ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM RHIZOSPHERE OF LOCAL FLORA OF D. I. KHAN

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Phosphates - Solubility.

Soil microbiology.

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FINAL APPROVAL

It is certificate that we have read and evaluated the thesis "Isolation and Characterization of Phosphate Solubilizing Bacteria from Rhizosphere of local flora of D.I.Khan" submitted by Ms. Samina Shah and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology

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DEDICATION

To

My Honourable Grandfather

S.Mohammad Hassan Shah

DECLARATION

It is hereby declared that the work present in the following thesis is our own effort, except where otherwise acknowledged, and that the thesis is our own composition.

No part of the thesis has been previously presented for any other degree.

Date_____

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In the name of ALLAH, Most Glorified, Most High

All praise to Almighty ALLAH, the eternal, the sovereign over everything and the only creator of the universe, who gave me courage, strength and patience to carry out this work. Who, I believe never spoils any effort of good deeds. May the blessings of ALLAH be upon his Prophet Muhammad (Peace Be upon Him), the source of knowledge and blessings for the entire creation, who has guided his Ummah to seek knowledge from cradle to grave, and enable me to win the honour of life.

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Samina Shah

LIST OF ABBREVIATIONS

Acronym	Abbreviation
°C	Degree centigrade
µg	Micro gram
µg/ml	Micro garm per millilitre
%	Percentage
ACC	1-aminocyclopropane-1-carboxylate
BNF	Biological Nitrogen Fixation
cm	Centimetre
CAS	Chrome azurol
HCl	Hydrochloric acid
HCN	Hydrogen Cyanide
IAA	Indole Acetic Acid
kb	Kilo basepairs
l	Litre
LB	Luria Broth
mm	Milimeter
mg	Miligarm
MgCl2	Magnesium Chloride
MgSO4	Magnesium Sulphate
min	Minute
ml	Mililiter
NARC	National Agricultural Research Centre
N2	Nitrogen molecule

NaOH	Sodium Hydroxide
OD	Optical density
P	Phosphorous
PGP	Plant Growth Promotion
PGPM	Plant growth promoting microorganisms
PGPR	Plant growth promoting Rhizobacteria
PSM	Phosphate solubilizing microorganisms
PSB	Phosphate solubilizing bacteria
rpm	Revolution per minute
LBA	Luria Bertani agar
PVK	Pikovskaya
PSI	Phosphorous Solubilizing Index
TCP	Tri Calcium phosphate
NH3	Ammonia

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ABSTRACT

The phosphorus content in soil (organic plus inorganic) is usually much higher than plant requirements, however bioavailability of phosphorus to plant is one of the major plant growth limiting constraints. Even the added P as phosphatic fertilizer may get unavailable because of its fixation/ precipitation in soil, particularly in calcareous and alkaline soils. Thus, there is dire need to mobilize this big pool of soil phosphorus for improving crop yields on sustainable basis and one of the strategies useful for this purpose is the use of specific microorganisms applied as inocula.

The objectives of this research was isolation and characterization of PSB from rhizosphere soil of Tomato, Rice, and Maize sample and evaluate their potential for P solubilization from insoluble P compounds for the effective plant growth promotion. These PSB isolates were further tested for the production of siderophores, antimicrobial secondary metabolites (HCN), production of hydrolytic enzymes (pectinase, proteases, and amylase), production of Ammonia and phytohormone production (IAA). Tomato is an important crop of Pakistan mainly grown in rain fed area. So, inoculation of phosphate solubilizing bacteria was studied to enhance the yield. Normal agronomic practices were followed till crop maturity. The study revealed that yield and fruiting of Tomato was significantly improved over control with PSB strains. All the isolates showed diverse enzyme activities. All strains showed good Protease and amylase activity while all strain exhibited no pectinase activity. Strains showed best results in case of ammonia production. The highest number of shoot dry weight/g (5.63 g) was detected in the plant treated with SM-3.While the highest number of plant height was observed when plant was treated with SM-3 and TS-4 102.66, 104.6 cm respectively. No significant differences were seen between treatments but only had significant differences compared with untreated plant. The highest number of fruit/plant was observed when plants were treated with SM-

13.The highest weight of tomato plant was observed with TS-1 and the lowest weight of plant was observed in plants treated with TS-6. Furthermore, SM-3, SM-13, TS-3, RS-5 showed increased root and root weight of a Tomato riogrande variety as compared to un-inoculated control.

Based on these results it can be concluded that these isolates have the potential to be used for plant growth promotion and needs to be further evaluated. All the morphological measurement showed a great response in PSB treated plants thus confirming the efficiency of the selected isolate PSB as a phosphate solubilizer.

Chapter 1
Introduction

1.0 INTRODUCTION

A noteworthy system to defeat the decrease in soil profitability is to grow new procedures for sustainable agriculture. There is need to undertake research on progression in biological perspectives to accomplish this task in an environment cordial way (Swaminath, 1991).Contemporary agriculture is relying on the use of chemical fertilizers, pesticides and growth regulators to upsurge yield. This reliance is linked with difficulties, such as environmental contamination, health threats, disruption of natural ecological nutrient cycling and damage to biological communities that otherwise maintain crop yield. Moreover numerous farming practices cause change of soil qualities that outcome in soil malfunction and eventually in the corruption of soil and water assets (Kloepper *et al.*, 1980., Enebak *et al.*, 1998).

Soil quality is a critical component of sustainable agriculture. It is an excellent niche of growth of many microorganisms: protozoa, fungi, viruses, and bacteria. Of these distinctive microorganisms, bacteria are by a wide margin the most well-known (i.e., ~95%). Research uncovered a cooperation between soil microorganisms and plants (from the perspective of the plant). This communication might be valuable, hurtful, or impartial. Nonetheless, the impact that a specific bacterium has on a plant might change as the conditions change. Plant growth in agricultural soils is prejudiced by numerous abiotic and biotic factors (Berthelet *et al.*, 1996).

New and novel solutions for plant growth improvements are mandatory to ease the burden imposed on our surroundings (Glick, 1995).Microorganisms in soils have a huge supply of hereditary material that can be used for creating novel anti-toxins, enzymes, bioremediation agents, biocontrol operators, plant development advancing operators, and dug for genes which can deliver transgenic plants and creatures, and further biotechnological applications (Berthelet *et al.*, 1996).

Plant growth promoting rhizobacteria (PGPR) are beneficial and have agricultural importance. These bacteria positively effects plant growth and health, suppress pathogenic microorganisms and increase nutrient accessibility and assimilation. Along these lines, keeping in mind the end goal to enhance soil fertility and product yield and to diminish the negative impacts of chemical fertilizer on the surroundings, there is a need to use PGPB for consistent valuable agriculture purpose (Rashid *et al.*, 2004).

Phosphorus is one of vital plant supplement and positioned after nitrogen in micronutrients. Its commitment to enhance plant biomass is perceived by exploration specialists (Goldstein, 1986). Soil might contain adequate measures of phosphorus yet generally it is not accessible to plants. For the most part the dissolvable P react with soil compound which brings about the development of insoluble compound (Saying *et al.*, 1990).

In Pakistan calcareous soil, metal cation encouraged edification of about 75–90% included P fertilizer. (Hinsinger, 2001). Further, it has additionally been theorized that the measure of phosphorus settled has expanded to such a degree in arable soils that are adequate to maintain most extreme crop yields worldwide for around 100 years (Goldstein, 1986).

PGPB are present as endophytes inside plants or as rhizobacteria in the rhizosphere. PGPR having the P-solubilizing limit are called as phosphate solubilizing bacteria (PSB).These accounted for to expand Phosphorous-accessibility by changing over insoluble structures to dissolvable ones by the release of organic acids (Rashid *et al.*, 2004) and consequently high the product yields (Zaidi, 1999).Soil inoculation with PSB is a promising methodology that might lessen the lack of phosphorus (Cakmakci, 2005).

The phosphate solubilizing capacity of creatures can change over the unsolvable phosphatic into dissolvable structures (Kang *et al.*, 2002; Pradhan and Sukla, 2005) in soil that make them accessible to the crops. This availability of inorganic phosphorus in the rhizosphere and nutritious quality of soil changes significantly with plant species (Hoflich *et al.*, 1994).

The effects of inorganic and organic sources of Phosphorous (symbol: P) on the growth of some plants in the laboratory experiments and after cultivation were investigated earlier (Zaidi *et al.*, 2009; Aslam *et al.*, 2010). Besides the role of other factors Phosphorous treatment significantly affected the yield of mungbean (*Vigna radiate* L.); Chickpea (*Cicer arietinum* L.); and wheat crop (*Triticum aestivum* L.) in different areas of the world (Afzal and Asghari, 2008; Aslam *et al.*, 2010; Bhattacharjee and Dey, 2014; Sarker *et al.*, 2014). Various concentrations of: poultry manure and chemical fertilizer were formulated and prepared to observe the effects of different treatments.

Phosphorous, organic and inorganic sources were established to play a significant role in attaining economical harvest. In a study, phosphate solubilizing bacteria (PSB) were found to enhance growth and nutrient uptake by wheat. It was concluded that different nutrients play a pivotal role on plant growth and the farmers were suggested the timely use such a practice which would also be an environmental friendly strategy (Aslam *et al.*, 2010; Sarker *et al.*, 2014).

The experiments on wheat plants also confirmed the role of P along with other nutrients (Afzal and Asghari, 2008).Promising results were observed on chickpea cultivar nodulation, grain yield and grain protein contents by rhizobium inoculation and fertilizer. The results indicated that phosphate solubilizing bacteria significantly improved tolerance against environmental stress and increased crop yield (Aslam *et al.*, 2010).

The rhizospheric bacteria are known to support a feasible growth of plants. It is traditionally believed that biofertilizers and control agents are essential for the improvement of agriculture world-wide. A number of plants and crops were investigated by different researchers and details were published and well circulated. The bacteria involved in this process were termed as "Plant Growth Promoting Rhizobacteria" abbreviated as 'PGPR' (Ashrafuzzaman, *et al.*, 2009).

It is worth mentioning that PSB were used as inoculants and interestingly they lead to increase P uptake by the plants. Later, the fact was substantiated in different crops in the field. The following genera species were found to be dominant to improve the crop yield: Pseudomonas species, Bacillus species, and Rhizobium species (Mahalakshmi and Reetha, 2009).

However, continued evidence based research work in different zones and regions confirmed the potential role of bacteria from different genera in promoting the availability of P which is otherwise, poorly available in the soil. The powerful active strains were repeatedly confirmed to improve the development of various plants then their mechanism of action was via solubilizing and availability of phosphorous (Aslam *et al.*, 2010).

Various plant development advancing microorganism in the have been isolated from rhizosphere soils have been comprising more than one systems for the advancement of plant development. At the point bacteria get into contact with plants where they might apply a scope of exercises that independently or in mix can bring about the advancement of plant development. (Mahalakshmi and Reetha, 2009).

Following the principal studies on PGPB in the 1950's, a few of applicant PGPB strains have been isolated and assessed in research facility, nursery and field contemplates far and wide. Today PGPB are as often as possible utilized as a part of rising states on a large number of hectares of area (Zehnder *et al.*, 2001).

Utilization of this biotechnology has been backed off by the absence of relentlessness and variety in reactions that are gotten in field trials (Lambert and Joos, 1989) however PGPB are nature friendly. Irregularity in exploratory results lies as an aftereffect of vacillations in natural conditions yet part of organic factor can't be discounted. Chemical fertilizer build high yield in farming however are costly and harming to the earth. They exhaust non-renewable vitality through draining out and polluting water basins, decimating smaller scale living beings and cordial bugs, making the crops more powerless to the assault of ailments, lessening soil fertility, in this way bringing about unsalvageable harm to the general framework. The utilization of PGPB could be a superior different option for chemical fertilizer and compound pesticides. They are sparing, ecofriendly and could without much of a stretch be found.

Aims and Objectives

- Isolation of phosphate solubilizing rhizobacteria from agricultural soils.
- Characterization of PSB.
- Assessment of PGP traits of bacterial isolates using *in vitro* studies
- Evaluation of PGP characteristics of bacterial strains on selected crop plant using in vivo studies.

1.1 Dera Ismail Khan

District Dera Ismail Khan (abbreviated as: D.I. Khan) is located in Khyber Pakhtunkhwa province of Pakistan where both Pashtu as well as Saraikiy are the languages spoken by the folk for communication (Fig. 1).

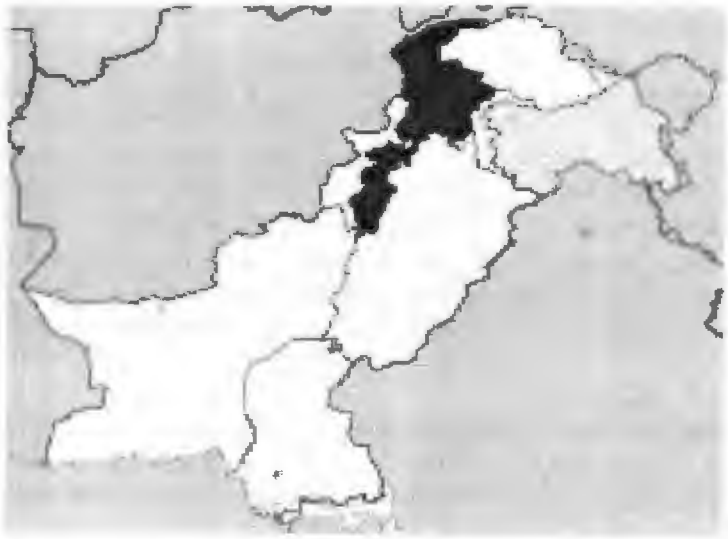


Figure. 1: Map of Pakistan with colored part and line are Representing Khyber Pakhtunkhwa province (KPK) of Pakistan.

1.1.1 D.I. Khan - Location and demographic data

According to official record of 2014, the total area of Dera Ismail Khan is 2,829 sq. miles (7,326 Km^2) with total population 1,939,000 and population density: 116/Km^2 (300/sq. miles). Saraiki and Pashtu are the languages of common folk. River Indus is guarding the eastern side of D.I. Khan where on the eastern bank of the river two cities of Punjab province (namely: Bhakkar city and Dera Ghazi Khan city) are located; South Waziristan on southwestern side; and Tank and Lakki Marwat districts are located on the northwest side, whereas Zoab region of Baluchistan is in the southern side of D.I. Khan. Sulaiman Mountain (the extension of Himalaya i.e. Hindukush mountainous range) is in the west serving as boarder-line between D.I. Khan, Pakistan and Afghanistan. The highest peak in Sulaiman Mountain is historically known as Takht-e-Sulaiman (Mehdi *et al.*, 2009).

The people of D.I. Khan Region are peace loving farmers and fond of agriculture and cattle rearing. Regrettably, D.I. Khan Area was neglected by the rulers earlier including the era of British Rule in Indian sub-continent. Hence, the developmental programs could not be initiated regarding food/agriculture, education, health and other sectors, which added to poverty elevation, lack of education and lack of facilities for agriculture in the region. No one could initiate any research project on resolving the role of phosphorous (P) and phosphorus solubilizing bacteria (PBS) in plant growth (Bernstein, 1978).

In the earlier documents and scripts, instruments used in agriculture, crops, vegetables, fruits and cattle products as well as the living style and dress of people living in and around Sulaiman Mountain are mentioned in the ancient record. But none of the old records could show any work on the role of P and PSB in the growth and performance of plants/crops (Bellew, 1880).

1.1.2 Current day location of D.I.Khan

The present day location of D.I. Khan City was selected and constructed in 15th century by Balouch tribes. D.I. Khan was part of 'Langah Dynasty' of Multan. D.I. Khan was named after Ismail Khan Balouch son of Sardar Malik Sohrab Khan (The area Subedar of Langah Rulers and Sardar of Balouch tribe). The land was called 'Daman' and it was mostly un-cultivated due to lack of water. However, rain water supported the traditional agriculture and lively hood of people in Daman area. Without knowing much, the local farmers used to put animal dung as manure and kept on discussing their field crop yield and possibilities for improvement (Mehdi *et al.*, 2009).

1.1.3 The barren land of D.I. Khan – Barani agriculture and folk traditions

The uncontrolled bed of river Indus was called 'Kachi' area whereas; the eastern part was a desert land. The land of District D.I. Khan in NWFP (now KPK) was fertile and agriculture was mostly dependent on rain (Barani) and other natural water resources. The lack of water resources kept the major area of the land uncultivated. Nevertheless, water from Indus River (Paharpur canal and right-bank canal), water from Sulaiman Mountain, Lur'ren and Zam water resources were tried throughout the history to be brought under control by making reservoirs followed by proper utilization, but tribal rivalry and lack of vision kept the area dry and no dams could be made (Bernstein, 1978)

Since ancient times, the land of D.I. Khan region was known to be suitable for the cultivation of wheat crop, maize, chickpea and rearing of animals. Unfortunately, a huge area of this region depended on rain water for crop cultivation (Barani area) (Khan, 2011).

The governmental grant and supervision is still needed to promote agriculture research, evidence based improved seed varieties and the supplies of seeds, other material (including machinery for agriculture), and training programs (modern techniques and research based farming). Such moves at a stage become the backbone of economy (Khan, 2011).

It is amazing to see that the common man and local farmers started participating in agriculture shows, agriculture elevation programs, and started open discussion on the output of the agriculture field work and crop model plots and other promotional schemes. Now it is well acceptable to ask the folk about modern agriculture because they got the information and attended the training programs regarding: improvements in different scientific fields of agricultural development. The farmer community of D.I. Khan is swiftly trying to use and understand the role of bacteria in improving agriculture (Ghulam *et al.*, 2012).

Dera Ismail Khan Agriculture and horticulture classified and other free online programs including radio and media related efforts are contributing in decimation of information: such as programs regarding education, training/skills development, promotion of agriculture, and timely use of fertilizers, pesticides, insecticides etc (Ghulam *et al.*, 2012).

1.2 Nature of Soil – D.I. Khan District (Soil texture)

The presence of certain essential macro and micro nutrients in the soil defines the fertility status of soil However, yield capacity, organic matter, pH, soil type and soil texture are also strong indicators of soil fertility (Cook, 1967).

The symptoms showing nutrient deficiency, field experiments, greenhouse experiments, and soil testing and plant analysis are the factors which help to find solution and requirements to bring the declining crop situation back to normal. Internationally, such investigations assist to conclude the need as well as quantity of fertilizer recommendations according to FAO procedures (Cottenie, 1980).

It is worth mentioning that the soil fertility in D.I. Khan is low due to poor levels of N, P, K and ther essential elements with eroded and light textured soil. Researchers in D.I. Khan are joining hands with other institutions to bring solutions to their problems and improve the performance of different crops in the region. In 'Rod Kohi' area of D.I. Khan: soil pH varies between: 7.3-8.2, and land is calcareous (Khan *et al.*, 2011).

On the other hand appropriate amount of K was found in 70.6% samples from the Rod-Kohi region of D.I. Khan; soil nutrients: presence of maco and micro nutrients have a significant role to play. However, the yield capacity and salinity along with electric conductivity and pH also measured as an indicator parameters (Cook, 1967; Khan *et al.*, 2011). It was also observed that effective use of N, P and K in balanced proportion had increased the yield of wheat under Rod Kohi conditions of D.I. Khan Division (Chaudhry, 1989).

Soil texture lies between: silty clay to silty clay loam along with low nutrients and problems of salinity/sodicity (Khan *et al.*, 2011). The soil testing (developed by earlier researchers) is the commonly used technique because it is found to be economical and accurate soil testing procedure (Gurmani and Bakhsh, 1989; Bajwa, 1992).

1.3. D.I. Khan - essential crops and weed control

How to control weeds on a modern fruit farm was a question rose earlier. It included many topics such as: insect control, pruning fruit trees, planting trees and fertilization were brought under discussion. Nevertheless, the danger of weed control and pest control is an interesting topic by itself because such factors seriously affect the crop productivity and affect fruit gardens. For weed control one has to bear in mind different points such as: organic agriculture, chemical poisoning, effects of pollution, environment and health of fruit-trees, crops, mulching and herbicides, human and animal health (Panhwar, 2005; Marwat *et al.*, 2013; Bakht and Khan, 2014).

Much work has been done on this issue; however, researchers at different institutions including D.I. Khan are working on this subject to keep the food situation (crops such as wheat) in control. Interestingly, some plant extracts were tested to be used for weed control in D.I. Khan under a research study entitled: Allelopathic effects of leaf water extracts of Eucalyptus, Acacia, Sorghum, Shishum, Sunflower, Poplar, Tobacco and Congress grass on weeds control and growth of wheat cv. The results obtained clearly demonstrated: the weed controlling potential of the plant extracts which in turn lead good crop performance and increase in the wheat yield. However, the limitation observed was that the extracts which could not be used for an unlimited area (Bakht and Khan, 2014).

Furthermore, the availability of phosphorous and other essential mineral in the soil still remained a challenging task because P contents are low in soil of district D.I. Khan (Khan *et al.*, 2015). It is also worth noteworthy that antagonistic bacteria that applied artificially affect the pervasiveness of all diseases of wheat in field experiments (Capper and Campbell, 1986; Steveni *et al.*, 1992; Marwat *et al.*, 2013).

Tomato farming and weed control by mulching and use of herbicides was investigated at Agriculture University, Peshawar (Bakht and Khan, 2014).

1.4 Integrated plant nutrient, Essential elements

Plants generally get their mineral from the soil solution. Six mineral components, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S), are prerequisite in high totals, while chlorine (Cl), boron (B), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) and molybdenum (Mo) are needed in more diminutive aggregates In geological domains of low phyto-accessibility, indispensable mineral parts are provided to crops by means of manures to achieve more paramount yields. Also, fertilizers comprises of indispensable mineral for human food are provided to crops to assemble their obsessions in consumable bits for the prosperity of humans (White *et al.*, 2010).

Copper is a crucial micronutrient for plants and creatures. It is included in numerous catalyst frameworks of plants and animal. Iron is a vital minor supplement for plants. It is included in chlorophyll synthesis in chloroplast. Zinc is fundamental for the change of sugars. It controls utilization of sugars. It is a piece of the enzyme system which direct plant development (Akenga *et al.*, 2014)

Diverse crops are known to rich with high level of PGPR which likewise upgrades filtering and capacity of certain crucial components, for example, zinc (Zn) which is vital both for human and also for plants. Zn levels are currently kept up by such organic systems and they are better than utilizing Zn-fertilizer (Abaid-Ullah *et al.*, 2015; Khan *et al.*, 2015).

Magnesium (Mg) is a vital component for growth of plant and development. Mg lacking is normally connected with interveinal chlorosis and development diminishment, though the more continuous idle insufficiency is regularly not obvious and barely to analyze but rather contrarily influences yield of products (Gransee and Führs, 2013).

Potassium "K" the essential - Potassium is the seventh endless metal and makes up around 1.5 % by weight of the universes outside. Potassium is a principal constituent for plant improvement and it is found in numerous soil. It is a key part in the human eating routine (Gumz *et al.*, 2015).

Nitrogen is acquired from the soil environment either as the ammonium (Ne) or nitrate (N4) particles, with nitrate being synthetically diminished inside of the plant to ammonium lead to the incorporation into organic molecule. An option of nitrogen for a few species is nitrogen (N2) in atmosphere, got through the procedure of nitrogen Fixation (Grusak, 2011).

Calcium is considered as one of secondary plant nutrient. Calcium in the form of divalent cation (Ca^{2+}), it is important for the rigidity of the cell wall and in controlling membranes structure, it act as a counter-cation in the vacuole for inorganic and organic anions, and as an intracellular messenger in the cytosol. Calcium deficiency is not very common in nature and high level of calcium limit plants communities on calcareous soil (White and Broadly, 2003).

Plants obtain from their ethereal and root area all constituents of their tissues. Specifically, soil richness oversees plant development and in this way the amounts of mineral supplements taken up by the roots. In natural environments, the minerals consumed by developing living beings come back to the soil after natural matter deterioration, and soil fertility kept up through supplement cycling. (Le Bot *et al.*, 1998).

1.5 Micronutrient-Phosphorous

Phosphorus (P) is a basic macronutrient mandatory for various function in plant, including generation of energy, synthesis oh nucleic acid, photosynthesis, glycolysis, respiration, stability of membrane, enzyme activation/inactivation, redox responses, flagging, carbohydrate breakdown system and nitrogen fixation (Abel *et al.*, 2002;Vance *et al.*, 2003). In the interim, Phosphorous less amount is considered as one of the major restrictions in agriculture yield (Schachtman *et al.*, 1998; Lynch and Brown, 2008). It has been assessed that 5.7 billion hectares of area worldwide are insufficient in P. Amount of phosphate in soil solution are in 10 mM, which are well underneath the basic level (Batjes, 1997).

Though in the soil the phosphorous be high, it is often present in insoluble forms or in forms that are only available outside of the rhizosphere. Generally, Phosphorous compounds in soil can be positioned into three classes: (i) inorganic compounds, (ii) organic compounds of the soil humus and (iii) organic and inorganic Phosphorous compounds linked with the cells of living matter that is desirable for the ideal performance of crops (Schachtman *et al.*, 1998).

In spite of the fact that an outsized bit of applied phosphorus is immediately immobilized being blocked off to plants. The substance of Phosphorus in soil is around 0.05% however just 0.1% of them are reachable to plants (Goldstein *et al*, 1986).

The phosphatic free particle present in soil and orthophosphatic particle is that exclusively particle which might acclimatize in an extensive amount by plants. Soil microorganisms assume as fundamental part in organic procedures e.g. included in the conversion of soil phosphorus. They are included in solubilization of soil phosphorus which in term have valuable impact on the development of plants (Beever *et al.* 1980).

Just around 20% of the phosphorus utilized as a part of horticulture achieves the food we devoured, the greater part of the rest lost in wasteful strides along the phosphorus cycle (Cordell et al. 2011). The expanding shortage of the phosphorus is of major concern. In numerous farming frameworks the utilization of Phosphorous to the soil is important to guarantee plant efficiency, the recuperation of applied P by crops in a developing season is low, in light of the fact, that in the soil 80% of the P gets to be fixed and distracted for plant uptake due to adsorption, precipitation, or transformation to the organic structure (Holford, 1997).

1.5.1 Soil Phosphorous Cycle

Phosphorus may be introduced into the soil by chemical weathering, mineralization of organic matter, added fertilizers and through agricultural, municipal and industrial wastes (Figure 2). It may be lost through runoff, erosion and leaching. Soil phosphorus is very reliant on pH and its lack issues are normal because of the way that, it responds with iron (Fe) and aluminum (Al) to frame insoluble Fe and Al phosphates in acidic soils and with calcium (Ca) to shape insoluble Ca phosphates in alkaline soils(Saleque *et al.*, 2004; Sims and Sharpley,., 2005).

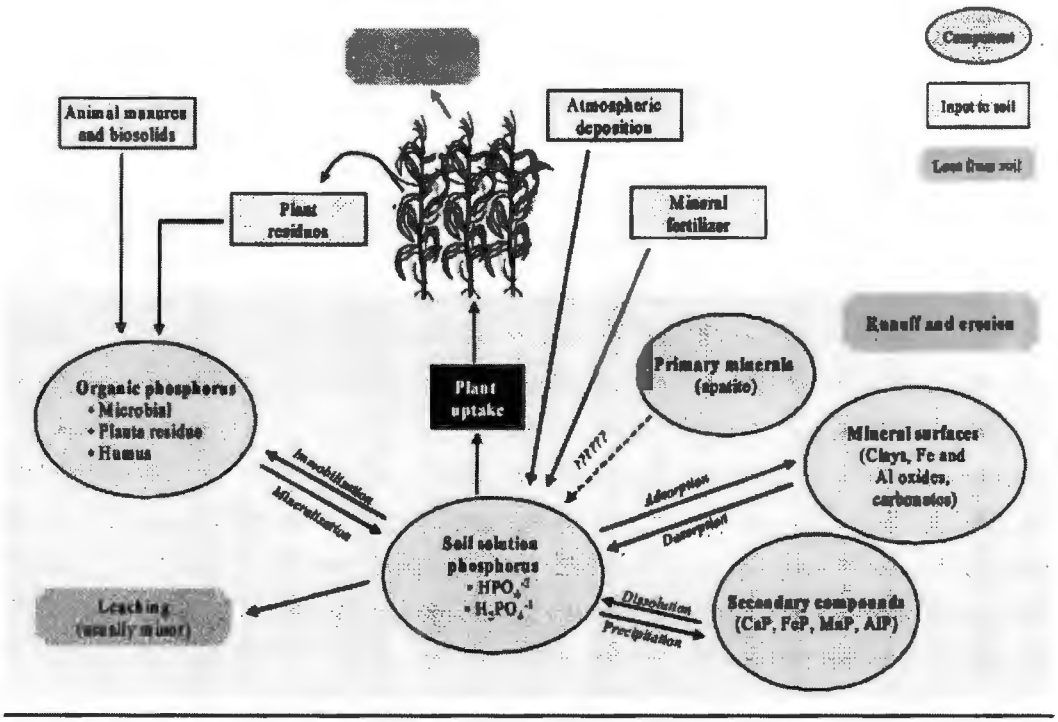


Figure 2. Soil phosphorous cycle (Pierzynski et al., 2005)

1.6 Phosphate solubilizing bacteria (PSB)

Microorganisms are an essential segment of soil and specifically or in a roundabout way impact the soil quality through their valuable or adverse exercises. Rhizospheric microorganisms intercede soil procedures, for example, decay, supplement activation and mineralization, discharge of supplements and water, nitrogen obsession and denitrification. Moreover, the microorganisms having a phosphate-solubilizing capacity can likewise change over the insoluble phosphatic compounds into soluble structures in soil and make them accessible to the crops (Khan *et al.*, 2007).

There are impressive populaces of microscopic organisms solubilize phosphorous in the soil and in the plant rhizosphere .These incorporate both aerobic and anaerobic strains, with a

pervasiveness of oxygen consuming strains in sub-merged soils. In recent times, phosphate solubilizing microorganisms have pulled in the consideration of agriculturists as soil inoculums to enhance the plant development and yield. Plant development advancing microbes and rhizosphere microorganism that can advance plant development by various systems and P solubilization capacity of the microorganisms is thought to be the most crucial qualities related with plant Phosphorous support. Although both bacteria and fungi are known to solubilize phosphorous, but bacteria are more successful in phosphorus solubilization than fungi (Behera *et al.*, 2014).

PGPR which solubilize phosphate are ordinarily present in the root range and can be utilized to illuminate this issue (Vessey, 2003). Many bacterial and fungal species have been accounted for as phosphate solubilizing microorganisms (Jain *et al.*, 2012).

Bacteria have enormous potential for phosphorus solubilization as compared to fungi.In the entire microbial populace in soil, PSB constitute 1 to 50 %, while phosphorus solubilizing fungi (PSF) are just 0.1 to 0.5 % and have P solubilization potential. Strains from bacterial genera Pseudomonas, Bacillus, Rhizobium and Enterobacter alongside Penicillium and Aspergillus organisms are the compelling P solubilizers (Khan *et al.*, 2009).

In wheat, A. chroococcum have been reported for improved growth and uptake of phosphorous (Kumar *et al.*, 2001), in peanut P. fluorescens (Dey *et al.*, 2004), in wheat Pseudomonas sp. (Babana and Antoun, 2005), in walnut Pseudomonas sp. and B. cereus (Yu et al., 2011), and in tomato Paenibacillus polymyxa and B. megaterium (El-Yazeid and Abou-Aly, 2011).

1.6.1 Mechanism of Phosphorous Solubilization

Many Bacterial species have potential for mineralization and solubilization of natural and inorganic phosphorus, subsequently. The solubilization of phosphorous is overseen by the potential of microorganisms to exudates metabolites, for case, organic acids, their functional hydroxyl and carboxyl group scavenge the cation bound to phosphate, changed over to soluble structure. Phosphate solubilization happen by various microbial system/structures include organic acid creation and proton ejection (sharma *et al.*, 2013).

The process of solubilization take place through phosphate solubilizing bacteria that are present in soil. This is a stepwise process (1) Microorganism produce mineral dissolving compounds e.g organic acid, Siderophore, OH ions,protons,and carbon dioxide.(2)utilization of extracellular enzymes that take part in mineraliation of phosphorous biochemically.(3)through P mineralization it release soluble form of phosphorous by substrate degradation(sharma *et al.*,2013).

Phosphorus solubilization is finished by boundless microorganisms and saprophytes getting up to speed with sparingly dissolvable soil phosphates, generally by chelation-mediated system. The process of solubilization of inorganic phosphorous take place by natural and inorganic acids released by PSB in which functional group of acids scavenge cations (Al, Fe, and Ca) and result in lowering the pH of soils. The PSB break up the soil P by creation of low molecular weight organic acids for the most part gluconic and keto gluconic acids, involve in bringing down the pH of rhizosphere. The pH of rhizosphere is brought down through biotical production of proton/bicarbonate release (anion/cation equalization) and gases (O2/CO2) conversation. Phosphorus solubilization ability of PSB has direct relation with medium pH. Roots release

1.7 Plant growth promoting attributes of PSM

Other than making solvent P available for plants, several studies have reported on plant growth promoting microorganisms (Gaur and Ostwal, 1972). PGM involved in the production of plant metabolites, for example, phytohormones, anti-microbials, or siderophores. Different PSM arrangements have been appeared to advance the development of numerous yields. Endophytic Bacteria secluded from rhizoplane of cacti plants developing in exposed pumice, not just altogether prepared Phosphate and different minerals (Puente *et al.* 2004a, 2009a) additionally advanced development of wild desert plant species.

1.7. 1 Biological nitrogen fixation

Plants play a vital function by enriching and choosing the varieties of bacteria by the elements of own root exudates. Hence, the community of microbes in the root area develops, relying on the concentration and nature of organic elements exudates through roots, also the capability of microbe to consume these as energy sources (Curl and Truelove, 1986).

Utilization of bio-enhancer and bio-fertilizer like nitrogen (N_2) fixing bacteria and helpful bacteria can reduce applications of chemical fertilizer and subsequently lower production cost. Exploitation of PGPB for the purpose of increasing the productivity aids in pollution reduction and environment stability in the soul of an ecological agriculture (Stefan *et al.*, 2008).

Commitment of natural nitrogen obsession is 180 X 106 metric tons/year around the world, into which cooperative connections produces 80%, the rest originates from affiliated frameworks or free-living (Sylvia *et al.*, 2005).

Microscopic organisms and Archea have the ability to start this critical measures of N2 from the reservoir of environmental soil nourishment (Youthful, 1992). These consist of mutual nitrogen-fixing forms, viz. *Rhizobium,* the *Frankia* in non-leguminous and trees and obligate symbionts in leguminous plants, and non-symbiotic (associative or endophytic, free-living) nitrogen-fixing forms such as *Azospirillum*, cyanobacteria, *Acetobacter diazotrophicus, Azotobacter*, and *Azoarcus*etc (Vessey, 2003).

Biological N_2 fixation (BNF) is limited to prokaryotic organisms. Rhizobia are the extensively studied and longest exploited plant growth promoting rhizobacteria (including *Allorhizobium, Rhizobium, Azorhizobium, Bradyrhizobium, Sinorhizobium and Mesorhizobium*) for their ability of nitrogen fixation in their leguminous host plants (Vessey, 2003).

1.7.2 Phytostimulation

1.7.2.1 Phytohormone production

In root development and morphology the positive impact of PGPR manifestly points to bacteria-mediated modifications so far (Vessey, 2003). PGPR promote plant development by producing different hormones like IAA (indole-3-acetic acid), cytokinins and gibberellic acid (Kloepper *et al.*, 2007). Various studies reported that PGPR as well as some symbiotic, pathogenic and free-living rhizobacteria are involved in the production of IAA and gibberellic acid in the rhizosphere and thus shows increase in root tips and root surface in numerous plants (Bhattacharyya and Jha, 2012). An large root surface zone permits the plant to make nutrients accessible from soil, therefore helps in plant growth promotion (Vessey, 2003).

The competency of phytohormone production is extensively dispersed amongst bacteria associated with plants and soil. Previous work have shown that PGPB can increase development of plant via auxins (indole acetic acid IAA) production (Spaepen *et al.*, 2008), cytokinins (Timmusk *et al.*, 1999), gibberellines (Bottini *et al.*, 2004), or through regulation of high levels ethylene in the plants.

1.7.2.2 Indole acetic acid (IAA)

IAA is the core plant hormone (auxin) and many significant plant-microbial associations focus on the auxins production. The plant division, rentiation and expansion of cells and tissues of plant and root elongation are attained by Indole acetic acid. In free living, pathogenic and symbiotic bacterial species IAA is reported by many (Costacurta and Vanderleyden, 1995; Tsavkelova *et al.*, 2006). Currently, auxin producing PGPR are extensively studied producers of phytohormone (Tsavkelova *et al.*, 2006; Spaepen *et al.*, 2007).

By using altered pathways these bacteria synthesize IAA from tryptophan, even though it also can be created through ndependent-tryptophan pathways, however in small quantity (Spaepen *et al.*, 2007). Phyto-pathogenic microbes primarily use the indole acetamide pathway for IAA production, which causes tumor induction in plants. Though it is not confirmed either it is used by advantageous microorganism. On the other hand PGPR have the main acid indole pyruvic pathway(Patten and Glick, 2002).

Azospirillum is well-studied IAA producers among other plant growth promoting rhizobacteria species. Further bacteria producing IAA belong to *Aeromonas* (Halda-Alija, 2003), *Bacillus* (Swain *et al.*, 2007), *Azotobacter* (Ahmad *et al.*, 2008), *Enterobacter* (Shoebitz *et al.*,

2009), *Burkholderia* (Halda-Alija, 2003), *Rhizobium* (Ghosh *et al.*, 2008) and *Pseudomonas* (Hariprasad and Niranjana, 2009) genera isolated from various soils of rhizosphere.

PGPR that produce IAA have been inoculated for stimulating seed germination, also accelerate growth of root, and alter the architecture of root system and to increase the biomass of root. Tsavkelova *et al.* (2007) have observed an increase in the orchid seeds (*Dendrobium moschatum*) germination, inoculated with *Sphingomonas* sp. and indole acetic acid producing *Mycobacterium* sp. Bacteria that produce IAA also help in tuber growth stimulation.

Swain *et al.* (2007) described a positive effect of *B. subtilis,* being IAA producer strains on the edible tubercle *Dioscorea rotundata* L. in their study. On plant surface they applied a *B. subtilis* suspension, which increased root and stem length, fresh weight of the stem and root, root: stem ratio and numbers of sprouts as compared to non-inoculated plants.

1.7. 3 Siderophores production

Microorganism like bacteria and parasites expend siderophores as iron (Fe) scavenging operators and are low molecular weight compound. If there should arise an occurrence of iron deficiency bacteria create these compounds and it ordinarily happens in basic to neutral pH soils, because of low solvency of iron at higher pH (Sharma and Johri, 2003). PGPR keep the plant pathogens from proliferation by sequestering Fe3+ in the root zone (Siddiqui et al., 2006).

In the past studies Bradyrhizobium, Pseudomonas have been segregated as siderophore maker (Boopathi and Rao, 1999), Rhizobium (Roy and Chakrabartty, 2000), Serratia and Streptomyces (Kuffner *et al.*, 2008) genera from the rhizospheric.

Reported that Rhizobium, Azospirillum and Pseudomonas that can create siderophore indicated constructive outcomes on development of alfalfA plant even in the limited iron accessibility. It likewise expanded seed germination and root/stem dry weight (Castañeda *et al.*, 2002).

1.8 Genetics of Phosphate Solubilizing Microorganism

In spite of the fact that information of the hereditary qualities of phosphate solubilization is still inadequate, and at the molecular level the end goal was to understand how definitely the PSM draws out the solubilization of insoluble P are uncertain (Sharma *et al.*, 2013). Functional genes with conserved nucleotide sequences also have a sympathetic role in bacterial identification and phylogenetic grouping of bacteria (Doty *et al.*, 2011). Preparatory achievements in the manipulation of these genes through genetic engineering and molecular biotechnology took after by their expression in rhizobacterial strains open a promising viewpoint for getting PSM strains with improved phosphate solubilizing limit, and in this way, a more successful utilization of these organisms as agriculture inoculants. The underlying accomplishment in cloning of genes required in P solubilization from the Gram negative microorganisms Erwinia herbicola was accomplished by Goldstein and Liu (1987). A development in extracellular phosphatase movement of the recombinant strain was perform (Sharma *et al.*, 2013).

1.9 Biological control

The utilization of living beings to restrain the development of pathogenic operators, along these lines lessening illness harm, is called biocontrol (Chinnasamy, 2006; Siddiqui, 2006). The utilization of PGPR as a biocontrol specialists could be an all the more ecologically agreeable option for supporting plant well-being as controlling plant pathogens utilizing chemical agents is immoderate and dangerous to the non-target environment and, in the long haul, might conceivably instigate pathogen resistance. PGPR might act against bugs, weeds or pathogenic microorganisms through opposing exercises coming about because of one or more particular activities, for example, rivalry for constrained supplements and niches on the root site, pernicious metabolite discharge or discharge of pathogen cell wall corrupting compounds (Barea *et al.*, 2005). The adversarial instruments for the most part include rivalry in the middle of PGPR and pathogens in the root environment (van Nut case and Bakker, 2003).

The most obvious indirect mechanism of PGP in soil bacteria is done by biological control agents (Glick, 2012). Generally, nutrient rivalry, niche segregation, and metabolites production against fungus are the main modes of biological control in PGPR (Lugtenberg and Kamilova, 2009).

Antifungal metabolites for example HCN, pyoluteorin, phenazines, pyrrolnitrin,2,4-diacetylphloroglucinol, tensin and viscosinamide are released by rhizobacteria (Bhattacharyya and Jha, 2012). Association of soil bacteria with roots of plants makes a plant resistant against pathogenic fungi, viruses and bacteria, the phenomenon is known as induced systemic resistance (ISR) (Lugtenberg and Kamilova, 2009).

Furthermore, ISR comprises signaling of ethylene and jasmonate in plant, and both of hormones motivate the plant's resistance strategies against a various pathogens of plant (Glick, 2012).

PGRP have been found to live and colonize plant roots. So, they are able to promote plant development, crop yield, diminishing diseases, and bug harm. In Pakistan, relative adequacy of plant growth promoting rhizobacteria was examined by a few scientists although, weed administration in Rice Yield was examined all the more as of late at Gomal University, D.I. Khan, Pakistan (Asim-Raza, 2014).

Micronutrients and rhizobacteria - Nutritional quality and medical advantages of chickpea (Cicer arietinum L.) were checked on and reported on WorldWideScience.com - The Global science Gateway (Jukanti *et al.*, 2012). Utilization of micronutrients (Zn, B, and Iron) for yield upgrade in rice was examined in D.I. Khan (Qadir, 2013).

PGPR likewise upgrade plant development by means of concealment of phytopathogens by an assortment of components, for example, anti-infection agents, fungi cell wall lysing chemicals or hydrogen cyanide which stifle the development of parasitic pathogens. Antagonistic microorganism connections intervened by Pseudomonas species are real drivers in the natural control of phytopathogenic parasites in the rhizosphere and might in a roundabout way advantage plant development and survival (Winding *et al.*, 2004). The union of particles includes in Antagonistic cooperations and ailment concealment, for example, the anti-infection 2, 4-diacetylphoroglucinal (2, 4-DAPG), pyoluteocin, and so on (Costa *et al.*, 2007).

1.10 Plant growth - Effect of PGRP strains

Different PGRP stains of *Pseudomonas putida* and *P. fluorescens* were demonstrated to affect elongation of shoots and roots of plants such as canola, tomato and lettuce (Hall *et al.*, 1996; Glick *et al.*, 1997). A substantial increase in the yield of wheat, potato, rice, sugar beet, apple, citrus, and other plants is reported (Kloepper *et al.*, 1988; Kloepper *et al.*, 1989).

Interestingly, *Azotobacter* inoculation increased the yield of wheat crop by 30% whereas yield increase reached 43% higher with *Bacillus* inoculants (Kloepper *et al.*, 1989). Earlier studies on *Bacillus megaterium* and *Azotobacter chroococcum* showed 10-20% increase in yield (Brown, 1974).

Recent reports from Malaysia suggested that *Azospirillum Spp.* (basically belonging to N_2-fixing bacterium) could significantly improve the growth of shoot, root and total biomass in different cereals in Tropical areas with less fertile soil. Sweet corn hybrid variety J-58 was inoculated with four different strains of Azospirillum species and overall best improvement was observed in vitro experiments (Faruq *et al.*, 2015). At large, Azospirillum Spp. were found to enhance the growth of vegetable and other crops (Zimmer *et al.*, 1988; Zahir *et al.*, 2003; Barassi *et al.*, 2007)

Plant Growth promoting rhizobacteria (PGPR) can enhance plant advancement capacity by extending seed rise, plant weight and yield. PGPR increase the advancement of different basic items, with a couple strains impelling systemic impenetrability to parasites, microorganisms, viruses and nematodes (Kloepper *et al.*, 2004a).

The majority of the strains utilized have a place with types of Pseudomonas or Bacillus (Ehret *et al.*, 2001). Various economically accessible PGPR-based items are accessible in various countries, the greater part of which contain strains of Bacillus spp (Kloepper *et al.*, 2004b).

1.10.1 Inoculation pattern - effect on some vegetables growth

Azospirillum spp. were found to expanded yield in crops like maize, sorghum, and wheat while Bacillus spp. were accounted for to expanded essentially the yield in peanut, potato, sorghum, and wheat (Burr *et al.*, 1978; Capper and Campbell, 1986). On the other side, *Azospirillum brasilense* was found to alter membrane potential and in proton efflux (Bashan and Levanony, 1991).

It is worth noteworthy that isolation of Azospirillum strains from water stressed conditions and as inoculants can mitigate drought effects. It was concluded that Azospirillum strain had significant effects as a strong inoculant for maize (*Zea mays*) that can support the crop to tolerate inadequate water availability (Bano *et al.*, 2013).

In generic, isolated bacteria from soils have Phosphate-solubilizing and -mineralizing abilities (Guang-Can *et al.*, 2008; Khan *et al.*, 2009). Bacteria are known to have an imperative part in sustaining the health status of soil ecosystem by preforming various biological processes. Azospirillum species and their role were further studied in wheat crops. Amongst PGPR, Azospirillum is closely-associated with plants and belongs to a significant genus and have potential to degrade organic contaminants, involve in advancing the plant healthiness and upsurge crops yield (Rasool *et al.*, 2015).

From wheat rhizosphere four bacterial strains were isolated. They were characterized on the basis of colony, morphology of cell, their shape, size and mobility (Bashan *et al.*, 2004). The strain Azo LR3 of *Azospirillum* was revealed to have mild pink color colony with: enviable (rod size 3μm), with typical helical motility. This strain is involved in production of indole-3-acetic acid and gibberellic acid. Additional, analysis of 16S rRNA genes sequence unveiled the isolated strain to belong to Azospirillum genus with similarity of 96% but differed from other members of the genus (Murumkar *et al.*, 2013; Rasool *et al.*, 2015).

In another study on the advance seedling development of vegetable like lettuce, tomato and cucumber was inquired with the target to assess the ability of three *Azospirillu brasilense* strains .According to the experimental design the before and after sowing of seeds, they were inoculated by soaking and drenching respectively. The experimental results exhibited that inoculation well affected; however, changes occurs due to different plant species, inoculation methods and PGPR strains used. The response was found to be dependent on: the concentration of inoculum and production of indole-3-acetic acid (IAA).1n generic, the magnitude of inoculation influence on the early growth of vegetables was more articulate and strongly increased root and shoot growth, germination and potency of tomato when inoculated by soaking. Although, there have been variances in effects on crops to the inoculation methods; however, soaking considered to be improved technique, and most of the strains showed concordant beneficial effects on tomato relatively more than lettuce and cucumber. These findings were in line with the earlier reports on the *Azospirillum brasilense* inoculation effect on morphology of roots and in tomato seedling respiration (Hadas, and Okon, 1987; Mangang *et al.*, 2015).

1.10.2 Inoculation of seeds – Results from University of Arid Agriculture, Pak.

Bacteria seem to be more active and effective for plant growth and protection. Hundreds of soil samples were collected from native soils of Pakistan and investigated at University of Arid Agriculture, Rawalpindi, Pakistan. Various genotypes showed different response with PGPR applications (Abaid-Ullah *et al.*, 2015).

Contrary to other macronutrients, phosphorous is taken up by plants from soil as phosphate anion. P is the least mobile element in soil and plant. It is known to precipitate as orthophosphate or absorbed by Al oxide and Fe oxide (via legend exchange). PSB play a vital role in solubilization and mineralization P and make it available to plants by acid phosphatases (Khan *et al.*, 2009).

Arid Zone - In general, the arid ecosystem is extended over the earth from the tropical to the sub-alpine zones (average sea level: 3000 m). The average rainfall in arid zone mostly varies between 100 to 400 mm with different drought intensities (Yadav *et al.*, 1989; Tripler *et al.*, 2007; Yadav and Verma, 2012). It is not possible to think of annual crop cultivation under severe drought conditions. In such cases, due to poor soil fertility the agriculture production is poor but the demands of grain, wood and fodder keep on increasing with growing population. To consider the use of chemical fertilizers is a risky proposition in such conditions of socio-economic burden. However, the use of PSB and P-fertilizers seem to possibly support the situation and increase the production of crop. The improved farming facilities (expensive/add to cost) and use of PSB meeting P requirements of crops are considered helpful (Barber, 1995).

D.I. Khan is known for Dates which are an iconic part of Muslim culture. Sandy loam and clayey loam soil are the best for their growth (Pasha *et al.*, 1972). The crop is known for its survival in stressful climatic conditions. Dhakki village dates (D.I. Khan) are much popular among folk:

Regular use of Dates is growth friendly and provide cure against various diseases: constipation, intestinal/digestive disorders, bones diseases, cardio-vascular diseases, nerves disorder, anemia, sexual dysfunction, diarrhea, abdominal cancer, anemia, allergy, expels toxic compounds, and many other disease conditions (Pasha *et al.*, 1972).

1.11 Scope of study

An unexpected increase, in agronomic practices to maximize crop production at extraordinary rate has directed towards the exploitation of technologies and approaches that are not effective for sustainability of soil health (Kumar *et al.*, 2010). Compelling and frequent use of agro-chemicals (fertilizer and pesticides) in agricultural lands is posturing serious threats to soil fruitfulness (Yu *et al.*, 2009). The dynamic lessening in the utilization of plant protectants and agrochemicals in cultivating practices without upsetting yield or nature of the crops must be possible with the infringement of new era advancements. In the few decades, the current progressions in biotechnology farming have unblocked new routes for the enlargement of agrarian efficiency in a reasonable mode and have made promising misuse of soil microorganisms for enhancing the soundness of yields (Khan *et al.*, 2010).

2.0 Materials and Methods

A systematic study was undertaken to isolate indigenous rhizobacteria from the farmer fields of Dera Ismail Khan in order to evaluate role of Phosphate solubilizing bacteria (PSB) and its effects on yield and growth parameters of Tomato plant. For these studies the details of methodology used being presented in the following three sections namely: isolation of bacteria, characterization and investigation of various plant growth promoting attributes under *in vitro* conditions and its effects on growth of plant. The work was carried out according to the following steps.

2.1 Isolation of phosphate solubilizing bacteria

2.1.1 Collection of samples

Samples of soil were collected from the rhizosphere of Tomato, maize and rice fields at a depth of 6 cm from the vicinity of the roots of plants. In August 2015 and September 2015. Each sample was taken in separate polythene bag, labelled and stored at 4 °C for further processing.

2.1.2 Isolation of rhizobacteria from soil

Isolation was carried out from rhizosphere soil of Tomato *(Solanum lycopersicum)*, Rice (*Oryz sativa*), and Maize (*zea mays*) by dilution plate method (Brierly, 1928). Soil (1 g) was properly mixed in autoclave distilled water (90 mL) for the isolation of indigenous rhizobacteria. After sedimentation of solid particles, dilution was made up to 10^{-6} was inoculated on Luria-Bertani (LB) medium(see appendix, Table 2.1) and incubated at 30°C for 24 hours (Aneja, 2002).

2.1.3 Colony Counting

Colony counting was done with the help of colony counter. Number of colonies present in the plate determines the number of cells present in the dilution.

2.1.4 Monitoring and purification of culture

Bacterial cultures were monitored regularly for up to 5-7 days. Isolated colonies were picked, streaked and re-streaked on LB media plates to get pure colonies. Distinct colonies were selected on the basis of shape, color, texture, size and other morphological characteristics.

2.2 Determination of phosphate solubilizing activity

Phosphate solubilizing activity was evaluated by inoculating the rhizobacteria on Pikovskaya agar plates. (Pikovskaya 1948) (See appendix, Table2.2) containing tri-calcium phosphate (TCP) and incubated for 30 ± 1 ℃ for 7 days. The colonies forming halo zone of clearance (Pikovskaya's medium) around them were counted as P-solubilizers. All the bacterial colonies exhibiting halo zones were selected, purified and maintained on LB agar plates for further studies (Pikovaskya, 1948).

2.2.1 Qualitative assay of phosphate solubilizing activity:

The colonies zone of solubilization were stocked. The solubilizing efficiency of the microorganisms was calculated using following formula:

$$PSI = \frac{\text{Colony diameter+ Halo zone diameter}}{\text{Colony diameter (mm)}}$$

2.2.2 Quantitative assay of phosphate solubilizing activity:

Quantitative assessment of phosphate solubilization of bacterial strains in broth was determined by the molybdenum blue method (Soltanpour and Workman ,1979).It is carried by utilizing Erlenmeyer flask (150 ml) containing 100 ml of medium inoculated in triplicate with the bacterial strain. Autoclaved un-inoculated medium considered as control. The flasks placed on shaker and incubated for 5 days at 30C. The cultures were collected by centrifugation at 10,000 rpm for 10 min. The pellet was discarded and supernatant of each culture was collected for this Dilute 0.1 mL aliquot of the extract to 9.9 mL with Distilled water. Add 2.5 mL **color developing reagent** carefully to prevent loss of sample due to excessive foaming and stir well. The blank and standards are developed the same way as described above. After ten minute the intensity of color of blank, standards, and samples were read on the **Spectrophotometer** at **880-nm wavelength**. A calibration curve for standards, plotting absorbance against the respective P concentrations. P concentration was read in the unknown samples from the calibration curve.

Calculations:

P (ppm) =ppm P (from calibration curve) X dilution factor.

2.2.3 Change in pH in PSM broth cultures

The pH of the growth medium was determined at regular intervals by using digital pH meter (Decibel India Ltd).

2.4 Morphological Characterization

Morphological characteristics of PSB colonies were observed on LB agar plates. The three old cultures were used to determine the following characteristics including form, elevation, margin, color, opacity and Gram reaction (Mudili, 2007).

2.5 Preservation of isolates

For short term storage slants were prepared in 15 ml falcon tubes by half strength Luria agar (see appendix, Table 2.1) and incubated for 24 hours at 30 °C to check contamination. Isolates were inoculated with sterile loop on media slants and incubated until a clear visible growth appeared in the medium tube, then stored at 4 °C. The isolates were refreshed every three months on fresh media slants. Glycerol stock was prepared for long term storage. Isolates were inoculated in Luria Agar (LA) and incubated overnight at 30 °C on shaking incubator at 200 rpm. Next day about 700 µl of this broth was mixed with 300 µl sterile 50% Glycerol stock in 2 ml cryovials. The resulting 15% glycerol stock was then stored at -80 °C (Pegg, 2006).

2.6 In-vitro tests for functional characterization of isolated PSB strains:

Selected isolates of Phosphate solubilizing bacteria were further characterized for plant growth promoting attributes like indol-3-acetic acid production (IAA), protease activity, siderophore activity, pectinase activity and Amylase activity, HCN Production activity, Ammonia production activity.

2.6.1 Amylase test

The single colony of bacterial isolates were point inoculated on starch agar medium(see appendix, Table 2.4) plates and placed in incubator at 30°C for 48 hour. After incubation period, the plates were flooded with iodine solution, kept for a minute and afterward poured off. Iodine reacts with starch to form a blue color compound. This blue color diminish rapidly. Hence the color less clear zone around colonies depicts the positive result for amylase (Namasivavan *et al.*, 2011).

2.6.2 Pectinase Test

Isolates having pectinase production activity were checked by using agar plates having pectin screening media. Media was prepared, its pH maintained and then autoclaved (See appendix, Table 2.5). After 48 hours incubation at 30 °C plates were ready to inoculate isolates. After inoculation plates were incubated for two to three days till the zones of clearance were produced. Clear zones were checked by flooding the plates with HCL solution (Namasivavan et al. 2011).

2.6.3 Protease Test

In this test agar media supplemented with skimmed milk was used in a media. Both broth and skimmed milk were made separately to avoid agglutination (See appendix, Table 2.3), then combined together after autoclaving in sterile environment under laminar flow hood to avoid contamination. Mixture was then poured in petri plates and allowed to cool. After that petri plates were incubated overnight for 48 hours at 30 °C. Strains were inoculated on plates and their activities checked by formation of halo zones (Kazempour, 2004).

2.6.4 Ammonia (NH_3) production

For detection of NH_3 production pure isolates were inoculated in test tubes having 10ml peptone water and then Nessler's reagent was added to each tube dropwise (See appendix, Table 2.6). And ammonia production was indicated by media color change from yellow to brown after 48 hours (Cappuccino & Sherman, 1992).

2.6.5 Hydrogen cyanide (HCN) production

Hydrogen cyanide production was detected by using TSA media having 4.4 g glycine which was autoclaved and poured into petri plates (See appendix, Table 2.7). Bacterial isolates were streaked on respected plates and Whatman No.1 filter paper soaked in a 2% sodium carbonate and 0.5% picric acid solution was placed top of each streaked isolates and placed in incubator at 30 °C for 4 days. The filter paper color turned from yellow to orange indicated HCN production (Ahmad *et al.*, 2008).

2.6.6 Siderophore production

Bacterial strain were point inoculated on Chrome azurol S (CAS) agar medium (see appendix Table 2.9) and incubated at 30° C for 48-72 hours to assess siderophore production. Positive result for siderophore production was indicated by the formation of yellow to orange zone surrounding the bacterial colony (Alexander & Zuberer, 1991).

2.6.6.1 Preparation of CAS agar medium

CAS agar media was prepared by mixing of four solutions which were sterilized separately and then mixed together and poured into sterilized petri plates in sterile environment under the laminar flow hood (Alexander & Zuberer, 1991).

2.6.7 Indole-acetic-acid activity test

Indole acetic acid producing bacteria were assessed by using a calorimetric method (Gordon & Weber, 1951). Bacterial isolates were inoculated in 5ml Nutrient Broth (see appendix, Table 2.10) incubated for 48 hours at 30 °C. Development of pink color indicated IAA production.

2.6.8 Catalase Test:

Catalase test was done by adding 3 drops of 3% hydrogen peroxide to the bacterial culture. Appearance of effervescence was taken as positive test for catalase activity (Cappuccino *et al.*, 2008).

2.7 Pot Experiment

The PSB strains performed better were selected from the experiment. Effect of PSB on Tomato was studied in green house of International Islamic University. Seedling of tomato were sown in 1st week of January in pots. The crop was allowed to grow till maturity with normal agronomic practices. In 1st week of April, harvest plants, data regarding growth parameter was collected from each plot.

2.7.1 Preparation of inoculum

Phosphate solubilizing bacteria isolated from farmer fields used as inoculant. Phosphate solubilizing bacterial strains sub-culture in LB stock incubated for 72 hours on a shaker at 120 rpm at 25°C. After incubation, culture broth centrifuge at 3000 rpm for 15 mins. Pelleted cells were re-suspended in sterile tap water and conformed to around 108 cells ml-1 in light of an optical thickness (OD660 = 0.08) (Bhuvaneawari et al., 1980).

2.7.2 Pot culture Study

Sterilized pots measuring (2 kg, 10 kg) were filled with autoclaved soil (soil, sand and humus in the extent of 2:1:1). Tomato seedling (Riogrande) obtained from the Horticulture Research Center (NARC) Islamabad. Seedling of Tomato were grown in each pot and in this way the pots were watered twice every week according to water holding limit of soil. Pots were arranged in completely randomized design in the green house of International Islamic University, Islamabad. The experiment was carried out with 10 PSB strains in 3 replicates.

Two millileters of either the single culture were infused into root zone by utilizing syringe when the youthful seedlings were at the mid first leaf stage. (Hadi, & Bano, 2010).Plants after 60 days transferred into 10 kg pots .Plants data gathered after 120 days of treatment and washed with water, Plant height and root length were measured with a centimeter scale from the base of stem to the highest point of the apical leaf and the root length from base to tip. Plants were detached into roots, stem and Fruits.

Table 2.1: Isolates Used for pot experiment

Rhizosphere soil sample	Isolate
Maize	SM2 SM3 SM13
Tomato	TS1 TS3 TS4 TS5 TS6
Rice	RS3 RS5

2.8 Statistical analysis

One way investigation of variance (ANOVA) was completed for affirmation of data variability ($p \leq 0.05$) and mean estimation of treatments were linked by Least significant difference (LSD) at $p \leq^{-} 0.05$.

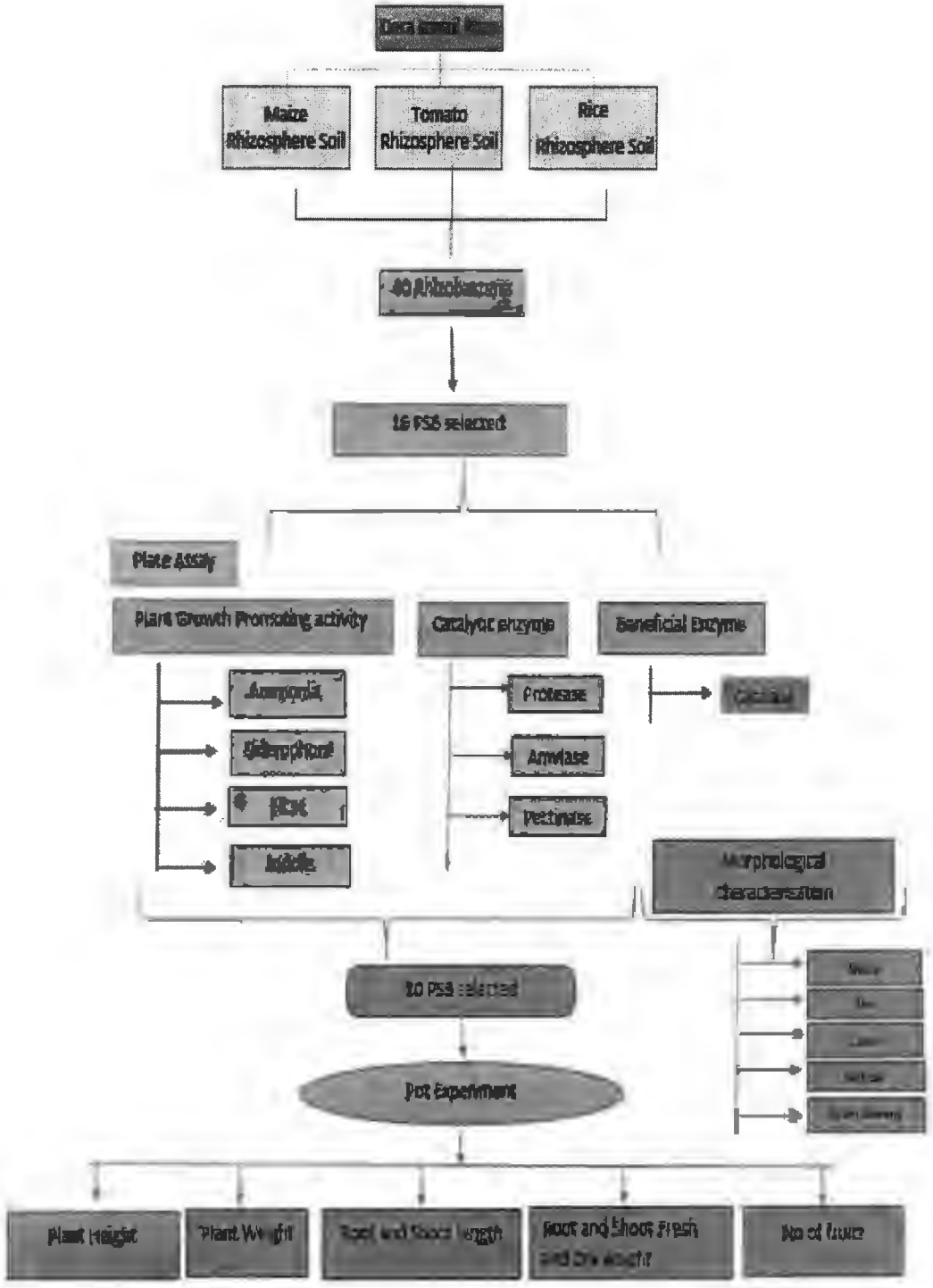


Figure 2.1: Scheme of study used for Isolation and Characterization of PSB

Chapter 3
Results

Results

In the present study, we isolated number of rhizobacterial strains from the rhizosphere of maize, Tomato and Rice plants fields. Rhizobacteria were first screened for phosphorous solubilization, and then further screening/evaluation for plant growth promotion activities was carried out. The performance of the most effective PSB isolates containing PGPR activity was then evaluated in soil culture by conducting pot experiment. Results of these experiments are discussed as under.

3.1 Isolation of Phosphate Solubilizing Bacteria

A total of 40 bacteria were obtained from agricultural soils mentioned above. The initial isolates obtained on Luria Agar medium and total bacterial counts from each soil type are shown in Table 3.1. The isolated rhizobacteria were screened for their phosphate-solubilizing ability on Pikovskaya (PVK) agar medium. Qualitative P-solubilization potential was anticipated by observing the large clear/ halo zones around the bacterial colonies on Pikovskaya agar media (Fig.3.1, 3.2). Out of 40 bacterial isolates tested, 16 isolates had P solubilizing ability on solid media amended with tri-calcium phosphate (TCP). The isolates exhibited different sorts of phosphate solubilizing index (PSI) ranging from 6.83 to 1.75. Elven isolates (SM3,SM4,SM7,SM13,TS1,TS3,TS4,TS5,TS6,RS3,and RS5) showed high PSI (Table 3.2). Maximum PSI (6.83) was observed in case of TS6 and minimum (1.75) in case of RS13 and SM2.

Table 3.1. Isolates initially obtained on LB medium

Rhizosphere Soil sample	Bacterial counts (CFU/ml)	No. of selected isolates
Maize Field	$9x10^5$	16
Tomato Field	$2.1x10^5$	7
Rice Field	$8.5x10^5$	17
	Total	**40**

Table 3.2. Isolates selected from various agricultural soil samples on basis of phosphate solubilizing bacteria.

Rhizosphere soil sample	Isolate
Maize	SM2 SM3 SM4 SM7 SM11 SM13
Tomato	TS1 TS3 TS4 TS5 TS6
Rice	RS3 RS5 RS7 RS13 RS15

3.1.2. Phosphate Solubilization in liquid medium

After confirming the Phosphate Solubilizing Activity on solid medium, the phosphorus solubilization in liquid medium (PVK Broth) was confirmed. Data presented in Table 3.3 summarizes the amount of "P" (µg/ml) solubilized in PVK liquid medium containing Tri calcium phosphate. All bacteria tested were found to be solubilizer of Tri calcium phosphate. The "P" content released into the medium from Tri calcium phosphate were given in the Table 3.3. Results showed that isolates TS-6, SM-3, RS-3, TS-1 and TS-5 showed maximum P solubilization in liquid medium. It was evident that in the medium with Tri calcium phosphate, the values of dissolved phosphate obtained with all the isolates were convincingly showing that the tested isolates have effectively converted the inorganic, insoluble phosphate into soluble form and were selected for further studies.

3.1.3 pH drop experiment

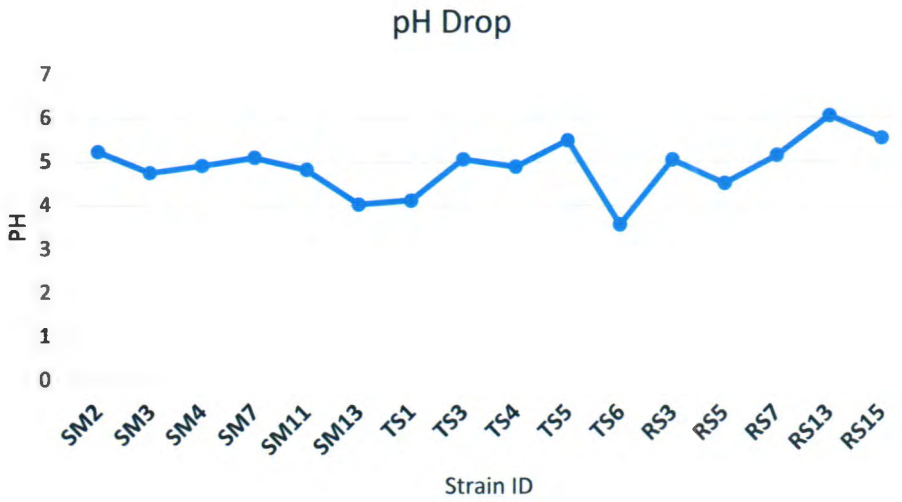
Rhizobacteria capable of solubilizing P are known to secrete different kinds of organic acids which result in lowering of pH of the media. To investigate pH changes, a pH drop experiment was conducted. In our pH drop experiment, it was found that after 7-Days of incubation period, 16 isolates were found most effective in decreasing pH of the cultured media between 6.05 to 3.47 (Table 3.3). Maximum decrease in pH was observed in the culture inoculated with TS-6 (3.47).

3.2 Morphological characterization

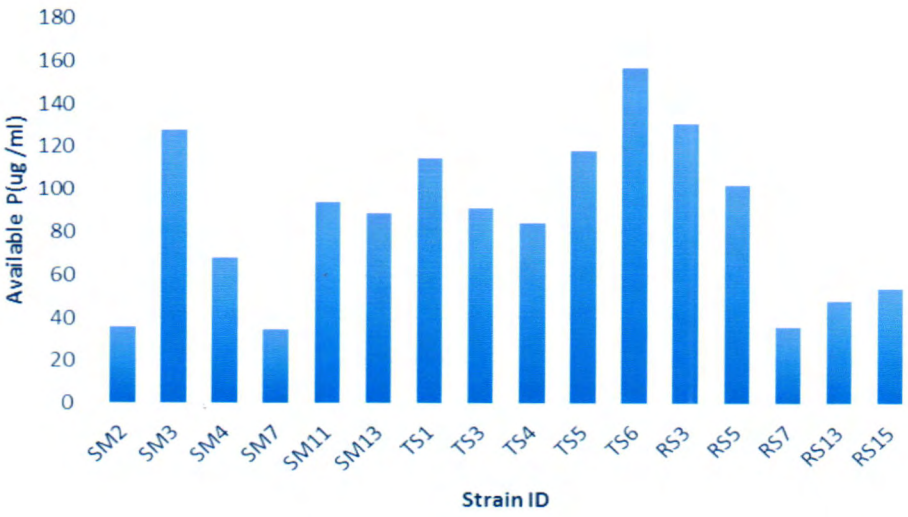
The PSB isolates were examined for their morphological features. The morphological characteristics were examined on their respective agar plates. The pure cultures from the slants were placed on the agar plates. After the growth of colonies morphological characters of the colonies like the form, elevation margin opacity, color, shape and gram staining were recorded. (Table 3.4)

Table 3.3: Phosphate solubilizing index and available P by selected bacterial isolates

Isolates	Halo zone Diameter(mm)	Colony Diameter (mm)	Phosphate solubilizing index	Available P (ug/ml)	pH Drop
SM2	2.3	1.75	1.75	36	5.23
SM3	4	0.95	5.21	128	4.75
SM4	2.7	0.5	6.4	68	4.91
SM7	1.9	0.7	3.71	35	5.1
SM11	2.1	1.45	2.44	94	4.82
SM13	3.1	1.1	3.81	89	4.03
TS1	2.6	0.9	3.8	115	4.12
TS3	2.9	0.5	6.8	91	5.06
TS4	3.1	1.1	3.81	84	4.89
TS5	3	1.3	3.30	118	5.49
TS6	3.5	0.6	6.83	157	3.57
RS3	2.8	1.1	3.54	131	5.05
RS5	3.3	0.85	4.8	102	4.51
RS7	1.8	1	2.8	36	5.15
RS13	1.2	1.6	1.75	48	6.05
RS15	1.3	0.95	2.36	54	5.54



Graph 3.1: Drop in pH by strains of phosphate solubilizing bacteria



Graph 3.2: Graphical representation of available P in PVK Broth

Fig.3.1: Results of plate assay indicating halo clear zone formation around bacterial colony

Fig.3.2: Results of plate assay indicating halo clear zone formation around bacterial colony

Strain ID	Form	Elevation	Margin	Opacity	Color	Shape	Gram Reaction
RS-3	Spindle	Flat	Undulate	Opac	Off-White	Cocci	+ive
RS-5	Irregulae	Pulvinate	Entire	Opac	Off-White	Rod	+ive
RS-7	Punctiform	Raised	Erose	Transparent	Off-White	Rod	-ive
RS-13	Circular	Convax	Entire	Opac	White	Cocci	-ive
RS-15	Filmentous	Raised	Erose	Opac	Off-White	Rod	-ive
SM-2	Irregular	Convax	Entire	Translucent	Yellow	Cocci	+ive
SM-3	Circular	Convax	Entire	Transparent	Off-White	Cocci	+ive
SM-4	Circular	Umbonate	Erose	Opac	Yellow	Cooci	-ive
SM-7	Punctiform	Convax	Erose	Opac	Yellow	Cocci	+ive
SM-11	Irregular	Pulvinate	Erose	Opac	Yellow	cocci	+ive
SM-13	Irregular	Convax	Undulate	Opac	Off-White	Rod	-ive
TS-1	Filamentous	Flat	Lobate	Opac	White	Rod	-ive
TS-3	Spindle	Flat	Erose	Opac	Yellow	Rod	+ive
TS-4	Rhizoid	Convex	Lobate	Opac	Yellow	Rod	-ive
TS-5	Irregular	Pulvinate	Filamentous	Opac	Off-White	cocci	+ive
TS-6	Circular	Flat	Entire	Opac	Yellow	Cocci	-ive

Table 3.4: Colony morphology and Gram staining

3.3 In-vitro tests for functional characterization of isolated PSB

3.3.1 Production of siderophore

All the selected PSB were tested for the production of siderophore. Among 16 isolates the eight isolates showed development of orange halo against dark blue background after 48 h of incubation suggests siderophore production (Table 3.5).

3.3.2 HCN production Test:

HCN production was checked in all isolates which showed significant results in phosphate solubilization. Out of these 16 isolates only 9 isolates showed HCN production after 48 and 72 h of incubation. Maximum HCN production was observed in TS-6 isolate. Presence or absence and intensity of HCN production can play a significant role in antagonistic potential of bacteria against phyto-pathogens (Table 3.5).

3.3.3 Indole acetic acid production:

The results of present study clearly showed that isolates (RS13,RS15,SM7,SM11) had no ability to produce IAA and consequently, while all other elven isolates considered as IAA producing (Table 3.5).

3.3.4 Ammonia Production Test:

All the selected PSB isolates from the rhizosphere of maize ,tomato and rice fields indicated ammonia production except SM11,SM2.RS15and TS5(Table 3.5).

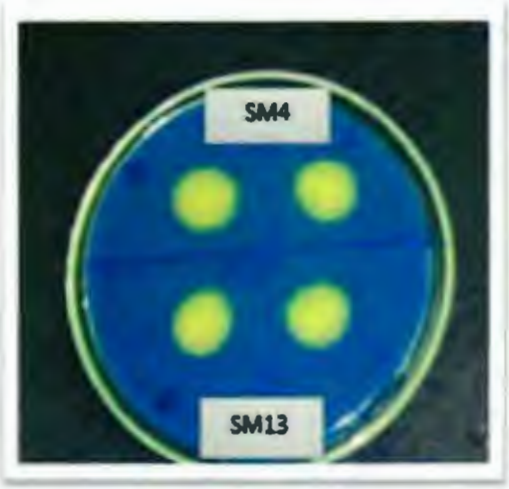


Fig 3.9. Medium plate showing siderophore activity of selected strain

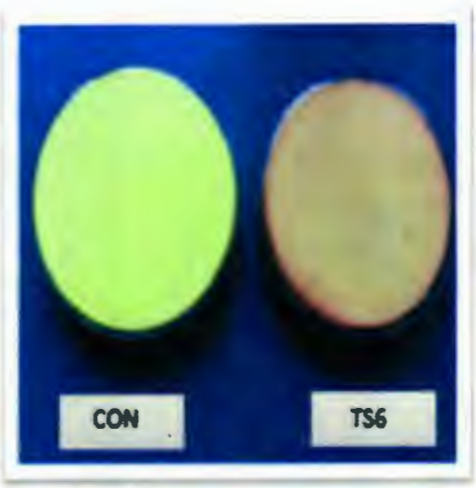


Fig 3.10: Medium plate showing HCN production of isolated strain TS-6

Fig 3.7. Color development showing Indole acetic acid production by isolated strains

Fig.3.8: Color development showing ammonia production by isolated strain.

3.4 Production of Hydrolytic enzymes (Protease, Pectinase, Amylase)

3.4.1 Protease activity.

The results presented in Table 3.5 revealed that isolated PSB showed protease activity as indicated by the development of clear zones around the colonies on skim milk agar. Among 16 isolates five (RS13, RS15, SM7, SM11, TS6) were tested negative for protease activity while elven isolates have shown protease activity.

3.4.2 Amylase test:

For Amylase activity result presented in graph 3.3, 12 bacterial isolates out of 16 showed positive result. Those isolates which gave better amylase activity are RS-3, RS-13 and TS-5,SM-3 which formed zone of diameter more than 30mm while TS-1, TS- 3, SM-13 and RS-5, RS-13 formed clear zones of more than 20mm diameter. Other isolate TS-4 formed clear zone of less than 1.While, SM-7, and SM-11, TS-6 did RS-15 not produce any clear zones.

3.4.3 Pectinase Test

All the selected PSB isolates from the rhizosphere of maize, tomato and rice fields tested negative for pectinase activity (Table 3.5).

3.5 Catalase Test

All the selected PSB isolates of all regions showed the catalase activity except RS7 (Table 3.5). Isolates from the rhizosphere of maize plants exhibited higher reaction intensity in catalase activity as compared to that from the rhizosphere of Tomato and rice.

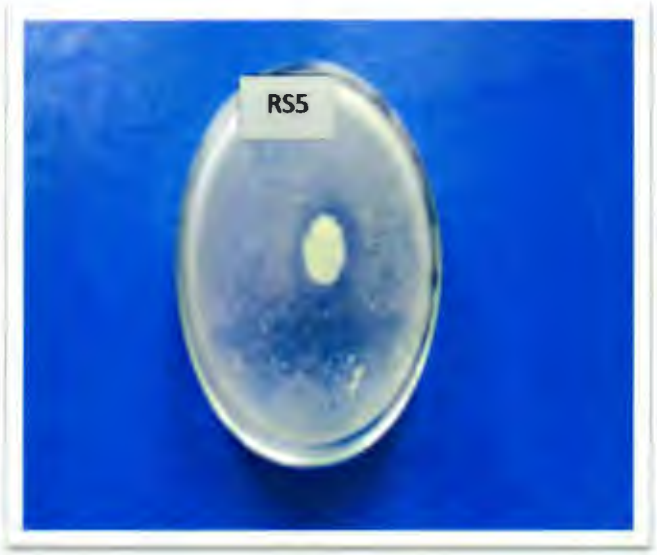


Figure 3.11: Medium plate showing protease activity of isolated strain

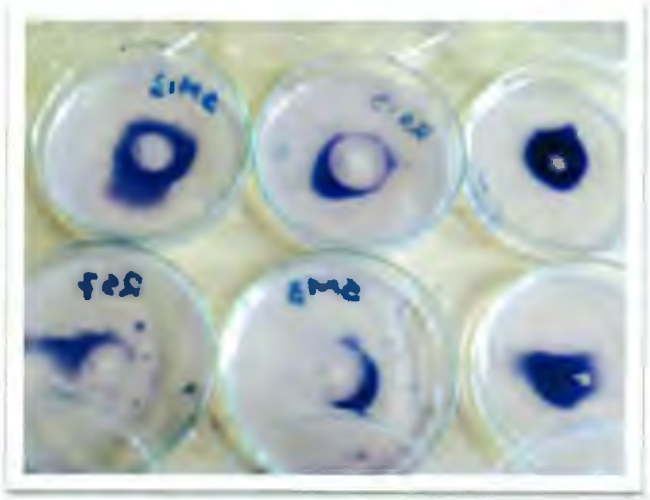
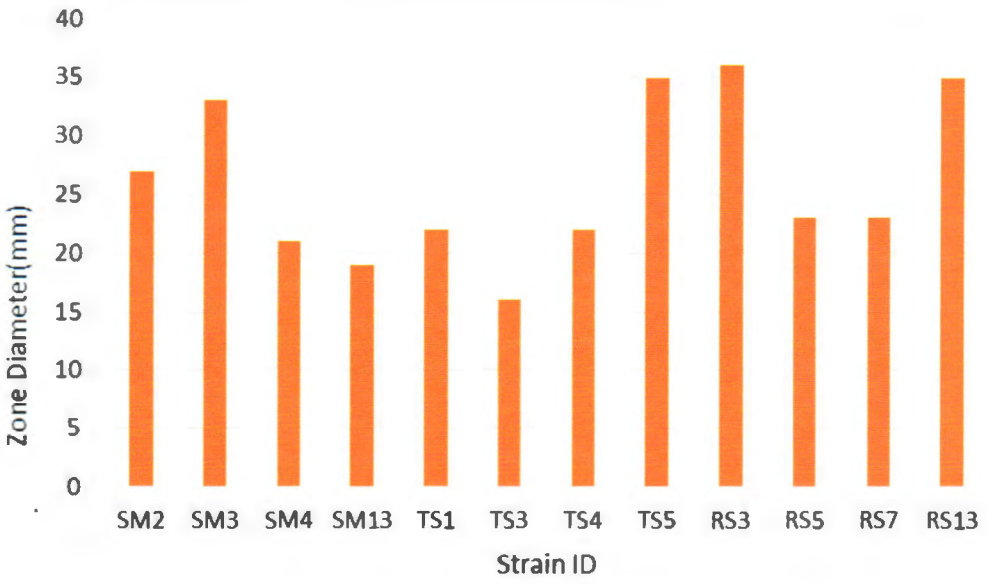


Fig.3.12. Medium plate showing Amylase activity of isolated strain (SM3, SM13.RS13.RS7) along with control



Graph 3.3: Graphical representation of amylase producing bacteria along with their zone diameter.

Strain ID	Amylase	Protease	Catalase	Pectinase	Indole	Ammonia	HCN	Siderophore
RS-3	+	+	+	-	+	+	+	+
RS-5	+	+	+	-	+	+	+	+
RS-7	+	+	_	-	+	+	-	-
RS-13	+	-	+	-	-	+	-	-
RS-15	-	-	+	-	-	-	+	-
SM-2	+	+	+	-	+	-	-	-
SM-3	+	+	+	-	+	+	+	+
SM-4	+	+	+	-	+	+	+	+
SM-7	-	-	+	-	-	+	-	-
SM-11	-	-	+	-	-	-	-	-
SM-13	+	+	+	-	+	+	+	+
TS-1	+	+	+	-	+	+	+	+
TS-3	+	+	+	-	+	+	+	-
TS-4	+	+	+	-	+	+	-	+
TS-5	+	+	+	-	+	-	+	-
TS-6	-	-	+	-	+	+	+	+

Table 3.5: In-vitro tests for functional characterization of isolated PSB strains

+ Sign shows positive activity of bacterial isolates whereas – sign shows no activity

- PSB Isolates from Maize Rhizosphere
- PSB Isolates from Tomato Rhizosphere
- PSB isolates from Rice Rhizosphere

3.6 Pot Experiment

The PSB strains were isolated from the rhizosphere of plants grown at rainfed area of Dera Ismail Khan. These strains were screened for growth promotion of Tomato under axenic conditions. So, in this way ten best isolates from each field were selected. The selected were studied in pot and for yield enhancement. The results achieved from the experiments are summarized as under.

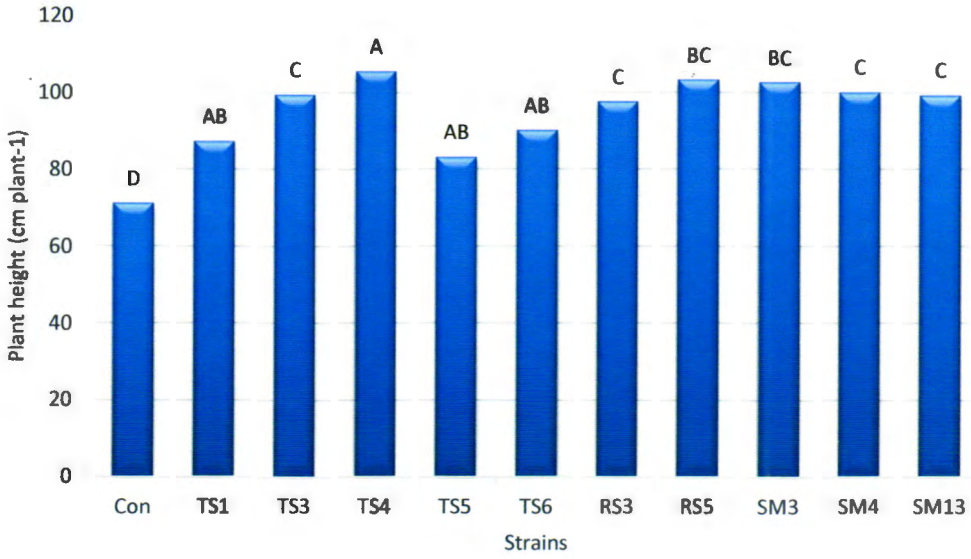
Strain ID	Plant Height(cm)	Plant weight(g)	Shoot length(cm)	Shoot fresh weight(g)	Shoot Dry weight(g)	Root length(cm)	Root fresh weight(g)	Root Dry weight(g)
Con	71.1	5.4	39.6	2.1	2.26	25 04	0.34	0.133
TS1	87.3	11.18	50.36	8.1	3.317	29 9	0.97	0.52
TS3	99.4	8.48	53.47	6.93	4.21	27.33	1.136	0.65
TS4	104.6	12.9	51.33	7.563	3 41	32 87	0.93	0.38
TS5	83.067	7.8	47 66	7.267	4.53	34.1	0.63	0.28
TS6	90.133	6.4	49.6	8.41	3.61	32.67	1.52	0.71
RS3	97.66	8.8	48.44	6.71	3 34	28 07	0.67	0 33
RS5	103.33	10.2	47.233	8.133	4.7	31.423	0.967	0.62
SM3	102.66	13.4	45 76	9.764	5.63	37.6	1.24	0.633
SM4	100.1	9.2	47.55	5.98	2.7	33.423	1.17	0.42
SM13	99.34	10.58	45.966	8.13	2.98	31.633	0.925	0.37

Table 3.6: Effect of inoculation on Growth parameters

*Values are mean of 3 randomly selected plants from each set.

3.6.1 Plant Height (cm)

The graph(3.4) pertaining the data of plant height showed that there was maximum plant height in case of plants inoculated with TS4, RS5,and SM3 strains (104.6 ,103.33 and 103.33cm, respectively) as compared to un-inoculated control. So, these three strains proved to be the most effective in producing increased plant height. While plant height in response to strains, TS5 and TS1 were 83.067 and 87.3 cm, respectively. However, untreated control treatment had the plant height of (71.1cm) conforming the role of inoculation.



Graph 3.4: Effect of inoculation of PSB strains on plant Height of *Solanum lycopersicum*

** Means sharing the same letter (s) do not differ significantly at P < 0.05 according to LSD.*

Pot Experiment views: Tomato plants after 60 days

Fig.3.13: Influence of ten PSB strains inoculation on *Solanum lycopersicum*

Fig.3.14: Effect of PSB strains (Tomato Rhizosphere) inoculation on *Solanum lycopersicum*

Pot Experiment views: Tomato plants after 60 days

Fig.3.15: Effect of PSB strains (Rice Rhizosphere) inoculation on *Solanum lycopersicum*

Fig.3.16: Effect of PSB strains (Maize Rhizosphere) inoculation on *Solanum lycopersicum*

Pot Experiment views: Tomato plants after 120 days

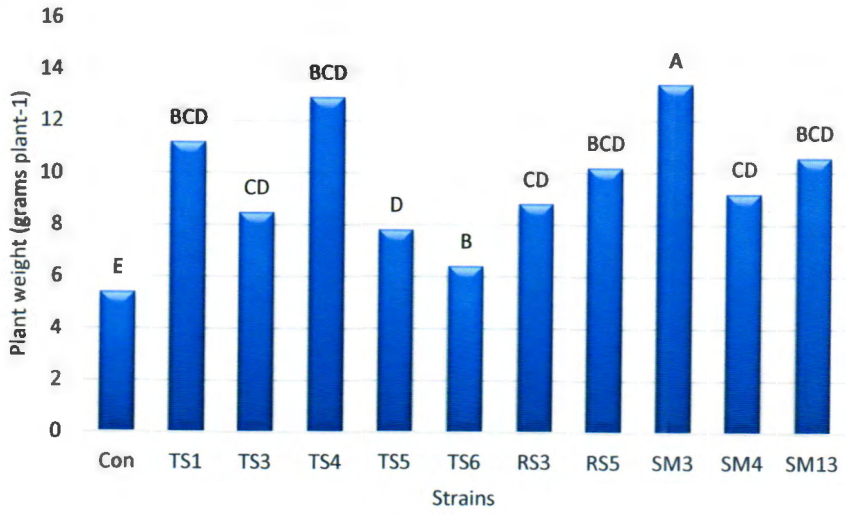
Fig 3.17: Influence of inoculation of PSB strains (Rice rhizosphere) on *Solanum lycopersicum*

Fig 3.18: Influence of inoculation of PSB strains (Maize rhizosphere) on *Solanum lycopersicum*

Fig 3.19: Influence of inoculation of PSB strains (Tomato rhizosphere) on *Solanum lycopersicum*

3.6.2 Plant Weight (g):

The results pertaining to plant weight revealed significant difference among all inoculated strains as shown in Graph (3.5). The highest plant weight was observed with TS4, RS-5 and SM3 isolates (1.31, 1.27 and 1.21g, respectively) as compared to the control and the other strains. Next to it, other five isolates SM4,SM13,RS5,RS3 and TS3 which were statically effective in increasing plant weight and showed 9.2,10.58,10.2,8.8 and 8.48g with increase over the control. Plant weight in response to TS6 and TS5 isolates were the lowest (6.4 and 7.8 respectively) among all strains. However, untreated control treatment had the lowest plant weight (5.4g), significantly lower from all the other inoculation treatments. It was noted from the data that all the isolates were found effective in the promotion of Plant weight but their potential varied with the isolates.

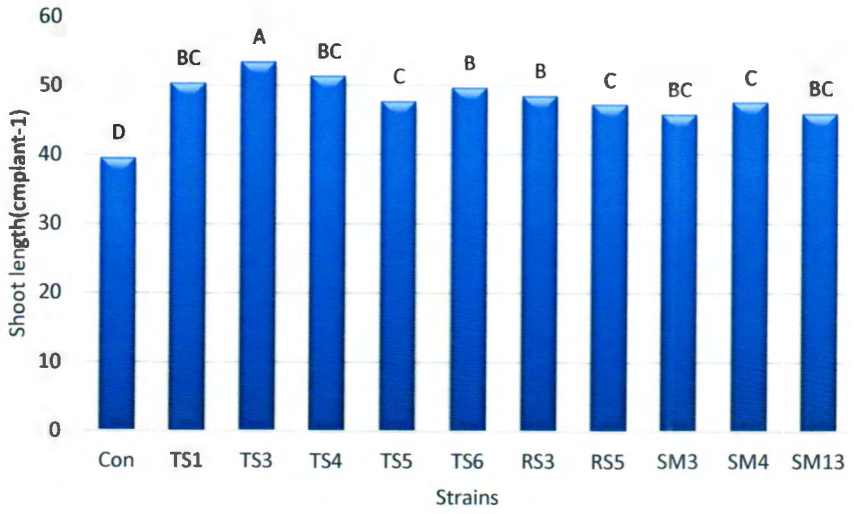


Graph 3.5: Effect of inoculation treatments on plant height of pot grown *Solanum lycopersicum*

**Means sharing the same letter (s) do not differ significantly at P < 0.05 according to LSD*

3.6.3 Shoot length (cm)

It is evident from the data (Graph 3.6) that application of PSB proved relatively better. Significant difference was found among all the treatments regarding shoot length. Maximum shoot length was recorded in plants inoculated with TS-3 treatment (53.47 cm) followed by value of (51.33 cm) in TS-4 and 50.36 cm in case of TS-1. These treatments showed increase, respectively over the control (39.6 cm).The three treatments with strains TS-6,TS-5,SM-3 and SM-4 produced the secondly most prolific shoot length that was 49.6, 47.66,45.76 and 47.55 cm with increase over control.



Graph 3.6: Effect of inoculation treatments on shoot length of pot grown *Solanum lycopersicum.*

**Means sharing the same letter (s) do not differ significantly at P < 0.05 according to LSD*

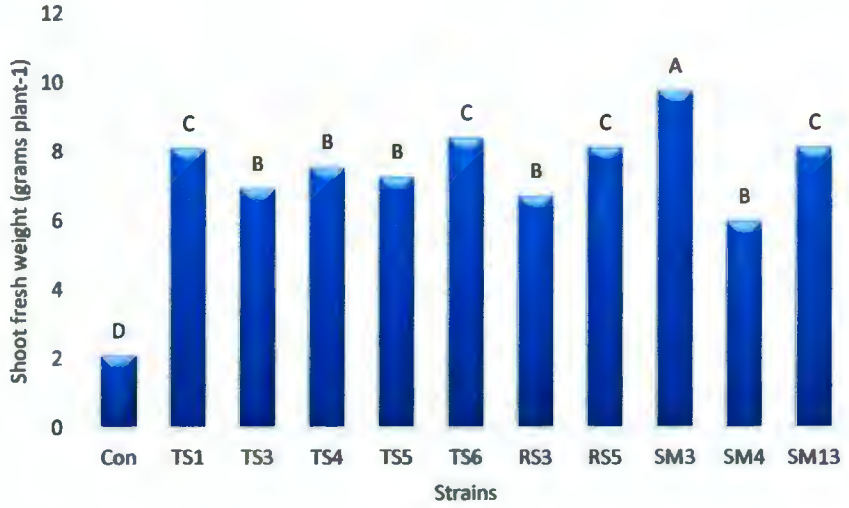
Figure 3.20: Effect of inoculation treatments on shoot length of pot grown *Solanum lycopersicum.*

3.6.4 Shoot fresh and dry weight (g)

Average shoot weight plant-1 increased significantly ($p < 0.05$) during growth in the Pot experiment as shown in Graph 3.7. Maximum fresh shoot weight (g plant-1) was recorded in plants inoculated with SM-3 (9.764g) followed by RS-5(8.133g) and TS-6 (8.41g) with increase over the control. As in case of shoot dry weight as shown in Graph 3.8, SM-3, RS-5 and TS-5, were the most efficient in producing dry mass plant-1 having values 5.63, 4.7 and 4.53 g plant -1, respectively .Shoot Fresh and dry mass formed in response to PSB strains SM-4 and SM-13 were not so impressive among all ten strains.

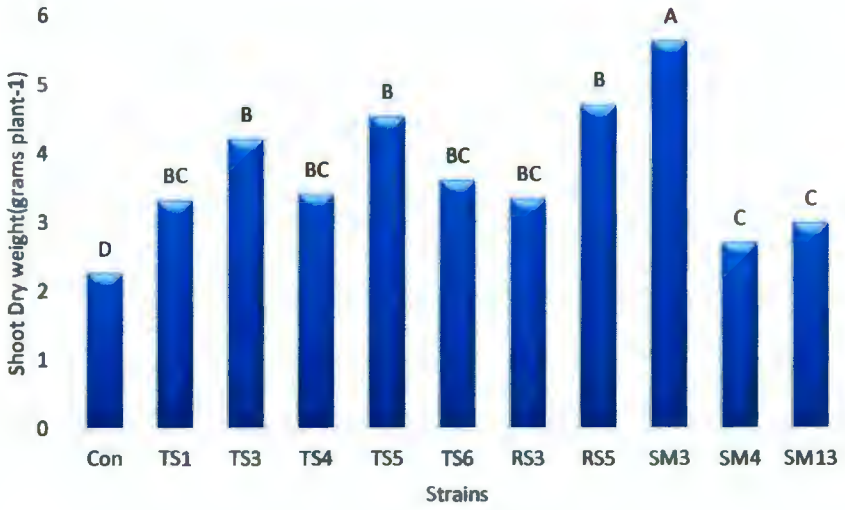
3.6.5 Root length (cm)

Regarding root length, significant difference was found among PSB treatments as shown in (Fig 3.12). Maximum root length (cm) was recorded in plants inoculated with SM-3 (37.6 cm) followed by other two combinations TS-5(34.1 cm) and SM-4 (33.423 cm). Plant root length produced as affected by strains TS1 and TS3 were minimum and statistically similar ranging from 29.9 to 27.33.In case of inoculation, root length increases, as compared to the control.



Graph 3.7: Effect of inoculation treatment on shoot fresh mass of *Solanum lycopersicum*

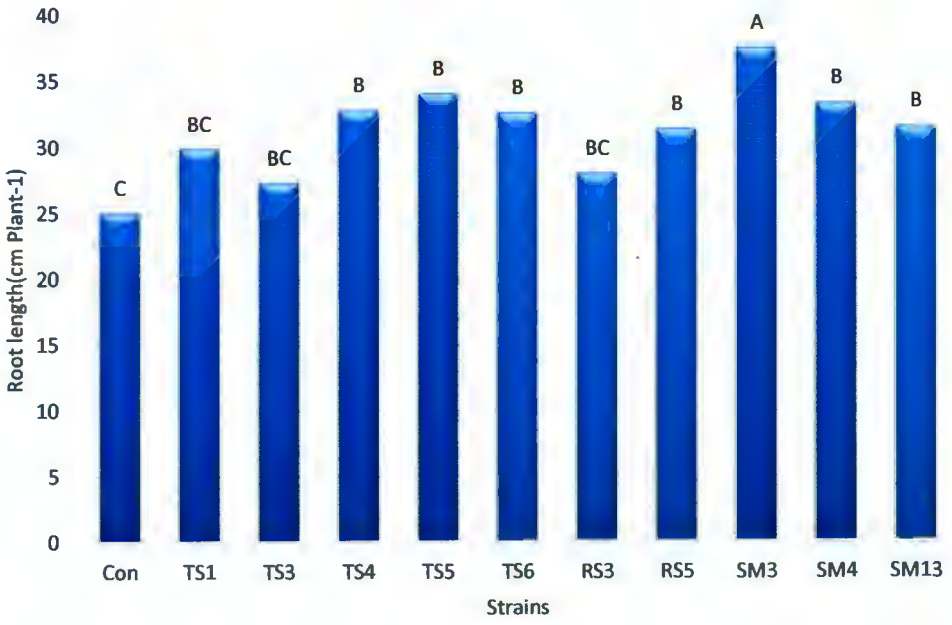
**Means sharing the same letter (s) do not differ significantly at P < 0.05 according to LSD*



Graph 3.8: Effect of inoculation treatment on shoot dry mass of *Solanum lycopersicum*

**Means sharing the same letter (s) do not differ significantly at P < 0.05 according to LSD*

Fig 3.21: Effect of inoculation on root length of pot grown *Solanum lycopersicum*.



Graph 3.9: Effect of inoculation treatments on root length of *Solanum lycopersicum*

**Means sharing the same letter (s) do not differ significantly at $P < 0.05$ according to LSD*

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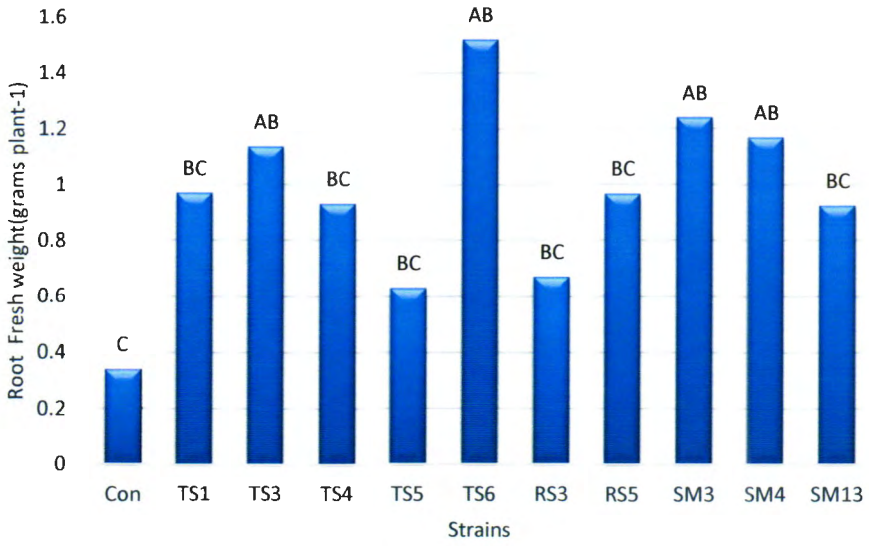
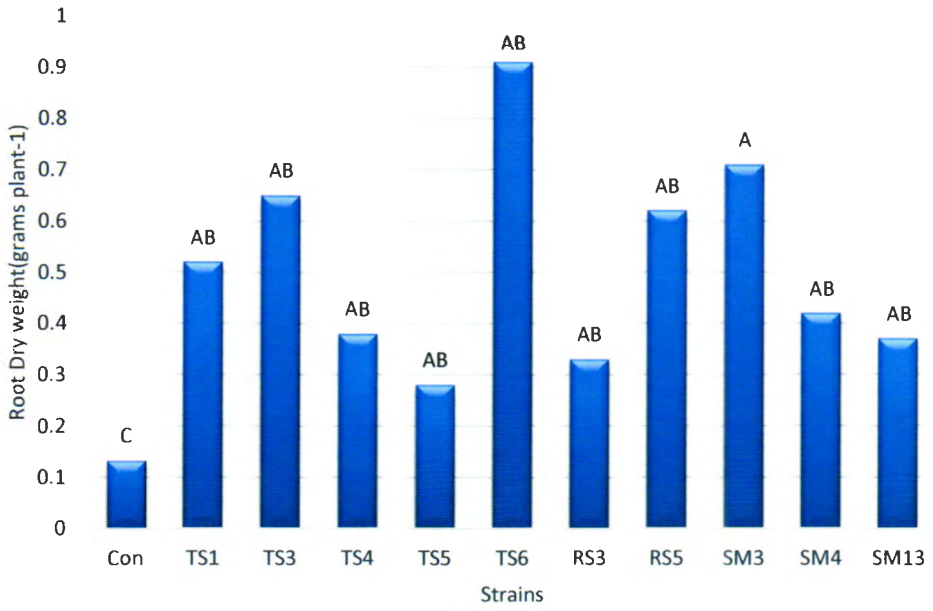
3.6.6 Root fresh and dry weight.

Average root weight plant-1 increased significantly ($p < 0.05$) during crop growth in the experiment as shown in (Graph 3.10, 3.11). The inoculation of PSB isolates had pronounced effect on root weight plant-1. The highest root weight (g plant-1) was recorded in plants inoculated with PSB strain SM-3 (1.24g) followed by other two combinations SM-4 (1.17) and TS-3 (1.136). These treatments showed increase in root weight more than control (0.34 g). The inoculation with TS-3,SM-3 and RS-5 which proliferated dry roots weights of 0.65, 0.63 and 0.62g plant-1and showed increase(0.133) over control.



Graph 3.10: Effect of inoculation treatments on fresh root weight of *Solanum lycopersicum*

**Means sharing the same letter (s) do not differ significantly at P < 0.05 according to LSD*



Graph 3.11: Effect of inoculation treatments on dry root weight of *Solanum lycopersicum*

**Means sharing the same letter (s) do not differ significantly at P < 0.05 according to LSD*

ISOLATES ID	NO OF FRUITS
CON	0
TS1	3
TS3	2
TS4	1
TS5	1
TS6	2
RS3	3
RS5	4
SM3	2
SM4	2
SM13	5

Table 3.7: Effect of inoculation treatments on Fruit no of *Solanum lycopersicum*

3.6.7 Number of Fruits

The data regarding the No. of Fruit plant -1 of Tomato shown in table 3.6 revealed that there was considerable variation in the number of fruit produced in response to bacterial strains used. Fruit number generally was the highest in the plants inoculated with *SM-13*, RS-5, and RS-3. The others strains were statically similar. Fruits formed in response to PSB strains TS-4 and TS-3 were the lowest but still significantly better than control. The significant increase in yield and fruit quality of Solanum lycopersicum might be attributed to improved uptake of Phosphorous.

Chapter 4
Discussion

4.0 Discussion

The plant rhizosphere is a versatile and enthusiastic biological environment of intense relation of organisms and plant for utilizing micro and macro-nutrients from lacking a supplement pool. In the period of agricultural production, the collaborations in the rhizosphere assume a principal part in change, activation, solubilization, and so on from a restricted supplement pool in the soil and resulting uptake of critical plant supplements by the crop plants to acknowledge full hereditary capability of the crop(Jeffries *et al.*, 2003). In current research, a total of sixteen strains from rhizosphere were isolated from agricultural soils of D-I-Khan. These isolates belonged to different bacterial genera. Some strains displayed multiple PGP traits which showed that they may stimulate plant growth by number of mechanisms that may be active at the same time or at different phases of plant life. Present study results are reported with those of Gupta *et al.* (2002), Ahmad *et al.* (2008). Rashid *et al.* (2012) also reported different levels of these activities. Kumar *et al.* (2011) stated that possession of multiple mechanisms for improving plant growth is necessary for a bacterial strain to be an effective PGPR. Soil microbes play important role in maintaining soil quality and health, and in regulating biogeochemical cycles of all nutrients in the soil (Jeffries *et al.*, 2003). Phosphorus is an essential macronutrient that perform imperative function in plant growth and development (Podile and Kishore, 2006; Ali *et al.*, 2012). Present study showed most of the isolates can solubilize good quantity of tricalcium orthophosphate, hence may help in

Increasing phosphorous availability to plants. Earlier reported researches confirmed that these bacterial genera were able to solubilize phosphate. Phosphate solubilization by *P. putida*, *Azotobacter*, *Klebsiella*, Fluorescent *Pseudomonas*, *Bacillus* and *S. maltophilia* was reported by Selvakumar *et al.* (2008), Farajzadeh *et al.* (2012), Rashid *et al.* (2012) respectively.

Many scientist considered the solubilization of phosphorous by microorganism result in the manufacture of organic acid. Consequently, Quantitative analysis for phosphorous solubilization by microorganism responsible for lowering of pH in surrounding medium. (Mehta and Nautiyal, 2001). After confirming the Phosphate Solubilizing Activity on solid medium, the phosphorus solubilization in liquid medium (PVK Broth) was confirmed. Results showed that isolates TS-6, SM-3, RS-3, and TS-1 showed maximum P solubilization in liquid medium. . Results showed that with increased in P solubilization pH was significantly decreased form its initial value 7.0 to 3.57 in all the different P sources (Tri calcium phosphate) suplimented in PKV broth. Significant ($P \leq 0.05$) declined in pH of the soil and high available phosphorous content of soil after inoculation of PSB strains P. agglomerans or B. anthina or coinoculation of isolates is in agreement with the study of Yu et al. (2011) who reported same results after soil inoculation with Pseudomonas chlororaphis and Pseudomonas fluorescens. (Walpola and Yoon, 2013).

Results of this research showed that members of isolated strain produced siderophores. Siderophore production by bacteria is a significant trait of PGPB, which may directly or circuitously effect the plant growth.

Siderophores get attach to the iron that are easily accessible in the rhizosphere, by doing so iron cannot be obtained by plant pathogens and hence plant is protected. These beneficial Siderophores producers enhance plant growth by reducing growth of bacterial and fungal populations in vicinity to plant roots (Gupta *et al.*, 2002). There is persuading confirmation to support an immediate part of Siderophore-intervened iron competition in the biocontrol capacity. Siderophore producing organism rapidly colonize plants roots and considerably increase plant yield (Haas and Défago, 2005).

Current results about siderophores production are similar to the earlier studies by Koo and Cho, (2009) who reported that some members of genus *Serratia* are able to produce IAA and siderophores. Ahemad and Khan, (2011) and Wani and Khan, (2010) reported that *P. putida, P. aeruginosa and Klebsiella* sp. produced IAA, siderophores, HCN, ammonia, exo-polysaccharides and could solubilize phosphate.

Biological control of phytopathogens and harmful microorganisms, by the production of lytic enzymes, antibiotics, siderophores and hydrogen cyanide or through competition for nutrient and space can significantly enhance health of plant and improve plant growth by increasing seedling emergence, yield and vigor (Antoun and Kloepper, 2001).

In agriculture, some microbes that have ability to produce protease play major role as biocontrol agents in suppression of some fungal phytopathogens. The screening of isolates for protease production on skimmed milk agar plate according to Cattelan *et al.* (1999) showed that isolates were positive for the proteolytic activities which supported the work done by our study.

In concurrence to our result are the previous findings, where the bacterial isolates like *P. aeruginosa, S, liquefaciens* produced protease (Patil and Chaudhari, 2011, Smita *et al.*, 2012).

Production of HCN by *Bacillus* (50%) and *Pseudomonas* (88.89%) in root nodules and in rhizospheric soil is a common trait (García *et al.*, 2004, Ahmad *et al.*, 2008). It is a severe environmental contaminant and a biological control agent of *Pseudomonas* sp. Saharan and Nehra, (2011) reported that rhizobacterial isolates of mustard belonging to genera *Bacillus* and *Pseudomonas* could produce HCN. Ahemad and Kibret, (2014) reported *P. putida, P. aeruginosa, Klebsiella* sp., *Bacillus* sp., PSB10, *Pseudomonas* sp., *S. marcescens, Rhizobium* sp. isolated from pea as HCN producers. Moreover *P. putida* was also reported as HCN producer by Karunya, (2011).

About 95% of rhizobacterial isolates of mangrove, rice and other contaminated soils are reported to produce ammonia (Samuel and Muthukkaruppan, 2011; Joseph *et al.*, 2012). Results reported in here are in agreement with them.

The role of PGPR in PGP is well recognized and the increase in shoot and root length under control conditions might be due to different plant growth promoting activities like synthesis of phytohormones (Zahir *et al.*, 2003) and nutrient availability (Peralta *et al.*, 2012). This improvement in uptake of nitrogen, phosphorus and potassium might be due to more availability of nutrients to plants by phosphorus solubilization (Ranjan *et al.*, 2013), siderophore production (Sayyed *et al.*, 2010) and improved root growth (Karnwal, 2012) which ultimately results in more uptake of nutrients from soil.

The expanded plant height and root length could be connected with cell elongation and duplication incited by more noteworthy assimilation of supplements, especially phosphorous (Walpola and Yoon, 2013).

The inoculants in present study had a higher plant stature than that of the control. The present results are in accordance with the investigation of Rudresh et al. (2005) and Gull et al. (2004) who researched phosphorous uptake and development advancement of chickpea plants (Cicer aritenium L.) in growth chamber and green house. Yu et al. (2011) reported phosphate solubilizing microscopic organisms amazingly expanded plant height, shoot and root dry weight, and phosphorous and nitrogen uptake of walnut seedlings. Furthermore, there are some comparable reports on improved dry matter substance of maize and groundnut because of inoculation of PSB (Hameeda et al., 2006a; Pandey et al., 2006).

The PSB inoculants brought about comparative or higher leaf numbers contrasted with the control. Likewise, different analysts reported that plant growth promoting rhizobacteria influenced the leaf quantities of soybean and fodder maize. (*Yolcu et al, 2012).*

PGPR strains identified in previous study of Kloepper *et al.* (1991) were *P. putida, P. fluorescens, S. liquefaciens*. In present study *Serratia* gave the best results for root length enhancement as reported previously. Some strains of *Pseudomonas* and *Bacillus* gave promising results too. Some of the tested strains negatively influenced plant growth which may be attributed to production of phytotoxins by these strains. Khalid *et al.* (2004) also reported similar cases.

4.1 Conclusion

Identification of different mechanisms involved in plant-rhizosphere microorganism interaction opened new possibilities to design strategies for improving crop yields. Literature describes many beneficial effects of PSB isolated from agricultural soils on plant growth from changing the root architecture and enhancing nutrient uptake to biocontrol .This study include screening of PSB and PGPR attributes for their growth-promoting activity under axenic conditions. The results demonstrated that both the bacterial isolates significantly improved the growth parameters in Tomato plants. However, the degree to which these inoculants imparted benefits to plant growth varied with the isolate. A PGPR strain with multifarious traits could be more useful under diverse conditions compared to a strain containing single trait. The idea is supported from the results that inoculation had promising positive effects on growth and yield of Tomato as compared with control. The inoculation also had a significant effect on the nutrient contents and uptake of Tomato plants. Thus, it can be concluded from the study that the inoculation of (PSB) could be the most effective and novel approach for promoting Growth, nutrient uptake and yield of tomato grown.

4.2 Future Directions

The successful use of PGPR and PSB as the best potential candidate for plant growth promotion can help in avoiding biosafety issues, as these are inoculated by natural means in these organisms, and hence can aid in eradicating the use of hazardous chemicals pesticides and fungicides. Most significantly, the rhizobacteria can be used commercially viable alternatives to chemical fertilizers and thus, enhance growth of economically

important crops. This study revealed a significant increase in this crop through inoculation of PSB. Therefore, these studies may be used as helpful tool for providing guidelines in future. However, further work is needed to explore the effectiveness of this approach to make it more useful for plant growth. In particular attention should be given to the following aspects.

Efforts should be done to increase the population of soil microbes by seed or soil inoculation.

- These rhizobium and PSB strains can be used for preparation of effective biofertilizer to enhance crop growth.
- Genetic manipulation of plant expressing microbial gene.
- To explore the physiological interaction between plant, microbes and stressors.

Chapter 5
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Appendix

Recipes of reagents, buffers and media

2.1. Luria Bertani Agar media

Contents	Quantity (Full strength) (g/L)
Yeast Extract	05
Tryptone	10
Agar	18
NaCl	05
Distilled water	1000
PH	7

2.2. Pikovaskya

Content	Quality (g/L)
Glucose	10
Na_2HPO_4	0.5
NaCl	0.2
$MgSO_4.7H_2O$	0.1
$Ca_3(HPO_4)_2$	5.0
KCL	0.2
Yeast Extract	0.5
$MnSO_4$	Trace
$FeSO_4.7H_2O$	Trace
Agar	15
Distilled Water	1000
PH	7

2.3. Protease media

Contents	Quantity
Glucose	1
Peptone	2
Yeast extract	5
K_2HPO_4	1
$MgSO_4.7H_2O$	0.2
Skimmed Milk	5
Agar	25
Distilled water	1000
PH	7

2.4. Amylase media

Contents	Quantity (g/L)
Yeast extract	1
$MgSO_4.7H_2O$	0.1
K_2HPO_4	7
KH_2PO_4	2
$(NH_4)_2SO_4$	1
NaCl	5
Starch	5
Distilled water	1000 ml
Agar	20
PH	7

2.5. Pectinase activity test

Contents	Quality (g/L)
$(NH_4)_2SO_4$	2
Na_2HPO_4	6
KH_2PO_4	3
Yeast Extract	1
Pectin	5
Agar	15
Distilled water	1000
PH	7

2.6. NH_3 production

Contents	Quantity
Peptone water	15 g/L
Nessler's reagent	0.5 ml

2.7. Hydrogen cyanide production

Contents	Quantity (g/L)
Nutrient Broth	21
Glycine	4.4
Agar	3.75
Picric acid	0.5%
Sodium carbonate	2%

2.9. CAS agar medium for Siderophore Production

CAS agar media was prepared from mixing of four solutions which were sterilized separately and then mixed together and poured in to sterilized petri plates.

2.9.1. CAS Dye (Solution 1)

	Contents	Quantity
Solution I	CAS dye	0.06g in 50 ml of ddH_2O.
Solution II	$FeCl_3$	0.0027g in10 ml of 10mM HCl.
Solution III	HDTMA (C-TAB)	0.073g in 40 ml of ddH_2O.

2.9.2. Buffer solution (solution 2)

Contents	Quantity
PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid])	30.24 g
KH_2PO_4	0.3 g
NaCl	0.5 g
NH_4Cl	1.0 g
Agar	15 g
ddH_20	800ml

2.9.3. Solution 3

This solution was prepared by dissolving following ingredients.

Contents	Quantity (70 ml)
Glucose	2 g
Mannitol	2 g
$MgSO_4.7H_2O$	0.473 g
$CaCl_2$	0.011 g
$MnSO_4.H_2O$	0.00117 g
H_3BO_3	0.0014 g
$CuSO_4.5H_2O$	0.00004 g
$ZnSO_4.7H_2O$	0.0012 g
$Na_2MoO_4.2H_2O$	0.001 g

2.9.4. Solution 4 (Casamino acid solution)

Contents	Quantity
Casamino acids	3 g
ddH_2O	27 ml
8-hydroxyquinoline.	3%
Chloroform	100 ml

2.10. Preparation of IAA stock solution

Contents	Quality (100 ml)
Tryptone	10
Yeast Extract	5
NaCl	5
Trypophane	1
Distilled water	1000
PH	7

2.11. Preparation of Salkowski's reagent

For Salkowski's reagent we added 2ml of 0.5 M $FeCl_3$ and 98 ml of 35 % $HClO_4$.

2.11.1. 0.5 M $FeCl_3$

Contents	Quantity (g/L)
$FeCl_3$	270.30
0.5 M $FeCl_3$	135.15

2.12 Gram Staining

Crystal Violet, Stain	**(g/l)**
Crystal Violet	**20**
Ethanol, Denatured	**200ml**
Ammonium Oxalate	**8**
Water, deionized	**800ml**
Iodine, Mordant	
Iodine	**3.3**
Potassium Iodine	**6.6**
Water, deionized	**1000**
Alcohol-Acetone, Decolorizer	
Ethanol, denatured	**500ml**
Acetone	**500ml**
Safranin, Counterstain	
Safranin O	**2.5**
Ethanol, denatured	**100ml**
Water, deionized	**900ml**
Basic Fuchsin, Counterstain	
Basic Fuchsine	**1**
Ethanol, denatured	**100ml**
Water, deionized	**900ml**