole, H_2S and methyl red negative (Table 5). So, these and other characteristics might render the isolates to belong to the genus *Pseudomonas*. On the other hand, EbPSB 9, which was found positive for endospore and gram stain might place it in to the genus *Bacillus*. The classification is mainly based on Bergey's Manual of Determinative Bacteriology. Many of the previous findings also showed that bacterial isolates that are frequently encountered in phosphate solubilization belong to the genus *Pseudomonas* and/ *Bacillus* (Krishnaveni, 2010). Wassie Haile (1999) and Assefa Keneni *et al*. (2010) also isolated rhizobacteria belonging to the genus *Pseudomonas* that was effective TCP solubilizers.

The result obtained in this study through physiological tests showed that isolate EbPSB 6 and 7 had a broad growth range of tolerance to high concentrations of NaCl (up to 7.5%), high temperature (41^0c), and pH range of 4.5-11 (Table 6). Kuhad *et al*. (2011) reported that most bacteria prefer to grow in neutral to alkaline (pH 7-8) reaction for maximum solubilization of phosphate, which is in accordance

with the current finding. Similarly, the optimum pH and temperature for growth of PSB was also reported 5.0 to 7.5 (Balamurugan *et al*., 2010) and 30-35^{0}C (Gaind and Gaur, 1999), respectively. All isolates also demonstrated diverse levels of intrinsically resistance to the antibiotics tested and the majority of the isolates were found to be more sensitive to streptomycin sulfate and Kanamycin monosulfate. On the contrary, almost all the isolates were tolerant to Erythromycin, Nalidixic acid and Neomycin sulfate at given concentrations. Among the isolates EbPSB 1, 2, 3 and 4 were the most tolerant. Although almost all the isolates in the present study belong to the same genus (*Pseudomonas*), they show heterogeneity with regard to physiological responses (*i.e.* pH, temperature and salt tolerance, and antibiotic resistance), which ensure that isolates other than EbPSB 9 were different species of the same genus. Tolerance of PGPR to salt, pH and temperature stresses, and resistance to several antibiotics have an ecological advantage in the survival, multiplication and spread of bacterial strains in rhizosphere soils when introduced as inoculum (Nautiyal *et al*., 2000). Survival of inoculant leads to bacterial colonization on roots and expression of their beneficial effect (Yasmin *et al*., 2009).

Results on qualitative measurement of PS revealed isolate EbPSB 6 and 7 as the best solubilizers in NBRIP plate assay followed by EbPSB 8, EbPSB 5 and EbPSB 3. Solubilization efficiency (%) of the nine persistent PSB isolates were ranged from 143-308 (Table 8). Similarly, Sharma *et al*. (2007) recorded solubilization efficiency values of 128% by *Bacillus megaterium* and 200% by *Pseudomonas fluorescence*. Measurement of SE (%) ranged from 96-228 (Kannapiran and Ramkumar, 2011) and 233-275 (Qureshi *et al*., 2012).

The above preliminary observation suggested the existence of rhizobacterial isolates exhibiting different degrees of PS efficiencies based on halo formation on agar plate. To confirm this observation, the isolates were again tested on NBRI-BPB broth medium, which was previously shown to be a reliable qualitative indicator of PS activity based on visual observation (Mehta and Nautiyal, 2001). EbPSB 7 (0.706) and EbPSB 6 (0.611) were again the best P-solubilizers in broth media (Table 9). The result also reveals PS activity of EbPSB 3 (0.525), which is higher than those recorded by EbPSB 8 (0.475) and 5 (0.434). Similarly, Perez *et al.* (2007) isolated a total of 130 bacterial isolates showing different degrees of TCP solubilizing activities from NBRIP plates, and further selected ten of the best solubilizers exhibiting OD_{600} shifts of more than 1.5 units.

Among the PSB isolates examined for IAA biosynthesis, isolates EbPSB 1, 3, 6 and 7 synthesized a significant quantities of IAA under conditions tested (13.3-26µgmL^{-1}) (Table 9). Similarly, Yadav *et al*. (2010) and Kannapiran and Ramkumar (2011) reported PSB capable of producing IAA ranged from 11.7-25.7µgmL^{-1} and 17.2-26.5µgmL^{-1}, respectively. Reports of similar nature on the production of IAA by PSB have been made by various workers (Khalid *et al*., 2004; Ahmad *et al*., 2005; Ng *et al*., 2012). The variation in the amount of IAA has been attributed to the variability in the metabolism, culture and medium conditions, species and growth stage of rhizobacteria (Khalid *et al*., 2004). IAA induce proliferation of lateral roots and root hairs and thus increase nutrient absorbing surfaces, and consequently nutrient absorption and plant growth (Yadav *et al*., 2010).

The *in vitro* results on the antagonistic activity of PSB isolates against *Fusarium oxysporum* revealed that five of the isolates caused a reduced mycelial growth of the test fungus. The highest percentages of growth inhibition (PGI) were obtained with EbPSB 1 and EbPSB 3 causing 69.75 and 67.03% reduction, respectively, which were significantly ($P< 0.05$) higher than the rest isolates (EbPSB 2, EbPSB 4 and EbPSB 9) (Table 9). Previous studies on biocontrol of *F. oxysporum* showed that many bacterial species totally or partially inhibited development of the fungus under *in vitro* and *in vivo* conditions (Liu *et al*., 1995; Ahmad Idris, 2007; Upadhyay and Srivastava, 2010). Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment (Agarwal *et al*., 2011).

Evaluation on the efficiency of isolates on solubilization of four different P-sources showed that all the PSB isolates solubilized phosphate at significantly ($P< 0.01$) higher rate than uninoculated control with a subsequent pH drop. There was also a progressive increment in solubilization of all P sources throughout the incubation period except in ErP, where EbPSB 3 showed a slight decrease after ninth day. An initial increase in P concentration followed by a gradual decrease in culture filtrate as observed in EbPSB 3 has also been well documented by other workers (Nautiyal, 1999; Selvi *et al*., 2011). A plausible reason for such slight inconsistency could be attributed to the availability of soluble P_i, which has an inhibitory effect on further solubilization (Narsian *et al*., 1995; Grover, 2003) or its co-precipitation of with some organic metabolites or metal ions (Perez *et al*., 2007; Selvi *et al*., 2011). According to Rodriguez *et al*. (2000), Seshadri *et al*. (2002) and Selvi *et al*. (2011), the fluctuation of PO_4^- concentration could be due to utilization of existing P for growth and development of the organism. Overall, EbPSB 7 is found to be the most effective solubilizer in almost all P sources, followed by EbPSB 6 (except in bone meal).

The data on solubilization of P-sources reveals the release of soluble phosphate as much as 41mg/100mL in TCP, 29.64 in ErP, 29.69 in FeP and 19.76 in bone meal (Figure 5-8). This indicates tricalcium phosphate as the most degradable substrate of all, and bone meal the least. Similar result was also obtained by Balamurugan *et al*. (2010) working with *Pseudomonas* sp. where $Ca_3(PO_4)_2$ was the best P source followed by $FePO_4$, $Mg_3(PO_4)_2$ and $AlPO_4$. Moreover, the solubilization of P from $FePO_4$ were also less effective when compared with $Ca_3(PO_4)_2$ (Perez *et al*., 2007). However, Wassie Haile (1999) demonstrated bone meal as the best P source followed by TCP and ErP. Yirgalem Kumelachew (2009) supports the highest solubilization tendency of TCP followed by bone meal > ErP. The poor solubilization of rock phosphates and bone meal might be due to the complex mineral composition and other non P content (Bardiya and Gaur, 1972), and/ the presence of a strong apatite bond (Gupta and Biswas, 1994).

A maximum soluble P concentration achieved from TCP, FeP, and ErP solubilization was achieved at the lowest values of the culture pH (3.25 and 3.2), which is in accordance with the results obtained by Ivanova *et al*. (2006) and Wassie Haile (1999). In bone meal, however, the rapid decrease in pH is not accompanied by high release of phosphorous. Gyaneshwar *et al*. (1999) established that high P starvation induces large scale production of organic acids which decrease the pH of the medium rapidly. Thus, PSB isolates when inoculated in those media release more acid than they did with TCP or other P containing media.

Regardless of the type of P source applied, a fall in pH (initially 7.0) in the culture filtrates was observed. A decrease in the pH of the medium from the initial value of 7.0 to a final value of 3.0-2.0 was recorded by many workers (Crespo *et al*., 2011). In this study, the decrease in pH of the medium by 3.83 units (*i.e.* pH 7.0 to 3.17) was observed. According to Seshadri *et al*. (2004), the pH reduction in BM amended media was negligible. The addition of rock phosphate also did not affect pH rather a minute increase is observed (Gupta *et al*., 2007). However, in the present study, a drastic drop in pH of the PSB inoculated medium supplemented with ErP or bone meal was resulted. Assefa Keneni *et al*. (2010) observed a similar result in both Egyptian and Bikilal rock phosphate, and the reason could be due to the highly insoluble nature of the phosphate sources.

In the present study, a direct significant ($P<0.01$) correlation between quantities of P-solubilized and a drop in pH of culture medium was observed. The values of Pearson correlation coefficients were from -0.961 (TCP) to -0.931 (bone meal). Similar inverse relationship between pH and soluble phosphate was reported earlier (Rashid *et al*., 2004; Assefa Keneni *et al*., 2010; Selvi *et al*., 2011). In few others, degree of solubilization was not always proportional to drop in pH (Gupta *et al*., 2007).

Even though isolates showed an increase in phosphate accumulation up to the final day of incubation, the rate of soluble P released per day by each PSB isolate in all phosphate sources tested were maximum in the first three days, and decreased with extension of incubation period (Table 10).

P-solubilizing ability of PSB is affected by many physiological factors some of which are C and N source in medium, mineral source, pH, incubation temperature, aeration and incubation period (Kuhad *et al*., 2011). The results on the effect of C and N sources on TCP solubilization by EbPSB 7 showed a diverse level of PS activity (mg/100mL) and pH change (Table 11).

Regardless of N source, maximum solubilization was achieved with glucose as a carbon source followed by fructose > starch. The preference of glucose for PS over other carbon sources has been reported (Sridevi *et al*., 2007; Chibuogwu and Nmesoma, 2011). Balamurugan *et al*. (2010) reported TCP solubilization by *Pseudomonas* sp. in the presence of ammonium sulphate, where glucose was the best C source followed by fructose, sucrose and starch in that order. According to Ahuja *et al*. (2007), maximum solubilization was achieved with fructose as the carbon followed by galactose, lactose, glucose and starch in that order. Nautiyal *et al*. (2000) reported xylose, glucose and lactose as the best C source.

In case of nitrogen, maximum solubilization of TCP and reduced pH occurred in ammonium sulphate containing medium, which in agreement with results reported by Narsian and Patel (2000), Sridevi *et al*. (2007), Balamurugan *et al*., (2010) and Scervino *et al*. (2011), whereas KNO_3 as the nitrogen source caused the least amount of PS. Similarly, Nahas (2007) reported that ammonium nitrogen salts when used as N sources in culture medium resulted in higher P-solubilization than organic or nitrate sources. This is contrary to the observations of Seshadri *et al*. (2004) and Relwani *et al*. (2008) in which NH_4NO_3 and KNO_3 were the best in promoting PS in that order, followed by ammonium sulphate. Nautiyal *et al*. (2000) observed an equally effective role of ammonium and nitrate source for phosphate solubilization. No significant change in pH and soluble P was observed amongst the N sources. Thus, for any given N, changes in carbon had a significant ($P< 0.05$) effect on PS and pH, but not *vis-versa*.

The best combination of C and N source for optimizing P-solubilization was glucose and $(NH_4)_2SO_4$ that enhanced TCP solubilization with the highest efficiency. This is supported by Sridevi *et al*. (2007) and Balamurugan *et al*. (2010) working with *Rhizobium* and *Pseudomonas* sp., respectively. The next best combinations of C and N source were glucose with ammonium nitrate followed by fructose with ammonium sulphate and starch with KNO_3 based medium.

Of the nine isolates tested for *in vitro* germination bioassay (Table 12), EbPSB 6, 7 and 8 showed the highest germination percentage (100%). EbPSB 6 also resulted the best performance in radicle length, plumule length and a vigor index. Ashrafuzzaman *et al*. (2009) noted a 2.3 to 14.7% increase in rice seed germination over control. Similar improvement of seed germination parameters by PGPR has been reported by other researchers such as Kumar, 1998 (tomato, eggplant, soybean and chick pea), Sharma *et al*., 2007 (chickpea), Aipova *et al*., 2010 (wheat), and Ng *et al*., 2012 (rice).

Among the selected isolates tested for greenhouse trial, EbPSB 6 was again non-significantly superior in promoting plant growth (shoot: 14.7cm, root: 13.8cm) compared to the other isolates (Table 13). The maximum shoot length, however, was recorded by the fertilized control (17cm). The next best isolate in promoting shoot length and root length were EbPSB 8 and EbPSB 3, respectively. Increments in shoot and root length by isolates was significantly ($P< 0.05$) higher than TCP application alone. The highest shoot and root dry weight was obtained in pots, which received EbPSB 7 (0.53gm) and EbPSB 3 (0.401gm) in the presence of TCP. Meanwhile pots containing EbPSB 8, EbPSB 3 and EbPSB 6 also showed superior dry matter production compared to control. The increments in root dry weight by isolates were highly significant at $P< 0.01$ over uninoculated plant with the exception of fertilized control. In general, shoot and root dry matter ranged from 0.129-0.53gm and 0.064-0.401gm per plant, respectively.

Plant growth promoting effects of PSB strains in different crops were clearly demonstrated and a large body of evidence suggests that seed bacterization by active strains of PSB enhance seed germination rate, plant growth and crop yield, improve seedling emergence, responses to external stress factors and protect plants from disease (Ashrafuzzaman *et al*., 2009; Aipova *et al*., 2010). Significant improvement on shoot length and dry biomass through rice seed bacterization with *Bacillus amyloliquefaciens* and *Enterobacter gergoviae* was attributed to production of IAA and solubilized phosphate (Ng *et al*., 2012). Numerous other studies have shown a substantial increase in seedling growth, dry matter accumulation and seed yield following inoculation with PSB to many crop plants (Peix *et al*., 2001;

Dobbelaere *et al*., 2003; Hameeda *et al*., 2008; Mishra *et al*., 2010; Yadav *et al*., 2010). However, field experiments have been rather inconsistent (Kucey *et al*. 1989). The varying success of PSM inoculations can be due to different reasons (Kucey *et al*. 1989; Gyaneshwar *et al*. 2002): (1) insufficient survival and colonization of inoculated strains, (2) competition with native microorganisms, (3) nature and properties of soils and plant varieties, (4) starvation of nutrients in the rhizosphere to produce enough organic acids to solubilize soil P and (5) inability of PSMs to solubilize soil phosphates.

In general, the present data pertaining to the effect of inoculation of PSB isolates along with TCP under greenhouse conditions brought about a significant increase on shoot length, root length and dry matter production of shoot and root of *Cicer arietinum* seedlings over control (P application alone), but statistically at par values with fertilized control (KH_2PO_4). These results are in agreement with the findings of Mishra *et al*. (2010) and Yadav *et al*. (2010) working on chickpea plant. A similar result was reported by Afzal and Bano (2008) who assessed the inoculation effect of PSB isolates on growth of wheat seedlings. They observed that inoculated plants with P resulted in a significant increase in plant height and grain yield of wheat, while PSB inoculation or P application alone did not improve grain yield of wheat.

CHAPTER SEVEN
CONCLUSION AND RECOMMENDATION

7.1. CONCLUSION

The present study proved the existence of culturable PS rhizobacteria associated with the rhizosphere soil of *Erythrina brucei*. Fifteen PSB isolates were retrieved from rhizosphere soil samples of which six of them lost their PS activity upon repeated sub culture. The isolates were tentatively identified based on phenotypic character, and classified to the genus *Pseudomonas* and *Bacillus*. By and large, gram-negative phosphobacteria showed superior activities over *Bacillus* in terms of abundance and P release. Although, isolates showed similarities, heterogeneity with regard to their physiological responses does exist, ensuring different species of the same genus. Most isolates were tolerant to most of the environmental factors like high pH and low pH, high and low temperatures, high salt concentrations, and also displayed a considerable resistance to many of the tested antibiotics.

Both the qualitative and quantitative estimation of phosphate solubilization efficiency reveled isolate EbPSB 6 and 7 as the best phosphate solubilizers. Quantitative estimation of IAA produced by the isolates revealed the existence of variation among isolates, and it was maximum in EbPSB 6. In addition, potent inhibitory effects were exhibited by five PSB isolates against deleterious wilt disease caused by *F. oxysporum* f. sp. ciceri. This was indicated by PGI (%) values ranging from 48.9-69.8. The highest percentage inhibition was recorded by EbPSB 1.

Results on the beneficial role of applying PSB isolates on seed germination and seedling growth under *in vitro* conditions reveled a general increase in chickpea seed germination, seedling growth with the formation of lateral roots and vigour index compared to control. In pot experiment, a distinct and statistically significant improvements in terms of root and shoot length and dry matter production were observed in case of pots amended with TCP with the test cultures over control (P application alone), but statistically at par values with fertilized control (KH_2PO_4).

7.2. RECOMMENDATION

Based on the present finding the following recommendations are forwarded.

For quick and reliable qualitative screening of the most potent and persistent PSB isolates NBRI-BPB broth in combination with NBRIP plate assay should be employed. But, for initial screening it is suitable to use NBRIP solid media since it has a closer representation to soil conditions. In addition, for optimum solubilization of insoluble phosphate by PSB in both the medium and the soil the effects of nutrients (like C and N) on cheaper and easily accessible sources should be done.

Many PSB species remain unknown and more studies are needed to reveal the real picture of their biodiversity and taxonomy. So, there is a need for analysis and identification of isolated strains on molecular basis by applying the techniques like PCR (polymerase chain reaction) amplification of 16S-rRNA gene, sequencing, plasmid profiling, and RAPD (random amplification of polymorphic DNA), for finding the diversity among the isolates. Bioinformatics tools can also be applied for the phylogenetic analysis of these isolates.

The stability of few PSB isolates with respect to their retention of original activity has been found to be weak (*i.e.* prolonged storage and periodical sub culturing has resulted in decreased SE). Hence, there is a need for concerted efforts to isolate competitive strain capable of stable PS activity. To this effect two approaches could be followed, *viz*., (i) to extensively screen PSB and select very efficient and competitive strains (culture dependent) (ii) to genetically manipulate the available PS strains to enhance the basal PS activity. Introduction of this trait in strains with other plant growth promoting effects is also a very attractive approach. Moreover, the mechanisms involved in bacterial PS should be studied. As our understanding of the mechanisms used by PSB advances, it becomes feasible to enhance their capacity to stimulate plant growth by modifying promising traits in both areas of biofertilizers and biocontrol agents.

Although, the trend and relative effectiveness of microorganisms in the soil are very complicated and unpredictable, it is suggested that for further studies, variation in plant, bacteria population and species, and the soil type be considered. Still more research is needed in this field such as field evaluation and interaction effect of PSB along with mycorrhizae and nitrogen fixing bacteria on growth of plants may also be studied.

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APPENDICES

APPENDIX A

QUALITATIVE PHOSPHATE SOLUBILIZATION ASSAY

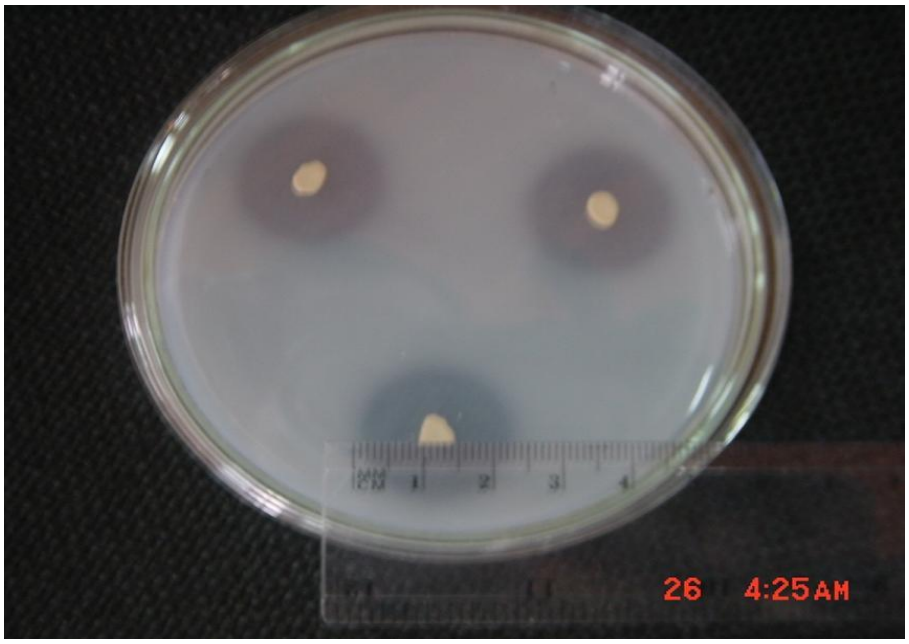

Figure A.1: *Solubilization of TCP by EbPSB 7 after 12 days of incubation in NBRIP agar*

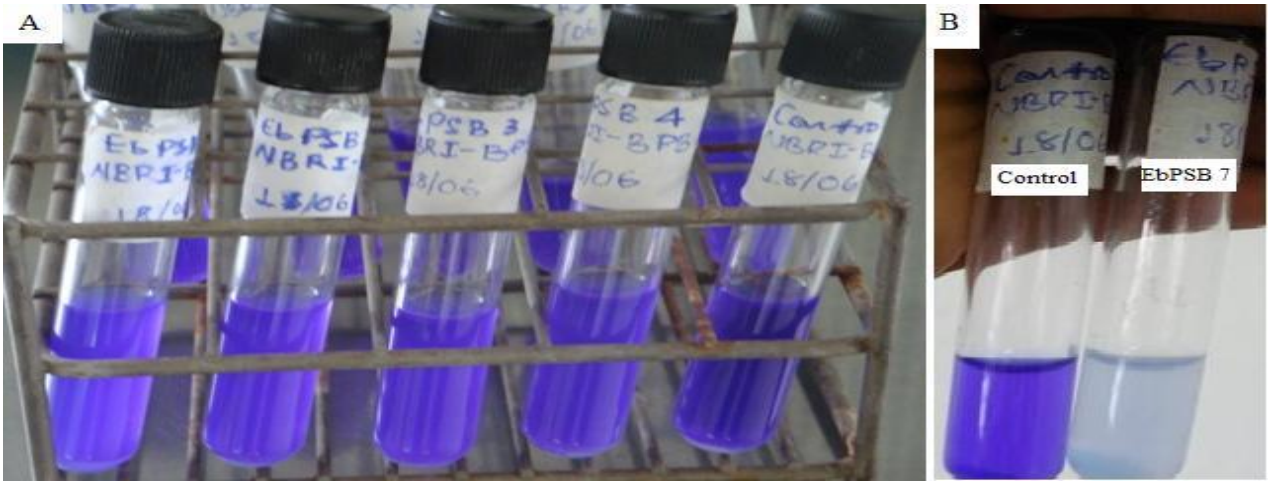

Figure A.2: *Qualitative analysis of PS activity (a) NBRI-BPB broth immediately after inoculation (initial OD) (b) broth inoculated with EbPSB 7 and control after 3 days of incubation (final OD)*

APPENDIX B

DETERMINATION OF SOLUBLE PHOSPHORUS IN GROWTH MEDIUM

Chlorostannous reduced molybdophosphoric acid blue method in sulfuric acid system

1. Reagents used for phosphorous estimation

Table B.1: *Reagents preparation instruction for estimating P concentration*

Reagents
Sulfomolybdic Acid Solution:
Exactly 6.25g of Ammonium Molybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$,is dissolved in 50.0mL distilled water slightly heated to dissolve, 70.0mL conc. $H_2SO_4$is added to this carefully(with mixing and cooling). The final volume was made to 250mL in volumetric flask. The reagent was stored in amber glass bottle in a cool & dark place. Thus stored, protected from light, the solution keeps indefinitely.
***Chlorostannous Acid Reductant*:**
Approximately 0.5g $SnCl_2$ is dissolved in 2.0 mL Conc. HCl, heated slightly to dissolve. This solution is diluted (with rapid stirring) to a final volume of 250 mL with distilled water. This was always prepared fresh just before use.
Standard P solution (50 ppm P)
Dissolve 0.2195g of dried KH_2PO_4 in 1000 mL of distilled water.
Working P solution (10 ppm P)
Dilute 10 ml of *standard P solution* to 50 mL of distilled water.

2. Procedure for estimation of soluble P in culture filtrate

- Take the broth of bacterial isolates, centrifuge and filter it.
- After centrifugation and filtration, 5.0 mL aliquot of each culture filtrate is transferred to 50 mL volumetric flask.
- To this add 2.0 mL of *Sulfomolybdic Acid Solution*.
- Next, add 1.0 mL of freshly prepared *Chlorostannous Acid Reductant*, and the solution is mixed thoroughly. The blue color intensity develops in 3 to 4 minutes.
- After mixing made the volume up to 50 mL as quickly as possible.
- Absorbance of the final solution is measured at 700nm**.**
- Check the sample's absorbance against the calibration curve and determine the concentration correct for dilution.

3. Standard calibration curve preparation for phosphorous estimation

Since the phosphate concentration is measured as a function of absorbance, a standard curve of absorbance versus known phosphate concentrations must be prepared. Six standard phosphorus concentrations and distilled water blank are treated with the same procedures as the samples **(**bacterial suspension**)**. Prepare 6 dilutions of the 10 PPM phosphate standard to result in the following final concentrations:

Table B.2*: Preparation of working standard phosphate concentration and absorbance value*

Volume of Standard	Final Volume in Flask	Final Concentration	Absorbance (OD_{700nm})
0.0 mL	50.0 mL	0.0 PPM	**0.0**
1.0 mL	50.0 mL	0.2 PPM	**0.145**
2.0 mL	50.0 mL	0.4 PPM	**0.223**
3.0 mL	50.0 mL	0.6 PPM	**0.311**
4.0 mL	50.0 mL	0.8 PPM	**0.398**
5.0 mL	50.0 mL	1.0 PPM	**0.496**
6.0 mL	50.0 mL	1.2 PPM	**0.579**

These ***7*** values are used to plot absorbance versus P concentration to give a straight line passing through the origin. Prepare all standards in duplicate. Use the average of absorbance for preparing the curve.

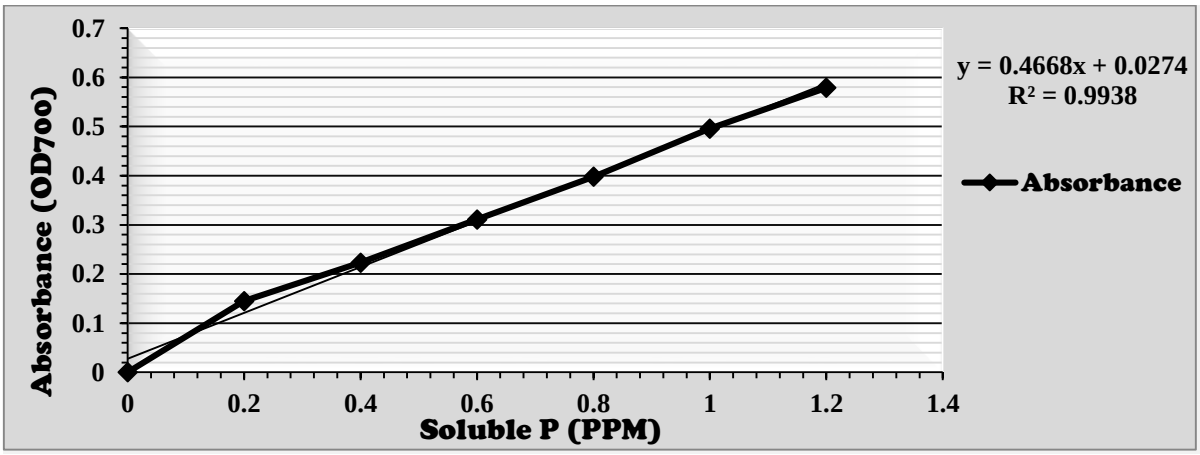

Figure B.1: *Standard calibration curve for P estimation*

Calculations:

The quantity of soluble-P in the culture filtrate (5mL) was determined using the above formula (*i.e.* **y= 0.466x – 0.027**, where **y**= *optical density at 700nm*, and **x=** *quantity of soluble-P in µg* mL^{-1}). The final P concentration of the sample (100mL) was determined by multiplying P concentration from the curve by 50mL and dividing it by initial volume used (*i.e.* 5mL).

APPENDIX C

PREPARATION OF STANDARD GRAPH OF IAA

Standard graph of IAA was prepared as mentioned by Sahasrabudhe, 2011. Different IAA concentrations are prepared as aqueous solution of IAA ranging from 12.5µg mL^{-1} to 100µg mL^{-1}. To each two drops orthophosphoric acid and 4mL of the standard, 1mL 0.5% FeCl in 35% perchloric acid *i.e.* Salkowaski's reagent is added and readings are taken after 30 minutes at 530nm by UV-Visible spectrophotometer.

Table C.1: *Standard concentrations for IAA and their absorbance value*

Standard for IAA	Absorbance (OD_{530nm})
0 PPM	**0.023**
12.5 PPM	**0.044**
25 PPM	**0.068**
50 PPM	**0.166**
100 PPM	**0.316**

Standard graph was prepared by plotting concentration of IAA in µg/mL vs. Optical Density at 530 nm. Straight-line graph indicates direct proportion between concentrations of IAA and the extent of red color developed. R^2 value of the graph was found to be 0.991 that showed the validity of the graph.

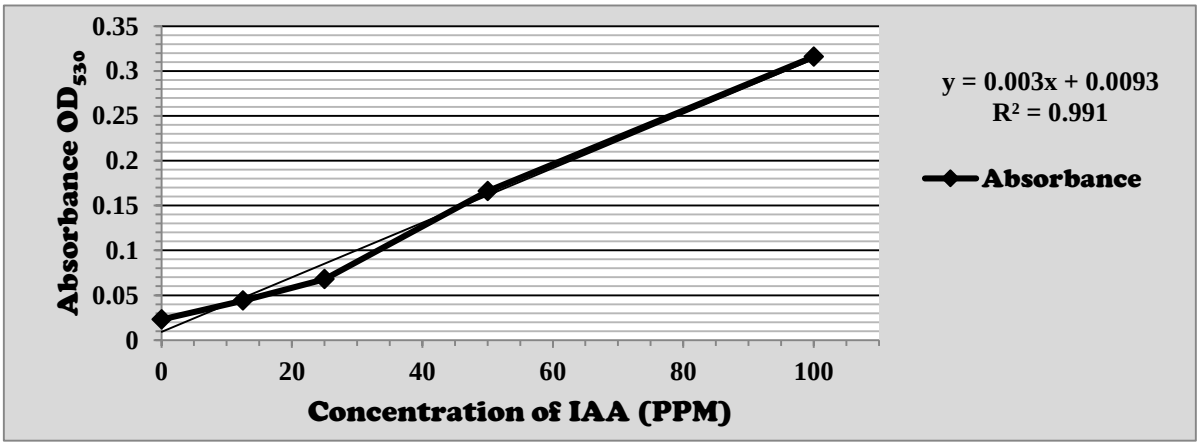

Figure C.1: *Standard graph of IAA*

Calculations:

The quantity of IAA in the culture filtrate was determined using the above formula (*i.e.* **y= 0.003x** + **0.009**, where **y**= *optical density at 530nm* and **x**= *quantity of IAA in µg* mL^{-1}) and expressed as PPM of the medium.

APPENDIX D

BIOCONTROL POTENTIAL OF PSB ISOLATES

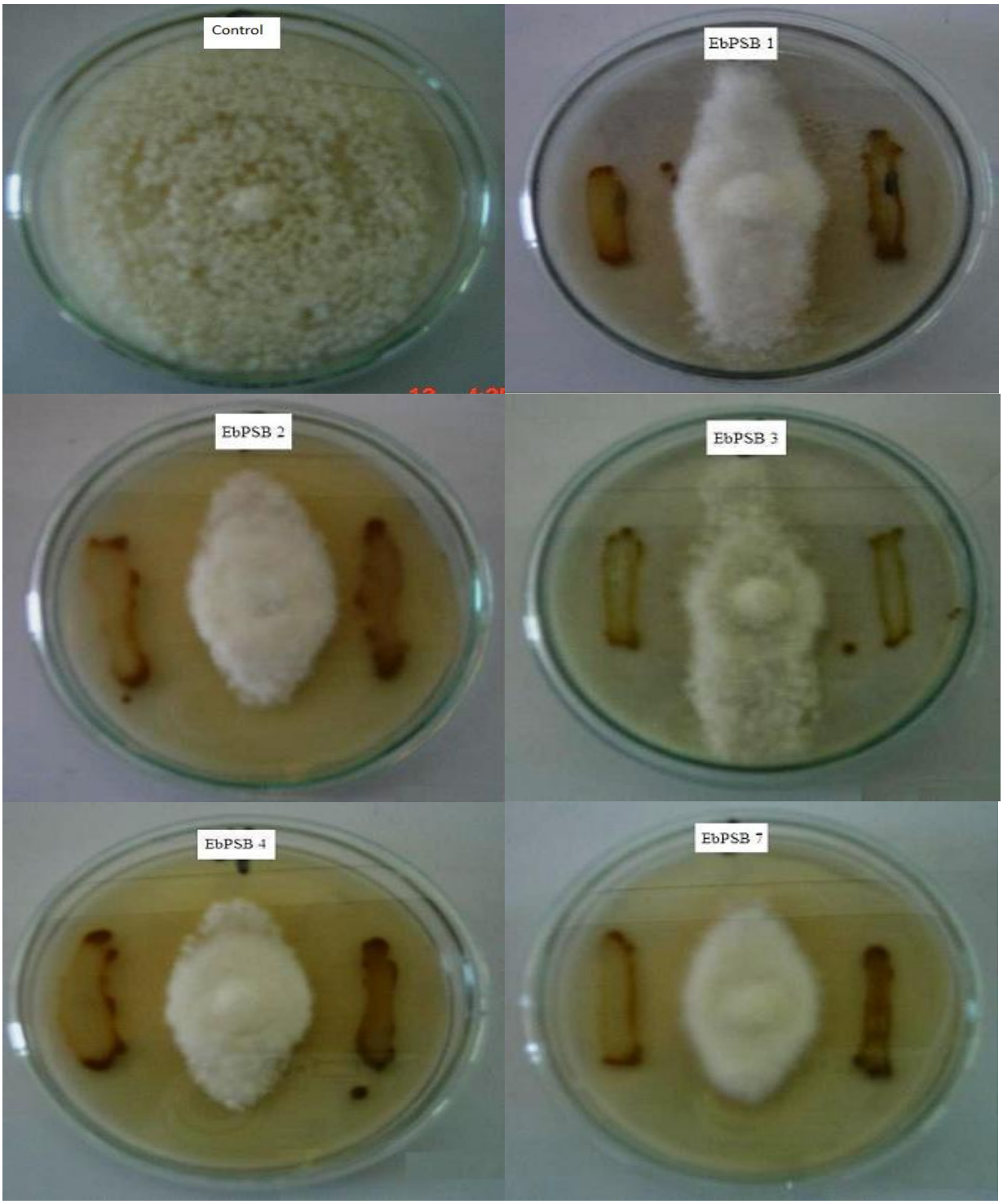

Figure D.1: *Antagonistic activity of PSB isolates against F. oxysporum* f. sp. *ciceri under in vitro*

APPENDIX E

GERMINATION AND GROWTH PROMOTING EFFICIENCY OF PSB ISOLATES ON CHICKPEA

1. Germination capacity of PSB isolates on chickpea seeds

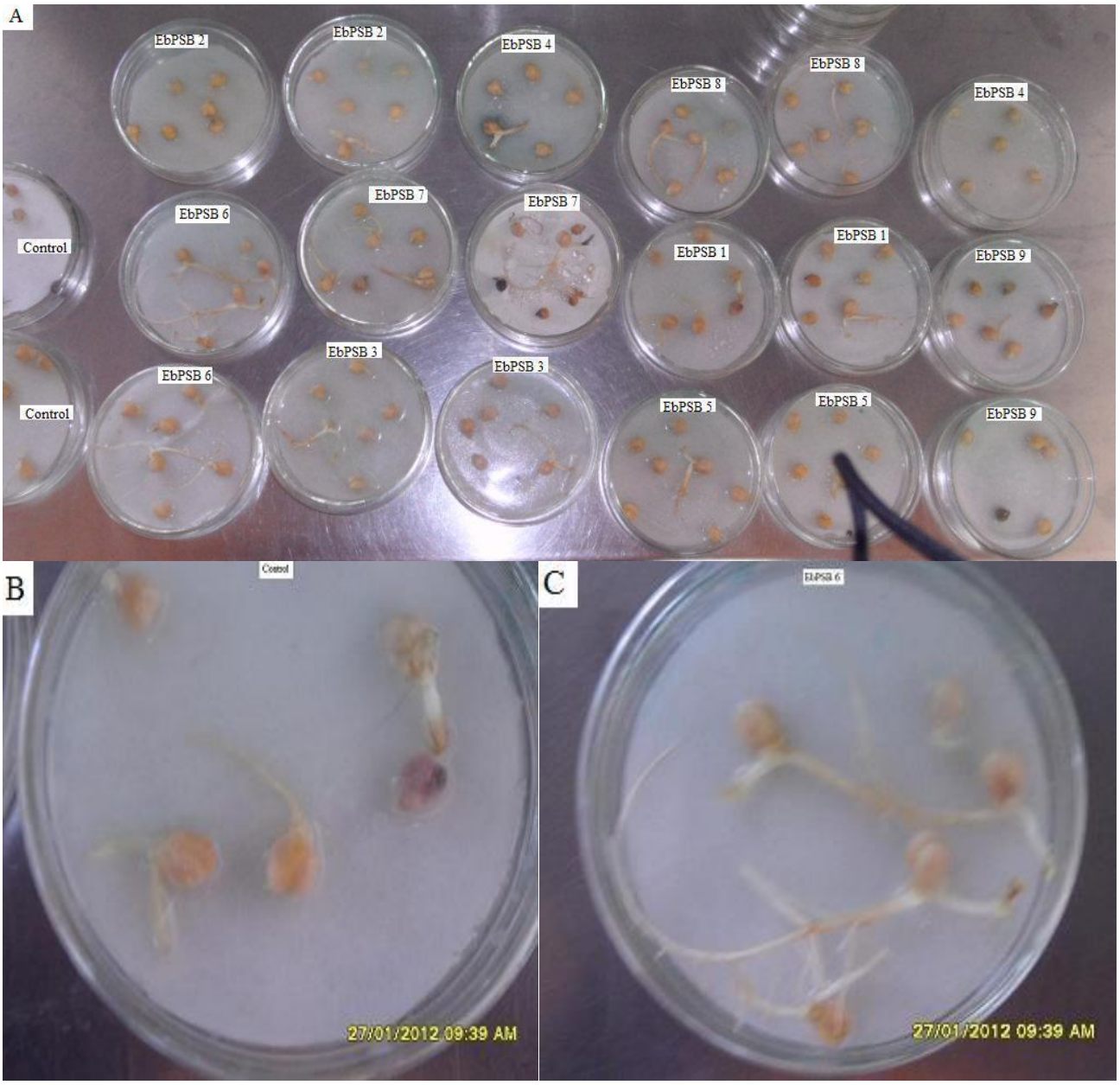

Figure E.1: *(A) Effect of PSB isolates on chickpea seed germination, (B) germination on control plate and (C) EbPSB 6 inoculated plate*

2. Green House evaluation of PSB isolates on chickpea plant

Declaration

I, the undersigned, hereby declare that this thesis is my original work and all information has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Name: Mulugeta Belay

Place: Addis Ababa University

Signature: _____

Date: _____