Improvement of wheat (*Triticum aestivum.* L) nutrients by zinc mobilizing plant growth promoting rhizobacteria (PGPR)

By

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CIIT/SP09-PBS-001/ISB

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It is certified that Muhammad Abaid-Ullah, CIIT/SP09-PBS-001/ISB has carried out all the work related to this thesis under my supervision at the Department of Biosciences, COMSATS Institute of Information Technology, Islamabad and the work fulfills the requirement for award of PhD degree.

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DEDICATION

To My Parents

Who inspired me to higher ideas of life

&

Gave me a chance to prove and improve myself through all walks of my life

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ABSTRACT

Improvement of wheat (*Triticum aestivum.* L) nutrients by zinc mobilizing plant growth promoting rhizobacteria (PGPR)

The main objective of the proposed PhD project was to get insight and depth of knowledge of deploying Zinc (Zn) solubilizer rhizobacteria to overcome Zn deficiency in wheat crop and ultimately to improve yield and quality of wheat. For this purpose, about 198 bacterial isolates were obtained from rhizosphere of wheat field. Out of 198, five strains FA-2, FA-3, FA-4, FA-9 and FA-11 were found effective for Zn solubilizing activity that was quantified through "Atomic Absorption Spectrophotometer" (AAS). The above mentioned five strains produced a clear zone with diameter of 27.5, 63, 59.5, 51.2, 63 and 17.2 mm, respectively on agar plate having $ZnCO_3$. Similar trend was also observed in ZnO modified agar plates. However, all strains did not show visible activity on ZnS. The aforementioned five strains solubilized Zn concentration by 135, 152, 164, 102 and 45 $\mu g\ mL^{-1}$ respectively, but all the strains showed little solubility in ZnS liquid broth. Based on variation in correlation between qualitative and quantitative screening, it can be stated that agar plate assay might not be so precise to detect the low level of solubilization with various Zn ores by Zinc solubilizing bacteria (ZSB). The ZSB showed positive response for indol acetic acid (IAA), exo-polysaccharides (EPS), siderophores activity, nitrogen (N_2) fixation, phosphate (P) solubilization, ACC deaminase, hemolytic and antifungal activities. The ZSB also produced gluconic acid in the range of 330-2500 ppm. The diversity among similar genera of ZSB was observed by amplifying intergenic regions (IGS) through PCR, and restriction length polymorphism (RFLP) analysis. Similarly as far as identification of the ZSB strains is concerned, FA-2, FA-3, FA-4, FA-

9 and FA-11 were characterized as *Serratia liquefaciens*, *Bacillus thuringiensis, S. marcescens*, *Pseudomonas aeruginosa* and *Enterobacter sp*. The *PqqC* (Co-factor for PQQ), *acdS and NifH* genes from ZSB were amplified, cloned and sequenced which also confirmed their identity as revealed by *16S rRNA* and *gyrB* gene analysis. The response of three promising ZSB, namely FA-2, FA-3, FA-4 and their consortium was further tested under diverse climatic field condition with four commercial wheat (*Triticum aestivum* L.) cultivars *viz*. Inqlab 91, Chakwal-50, Lasani-08 and SH-2002. A significant increase of 54, 68, 57 and 46%, respectively in wheat Zn contents was recorded over chemical Zn fertilizer. Similarly the above-mentioned three ZSB strains and their consortium increased grain yield by 2.4, 0.7, 2.2 and 8.6% over chemical Zn fertilizer. The bacterial strains showed significant interaction with wheat crop cultivars and locations of the field. Moreover, the three strains showed less survival in rhizosphere (45, 38 and 47%) as compared in endophytic environment (91, 90 and 83%).

The results of present study are very encouraging and prove that the effect of plant growth promoting rhizobacteria (PGPR) in wheat crop is at least partly. Maintaining suitable density of Zn solubilizers in the soil through field inoculation might be a promising strategy to enhance grain yield and Zn content of wheat. It would also appear as a natural, inexpensive and eco-friendly approach which might reduce the use of synthetic fertilizer to minimize environmental hazards. Commercial field application of this approach among farmers is recommended.

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LIST OF ABBREVIATIONS

PGPR	Plant Growth Promoting Rhizobacteria
ZSB	Zinc Solubilizing Bacteria
DALY	Disability-Adjusted Life Year
AARI	Ayub Agriculture Research Institute, Faisalabad
BARI	Barani Agriculture Research Institute, Chakwal
CRS	Cotton Research Station, Multan
BLAST	Basic local alignment search tool
PQQ	Pyrroloquinoline quinone
Gyr	Gyrase
bp	Base pair
kbp	Kilo base pair
CIIT	COMSATS Institute of Information Technology
DNA	Deoxyribo nucleic acid
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
$MgCl_2$	Magnesium chloride
min	Minute
Sec	Second
h	Hour
mL	Milliliter
mM	Milli molar
ºC	Degree centigrade
PCR	Polymerase chain reaction

pH	Potential hydrogen
CIMMYT	Centro Internacionale de Mejoramiento de Maiz y Trigo
FAO	Food and Agriculture Organization
RFLP	Restriction Fragment Length Polymorphism

Chapter 1

Introduction

1. Introduction

Zinc (Zn) is an essential element necessary for plants, humans and microorganisms (Broadley *et al.,* 2007; Prasad, 2008b; Cakmak, 2008). Humans and other living things require Zn throughout life in little quantities to orchestrate a complete array of physiological functions. Zinc is a vital mineral of "exceptional biological and public health importance" (Hambidge and Krebs, 2007). Furthermore 100 specific enzymes are found in which Zinc serves as structural ions in transcription factors and is stored and transferred in metallothionein (United States National Research Council, 2000; Silvera and Ronan, 2001). It is typically the second "most abundant transition metal in organisms" after iron and it is the only metal which appears in all enzyme classes (King 2006; Broadley *et al.,* 2007).

Biofortification is a current approach aimed at increasing the bioavailability of micronutrients such as Zn and Fe in the staple crops of specific region (Stein, 2010). In this regard beneficial free-living soil bacteria which have been shown to improve plant health or increase yield can also mobilize micronutrients. In this chapter, the collective results highlight the importance of Zn with comparison to various strategies to meet its required quantity in major food crops. The next technological revolution to eradicate Zn malnutrition would be the Plant Growth Promoting Microorganisms enabling better availability of Zn and other micronutrients through their economical, beneficial and eco-friendly nature.

1.1. Role of Zinc in plants:

Zinc is important micronutrient for plants which plays numerous functions in life cycle of plants (Hirschi, 2008). Crop growth, vigor, maturity and yield are very much reliant upon essential micronutrient such as Zn. It is involved in many physiological functions in plants. It is responsible for synthesis of auxin and catalyzes the photochemical reaction of chlorophyll. Zn is also required for the stability of biological membranes and is important for the activity of various enzymes e.g. Cu/Zn superoxide dismutase (SOD), carbonic

anhydrase which contain structurally bound Zn and plant growth regulator Indole Acetic Acid (IAA). It influences the synthesis of nucleic acid, lipids and proteins by which the grain quality become superior (Seilsepour, 2006; Hershfinkel, 2006; Kramer and Clemens, 2006). Physiologically it activates metabolism of carbohydrates, auxin, RNA and ribosome's functions. Zn has also been reported for increased growth, yield and yield components as well as improved leaves and flowers nutrients content and plant chemical constituents, i.e. pigments, carbohydrates and flowers oil concentration (Khalifa *et al.,* 2011) as shown in Fig. 1.1.

It has been proved that Zn application to wheat increase its concentration in flag leaves and grains (Ranjbar and Bahmaniar, 2007; Cakmak, 2008; Waters *et al.,* 2009). Higher absorption of Zn produced higher grain yield (Han *et al.,* 2006).

1.2. Role of zinc in humans

Humans cannot attain normal vigorous growth without essential elements like Zn (Calder and Jackson, 2000). In developing countries, supplementation with Zn was found to lower frequency and severity of infections like diarrhea and pneumonia and decrease mortality (Black *et al.,* 2008).

Biologically Zn plays catalytic, co-catalytic or structural roles in more than 300 enzymes. The six enzyme classes namely oxido-reductases, transferases, hydrolases, lyases, isomerases and ligases depend on Zn for their activity. Although 86% is in skeletal muscle, there are certain parts prostate, hippocampus, pancreas, and kidney cortex where zinc concentration is particularly high and may represent functional significance (Vallee and Falchuk, 1981).

Furthermore, in the synthesis of proteins and metabolism of DNA, RNA and metabolic homeostasis in human body zinc is critically involved. Strong evidence indicates the presence of a number of zinc-containing proteins, which directly influence gene expression (Welch, 2001). Exposure to high doses has toxic effects but intoxication by excessive exposure is rare.

Figure 1.1 Growth and vigor of rice plant at different concentration of Zn (http://www.harvestzinc.org/pictures/zinc-deficiency-symptoms)

1.3. Zinc deficiency and malnutrition

Zn deficiency is among the top micronutrient deficiencies reported in human beings which influences almost one third of the world's populations (Hotz and Brown, 2004; Stein, 2010; Zhang *et al.,* 2012).

Zn is an essential metal element for human health. Its deficiency caused by malnutrition is the 11th major risk factor of disease trouble in the global distribution linked with 1.8 million deaths yearly (WHO, 2002). About 100 million people mainly living in rural areas undergo Zn deficiency in China (Ma *et al.,* 2008; Zhang *et al.,* 2010). Studies of inhibition of spermatogenesis and different abnormalities of sperm production in human have shown Zn deficiency (Prasad, 2008a). It is estimated that globally two billion people are at threat of zinc deficiency (Gibson and Ferguson, 1998). Additionally 37% children of less than five year age are at risk of zinc deficiency in Pakistan (HarvestPlus, 2012). The rate of deaths due to deficiency of Zn is shown in Table 1.1.

In vitro trials have illustrated that zinc supplementation can decrease the brutality of morbidity from a numeral common babyhood infections (HarvestPlus, 2012), as a result WHO recommended Zn supplementation during diarrheal infection and for treatment of severe malnutrition (WHO, 2004). A study in China has proved that zinc fortified flour could improve its deficiency in women of childbearing age (Brown *et al.,* 2010). Zn absorption is influenced by various factors i.e. binding to a ligand secreted by the pancreas increases absorption, luminal amino acids bind Zn and prevent its precipitation by substances such as phosphate and phytate, whereas pregnancy, corticosteroids, and endotoxin all enhance absorption while phytate, phosphate, iron, copper, lead, and calcium hinder absorption of Zn (Davies, 1980). Zn deficiency is widespread and has a detrimental impact on growth, neuronal development, and immunity (Plum *et al.,* 2010). The reason behind Zn deficiency is insufficient nutritional ingestion of Zn and Fe in majority of the cases

Table 1.1 Zn deficiencies in children under age 5 year, from different regions

Region	Prevalence (%)	Deaths('000)	*DALYs lost('000)
East Asia & Pacific	7	15	1,004
East Europe & Central Asia	10	4	149
Latin America & Caribbean	33	15	587
Middle East & North Africa	46	94	3,290
South Asia	79	252	8,510
Sub-Saharan Africa	50	400	14,094
High Income Countries	5	0	2

(Source: Disease Control Priorities in Developing Countries, 2nd edition, 2006) (Harvestplus, 2011); *Disability Adjusted Life Years

(Welch and Graham, 2004; Cakmak *et al.,* 2010). Conversely the concentration of a number of minerals especially zinc, iron, iodine and selenium are inherently poor in plants as compare to animal derived foods. As a consequence more than three billion people globally suffer from micronutrient starvation (Cakmak, 2008; White and Broadley, 2009). As reported by the Alloway (2004) most of the wheat crop was harvested on the zinc deficient soils which resulted in lower zinc content of wheat grain. The development of high-yielding genotypes have aggravated this dilemma (Zhao and McGrath, 2009; Cakmak *et al.,* 2010; Stein, 2010). Moreover, the processing of wheat significantly decreases the concentration of Zn as well as other minerals, which promote Zn deficiency (Zhang *et al.,* 2010; Kutman *et al.,* 2011). To overcome this problem, improvement of Zn bioavailability in cultivated soils may enhance Zn contents in the staple food grains which would possibly diminish the major health risks attributable to this micronutrient deficiency.

1.4. Zinc status of soil

Most of the soils are either Zn deficient or contain Zn in fixed form i.e. unavailable to the plant. According to FAO reports, 50% of the soils are deficient in Zn (FAO and WHO, 2002). Deficiency of Zn is frequent in calcareous and neutral soils, paddy soils, intensively harvested soils and inadequately drained soils, saline and sodic soils, peat soils, soils with elevated level of phosphorus and silicon, sandy soils, extremely weathered acid and coarse-textured soils (Sillanpaa, 1982; Alloway, 2008) as illustrated in figure 2.1. Zn deficiency may also be related with the nature of soil such as in calcareous soils; Zn^{2+} may exist as low as 10^{-11} to 10^{-9} M and can reduce crop growth (Hacisalihoglu and Kochian, 2003).

Approximately half of the agricultural soils in China has been affected by zinc deficiency while in India zinc-deficient soils has engaged almost 50% of the agricultural part and the

same is the situation in Turkey (FAO and WHO, 2002). In Pakistan, 70% of agricultural land has been reported as Zn deficient as shown in Fig. 1.2 (Hamid and Ahmad, 2001; Kauser *et al.,* 2001).

Occurrence of Zn in soil is found as ZnS (sphalerite), further less frequent Zn containing mineral ores include: smithsonite ($ZnCO_3$), zincite (ZnO), zinkosite ($ZnSO_4$), franklinite ($ZnFe_2O_4$) and hopeite [$Zn_3(PO_4)_2.4H_2O$]; however, availability of Zn from these sources depends on various factors. The natural sources of zinc to soil include

a) Chemical and physical weathering of parent rocks (Alloway, 1995).
b) Atmospheric contribution of zinc to soils (e.g. volcanoes, forest fires, and surface dusts) (Friedland, 1989; International Zinc Association, 2011).

Micronutrients uptake from the rhizosphere is the primary step for its accumulation into the plant before translocation to seeds (Giehl *et al.,* 2009). Plant roots, uptake Zn as Zn^{2+} cation which is constituent of synthetic and organic compounds (Havlin *et al.,* 2005; Oliveira and Nascimento, 2006). Plants adsorb available zinc from the soil solution in a reactive form.

Accessible amount of zinc to plants is controlled by the soil factors e.g. the total zinc concentration, pH, organic matter, clay and calcium carbonate, redox conditions, microbial activity in the rhizosphere, soil moisture, concentrations of other trace elements, concentrations of macro-nutrients, especially phosphorus and climate as explained in Fig. 1.3 (Alloway, 2008).

Zn supply is mainly affected by the soil pH in soil pools; in view of the fact that this element is readily adsorb in exchange cation sites at over-neutral pH and made accessible at low pH values (Havlin *et al.,* 2005; Broadley *et al.,* 2007). Cereals have very little Zn concentration in grains as compared to animal-based foods or pulses. Currently, Indian soils are Zn-deficient particularly in wheat cropping system and it will further decrease grain Zn concentration in cereals (Prasad, 2005; Gupta, 2005). Generally cereal grain contains low concentration of Zn due to the existence of anti-nutrition factor such as

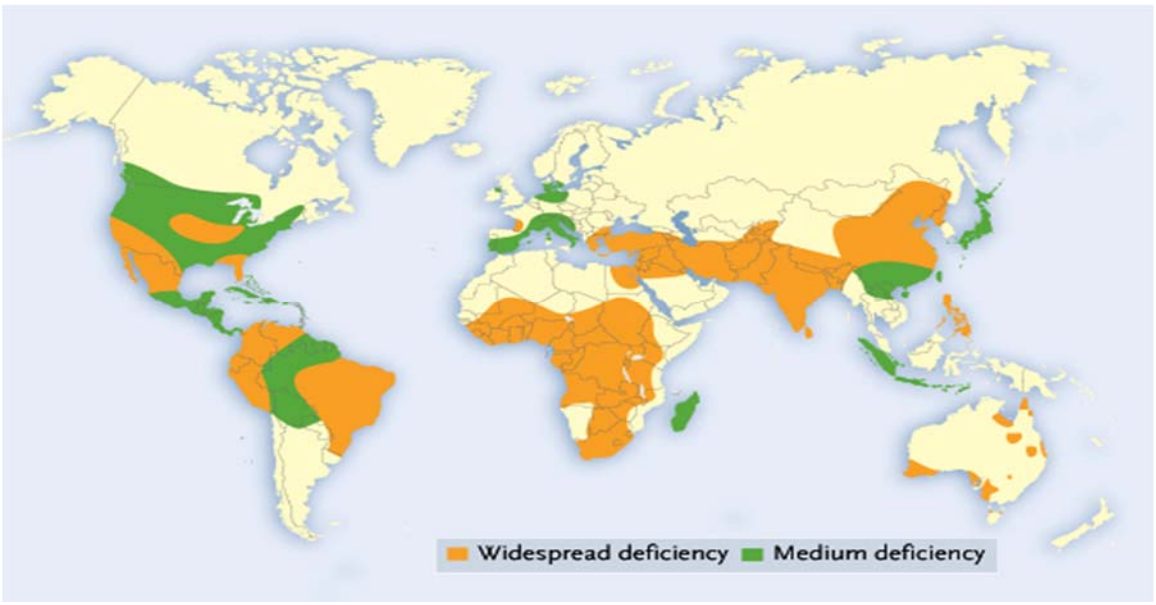


Fig. 1.2 World soil zinc distribution (Alloway, 2008)

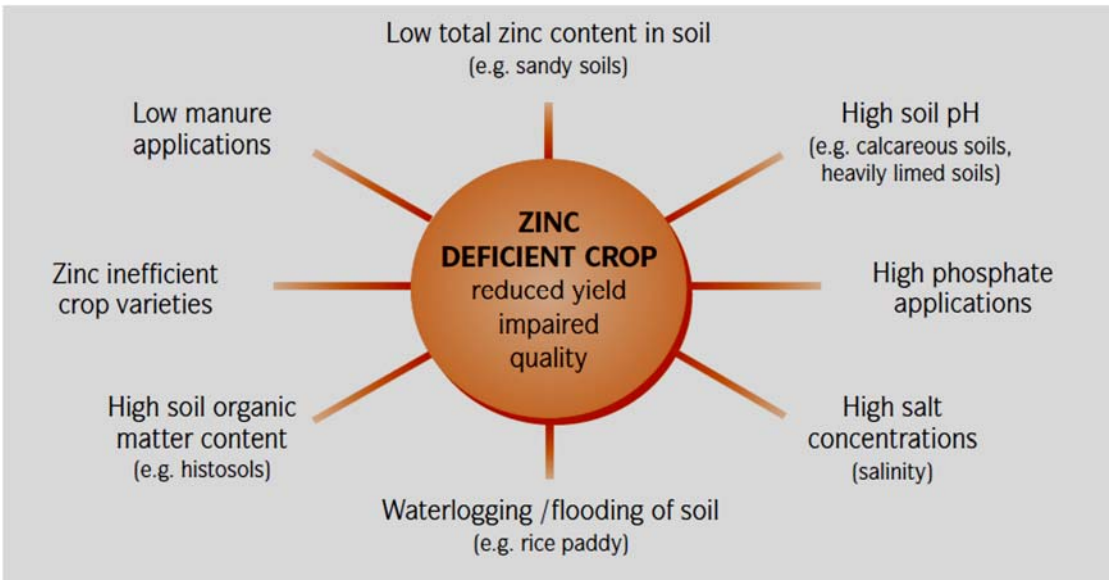


Fig. 1.3 Major reasons of Zinc deficiency in crops (Alloway, 2008)

phytic acid (PA) which reduces the mineral bioavailability (Pahlvan-Red and Pessarakli, 2009). The lesser bioavailability of soil zinc directly affects grain zinc concentration and human health.

1.5. Strategies to overcome the Zinc deficiency

To address the problem of Zn deficiency, micronutrients biofortification of grain crops is gaining increased interest in developing countries (Cakmak, 2008; Zhao and McGrath, 2009; Bouis and Welch, 2010). Several approaches have been projected and practiced for fortification of cereals (Bouis, 2003; Pfeiffer and McClafferty, 2007). Enhancing Zn concentration of cereal grains has been recognized as an approach of tackling humane Zn deficiency (Pahlvan-Red and Pessarakli, 2009). Plant scientists are formulating different methodologies to tackle the Zn deficiencies in crops through fertilizers applications and/or by means of plant breeding strategies to augment the absorption and/or bioavailability of Zn in grain crops (Cakmak, 2008; White and Broadley, 2009). Various dietary factors e.g. organic acids (citrate), amino acids (histidine and methionine), and chelators such as EDTA seem to support the bioavailability of zinc whereas fibers and some minerals such as copper, iron, and calcium may decrease it in some situations (Lonnerdal, 2000). Recent studies have also demonstrated that enhanced Zn bioavailability reduces the phosphorus and the phytic acid concentration in grain (Cakmak *et al.,* 2010). Absorption of Zn is improved by citric acid, malic acid, lactic acid and ascorbic acid. EDTA can assist to solubilize Zn from more insoluble phytate-Zn compound, forming Zn-EDTA. Crop fortification is the best approach and it is aimed to increase the average Zn contents of wheat from 25 ppm in Pakistan (HarvestPlus, 2012). It involves two strategies; genetic biofortification and agronomic biofortification.

1.5.1. Genetic biofortification

Genetic biofortification comprises the developing varieties with increase Zn content of grain through conventional breeding and genetic engineering.

1.5.1.1. Breeding Practices

Altering the genetics of plants with the intention to produce desired characteristics is known as plant breeding. Breeding and biotechnology are the most important tactics of the plant biofortification.

Genetic strategies are powerful approaches for altering the nutrient balance in the food crop. In the earlier period, agronomists and policy makers focused on yields only without considering the nutritional worth of the crops, thus generating the mineral malnutrition in humans (Khoshgoftarmanesh *et al.,* 2009). It has been reported that increase in yield of crops is found to associate for reduction in micronutrients. The zinc concentration is low in the edible tissues of higher yielding varieties as compared to the low yielding varieties (Fig. 1.4 and Fig. 1.5) (Monasterio and Graham, 2000; White and Broadley, 2009; Zhao and McGrath, 2009). Therefore it is fundamental to believe whether any enhancement in tissue zinc concentration is just the result of slower growth or low yields (White and Broadly, 2011).

Despite the fact that the improvements achieved in development of novel genotypes consisting of high zinc are admirable (Pfeiffer and McClafferty, 2007; Bouis and Welch, 2010; Cakmak *et al.,* 2010), but there exist some issues with this strategy. Similar to Zn, toxic metals like Cadmium can be translocated in the same pathway (Intawongse and Dean, 2006), and has a bioavailability that is much greater than that of other heavy metals (Reeves and Chaney, 2008). Moreover many ZIP family proteins (metal transporter in plants) can transport Cadmium (Yang *et al.,* 2009; Assunc *et al.,* 2010), which makes it difficult to ignore the risk in breeding programs.

The important issue associated with these breeding strategies is the instability of newly incorporated Zn trait in different genotypes and relatively limited genotypic variation for grain Zn concentration among wheat cultivars of cereals (Welch and Graham, 2004).

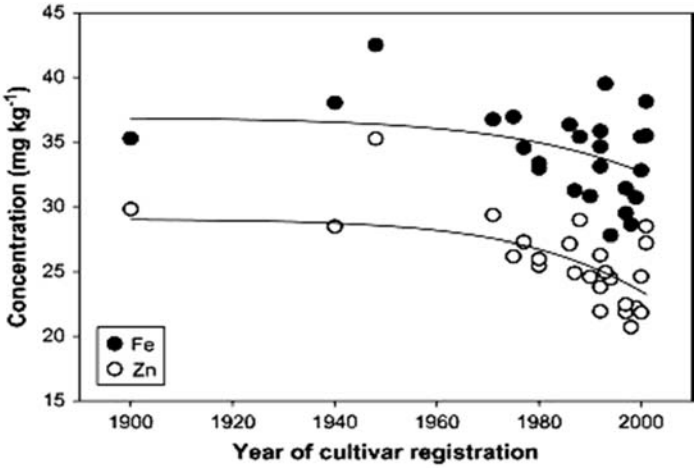


Fig. 1.4 Zn concentrations in wheat cultivars (Monasterio and Graham, 2000)

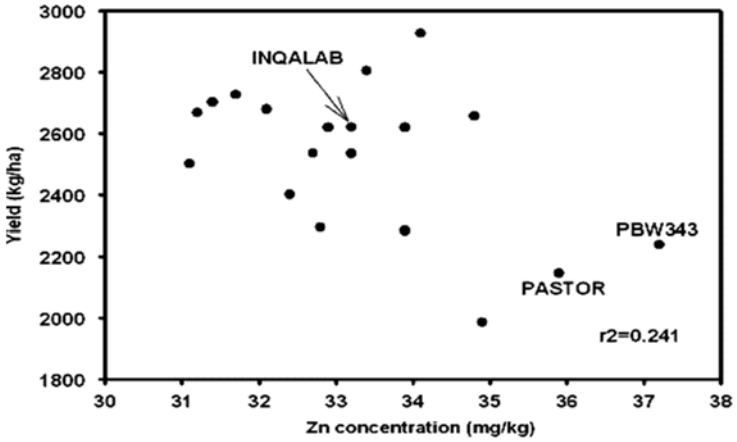


Fig. 1.5 Relationship between mean Zn concentration and mean grain yield in Pakistan (Trethowan, 2007).

It has been proved that Zn translocation in the wheat grain was highly influenced by the genotype, climate, and their interactions (Gomez-Becerra *et al.,* 2010; Zhang *et al.,* 2010).

Secondly, it is time consuming approach and takes several years to develop a biofortified variety (Cakmak, 2008) and further breeding program is also constrained by high cost and complexity of laboratory analysis (Monasterio *et al.,* 2007).

1.5.1.2. Transgenic approach

Transgenic approach is also contributing in developing the biofortified crops. Numerous transgenic food crops have been produced with the better zinc concentrations in the edible parts than conventional cultivars. Studies have shown that constitutive expression of transcription factors *bZIP19* and *bZIP23* could be used to enhance the Zinc accumulation in the edible parts of food crop plants (Assunc *et al.,* 2010). Different transport proteins of plasma membrane are the targets for the manipulations of zinc concentrations in the different portions of plants. These transport proteins make possible the uptake and sequestration of zinc in the vacuole together with enzymes concerned in the synthesis of substances that bind Zn^{2+} in the rhizosphere. Over-expressing genes encoding a transport protein of root will specifically increase the uptake of Zn^{2+} in the root portion (Gustin *et al.,* 2009). Wheat grain zinc concentration can be raised by the over-expression of NAC-transcription factor (NAM-B1) which is responsible to increase the remobilization of mineral elements from leaves to the developing grain and the senescence (Uauy *et al.,* 2006).

1.5.2. Agronomic practices

Different authors have evaluated agronomic strategies in the perspectives of both global health and sustainable economic progress to increase the concentrations of Zinc in edible parts of major crops. Many studies have described profit for edible crop production and human health by agronomic zinc biofortification of grain crops (Cakmak, 2008; White and Broadley, 2009). Zinc Fertilization of food crops signifies a short-term remedy of ensuring Zn translocation (Cakmak, 2008). Different genotypes have different capability

for the zinc accumulation. Zn is transportable and different fertilizers such as zinc sulphate ($ZnSO_4$) can enhance the yield of grain crops in zinc deficient soils, and can raise zinc concentration in the grain (White and Broadley, 2005). Combined application of zinc through soil as well as through foliar considerably enhanced the concentration of zinc in wheat grain (Cakmak, 2008; Zhang *et al.,* 2012; Zhao *et al.,* 2011). Zn fertilization also diminishes the anti nutrient concentration in grain and decrease phytic acid (PA) to Zn molar ratio, which is generally expressed as an indicator of Zn bioavailability in diets (Cakmak *et al.,* 2010). The water soluble Zn is low in soil solution and even in Zn-contaminated soils (Knight *et al.,* 1997). Chelate-mediated bioavailability involves the utilization of synthetic chelators, e.g. ethylene diamine tetraacetate (EDTA) (Sahi *et al.,* 2002; Piechalak *et al.,* 2003).

In nutrient-deficient ecosystems the application of Nitrogen-Phosphorous-Potassium (NPK) fertilizers is necessary for obtaining the high yield of field crops. These three macronutrients simultaneously augment root growth and result in an elevated transport of micronutrients from the soil to the plant. Conversely availability of zinc applied to the soil also depends on pH of soil e.g. NH^+_4 causes acidification of the rhizosphere which increases transfer of Zn from the soil to the plant, while NO^-_3 causes more alkalinity to the soil, dropping this transfer rate. If the efficient absorption of minerals occurs it can raise mineral levels in leaves but not essentially in fruits or seeds, for the reason that the relative efficiency of mineral transfer differs depending on the different parts of plant (Hartikainen, 2005). Application of Zn- containing fertilizers seem to be a quick and easy solution to the Zn deficiency problem, but resource-poor farmers especially in the developing countries, cannot afford application of micronutrient fertilizers. On the other hand it is reported that several synthetic chelators are costly and cause a hazard to the soil quality and groundwater (Kos and Lestan, 2003). Plant growth can be restricted and also influence the other soil organisms if large amount of metal is applied to soils. Abandoned use of chemical inputs in cultivation has escalated the expenses of production, damaged the soil, water and biological resources globally. There is also a dire need to improve Zn application methods in provisions of form, dose and effective time

for the application of Zn fertilizers. The Table 1.2 shows the comparative prospective of diverse strategies to assuage the micronutrients deficiency in cereals.

1.6. Plant Growth Promoting Rhizobacteria (PGPR) as Zn mobilizers

Plant Growth Promoting Rhizobacteria (PGPR) is one of the key factors that have important function in sustainable agriculture. PGPR are a diverse group of bacteria that can be found in the rhizosphere on root surfaces as well as in association with roots (Ahmad *et al.,* 2008; Maheshwari *et al.,* 2012). These bacteria move around from the bulk soil to the living plant rhizosphere and antagonistically colonize the rhizosphere and roots of plants (Kloepper and Schroth, 1978; Hafeez *et al.,* 2001; Yasmin *et al.,* 2004). Soil bacteria which are important for plant growth are termed as plant growth promoting rhizobacteria (PGPR) (Hafeez *et al.,* 2001; Yasmin *et al.,* 2004; Hayat *et al.,* 2010). PGPR can be alienated into two groups according to their relationship with the plants: symbiotic bacteria and free-living rhizobacteria (Khan, 2005; Maheshwari *et al.,* 2012). These are comprised of naturally occurring beneficial microorganisms in soil that make available nutrients to plants through several mechanisms by fixing atmospheric nitrogen, solubilize the nutrients fixed in soil and by producing phytohormones (Hafeez *et al.,* 2005; Jilani *et al.,* 2007; Jacob *et al.,* 2008; Yao *et al.,* 2008; Siddiqui *et al.,* 2008; Hafeez, 2009; Maheshwari *et al.,* 2012).

In addition to phosphate mobilization they are responsible to play key role in carrying out the bioavailability of soil phosphorous, potassium, iron, zinc and silicate to plant roots (Tariq *et al.,* 2007; Ahmad, 2007; Abaid-Ullah *et al.,* 2011; Saravanan *et al.,* 2011; Hafeez and Hassan, 2012).

Table 1.2 Comparative look of the strategies used for the improvement of Zn deficiency in plants

Strategies	Merits	Demerits	References
Chemical Fertilizations	• Short time solution • Foliar Zinc application is also effective • Readily diminish the phytic acid concentration in the grains	• Expensive • Extremely reliant upon crop & cultivar • Not promising to target edible parts • Not eco-friendly	Cakmak, 2008; 2009 Galloway *et al.,* 2008 Smith *et al.,* 2008
Conventional Breeding	• Exploits inherent properties of crops • Feasible to improve the Zinc deficiency • Increase micronutrients density in the edible parts of plants	• Depends on existing trait diversity of gene pool • Long term strategy • Traits might need to be introgressed from wild relatives • Possible intellectual property restraint	Raboy, 2002 Bouis and Welch, 2010 Cakmak *et al.,* 2010 Pfeiffer and McClafferty, 2007
Transgenic Techniques	• Rapid • Independent of gene pool • Targeted expression in edible parts • Applicable	• Similar to Zinc translocation many ZIP family proteins can transport toxic metals (e.g. Cd) • Regulatory landscape • Socio-economic and	Perfus-Barbeoch, 2002 Yang *et al.,* 2009 Assunc *et al.,* 2010

	directly to elite cultivars	political issues concerning with transgenic plants • Possible intellectual property restraint	Johnson *et al.,* 2011 Lee *et al.,* 2011 Chowdhury *et al.,* 2010
Biofertilizer Application	• Economical • Eco-friendly • Increase macro and micro-nutrients uptake (P, Fe, Zn, Si …) • Natural • Helpful against pathogenic micro-organisms • valuable for bioremediation • Significantly increases the yield of crops	• Limited shelf life • Slow action • Affected by environment • More translocation of Zn to grain/edible parts	Hafeez *et al.,* 2002; 2006 Ahmed *et al.,* 2011 Bahrani *et al.,* 2010 Badr *et al.,* 2009 Metin *et al.,* 2010 Kaewchai *et al.,* 2005 Tariq *et al.,* 2007

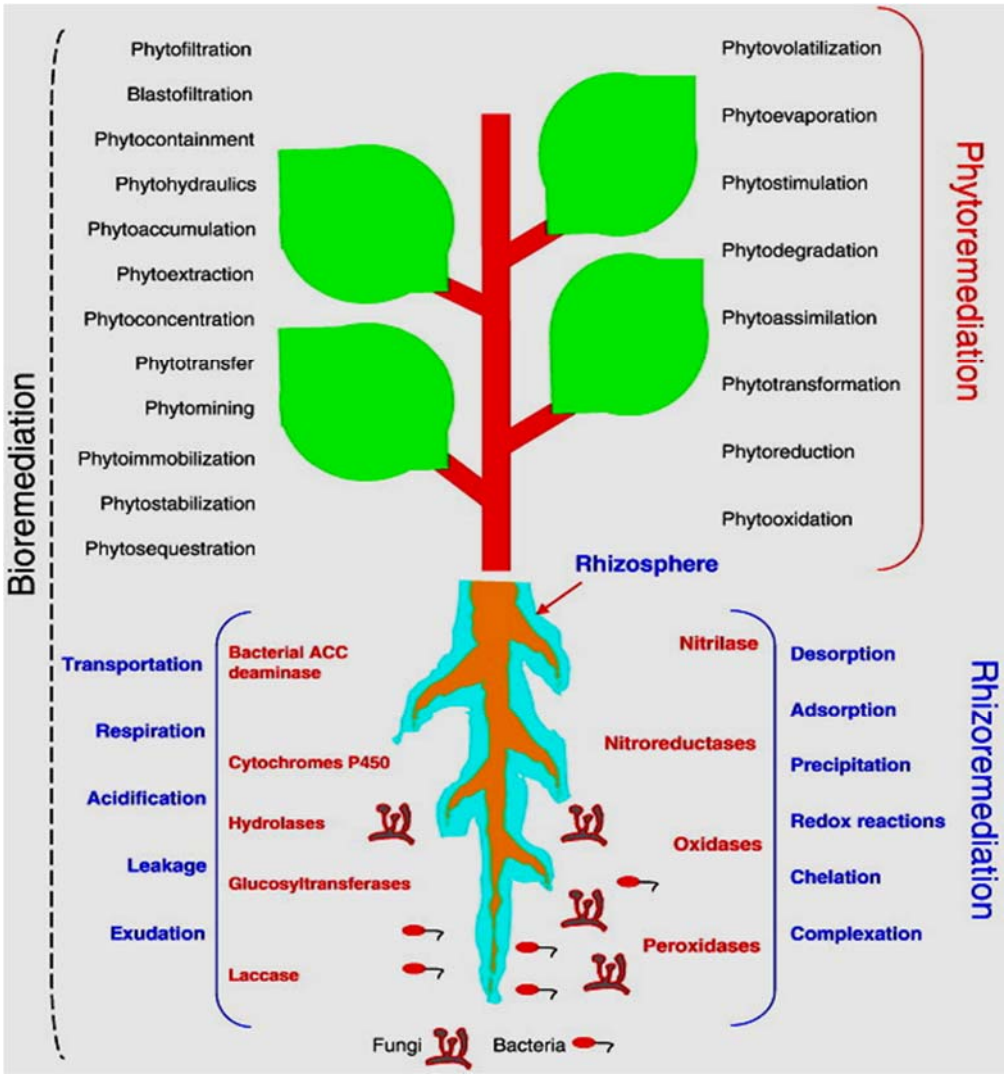


Figure 1.6 An overview of the microbes-soil-plant interaction (Ma *et al.*, 2011)

Many studies have revealed that inoculations of potent strains of Zn mobilizer rhizobacteria increased the yield of field crops such as rice, wheat, barley and maize. Tariq *et al.,* (2007) has described the effect of Zn mobilizing PGPR which significantly alleviated the deficiency symptoms of Zn and regularly increased the total biomass (23%), grain yield (65%) and harvest index in addition to Zn concentration in the grain of rice. Furthermore inoculation of Zn mobilizing PGPR had a notably positive impact on root weight (74%), root length (54%), root area (75%), root volume (62%), shoot weight (23%), panicle emergence index (96%) and exhibited the maximum Zn mobilization efficiency as compared the un-inoculated control. Besides it was also confirmed that PGPR strains can efficiently solubilize the Zn in liquid culture which was accessible for rice plant. Interestingly, the yield data has indicated that the PGPR contributed larger storage of assimilates in rice grains (Tariq *et al.,* 2007). Ahmad (2007) screened the best Zn mobilizing strain, out of fifty strains on the basis of clear zone formation by plate assay isolated from maize rhizosphere. Similar work accomplished by Yasmin (2011), determined the Zn mobilizing ability of *Pseudomonas* sp Z5 isolated from rhizosphere of rice plants.

Endophytes bacteria have received increasing attention as bio-fertilizers and/or bio-pesticides due to close contact with plant root. These microbes could not only ensure the availability of nutrients to plants but also improve nutrient use effectiveness (Khalid *et al.,* 2009). Endophytic bacteria may be even more imperative than rhizosphere bacteria in future in promoting crop yield and quality, because they break away from competition with rhizobacteria and get a more close contact with plant tissues. Different mechanisms has been proposed by which endophytic bacteria stimulate plant growth such as biological nitrogen fixation, environmental stress relief, production of phytohormones, synergism with other plant-microbe interactions, discretion of plant ethylene synthesis in addition to increasing accessibility of macro and micronutrients like phosphorus, iron, Zinc and other essential nutrients, and growth promotion by volatile compounds (Compant *et al.,* 2010; Mitter *et al.,* 2013).

Viable applications of PGPR are being tested and are repeatedly promising; however, a good understanding of the microbial interactions that result in plant growth increase will significantly raise the success rate of field applications (Burr *et al.,* 1984: Saharan and Nehra, 2011; Saravanan *et al.,* 2011).

1.6.1. Mechanism of Zinc mobilization by PGPR

Approximately 50% of the cereal-cultivated soils are Zn deficient globally following diminished crop yields and grain nutritional value (Welch, 2013) and about 90 % of it is present in insoluble form and consequently inaccessible for plant uptake (Barber, 1995) while ensuring their availability to plant is a crucial characteristic of PGPR. Mobilization of metal salts is an imperative feature of PGPR, as mobilized compound are accessible for the plants. Bacterial comparative and functional genomics research has opened new avenues for exploring these underlying mechanisms at biochemical and molecular level. Various studies have been conducted to explore the mechanisms of Zn mobilizing PGPR. Generally PGPR solubilize the nutrients (Essential trace elements) through acidification, chelation, exchange reactions, and release of organic acids (Chung *et al.,* 2005; Hafeez *et al.,* 2005). Soil-plant-microbe interactions are complex and there are lots of ways in which the outcome can influence the crop vigor and yield (Hafeez *et al.,* 2002; Pieterse *et al.,* 2003). It is found that mobilization mechanism of Zn and iron possibly involve the siderophore production (Burd *et al.,* 2000; Tariq *et al.,* 2007; Wani *et al.,* 2007; Saravanan *et al.,* 2011), gluconate or the derivatives of gluconic acids e.g. 2-ketogluconic acid (Fasim *et al.,* 2002), 5-ketogluconic acid (Saravanan *et al.,* 2007) and various other organic acids by PGPR (Di Simine *et al.,* 1998; Tariq *et al.,* 2007; Wani *et al.,* 2007) as described in Table 1.3.

The studied mechanism of Zn solubilization by gram-negative bacteria suggested that secretion of gluconic acids (GA) produced by bacteria, are involve through direct oxidation of glucose via the membrane bound quinoprotien glucose dehydrogenase (GDH) (Patel *et al.,* 2008). GDH requires pyrroloquinoline quinine (PQQ) as a cofactor.

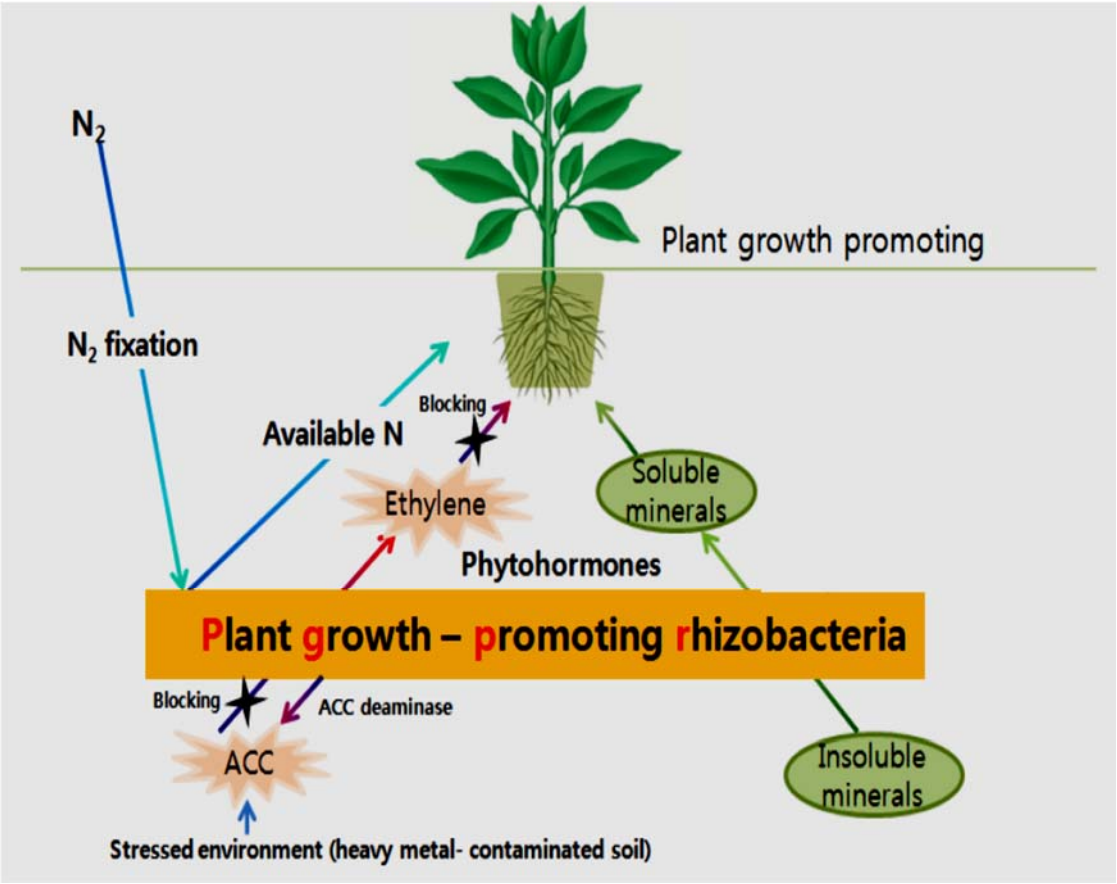


Fig. 1.7 Soil PGPR assisted uptake of macro and micronutrients for plant.
(Environmental Management Research Centre, www.envitop.co.kr)

The genes accountable for biosynthesis of PQQ involves an operon of 11 genes, *pqqA, B, C, D, E, F, H, I, J, K*, and *pqqM* while all these genes have been cloned and sequenced in a number of bacterial genera, including *Pseudomonas, Methylobacterium, Acinetobacter, Klebsiella, Enterobacter* and *Rahnella* (Schnider *et al.,* 1995; Han *et al.,* 2008; Guo *et al.,* 2009). *PqqC* is a cofactor that catalyzes the final step of PQQ biosynthesis and the reaction catalyzed by *PqqC* has been characterized as an oxidase which does not involve further cofactor (Magnusson *et al.,* 2004).

1.6.2. Characterization of Zn solubilizing PGPR

PGPR can be identified by using various PCR based molecular markers and phylogenetically categorized and re categorized into new genera. Targeting 16S rRNA (Olsen and Woese, 1993), *16S-23S rRNA*, *gyrA*, *gyrB* gene has been designed and appraised as a very sensitive and precise assay e.g. identification of *Pseudomonas* sp. via PCR-based methods, various markers such as *16S rRNA* (Relman *et al.,* 1992) and *gyrB* (Qin *et al.,* 2003) have been reported when tested with *P. aeruginosa* strains and species of pseudomonads closely related to *P. aeruginosa* (Lavenir *et al.,* 2007). Furthermore, analysis of *PqqC* gene exposed that it is highly conserved in many gram negative bacteria hence; it can be used as a molecular marker to identify the unknown gram negative bacteria (Meyer *et al.*, 2011).

1.6.3. Screening of Zinc mobilizing PGPR

Zinc mobilizing PGPR can be screened by plate assay and their relative Zn mobilizing capacity can be quantified through Atomic Absorption Spectrophotometer (AAS) analysis. Screening of Zn mobilizing PGPR can be made qualitatively by agar plate assay. The mobilizing potential can be visualized by the zone formation on the modified Bunt and Rovira (1955) media containing insoluble Zn ores (ZnO, $Zn_3(PO_4)_2$, ZnS, $ZnCO_3$ …). Quantification of Zn mobilizing ability of PGPR is also obtainable by LB-media amended with different insoluble Zn ores (ZnO, $Zn_3(PO_4)_2$, ZnS, $ZnCO_3$ …) through AAS (Hafeez and Hassan, 2012; Abaid-Ullah *et al.,* 2010).

Table 1.3 Zn mobilizing Plant Growth Promoting Rhizobacteria (PGPR) producing various compounds helpful for plant growth.

No	Zinc Mobilizing PGPR	Plant/Source	PGPR Traits	References
1	*Pseudomonas fluorescens*	Forest soil	Zn, P solubilizer, citric acid and gluconic acid production	Di Simine *et al.,* 1998
2	*Pseudomonas aeruginosa*	Air environment of a tannery	Zn solubilizer, low gluconic acid but higher amount of 2-ketogluconic acid production	Fasim *et al.,* 2002
3	*Gluconacetobacter diazotrophicus*	*Saccharum officinarum*	Zn solubilizer, 5-ketogluconic acid production	Saravanan *et al.,* 2007a,b
4	*Rhizobia* spp.	*Pisum sativum*	Zn solubilizer/tolerant, phytohormones production	Wani *et al.,* 2008
5	*Pseudomonas sp.* PsM6, *P.jessenii* PjM15	*Ricinus Communis*	Zn, Ni and Cu mobilizers, ACC De aminase, siderophore and IAA production, biosorption	Rajkumar and Freitas, 2008
6	*Flavobacterium* sp.	*Orychophragmus violaceus*/Sewage sludge	Zn mobilization and accumulation,	He *et al.,* 2010
7	*Pseudomonas* sp. Z5	*Oryza sativa*	Zn mobilization	Yasmin, 2011
8	*Serratia* sp.	*Triticum aestivum*	Zn and P solubilization	Abaid-Ullah *et al.,* 2011

An effective PGPR, bacteria must be able to colonize roots in the presence of the indigenous micro flora because bacteria need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects. Conversely, low population of an efficient strain in rhizosphere of plant may cause inconsistent performance *in vitro* and *in vivo* (Smyth *et al.,* 2011). Endophytic bacteria might in future be even further significant than rhizosphere bacteria in promoting plant growth and nutrition, as they break out competition with rhizosphere microorganisms and attain a more close contact with plant tissues.

1.6.4. Formulation and delivery of Zn mobilizing PGPR

Biofertilizer formulation is an industrial art of converting a promising laboratory proven bacterium in to a commercial field product (Bashan, 1998; Hafeez *et al*., 2006). Formulations of PGPR are composed of the active component i.e. rhizobacteria which are carried by an inert stuff used to deliver the active ingredients to the target (Hafeez and Hassan, 2012). These microbial inoculums not only defeat loss of viability for the period of storage, but also have longer shelf life and strength over a range of temperature -5 - 30°C as in the marketing supply change (Hafeez, 2009; Bashan, 1998).

The PGPR formulated in solid carrier materials and commercialized with different trade names throughout the world. The Biofertilizers with trade name Azotobakterin^{TM} consisting of *Azotobacter chroococcum,* a Phosphobacterin^{TM} consisting of *Bacillus megaterium* var. *phosphaticum* are being used as seed treatment and soil drenching. The Liquid based formulations of biofertilizers such as Nitragin^{TM} containing *Rhizobia* cells and Nitro^{TM} containing *Azotobacter* cells are also being used as seed treatment in USA since 1885. Solid formulation based biofertilizer Gmax^{TM} and Nitromax^{TM} consisting of *Azotobacter* cells and *Azospiriilum* cells respectively are being commercialized. These carrier materials are used as seed treatment as well as for N, P fertilizer and IAA production.

In Pakistan, mixture of potent PGPR has been formulated in sugarcane filter-cake and marketed with the trade name of *Biopower* as Nitrogen, phosphatic fertilizer and growth

stimulator for different field crops by National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad-Pakistan in collaboration with public sector (Hafeez *et al.,* 1989; Hafeez *et al.,* 2002; Hafeez, 2009).

Certain other phosphate-solubilizing PGPR has been formulated in humic acid carrier by the COMSATS Institute of Information Technology, Islamabad-Pakistan and being marketed with the trade name of *Humiphos* and *Biophos* by the private sector ; AURIGA Chemical Enterprises, Lahore, Pakistan (Khan *et al.,* 2010; Hafeez and Hassan, 2011; Hafeez and Hassan, 2012).

Zn mobilizing PGPR inoculants exploited as biofertilizers can accelerate rehabilitation of degraded land and improvement of soil fertility, enhancing survival and growth of plants, increase grain yield, lowering malnutrition rates and reduce dependence on chemical fertilizers (Hafeez *et al.,* 2001). Moreover biofertilizers are economical, eco-friendly and its use can augment crop productivity (Hafeez *et al.,* 2002). Hence biofertilizer formulation for Zn deficiency in cereal crops may represent a natural, environment friendly and inexpensive alternate to replace already existing chemical fertilizer hazard. Using Zn mobilizing rhizobacteria together with all other beneficial traits will be a key advantage for the formulation of effective biofertilizers. It will be their binary beneficial nutritional effect resulting together from phosphate solubilization, N_2-fixation (Gull *et al.,* 2004; Zaidi and Khan, 2006) and their well-documented synergistic interactions with Arbuscular Mycorrhizal fungi (Ordookhani *et al.,* 2010). The exploitation of PGPR inoculants as biofertilizers and/or antagonists of phytopathogens offer a promising alternative to chemical fertilizers and pesticides. It was practically determined that the application of PGPR (*Pseudomonas fluorescens* and *Paenibacillus polymyxa*) to rice plant enhanced the induced systematic resistance in a pot experiment (Naureen *et al.,* 2009; Naureen and Hafeez, 2011; Umashankari and Sekar, 2011).

Current trends in agriculture are focused on the reduction of the pesticides and inorganic fertilizers utilization, forcing the study for alternative ways to progress a more sustainable agriculture (Kloeper *et al.,* 1989; Hafeez and Gull, 2009; Mahdi *et al.,* 2010). The

outcome of studies have shown that a *Bacillus* sp. (Zn mobilizing bacteria) can be exploited as bio-fertilizer for zinc or in soils where native zinc is elevated or in conjunction with insoluble cheaper zinc compounds like zinc oxide (ZnO), zinc carbonate ($ZnCO_3$) and zinc sulphide (ZnS) as an alternative of expensive zinc sulphate (Mahdi *et al.,* 2010).

Subsequent studies of PGPR have shown that several best strains are multifunctional, and secondly, PGPR traits are frequently disseminated among various different species and genera of microorganisms, many of which are native members of the soil microbial community. Generally individual strains differ significantly in performance. Native PGPR can affect the performance of introduced PGPR inoculants comparatively. Hence lack of information about the background PGPR function; it is not easy to predict the response to soil inoculations. A lot of PGPR frequently solubilize nutrients (phosphorus, iron, zinc, silicate…); produce auxins which stimulate root development, produce siderophore and antibiotics that may help in inhibition of root infection. During environmental stress the plants produces ethylene or hydrogen cyanide and Reactive Oxygen Species (ROS) that may be degraded by substances (enzymes) produced by these PGPR.

1.7. Purpose of the present study

Use of Plant Growth Promoting Rhizobacteria (PGPR) is becoming an effective approach to substitute synthetic fertilizer, pesticides, and supplements. Many nutrients mobilizing bacteria belongs to the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Serratia*, *Pantoea*, *Rhizobium* and *Flavobacterium* (Buch *et al.,* 2008). PGPR mobilize the nutrients by various mechanisms such as acidification, chelation, exchange reactions, and release of organic acids (Chung *et al.,* 2005; Hafeez *et al.,* 2005). The PGPR inoculants as biofertilizers or antagonists of phyto-pathogens are believed as a promising alternative to replace chemical fertilizers and pesticides. However, the inoculants should be applied according to their adaptation to a specific plant genotype and soil in an ecological unit.

The root colonization of native PGPR might show better effects on crop plants. Their effectiveness has been found to be dependent upon population density (Smyth *et al.,* 2011). Moreover, the endophytic bacteria might appear more effective for plant growth and nutrition as compare to rhizospheric bacteria due to more close contact with plant tissues. The dual beneficial effects of PGPR on nutrient mobilization and biofortification of crop plants is also well documented (Hafeez *et al.,* 2006; Hayat *et al.,* 2010; Metin *et al.,* 2010; Rana *et al.,* 2012).

Similarly native PGPR might respond differently with different genotypes of a crop species. To the best of our knowledge, very little information is available on this aspect. We designed a study to use locally isolated Zn-solubilizing rhizobacteria as model strains to see their effects on wheat yield and Zn contents under lab and field conditions. The Zn mobilizing rhizobacterial strains were inoculated on four commercial wheat varieties to observe colonization ability and Zn mobilization in wheat grain. The overall objective of the proposed study was to gain in depth knowledge of response of Zn-solubilizers towards growth, yield and quality of various wheat genotypes under field conditions.

1.8 Research objectives

- **Selection of the Zn solubilizing PGPR and elucidation of mechanism of Zn solubilization**
  - Qualitative and quantitative screening of PGPR collected from PCMC and soils.
  - Morphological and biochemical characterization of Zn solubilizing PGPR.
  - Characterization of Zn solubilizing rhizobacteria for stress tolerance.
  - Characterization of Zn solubilizing rhizobacteria for plant growth promotion.
  - Characterization of Zn solubilization PGPR as biocontrol agent.

❖ **Field evaluation of Zn solubilizing PGPR on wheat**

- Effect of Zn solubilizing PGPR on qualitative and quantitative parameters of wheat under rain fed and irrigated conditions.
- Rhizospheric and endophytic root colonization with wheat under rain fed and irrigated conditions.
- ZSB mediated Zn translocation in various wheat genotypes under rain fed and irrigated conditions.

❖ **Molecular characterization of ZSB**

- IGS (*16-23S rRNA gene*) region amplification and RFLP analysis.
- Identification of Zn solubilizing PGPR by *16S rRNA*, *gyrB* and *gyrA* gene analysis.
- Characterization of Zn-mobilizing PGPR for nitrogen fixation and ACC deaminase activity by *nifH* and *acdS* gene amplification respectively.
- Cloning and sequencing of *PqqC* genes involved in the biosynthesis of PQQ.

Chapter 2

Materials and Methods

2 Materials and methods

2.1. PGPR source and culture conditions

In this study 198 bacterial strains were used. About 98 strains were obtained from Pakistan Collection of Microbial cell Culture (PCMC) previously collected from wheat fields (Table 2.1) while 100 bacteria were isolated from the wheat rhizosphere growing at different climatic conditions of Pakistan i.e. Chakwal (Table 2.2). The strains were grown and purified on LB agar (Appendix I). After sufficient growth, single colony of bacteria was transferred to 30 mL of LB broth in test tubes. Bacterial growth was obtained by incubating the culture tubes in shaking incubator at 120 rpm at 30 ± 2°C for 1-3 days. The sub-culturing was done in a sterilized laminar flow. The bacterial strains used in this study from PCMC and soils (Chakwal) are given in Table 2.1 and Table 2.2. Various characteristics of isolates were detected on specified medium.

2.1.1. Selection of Zinc (Zn) solubilizing rhizobacteria

The isolates were initially screened by inoculation into Bunt and Rovira agar medium (Bunt and Rovira, 1955) containing 0.1% of the insoluble zinc compounds zinc oxide (ZnO), zinc sulphide (ZnS) and zinc carbonate ($ZnCO_3$) incubated at 30ºC. The isolates showing Zn mobilizing activity efficiently were again inoculated into Bunt and Rovira agar medium (Bunt and Rovira, 1955; Ahmad, 2007) containing 0.1% of the different insoluble zinc compounds as mentioned above and incubated at 30ºC for various time intervals of 36 to 96 h. Appearance of halo zone around the colonies indicated their potential to solubilize Zn which was estimated by measuring the zone diameter at different time intervals. The recipe of the Bunt and Rovira medium is given in Appendix II.

Table 2.1 PGPR obtained from PCMC

Serial No.	Strain code	Agar plates			
		ZnO	**ZnS**	**$ZnCO_3$**	**Growth of PGPR**
1	WBPS-1	-	-	-	+
2	WBPS-2	-	-	-	-
3	WBPS-3	-	-	-	-
4	WBPS-4	-	-	-	-
5	WBPS-5	-	-	-	+
6	WBPS-6	-	-	-	+
7	WBPS-7	-	-	-	-
8	WBPS-8	-	-	-	-
9	WBPS-9	-	-	-	-
10	WBPS-10	-	-	-	+
11	5-1-A	-	-	-	+
12	Z-2-4	-	-	-	+
13	4-2-1-A	-	-	-	-
14	F-91	+	-	+	+
15	F-18	+	-	+	+
16	Xan	+	-	+	+
17	18A-1	+	-	+	+
18	SPR7	-	-	-	-
19	Z-2-7	-	-	-	-
20	BPS-12	-	-	-	-
21	SPR-4	-	-	-	-
22	BPS-10	+	-	-	+
23	BPR1	+	-	+	+
24	SPS-2	+	-	+	+
25	SPR-5	+	-	+	+
26	4-1-1.B	+	-	+	+
27	MZ-12	+	-	+	+
28	18A-1-1	+	-	+	+
29	17A-1	+	-	+	+
30	MS3Y	-	-	-	+
31	6-1-1-B	+	-	+	+
32	IJ03	-	-	-	-
33	IMG111	-	-	-	-

34	PF	-	-	-	-
35	BS	-	-	-	-
36	SE	-	-	-	-
37	1(2)	-	-	-	-
38	17	-	-	-	-
39	16	-	-	-	-
40	NH-5	-	-	-	-
41	CHAO	-	-	-	-
42	AP4	-	-	-	-
43	AF3	-	-	-	-
44	AR	-	-	-	-
45	51	-	-	-	-
46	MMP9A	-	-	-	-
47	LOX-P	-	-	-	-
48	98F	-	-	-	-
49	MMP4BR	-	-	-	-
50	MMP9AF	-	-	-	-
51	LOX-F	-	-	-	-
52	AP2	-	-	-	-
53	S3	-	-	-	-
54	S4	-	-	-	-
55	AF4	-	-	-	-
56	AP1	-	-	-	-
57	AP5	-	-	-	-
58	AP6	-	-	-	-
59	S2	-	-	-	-
60	29GP	-	-	-	-
61	25GP	-	-	-	-
62	R13	-	-	-	+
63	R2	-	-	+	+
64	R24	-	-	-	-
65	LB41	-	-	-	-
66	LB44	-	-	-	-
67	LB53	-	-	-	-
68	JA1	-	-	-	-
69	2.2.4	-	-	-	-
70	Xan	+	-	+	+
71	F11	+	-	+	+
72	WBR8	+	-	+	+

73	SPS2	+	-	+	+
74	BPR1	-	-	-	-
75	ME-42	-	-	-	-
76	ME-44	-	-	-	-
77	L1	-	-	-	-
78	L2	-	-	-	-
79	L3	-	-	-	-
80	L4	-	-	-	-
81	L5	-	-	-	-
82	L6	-	-	-	-
83	R21	-	-	-	-
84	R5	-	-	-	+
85	R7	-	-	-	-
86	L11	-	-	-	-
87	L21	-	-	-	-
88	G-10	-	-	-	-
89	G-12	-	-	-	-
90	NH-A1	-	-	-	+
91	NH-A5	-	-	-	-
92	BR-J1	-	-	-	-
93	BRJX	-	-	-	-
94	K1A	-	-	-	-
95	ZH3	-	-	-	-
96	ZH4	-	-	-	-
97	ZH9	-	-	-	-
98	PP3	-	-	-	-

Table 2.2 PGPR isolated from endosphere and rhizosphere of soil in BARI, Chakwal

Serial No.	Strain code	LB agar			
		ZnO	**ZnS**	**$ZnCO_3$**	**Growth of PGPR**
1	CH-1	-	-	-	-
2	CH-2	-	-	-	-
3	CH-3	-	-	-	-
4	CH-4	-	-	-	-
5	CH-5	-	-	-	-
6	CH-6	-	-	-	-
7	CH-7	-	-	-	-
8	CH-8	-	-	-	-
9	CH-9	-	-	-	-
10	CH-10	-	-	-	-
11	CH-11	-	-	-	+
12	CH-12	-	-	-	+
13	CH-13	-	-	-	-
14	CH-14	-	-	+	+
15	CH-15	-	-	+	+
16	CH-16	-	-	+	+
17	CH-17	-	-	+	+
18	CH-18	-	-	-	-
19	CH-19	-	-	-	-
20	CH-20	-	-	-	-
21	CH-21	-	-	-	-
22	CH-22	-	-	-	+
23	CH-23	-	-	+	+
24	CH-24	+	-	-	+
25	CH-25	+	-	-	+
26	CH-26	+	-	-	+
27	CH-27	-	-	-	+
28	CH-28	-	-	-	+
29	CH-29	-	-	-	+
30	CH-30	-	-	-	+
31	CH-31	-	-	-	+
32	CH-32	-	-	-	-
33	CH-33	-	-	-	-

34	CH-34	-	-	-	-
35	CH-35	-	-	-	-
36	CH-36	-	-	-	-
37	CH-37	-	-	-	-
38	CH-38	-	-	-	-
39	CH-39	-	-	-	-
40	CH-40	-	-	-	+
41	CH-41	-	-	+	+
42	CH-42	-	-	+	+
43	CH-43	-	-	+	+
44	CH-44	-	-	-	+
45	CH-45	-	-	-	+
46	CH-46	-	-	-	+
47	CH-47	-	-	-	+
48	CH-48	-	-	-	-
49	CH-49	-	-	-	-
50	CH-50	-	-	-	-
51	CH-51	-	-	-	-
52	CH-52	-	-	-	-
53	CH-53	-	-	-	-
54	CH-54	-	-	-	-
55	CH-55	-	-	-	+
56	CH-56	-	-	-	+
57	CH-57	-	-	-	+
58	CH-58	-	-	-	-
59	CH-59	-	-	-	-
60	CH-60	-	-	-	-
61	CH-61	-	-	-	-
62	CH-62	-	-	-	+
63	CH-63	-	-	+	+
64	CH-64	-	-	-	-
65	CH-65	-	-	-	-
66	CH-66	-	-	-	-
67	CH-67	-	-	-	-
68	CH-68	-	-	-	-
69	CH-69	-	-	-	-
70	CH-70	-	-	+	+
71	CH-71	-	-	+	+
72	CH-72	-	-	+	+

73	CH-73	-	-	+	+
74	CH-74	-	-	-	-
75	CH-75	-	-	-	-
76	CH-76	-	-	-	-
77	CH-77	-	-	-	-
78	CH-78	-	-	-	-
79	CH-79	-	-	-	-
80	CH-80	-	-	-	-
81	CH-81	-	-	-	-
82	CH-82	-	-	-	-
83	CH-83	-	-	-	-
84	CH-84	-	-	-	+
85	CH-85	-	-	-	-
86	CH-86	-	-	-	-
87	CH-87	-	-	-	-
88	CH-88	-	-	-	-
89	CH-89	-	-	-	-
90	CH-90	-	-	-	+
91	CH-91	-	-	-	-
92	CH-92	-	-	-	-
93	CH-93	-	-	-	-
94	CH-94	-	-	-	-
95	CH-95	-	-	-	-
96	CH-96	-	-	-	-
97	CH-97	-	-	-	-
98	CH-98	-	-	-	-
99	CH-99	-	-	-	+
100	CH-100	-	-	-	+

2.1.1.1 Quantification of Zinc (Zn) solubilizing ability

The Zn solubilizing ability of the strains was quantified by growing them in Bunt and Rovira broth at 30 ± 2°C for 48 h with shaking continuous at 160 rpm. The cell free supernatant was obtained by centrifuging the culture at 7000 rpm for 15 min followed by passing through a 0.22 µm filter and analyzed on Atomic Absorption Spectrophotometer (AA-6300, Shimadzu) at Auriga Group of Companies, Lahore. Amount of Zn solubilized by strains was quantified by comparing with the un-inoculated broth as control sample. Experiment was conducted in three replicates.

Bacterial strains on the basis of their Zn solubilizing ability were selected for further study. These Zn solubilizing PGPR were preserved in 20% glycerol and stocks were stored at -80°C for two to three years. Each PGPR was stocked in five replicates.

2.1.2 Morphological and biochemical characterization of ZSB

The Zn solubilizing strains were characterized for Gram's reaction, colony color and colony morphology by standard procedures (Vincent, 1970). Pure culture of each Zn solubilizing bacteria was observed on LB agar plates for colony morphology. A colony of individual bacterial strain was streaked on agar plates and incubated at 37°C for 24 hours. Growing colonies were visualized for their color, shape and texture under light microscope. Zn solubilizing PGPR were stained by using methods described by Vincent (1970) and reagents were prepared as given in Appendix III.

2.1.3 Characterization of ZSB for stress tolerance

2.1.3.1 Growth curve analysis

The growth kinetics of Zn solubilizing rhizobacteria was checked across various Zn compounds such as zinc chloride ($ZnCl_2$), zinc sulphate ($ZnSO_4$), zinc oxide (ZnO) and zinc nitrate ($ZnNO_3$). Tolerance of Zn solubilizing PGPR was also observed with various concentrations of ZnO. An automated 96 well plate microtitre plate reader was used for the growth curve analysis. In a two step experiment, the Tryptic soy broth (TSB) (Appendix IV) supplemented with different Zn ores, and LB broth various concentrations

of Zinc oxide 0.1, 0.2 and 0.3% were inoculated with overnight grown fresh culture of Zn solubilizing bacterial strains. The growth conditions comprises of 30°C with 10s of shaking for every 20 min and reading with 30 min intervals for 24 h.

2.1.3.2 Exopolysaccharide activity (EPS)

Exo-polysaccharide (EPS) activity of the strains was assessed qualitatively by growing them on Weaver mineral medium supplemented with glucose. The EPS production was observed visually as fluffy material on the plates after 48 h of incubation at 28 ± 2°C (Weaver *et al.,* 1975).

2.1.4 Characterization of ZSB for plant growth promotion

2.1.4.1 Phosphorus solubilization

Phosphorous mobilization ability of strains was tested on Pikovskaya agar medium (Pikovskaya 1948) and semi quantified by measuring the zone diameter. The recipe of Pikovskaya medium is given in Appendix V.

2.1.4.2 Detection of organic acids by HPLC

Identification and quantification of organic acids was achieved using high performance liquid chromatography (HPLC). For this, Bunt and Rovira broth amended with various zinc ores such as $ZnCO_3$, ZnO, $Zn_3(PO_4)_2$ and ZnS was inoculated with freshly grown PGPR. Pikoviskaya broth was also inoculated with PGPR and control was also maintained without PGPR inoculation. Supernantant was separated by centrifugation of tubes at 10000 rpm after 48 hr growth. Sample volume of 10-20 µL, filtered through 0.21 mm filter was injected and chromatograms recorded over a period of 10 min at a wavelength of 210 nm. The column was C18 Supelco, reverse phase. Elution was carried out isocratically (0.1M Na_2SO_4, pH 2.7, CH_3CN) at room temperature. Organic acids were identified by comparison with authentic standards of gluconic acid, acetic acid, citric acid, oxalic acid and lactic acid.

2.1.4.3 ACC-deamminase activity

Production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase was detected by observing the growth of strains on Brown & Dilworth (BD) minimal medium (Brown and Dilworth 1975) containing 0.7 g L^{-1} ACC as a sole nitrogen source. The BD petri plates containing 0.7 g L^{-1} NH_4Cl were kept as positive control, and BD plates containing no nitrogen source were used as negative control.

2.1.4.4 Auxin production

Auxin Production was assessed both in the presence and absence of L-tryptophan as described by (Sarwar *et al.,* 1992; Yasmin *et al.,* 2004). IAA was determined by bacterial isolates grown in LB broth (28±2°C, 180 rpm) supplemented with 1 % L-TRP. The cells were harvested by centrifugation (10,000^{x}g for 10 min) and three milliliters of the supernatants were mixed with 2 mL Salkowski's reagent. (12 g L^{-1} $FeCl_3$ in 429 mL of H_2SO_4) the mixture was incubated at room temperature for 30 min for color development and absorbance at OD_{535} nm was measured using microplate reader. Auxin quantity produced by bacterial cells was determined using standard curve for IAA prepared from serial dilutions of 10 - 100 µg mL^{-1}.

2.1.5 Characterization of ZSB as biocontrol

2.1.5.1 Antagonism

The antagonistic activity of Zn solubilizing rhizobacteria was tested on potato dextrose agar (PDA) and yeast malt agar (YMA) against phytopathogenic fungi; *Fusarium caulimons, F. graminarium, F. oxysporum* and *F. solani* obtained from Austrian Institute of Technology (AIT-Vienna) strain collection by the dual culture technique (Tambong and Höfte, 2001). A 5 mm disk of each fungus was positioned in the midpoint of petri plates and 10 µL of bacterial culture grown for overnight in tryptone soy agar (TSA) was speckled 2 cm away from the fungus. Petri dishes were incubated at 27 ± 2 °C for 12 days and inhibition of fungal mycelium was observed. The antagonistic activity of the strains was scored according to Perneel *et al.* (2007) and Hassan *et al.* (2010). Growth of

fungal mycelium over bacterial colony = 0, growth of fungal mycelium near edge of bacterial colony = 1, Growth of fungal mycelium away from bacterial colony = 2.

2.1.5.2 Siderophore production

Siderophore production was qualitatively assessed by plate assay described by Schwyn and Neilands (1987). The bacterial strains were inoculated to the plates containing Chrome azurol S (CAS) agar and incubated at 28 ± 2 °C for 4-5 days. A change in blue to orange colour depicted production of siderophores.

2.1.5.3 Resistance against different antibiotics

Rhizobacteria were also tested for inherent resistance to various antibiotics namely ampicillin, amikacin, tetracycline and kanamycin (Alvarez and Brodbelt, 1995). Each bacterial strain was spread on LB agar plate and disc of each antibiotic (Oxoid) with a concentration of 30 µg / mL except streptomycin with a concentration of 10 µg / mL was placed on the inoculated plate. The plate was incubated at 28 ± 2°C for 24 h. The bacterial strains were characterized as resistant or non-resistant by measuring the zone of inhibition and comparing with the manufacturer's recommendations.

2.1.5.4 Surfactants production

Zn solubilizing PGPR were tested for their ability to produce surfactants. For this, hemolytic assay was carried out by inoculating 1 µL of freshly grown bacterial culture on Soy Agar with 5% Sheep Blood (BBL™ Trypticase™; TSA II) and incubated at 30-37°C. After 12-72 h incubation, blood agar plates were observed and the bacteria with halo zone were marked as surfactants producing PGPR (Monteiro, 2005).

2.2 Molecular characterization of ZSB

2.2.1 Microbial DNA extraction

2.2.1.1 DNA isolation using kit

The genomic DNA was extracted using the FastDNA® Spin kit (MP Biomedicals, Solon, OH, USA) as described by the manufacturer. Extracted DNA was stored at -80 °C.

2.2.1.2 DNA isolation using boiling method

Bacterial cells were suspended in eppendorf tube containing TE buffer and exposed to heat shock at 100°C for 20 min. Then, the tubes were placed in ice to give cold shock for 2 min. Cells were separated by centrifugation at 8000 rpm for 5 min at 4°C. A 100 µL of supernatant was collected in the fresh eppendorf tubes (Cao *et al.,* 2003).

2.2.2 Quantification of DNA

Isolated DNA was quantified and estimated by the spectrophotometer and yield gel electrophoresis. Methodology for both techniques is given below.

DNA was quantified using Nanodrop spectrophotometer (ND-1000, USA). For this, sensor of Nanodrop was wiped with the kimwipe. After this, 2 µL of elution buffer was pipetted on sensor and considered as blank reading. Isolated DNA sample was loaded on sensor after wiping it again kimwipe and absorbance was noted at both 260 and 280nm. DNA peak was at 260nm and 260/280 ratio was between 1.8 and 2.

Isolated DNA was further quantified by using 1 % agarose and agarose gel electrophoresis was run at 120 V and 47 mA for 1 h. DNA was visualized by using UV trans-illuminator (Bio Rad, UK).

Extracted DNA was diluted in double distilled water in a 1:9, mixed and stored at -20 °C to use as working concentration.

2.2.3 Amplification of IGS region and RFLP analysis

The diversity among isolates was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region (IGS) (Rasche *et al.*, 2006). For this, amplification of 16S-23S intergenic region was done by set of primers as mentioned in Table 2.3. Single colony was picked from pure culture of bacterial strain by sterilized needle tip and suspended in the eppendorf tube containing 70 µL of distilled water. This eppendorf tube was kept at 94°C for 10 min. 2 µL of supernant was collected after centrifugation at 8000 rpm for 5 min at 4°C and added to the PCR master mixture

containing 0.6 µL of 15 µM of each primers (IGS_F and IGS_R), 2 µL of 10x buffer BD, 2 µL of 2 mM deoxynucleoside triphosphate (dNTP), 1.8 µL of 25 mM $MgCl_2$, 0.2 µL of 5 µL FIREPol® DNA Polymerase (Solis Biodyne). Amplification was performed in a thermocycler programmed as mentioned in Table 2.3. PCR primers for IGS amplifications are listed in Table 2.3. Amplified product was kept at 4°C.

2.2.3.1 RFLP enzymes

PCR amplified 16S-23S rRNA intergenic spacer region was treated with restriction endonuclease enzymes with compatible buffers as mentioned in Table 2.4. For RFLP analysis, 5 µL of PCR amplified sample was added with 2 µL buffer, 5 µL H_2O and 1 µL of restriction endo-nuclease enzyme. The eppendorf tube containing the mixture was kept at 37 °C for 4 h and at 65 °C for 20 min. The RFLP was detected on 1.5% Gel electrophoresis.

2.2.4 Amplification of *16S rRNA* gene and *gyrB* gene

Zn mobilizing rhizobacteria were identified by partial amplification of *16S rRNA*, *gyrB* and *gyrA* genes analysis. The *16S rRNA* gene was amplified by primers 8F (5'-AGA GTT TGA TCC TGG CTC AG- 3') (Weisburg *et al.* 1991;) and 1520rev (5'-AAG GAG GTG ATC CAG CCG CA-3') (Edwards *et al.* 1989) (Table 2.3) PCR reaction mixture (20 µL) was prepared by mixing 0.6 µL of 15 µM of each primers (forward and reverse), 2 µL of 10x buffer BD, 2 µL of 2 mM deoxynucleoside triphosphate (dNTP), 1.8 µL of 25 mM $MgCl_2$, 0.2 µL of 5 µL FIREPol® DNA Polymerase (Solis Biodyne) and 1 µL of genomic DNA. Amplification was performed in a thermocycler programmed as mentioned in Table 2.3.

The *gyrB* gene was amplified by using double primers UP1_S (5'-GAA GTC ATC ATG ACC GTT CTG CA-3') and UP2r_S (5'- AGC AGG GTA CGG ATG TGC GAG CC -3') (Yamamoto and Harayama 1995). PCR reaction mixture (20 µL) was prepared by mixing 2.0 µL of 2 µM of each primers (forward and reverse), 1 µL of H_2O, 10 µL of pre-mix E, 0.16 µL of 5 U / µL FIREPol® DNA Polymerase (Solis Biodyne) and 1 µL of genomic DNA. Amplification was performed in a thermocycler programmed as mentioned in Table 2.3.

Table 2.3 Sequences of oligonucleotides used as primers to amplify various genes

Primer	Sequence (5'-3')	Target gene (s)/ size	PCR profile	Reference
8f	AGAGTTTGATCCTGGCTCAG	*16S* 1000 bp	95°C for 5 min, 36 cycles of 95°C for 30 S, 55°C for 45 S, 72°C for 90 S, elongation at 72°C for 10 min	(Weisburg *et al.*, 1991)
1520rev	AAGGAGGTGATCCAGCCGCA			(Edwards *et al.*, 1989)
UP1_S	GAAGTCATCATGACCGTTCTGCA	*gyrB* 1100 bp	95°C for 5 min, 35 cycles of 95°C for 60 S, 58°C for 60 S, 72°C for 120 S, elongation at 72°C for 10 min	(Yamamoto and Harayama, 1995)
UP2r_S	AGCAGGGTACGGATGTGCGAGCC			
GyrAF	CAGTCAGGAAATGCGTACGTC	*gyrA* 800 Bp	95°C for 3 min, 35 cycles of 95°C for 30 s, 56°C for 45s, 72°C for 60s, elongation at 72°C for 10 min	(Roberts *et al.*, 1994)
gyrAR	CAAGGTAATGCTCCAGGCATT			
PolF	TGCGAYCCSAARGCBGACTC	*nif*H 360 bp	94°C for 4 min, 30 cycles of 94°C for 60 s, 55°C for 60s, 72°C for 120s, elongation at 72°C for 10 min	(Poly *et al.*, 2001)
PolR	ATSGCCATCATYTCRCCGGA			
DegACCf	GGBGGVAAYAARMYVMGSAAGCTYGA	*acdS* 535 bp	95°C for 3 min, 30 cycles of 95°C for 30 s, 46°C for 60s, 72°C for 60s, elongation at 72°C for 5 min	(Nikolic *et al.*, 2011)
DegACCr	TTDCCHKYRTANACBGGRTC			
IGS-f	TGCGGCTGGATCACCTCCT	*16S-23S* 1100 bp	95°C for 5 min, 30 cycles of 95°C for 60 S, 55°C for 90 S, 72°C for 120 S, elongation at 72°C for 10 min	(Massol-Deya *et al.*, 1995)
IGS-rev	GGCTGCTTCTAAGCCAAC			
SmpqqC_Fw	GCGAGCAGATCCAGGGCTG	*PqqC* 600 bp	95°C for 6 min, 36 cycles of 95°C for 30 s, 58°C for 45s, 72°C for 60 S, elongation at 72°C for 10 min	This study
SmpqqC_Rev	GTCCAGCATGCTCCACAGAA			

Genpqqc_Fw	CCCGCGAGCAGATCCAGGGCTGGGT	*PqqC* 750 bp	95°C for 10 min, 36 cycles of 95°C for 30 S, 62°C for 45 S, 72°C for 60 S, elongation at 72°C for 10 min	This study
Genpqqc_Rev	TAGGCCATGCTCATGGCGTC			
Paepqqc_Fw	CGACAAGGGACGCTACTACCATA	*pqqC* 650 bp	95°C for 6 min, 36 cycles of 95°C for 30 S, 56°C for 45 S, 72°C for 60 S, elongation at 72°C for 10 min	This study
Paepqqc_Rev	CCGATAGTGCTCCAGGGTGA			

S = C or G, Y = A or T, K = G

Table 2.4 List of restriction endo-nuclease enzyme

Serial no.	**Endo-nuclease enzyme**
1	Acc65I
2	SalI
3	HaeIII
4	EcoRV
5	HhaI
6	AluI
7	PvuII
8	NotI
9	HindIII
10	RsaI
11	DraI
12	EcoRI

2.2.5 Amplification of *nifH* gene and *acdS* gene

The *nifH* gene associated with N_2-fixing ability of strains was amplified by primers PolF (5'-TGC GAY CCS AAR GCB GAC TC-3') and PolR (5'-ATSGCCATCATYTCRCCGGA-3') (Poly *et al.* 2001). PCR reaction mixture (50 µL) was prepared by mixing 25 µL of 10X premix E, 5 µL of each 1.5 µM primer (forward and reverse), 0.4 µL of 5U / µL Taq (Invitrogen) ® DNA polymerase and 2 µL of genomic DNA. Amplification was performed in a thermocycler programmed as stated in Table 2.3.

The *acdS* gene for ACC deaminase was amplified to confirm this activity by primers DegACCf (5'-GGB GGV AAY AAR MYV MGS AAG CTY GA-3') and DegACCr (5'-TTD CCH KYR TAN ACB GGR TC-3') (Nikolic *et al.* 2011). PCR reaction mixture (20 µL) was prepared by mixing 0.50 µL of 100 µM of each primers (forward and reverse), 5 µL of 10x buffer BD, 5 µL of 2 mM deoxynucleoside triphosphate (dNTP), 0.25 µL of Dimethyl sulfoxide (DMSO), 4.4 µL of 50 mM $MgCl_2$, 1µg of Bovine Serum Albumin (BSA), 0.25 µL of FIREPol® DNA Polymerase (Solis Biodyne) and 1 µL of genomic DNA. Amplification was performed in a thermocycler programmed as mentioned in Table 2.3.

2.2.6 Primers designing for amplification of *PqqC* gene

PqqC gene sequence was retrieved from the GenBank and alignment of the *pqqC* regions was performed using the multiple sequence alignment programme BioEdit Sequence Alignment Editor (BioEdit version 7.2.5) to determine regions conserved within the *pqqC* gene among various genera i.e. genus *Pseudomonas*, *Serratia* and *Enterobacteria* etc (Fig. 2.1). The set of primers Gen_PqqC, Pa_PqqC and Sm_PqqC which amplify 610-bp, 593-bp and 553-bp long *PqqC* gene fragment respectively were designed and shown in Table 2.2. The primers were tested for specific amplication by using BLAST and their specificity was checked by using DNA calculator (http://www.sigma-genosys.com/calc/DNACalc.asp). The oligonucleotides were manufactured by Microsynth, company (http://www.microsynth.ch).

PCR amplification of *PqqC* gene was carried out from bacterial DNA in a 20 µL mixture containing 0.6 µL of 10 µM of each primers (forward and reverse), 2 µL of 10x buffer BD, 2 µL of 2 mM deoxynucleoside triphosphate (dNTP), 1.8 µL of 25 mM $MgCl_2$, 0.2 µL of 5 µL FIREPol® DNA Polymerase (Solis Biodyne) and 1 µL of genomic DNA. mplification was performed in a thermocycler programmed as given in Table 2.3.

2.2.6.1 Cloning of *PqqC* gene

PCR cloning protocol was proceeded in four step as shown in Fig. 2.2. In first step, Genomic DNA was amplified by PCR using *Taq* DNA polymerase and an aliquot of the PCR reaction was analyzed on an agarose gel to verify production of the expected fragment. Amplified *pqqC* gene fragments were cloned using the Strata Clone PCR cloning vector pSC-A-amp/kan (Agilent Technologies, Catalog #240205, 11011 North Torrey Pines Road La Jolla, CA 92037). The constructs were transformed into chemically competent *E. coli* (SoloPack competent cells) according to the manual instructions (Appendix).

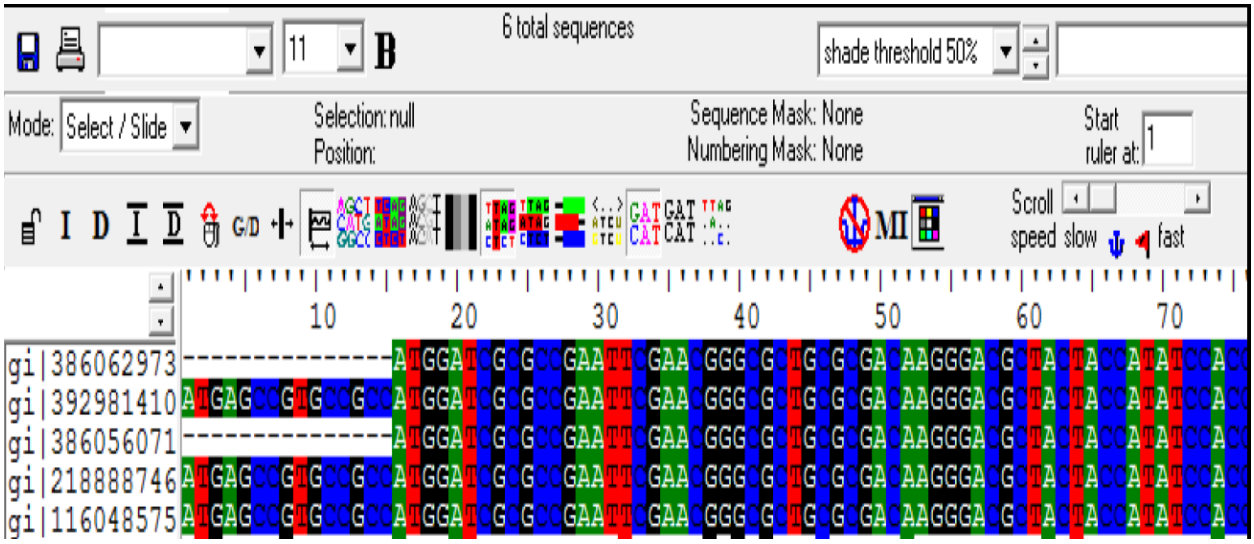


Fig. 2.1 Primer designing for *PqqC* gene amplification by using BioEdit v7.1.11

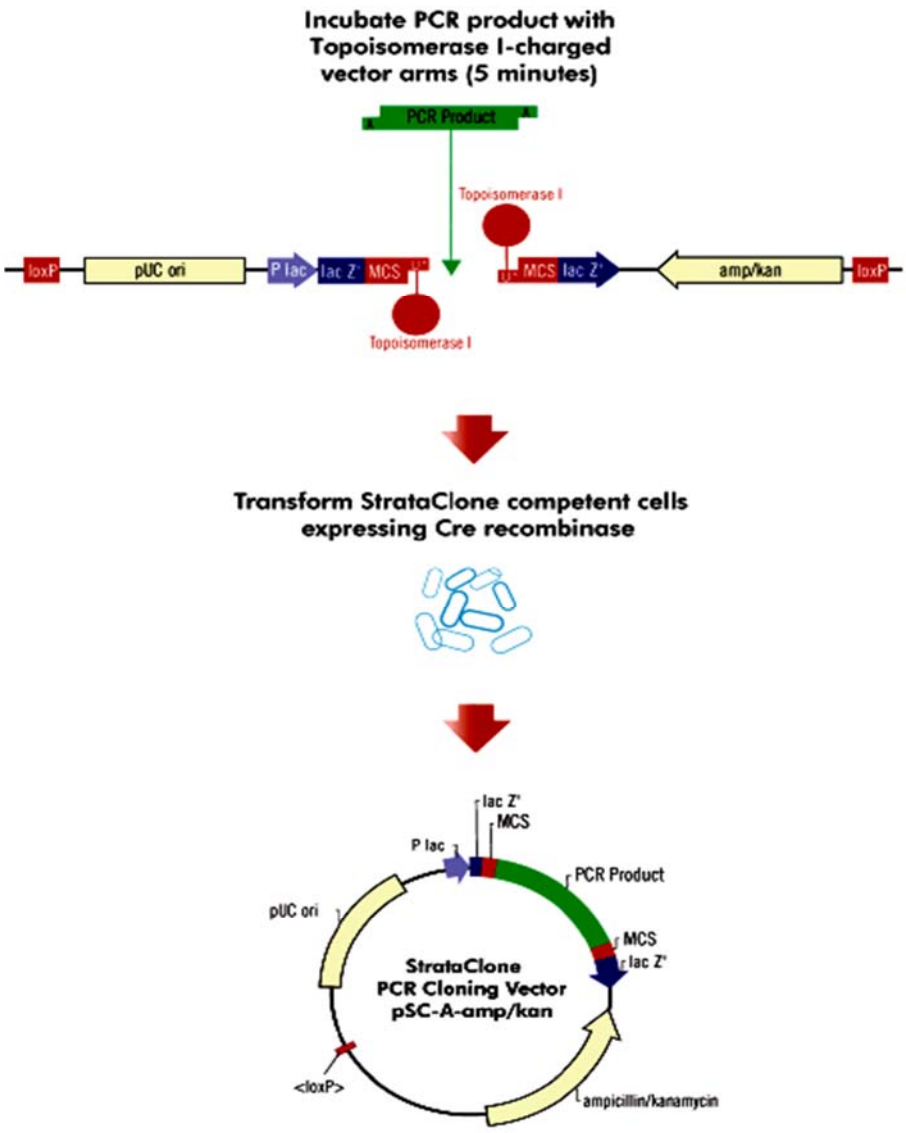


Fig 2.2 Overview of the StrataClone PCR cloning method

2.2.7 Sequencing, BLAST search and sequence submission to NCBI

PCR product was detected on 1% gel electrophoresis at Bio-resource lab, AIT and sequenced by LGC genomics (LGC Genomics GmbH, 12459 Berlin-Germany). The sequences were analyzed on BLAST and identified on the base of homologous gene. The percent (%) identity of each strain were compared with GENBANK entries and 16S rRNA and *gyrB* gene sequences have been submitted to National Center for Biotechnology Information (NCBI) GENBANK database.

2.3 Field evaluation of ZSB on wheat

The Zn mobilizing strains were evaluated for their ability to colonize the roots of different wheat varieties to enhance growth, yield and zinc content of grains under diverse climatic field conditions. The experiments were conducted on wheat at three sites with wheat crop.

Experiment I

2.3.1 Ayub Agriculture Research Institute (AARI), Faisalabad

Ayub Agriculture Research Institute (AARI), Faisalabad (situated between 31° - 25 N latitude, 73° - 06 E longitudes and 214 m altitude)-Pakistan. Field area of AARI is a well irrigated zone. The experiment was conducted from October 2010 to May 2011.

Experiment II

2.3.2 Barani Agriculture Research Institute (BARI), Chakwal

The experiment was conducted at the fields of Barani Agriculture Research Institute (BARI), Chakwal which is considered a stress prone area because it is rain fed zone. Field trial was conducted from October 2010 to May 2011.

2.3.3 Cotton Research Institute (CRS), Multan

Cotton Research Institute (CRS), Multan is irrigated with very hot climatic condition. Field trial was conducted from October 2010 to May 2011.

2.3.4 Plant material and preparation of bacterial inocula

For assesment the effects of selected PGPR, wheat (*Triticum aestivum* L), the major staple food of Pakistan, was selected. Four varieties of wheat were used for experiments which were screened from commercially approved genotypes with best agronomic traits. These commercial cultivars of wheat were selected on the basis of zinc content viz. Two varieties with low zinc contents while two varities with highest zinc contents. These are given below,

Variety 1 (V1)	Chakwal-50	High Zn varieties
Variety 2 (V2)	SH-2000	
Variety 3 (V3)	Inqilab-91	Low Zn varieties
Variety 4 (V4)	Lasani-08	

The Zn solubilizing rhizobacteria were grown in LB broth in a 250 mL Erlenmeyer flask on a shaking incubator at 100 rev min^{-1}, 28 ± 1°C for 48 h until a cell population reached up to 10^9 CFU mL^{-1} (OD_{595} nm) (Hassan *et al.,* 2010). Five mL of this bacterial culture was poured into polythene zipper bag containing 20 g of wheat seed, 2 g of soil and mixed well to maintain the cell population of 10^8 CFU mL^{-1}.

2.3.5 Experimental design, lay out and sowing

The experiment was laid out as a completely randomized split-plot design with four blocks as replicates. There were seven treatments viz; ZSB FA-2 (T1), FA-3 (T2), FA-4 (T3), the combination of strains FA-2, FA-3 & FA-4 (T4), positive control with chemical Zn (20% $ZnSO_4$ @ 30 kg ha^{-1}) (T5), negative control-1 with the non-ZSB FA-1 (T6), negative control-2 without fertilizer and amendment of ZSB (T7). Treatments assigned in the main plot and the four wheat varieties Inqlab 91, Chakwal-50, Lasani-08 and SH-2002 were allocated in the sub-plots. Size of plot and sub plot was (6.9 m^2) and (1.7 m^2) respectively. The soil samples were collected from 0-30 cm depth and analyzed.

Soil was ploughed at desirable field condition followed by fertilizer NPK application @ 90:60:60 kg ha^{-1}. All the P, K and $1/4^{th}$ N was applied at the time of sowing and

remaining N was applied in 2-3 split doses at tillering and dough stages. The seeds treated with ZSB were sown as recommended cultural practices (Arshad *et al.*, 2008) on 15th October, 2010 with Row x Row distance = 0.30 m and Plant x Plant distance = 0.8m. The plants were irrigated by five times as the earliest irrigation was done 30 days after crop emergence and later irrigations were applied at specific crop stages i.e. tillering, booting, anthesis and grain development at AARI, Faisalabad and CRS, Multan (Wajid *et al.,* 2002).

2.3.6 Harvesting

The crop was harvested manually at CRS, Multan upon physiological maturity in the month of May, 2011 while at AARI, Faisalabad and BARI, Chakwal the harvesting was done mechanically with wheat thresure. This was done when the green color from the glumes and kernels disappeared completely. Threshing of each plot was done separately.

2.3.7 Characteristics of the field soil

Soil samples from all three research stations under which experiment were carried were collected before seed sowing up to the depth of 15 cm and 15-30 cm and were analyzed for different physico-chemical properties by Soil & Water Testing Laboratory for Research, Rawalpindi. Soil analysis is given in the Tables 2.5, 2.6, 2.7.

The meteorological data during cropping season of all three experimental sites was collected from respective research station and shown graphically in Fig 2.3.

2.3.8 Root colonization of inoculated Zn rhizobacteria on wheat roots

Roots of nine plants from each replication were sampled at the germination and harvesting of wheat. The plucked plants were shifted to the lab. In the lab, plants were shaken vigorously to remove adhered soil The root adhered soil was collected by shaking the roots gently and mixed well. For endophytic bacterial population, the roots of nine plants replication^{-1} were mixed, sterilized with 0.1 % mercuric chloride and blended in a mortar pestle. The rhizobacteria were isolated by making serial dilution method as described by Yasmin *et al.* (2004) and Gull *et al.* (2004). The inoculated strains were identified on the basis of their morphological and biochemical

Table 2.5 Characteristics of soil sample taken from the fields of Chakwal

Chemical Analysis of Soil								
Depth	E.C (dSm-1)	pH	Organic Matter	Available P (mg/kg)	Available K (mg/kg)	Saturation	Texture	Available Zn (mg/kg)
0-15 cm	0.32	8.1	0.65	5.1	220	28	Sandy Loam	0.38
15-30 cm	0.36	8.0	0.30	3.0	240	29		0.42

Table 2.6 Characteristics of soil sample taken from the fields of Multan

Chemical Analysis of Soil								
Depth	E.C (dSm-1)	pH	Organic Matter	Available P (mg/kg)	Available K (mg/kg)	Saturation	Texture	Available Zn (mg/kg)
0-15 cm	1.70	8.9	0.76	15	121	34	Loam	0.36
15-30 cm	1.23	9.0	0.63	13	140	36		0.38

Table 2.7 Characteristics of Soil Sample taken from the fields of Faisalabad

Chemical Analysis of Soil								
Depth	E.C (dSm-1)	pH	Organic Matter	Available P (mg/kg)	Available K (mg/kg)	Saturation	Texture	Available Zn (mg/kg)
0-15 cm	0.51	7.8	0.88	18	230	46	Sandy Loam	0.52
15-30 cm	0.38	7.9	0.83	19	240	48		0.54

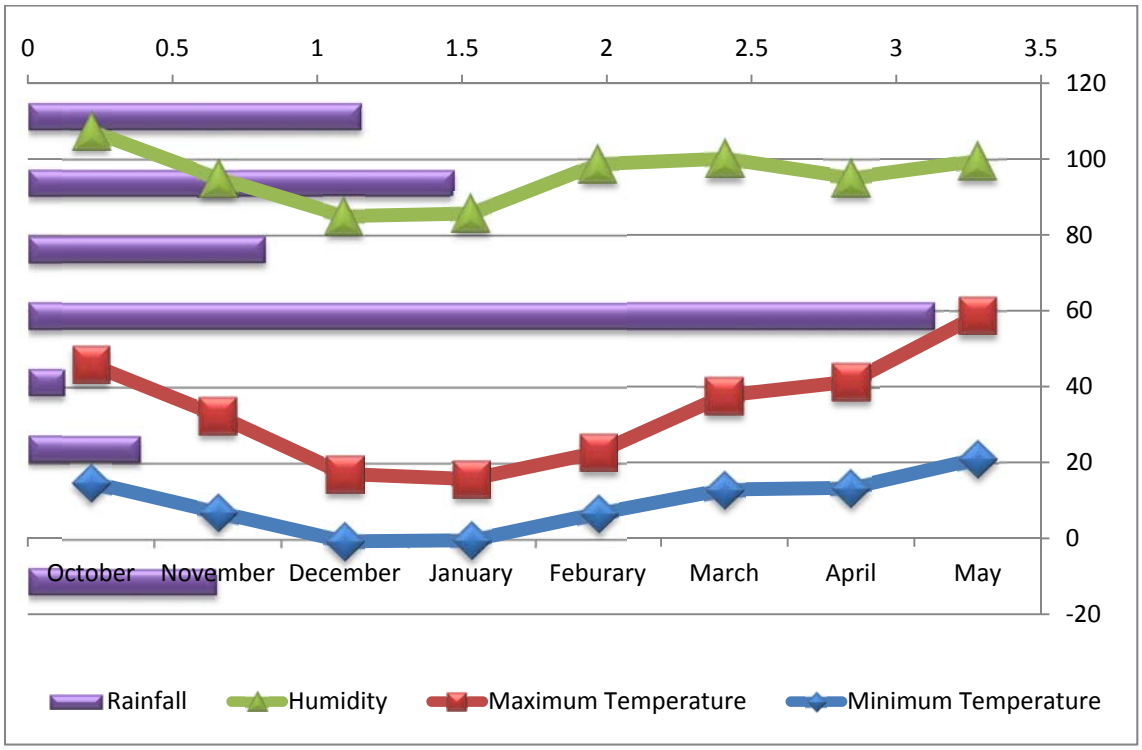


Fig. 2.3 Graphical representations of Meteorological data of Chakwal

markers such as colony color, Zn solubilizing ability, antagonistic activity and antibiotic resistance. The average number of bacterial colony forming units was calculated and presented as mean colony-forming unit mL^{-1} of root rhizosphere and root endosphere. The CFU mL^{-1} was converted into log_{10}CFU.

2.3.8.1 Percent (%) survivability

Percent survivability was calculated for both rhizospheric and endophytic population by using formulae given below,

% survivability = CFU mL^{-1} at harvesting / CFU mL^{-1} at germination × 100

2.3.9 Growth parameters, yield and zinc analysis of grains

Different growth parameters such as flag leaf area (cm^2), days to headings, spike length (cm) were observed. Total biomass (t ha^{-1}), grains $spike^{-1}$, thousand seed weight (g), tillers $plant^{-1}$ and grain yield (t ha^{-1}) were recorded as yield parameters.

After harvesting, the wheat grains were analyzed for Zn analysis by tri acid (a mixture of $HClO_4$, HNO_3, H_2SO_4 at 1:2:5) digestion (Allen *et al.* 1986) using Atomic absorption spectrophotometer (AAS) at AURIGA Group of Companies, Lahore.

2.3.10 Translocation efficiency of Zn

Zinc translocation efficiency was calculated by using formulae given below,

Translocation efficiency = Grain Zn concentration / Straw Zn concentration

2.4 Statistical analysis

The Genstat 9.2 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK) statistical package was used for ANOVA on the data of plate assay, AAS analysis, wheat growth & yield, grain Zn concentration, growth curve analysis and root colonization parameters, followed by a post hoc least-significant difference (LSD) test for comparison of means. Yield versus treatment data analysis was performed in CANOCO 4.5 for windows (Ter Braak, 1988).

Chapter 3
Results

3. Results

3.1.1. Screening of Zinc (Zn) solubilizing rhizobacteria collected from PCMC

Among 98 PGPR collected from PCMC, initially fifteen (15) PGPR were found to solubilize Zn while only four strains were found efficient upon further screening. All the selected isolates FA-4, FA-3, FA-2 and FA-8 showed considerably diverse zone for Zn solubilization (59.50, 51.17, 27.5 and 17.17 mm) gradually on $ZnCO_3$ (Fig. 3.1). Less difference was found between FA-3 and FA-4 for the zone formation on ZnO however, both (FA-3 and FA-4) isolates showed distinctly higher zone as compared to FA-2 and FA-8. A similar trend was observed on $Zn_3(PO_4)_2$. The isolates FA-2, FA-3, FA-4 and FA-8 formed no zone on ZnS in initial screening. The bacterial strains FA-4 as well as FA-3 appeared best and efficient in zone formation than other strains. The strain FA-8 showed less effectiveness so it was skipped in further characterizations. The isolates showed no visible activity on ZnS ore till 18th day of inoculation. The solubilization activity of the strains was maximum on 3rd day which almost remained constant until 18th day of inoculation except on $Zn_3(PO_4)_2$ ore which significantly increased between 4th day to 18th day (Fig. 3.1; Fig. 3.2).

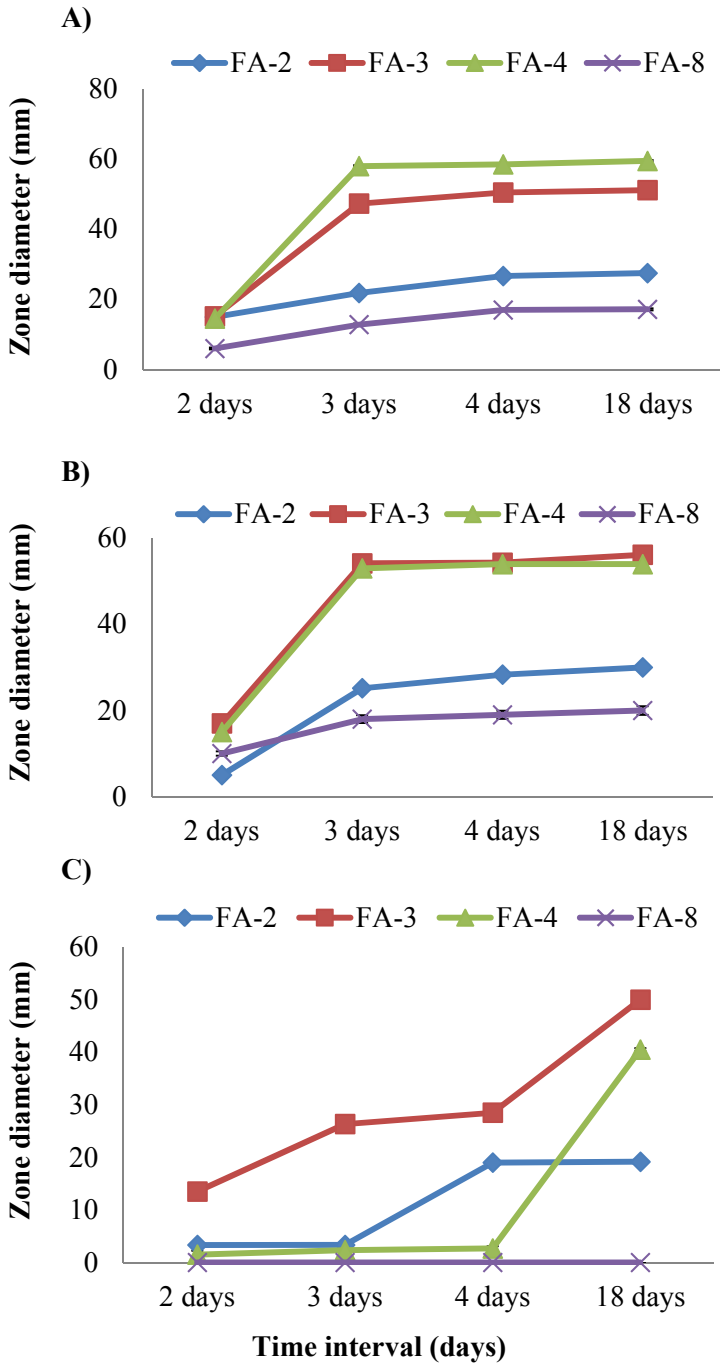


Fig. 3.1 Zn solubilization potential of ZSB on the basis of halo zone formation with different insoluble Zn ores (a) Zn carbonate (b) Zinc oxide (c) Zinc phosphate at different intervals of time. Data are mean values of three replicates; Error bars indicate the standard error.

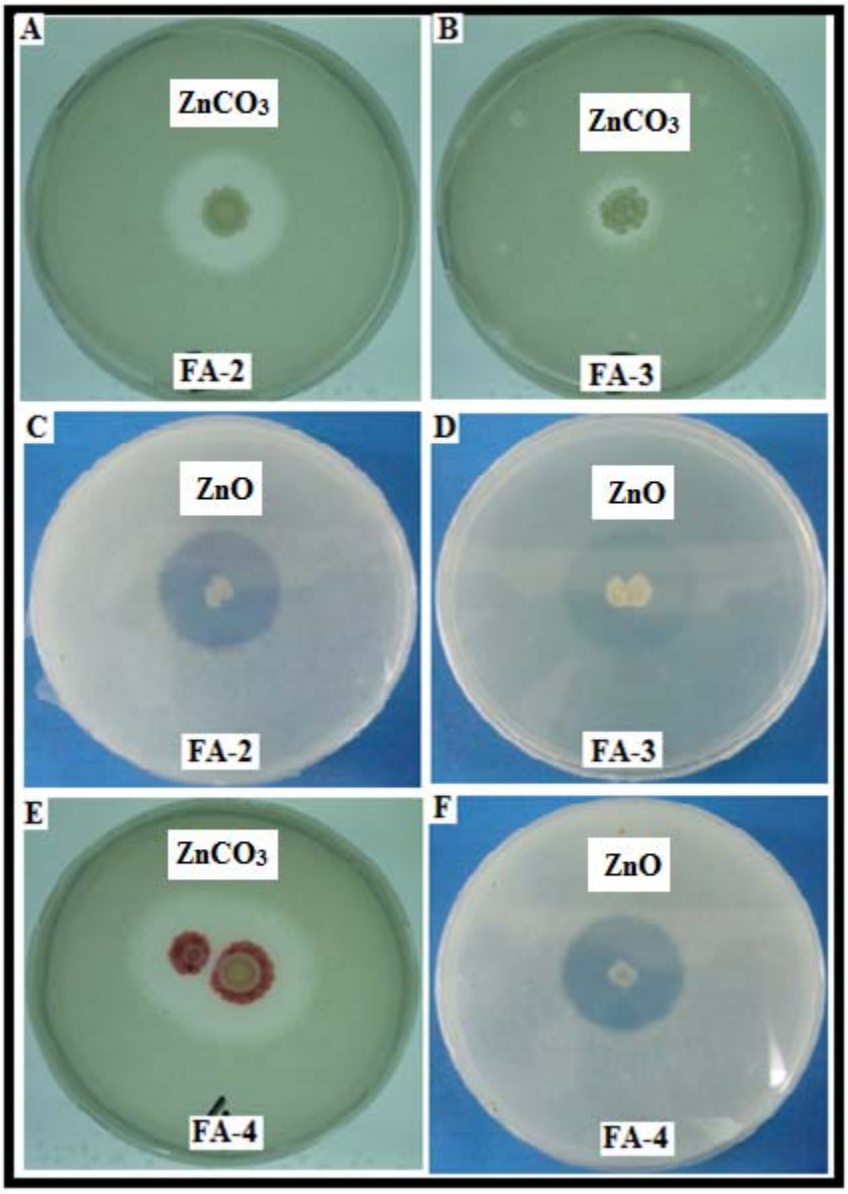


Fig. 3.2 Zn solubilization of different Zn ores $ZnCO_3$ and ZnO by ZSB *S. grimesii* FA-2, *B. thuringiensis* FA-3 and *S. marcescens* FA-4.

3.1.2. Quantification of zinc solubilizing ability of rhizobacteria collected from PCMC

Activity of three strains with maximum solubilization on agar plates based on zone diameter was further quantified by atomic absorption spectrophotometer (AAS). In AAS analysis, the strain FA-3 solubilized maximum Zn from ZnO while FA-4 solubilized maximum Zn from $ZnCO_3$. All the strains FA-4, FA-3, FA-2 showed less potential to solubilize zinc from the ZnS ore (0.35 µg mL^{-1}, 0.46 µg mL^{-1} and 1.17 µg mL^{-1}) as mentioned in Table 3.1.

3.1.3. Screening of Zinc (Zn) solubilizers rhizobacteria isolated from BARI, Chakwal

Among hundred strains obtained from wheat rhizosphere, only two were found efficient as Zn solubilizer. The strain *P. aeruginosa* FA-9 produced 63 mm zone of clearance on the plate containing $ZnCO_3$, whereas *Enterobacter* sp. FA-11 produced 17.2 mm zone (Fig. 3.3). The similar trend was observed on ZnO (Fig. 3.4). The isolate *Enterobacter* FA-11 produced no zone on the plate containing $Zn_3(PO_4)_2$ while *Pseudomonas* FA-9 produced a zone of 51 mm diameter. It is clear that FA-9 was efficient in Zn solubilization as compared to FA-11 (Fig. 3.3). Both strains showed no visible activity on ZnS ore up to 18th day of inoculation.

Table 3.1 *In vitro* quantification of Zn through AAS solubilized by the selected ZSB

Strains	$ZnCO_3$ (mg kg^{-1})	ZnO (mg kg^{-1})	ZnS (mg kg^{-1})
Control	10 ^{D}	9.6 ^{E}	0.14 ^{C}
S. liquefaciens FA-2	135 ^{C}	18 ^{D}	0.20 ^{C}
B. thuringiensis FA-3	152 ^{B}	126 ^{A}	1.17 ^{A}
S. marcescens FA-4	164 ^{A}	93 ^{C}	0.35 ^{B}
LSD	11.93	5.99	0.41

Mean values (n=3); Means values with different letters in the same column differ significantly at $P<0.05$ based on LSD.

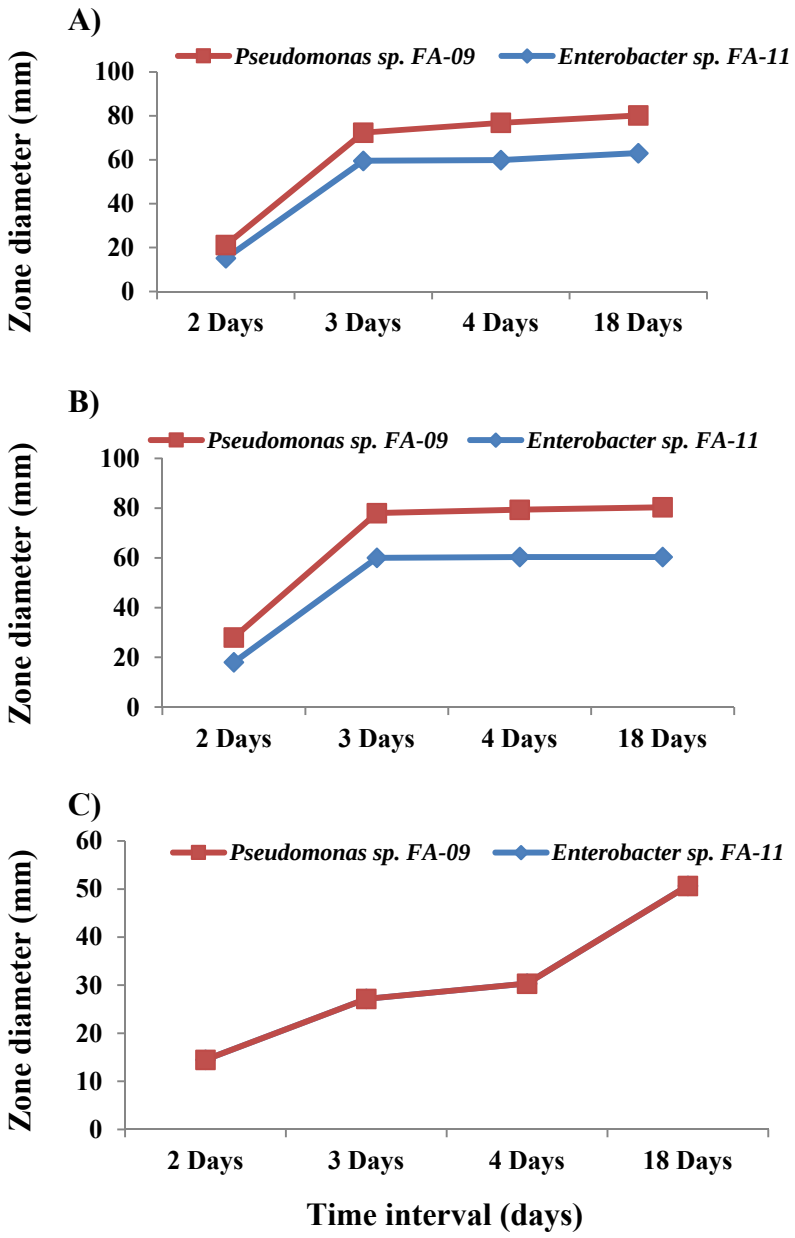


Fig. 3.3 Zn solubilization efficiency of ZSB on the basis of clear zone formation with various insoluble Zn ores (A) Zn carbonate (B) Zinc oxide (C) Zinc phosphate at a range of intervals of time. Data are mean values of three replicates.

3.1.4. Quantification of Zn solubilizing activity of rhizobacteria isolated from BARI, Chakwal

In quantitative assay, the strain FA-9 and FA-11 solubilized maximum Zn (168 µg mL^{-1} & 59 µg mL^{-1}) from $ZnCO_3$ ore as compared to the ZnO ore (102 µg mL^{-1} & 45 µg mL^{-1}) as shown in Fig. 3.5. The potential of both bacterial strains to solubilize zinc from ZnS ore was less i.e. 7 µg mL^{-1} and 0.57 µg mL^{-1} respectively as compared to that of other compounds. A noteworthy correlation was observed between the zone diameter (mm) and zinc quantification (µg mL^{-1}) depending on the zinc source and Zn solubilizing bacteria (Fig. 3.6).

3.1.5. Morphological and biochemical characterization of Zn solubilizing PGPR

Among the Zn solubilizing PGPR only one strain was found Gram positive while all others were Gram negative and fast grower. The cell shape and colony morphology and Gram's reaction is given in Table 3.2.

3.1.6. Characterization of Zn solubilizing rhizobacteria for stress tolerance

3.1.6.1. Growth curve analysis

Zn solubilizing PGPR showed little bit similar pattern for growth after inoculation with tryptic soy broth amended by 0.1% of various zinc compounds such as zinc chloride, zinc oxide, zinc nitrate and zinc sulphate (Fig. 3.7).

Growth curve analysis of all the five strains FA-2, FA-3, FA-4, FA-9 and FA-11 showed the clear exponential phase with 0.1% and 0.2 % of Zn quantity while growth became parallel to control at 0.3% of Zn concentration except FA-4 which showed growth activity even at the Zn level of 0.3 %. All the PGPR showed lag phase initially for 2 h and the next 12 h showed signs of exponential growth phase while upon diminishing growth phase, last 2-3 h revealed the stationary phase as exhibited in Fig.3.8.

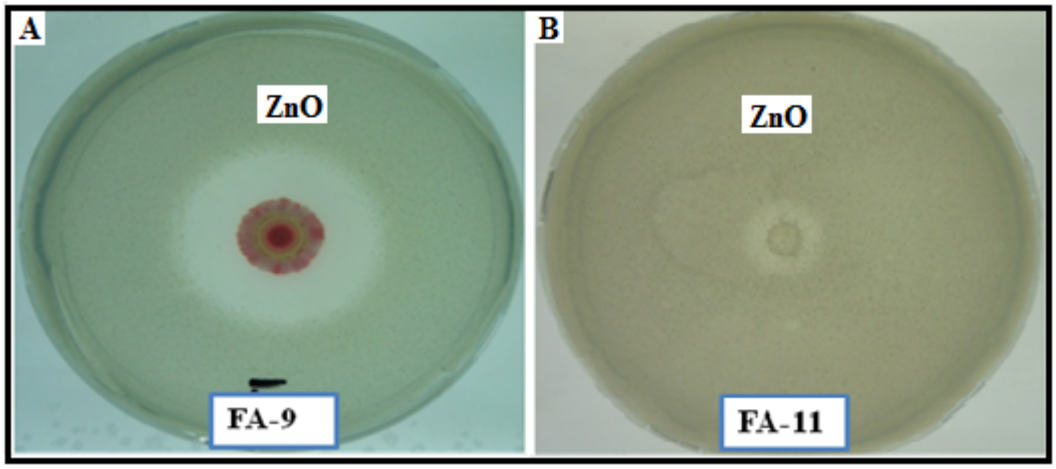


Fig. 3.4 Solubilization of ZnO by (A) *P. aeruginosa* FA-9 (B) *Enterobacter* sp. FA-11

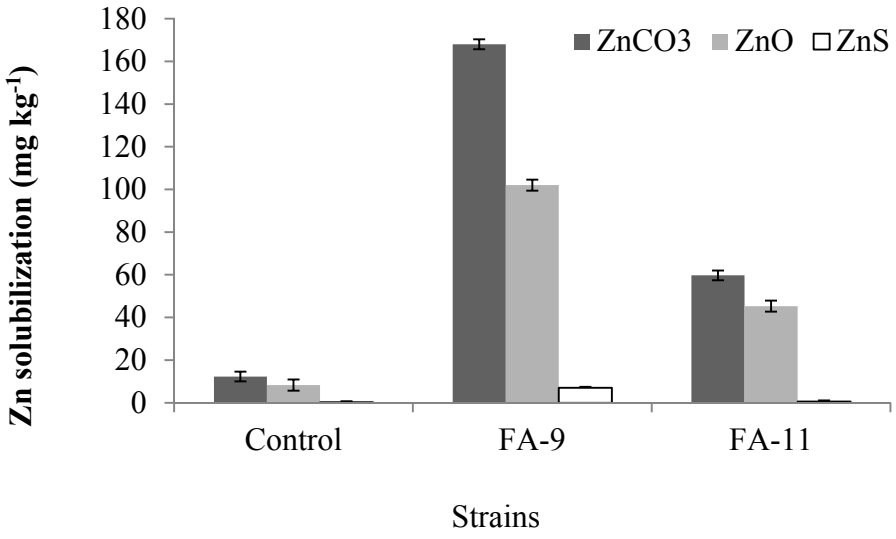


Fig. 3.5 AAS quantification of Zn solubilized by ZSB *P. aeruginosa* sp. FA-9 and *Enterobacter* sp. FA-11. Data are mean values of three replicates; Error bars indicate the standard error.

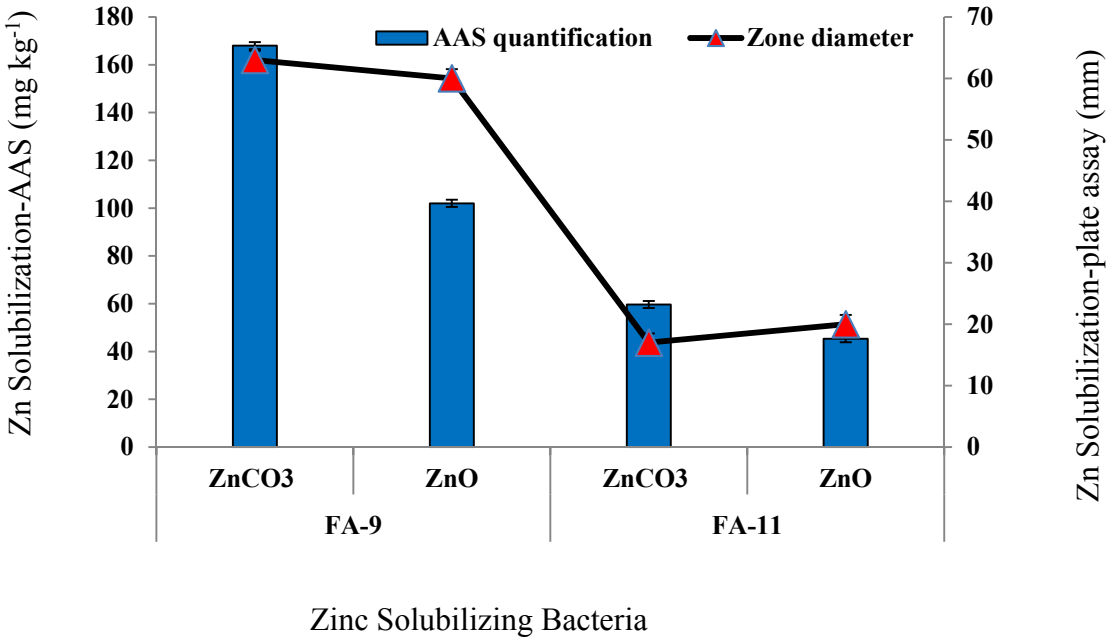


Fig. 3.6 A correlation between plate assay (mm) and AAS quantification (µg mL^{-1}) of Zn solubilized by ZSB *P. aeruginosa* FA-9 and *Enterobacter* sp. FA-11. Data are mean values of three replicates; Error bars indicate the standard error.

Table 3.2 Morphological characterization of ZSB

PGPR	**Gram's reaction**	**Cell shape**	**Colony color**
S. liquefaciens FA-2	-	Rod	White
B. thuringiensis FA-3	+	Rod	Creamy white
S. marcescens FA-4	-	Short rod	Orange red
P. aeruginosa FA-9	-	Rod	Blue green
Enterobacter sp. FA-11	-	Rod	Off white
Un-identified strain FA-8	-	Rod	white

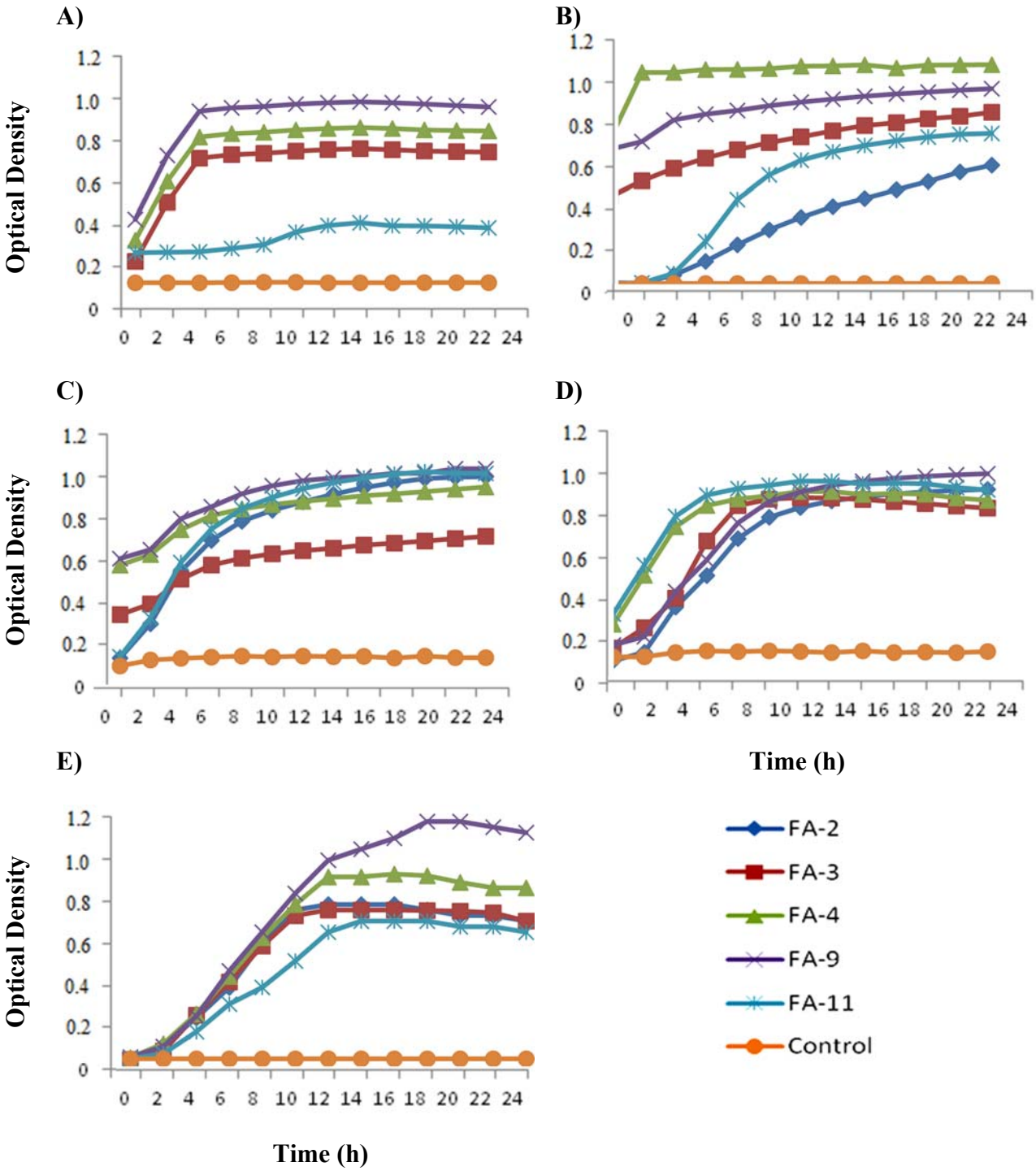


Fig. 3.7 Growth curve of ZSB in tryptone soy broth (TSB) amended with various Zn compounds (A) zinc chloride (B) zinc nitrate (C) zinc oxide (D) zinc sulphate (E) No Zn

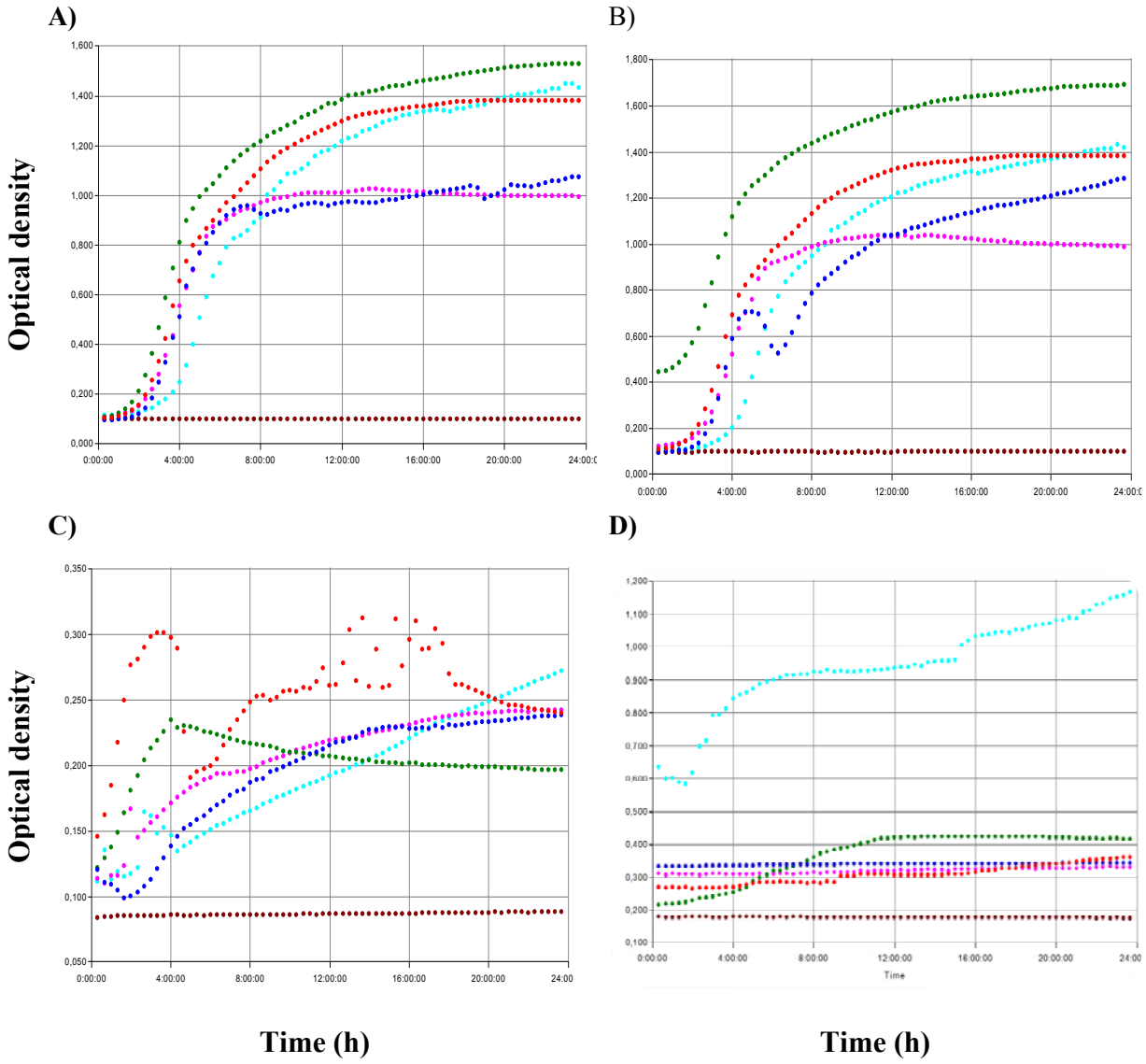


Fig. 3.8 Growth curve of different Zn solubilizing rhizobacteria inoculated in LB broth amended with (A) Zn = 0% (B) Zn = 0.1% (C) Zn = 0.2% (D) Zn = 0.3%

3.1.6.2. Exo-polysaccharide (EPS) activity

All the Zn solubilizing PGPR, FA-2, FA-3, FA-4, FA-9 and FA-11 showed positive EPS activity as mentioned in Table 3.3.

3.1.7. Characterization of Zn solubilizing rhizobacteria for plant growth promotion

3.1.7.1. Phosphorus solubilization

The Zn solubilizing PGPR were positive for P solubilization. The strain FA-9 produced highest clear zone diameter of 15 mm followed by strain FA-3 (14.7 mm), FA-4 (13.4 mm), FA-2 (13 mm) and FA-11 (5 mm) respectively (Table 3.4).

3.1.7.2. ACC-deaminase activity

All the selected Zn solubilizing PGPR were positive for ACC-deaminase activity on plate assay as mentioned in Table 3.3.

3.1.7.3. IAA production

The Zn solubilizing PGPR showed diverse pattern of IAA production with and without L-tryptophan (ranging from 1.2 to 4.2 μg mL^{-1}). Maximum IAA production was observed for FA-2 with L-tryptophan (4.20 μg mL^{-1}) and without L-tryptophan (1.23 μg mL^{-1}) followed by FA-9 with L-tryptophan (3.25 μg mL^{-1}) and without L-tryptophan (2.86 μg mL^{-1}), FA-3 with L-tryptophan (2.62 μg mL^{-1}) and without L-tryptophan (1.09 μg mL^{-1}), FA-4 with L-tryptophan (2.57 μg mL^{-1}) and without L-tryptophan (1.08 μg mL^{-1}), FA-11 with L-TRP (1.23 μg mL^{-1}) and without L-TRP (1.02 μg mL^{-1}) (Table 3.4).

Table 3.3 Plant growth promoting and biocontrol attributes of ZSB

ZSB	*ACC-deamminase activity		N_2 fixation	Exo-polysaccharide activity	Siderophore activity	Hemolytic activity
	Plate assay	*acdS* gene amplification	*nif* gene amplification	Plate assay	Plate assay	Plate assay
Control FA-1	-	-	+	+	-	-
S. liquefaciens FA-2	+	√	+	+	+	-
B. thuringiensis FA-3	+	√	--	+	+	+
S. marcescens FA-4	+	√	+	+	+	-
P. aeruginosa FA-9	+	√	+	+	+	+
Enterobacter sp. FA-11	+	√	+	+	+	-

*ACC = 1-aminocyclopropane-1-carboxylic acid; +++ = Very good activity, ++ = Good activity, + = Fair activity, - = No activity

Table 3.4 Plant growth promoting attributes of ZSB

ZSB	P solubilizing activity	IAA** production	
	Plate assay (mm)	With L-TRP ($\mu g\ mL^{-1}$)	Without L-TRP ($\mu g\ mL^{-1}$)
Control FA-1	-	-	-
S. liquefaciens FA-2	13.00±0.2	4.20±0.01	1.23±0.01
B. thuringiensis FA-3	14.66±0.2	2.62±0.01	1.09±0.01
S. marcescens FA-4	13.33±0.2	2.57±0.01	1.08±0.01
P. aeruginosa FA-9	15.00±0.2	3.25±0.02	2.86±0.03
Enterobacter sp. FA-11	5.00±0.2	1.23±0.03	1.02±0.03

**IAA= Indole acetic acid; Values are average of three replicates ± standard error

3.1.8. Detection of organic acid by HPLC analysis

Among various organic acids, only gluconic acid was detected by comparing against the standards (Fig. 3.9). Highest value of gluconic acid production was noticed by FA-2 (2500 ppm) with zinc carbonate while FA-3 (1640 ppm) with zinc oxide. All other strains were positive for gluconic acid production with varying potential. The strains FA-3 and FA-4 produced gluconic acid of 1640 and 1285 ppm with zinc carbonate while FA-3 produced higher concentration (1330 ppm) of gluconic acid with zinc phosphate followed by FA-4 (420 ppm) and FA-2 (330 ppm), respectively. Lowest values for acid productions were quantified with ZnS amended and pikovskaya broth.

3.1.9. Characterization of Zn solubilizing PGPR as biocontrol agent

3.1.9.1. Siderophore production

The production of low molecular weight, iron-chelating siderophores by Zn solubilizer bacterial strains was detected on blue agar (Fig. 3.10). The Zn solubilizing bacterial strains expressed the capability to chelate Fe but with various strength. The highest orange halo zone was produced by strain FA-9 followed by the FA-11, FA-3, FA-2 and FA-4 respectively.

3.1.9.2. Antifungal activity

The Zn solubilizing PGPR suppressed the growth of *Fusarium* species, a fungal phyto-pathogen as shown in Fig 3.11. Antagonistic activity of strain FA-3 was observed maximum (scored = 2) against *F. graminearum, F. caulimons* and *F. solani* while it was less (scored = 1) against *F. oxysporum* (Table 3.5). The efficiency of FA-4 was found to be more antagonistic (scored = 2) against *F. graminarium* as compared to *F. oxysporum, F. caulimons* and *F. solani* (scored = 1). The strain FA-9 expressed maximum (scored =

2) antagonism against all the four species of *Fusarium* while moderate antagonistic activity of FA-2 and FA-11 was observed against all these species of *Fusarium.*

3.1.9.3. Hemolytic activity

The ZSB FA-3 and FA-9 were positive for hemolytic activity as shown in Fig. 3.12 and Table 3.3.

3.1.9.4. Antibiotic resistance

The pattern intrinsic resistance of antibiotic against ZSB was found variable depending upon strains. The antibiotic ampicillin was found to be susceptible against both the strains FA-4 and FA-9. All other antibiotics such as amikacine, kanamycine, streptomycine and tetracycline showed a clear zone of inhibition against zinc solubilizing bacteria as shown in Table 3.6.

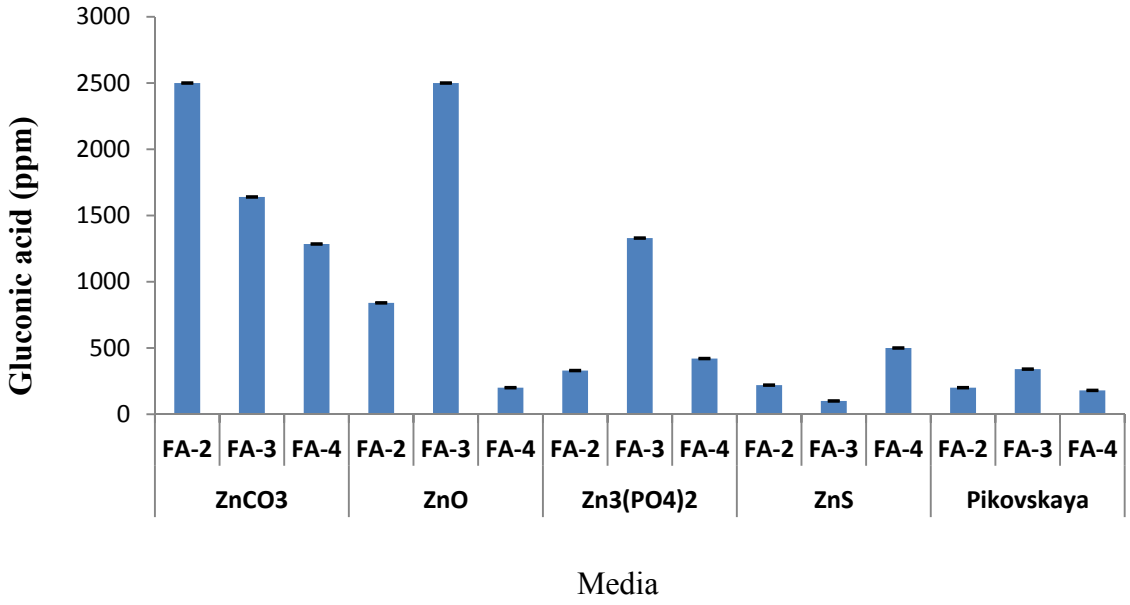


Fig. 3.9 Comparative potential of ZSB to produce gluconic acid with LB broth amended with different Zn ores and Pikovskaya broth; Data are mean values of three replicates; Error bars indicate the standard error.

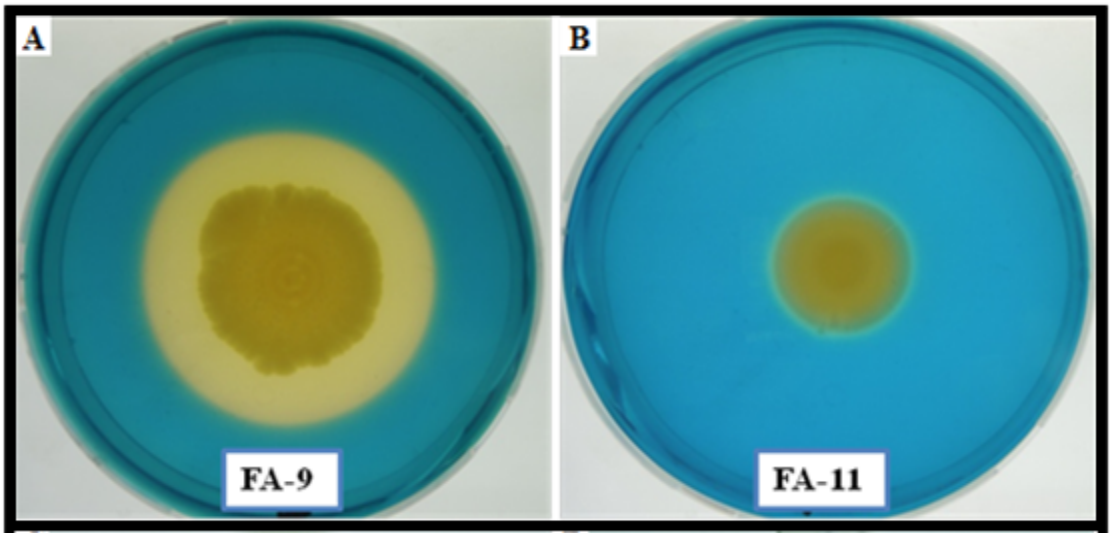


Fig. 3.10 Siderophores production by (A) *P. aeruginosa* FA-9 (B) *Enterobacter* sp. FA-11

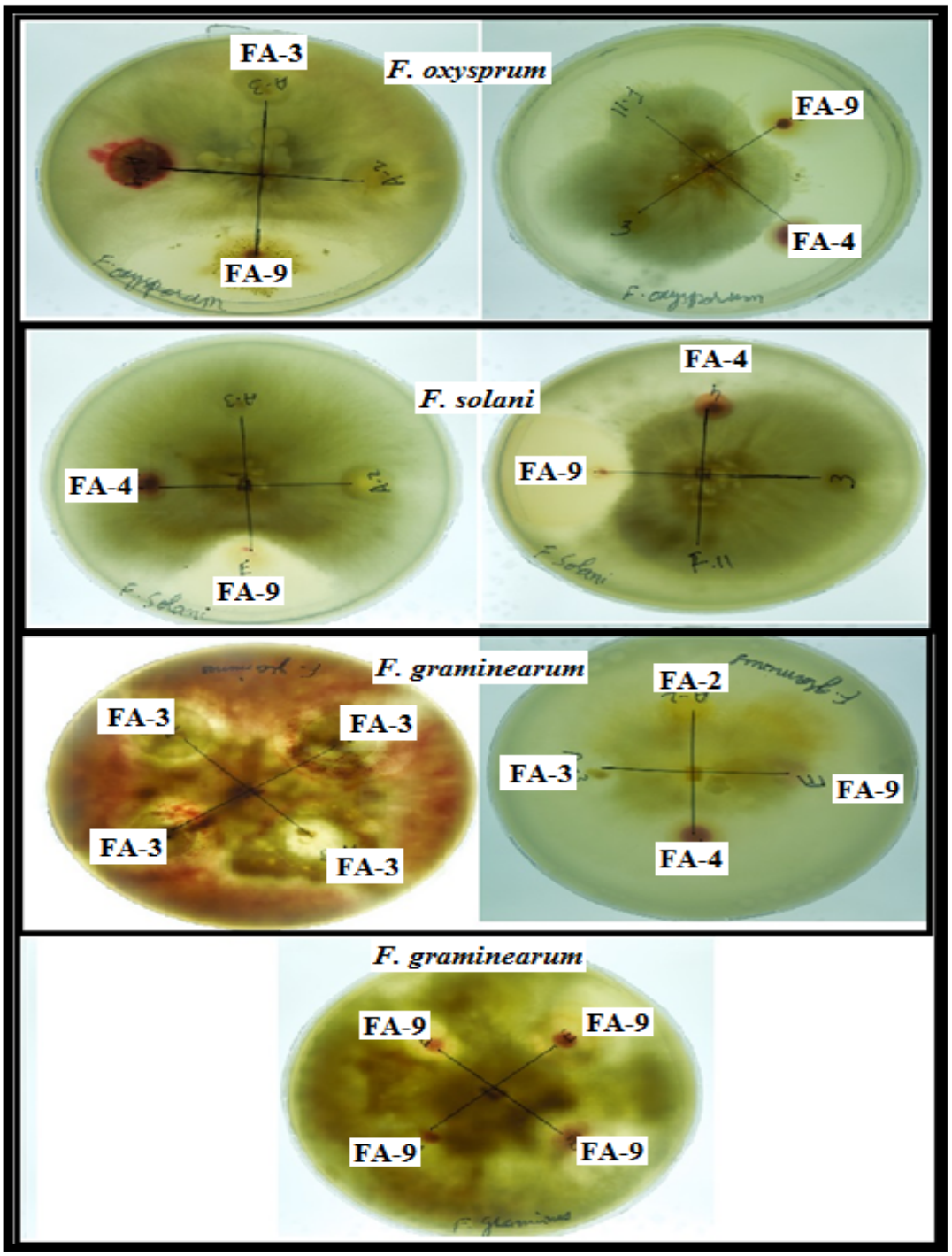


Fig. 3.11 Antifungal activity of *P. aeruginosa* FA-9, *B. thuringiensis* FA-3, *S. marcescens* FA-4 and *S. liquefaciens* against fungi; *F. solani*, *F. oxysporum* and *F. graminearum*

Table 3.5 Antifungal effects of ZSB against *Fusarium* species

ZSB	Antifungal activities			
	F. caulimons	*F. graminearum*	*F. oxysporum*	*F. solani*
Control FA-1	-	-	-	-
S. liquefaciens FA-2	++	+	++	++
B. thuringiensis FA-3	+++	+++	+	+++
S. marcescens FA-4	++	+++	++	++
P. aeruginosa FA-9	+++	+++	+++	+++
Enterobacter sp. FA-11	+	+	+	+

Growth of fungal mycelium over bacterial colony = 0, growth of fungal mycelium near edge of bacterial colony = 1, Growth of fungal mycelium away from bacterial colony = 2.

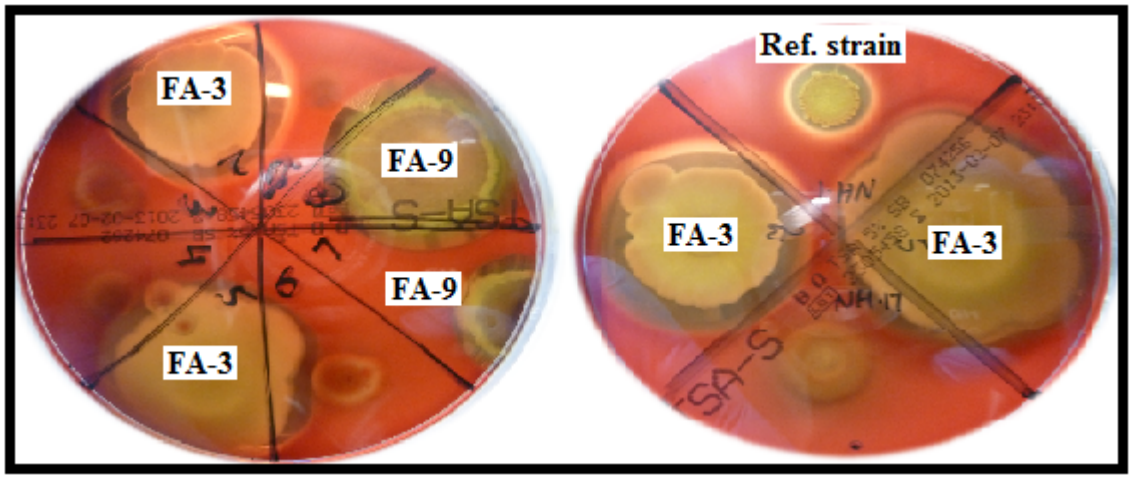


Fig. 3.12 Hemolytic activity of *P. aeruginosa* FA-9 and *B. thuringiensis* FA-3

Table 3.6 Zone of inhibition (mm) of intrinsic antibiotics resistance against ZSB on plate assay

ZSB	Zone of inhibition (mm) of intrinsic antibiotics resistance					
	Chl.	**Amp.**	**Amk.**	**Str.**	**Ken.**	**Tet.**
S. grimesii FA-2	15	12	19.5	17	19	13
B. thuringiensis FA-3	14	10	17	19	14	18
S. marcescens FA-4	13	S	17	8	16	11
P. aeruginosa FA-9	11	S	9	6	11	12

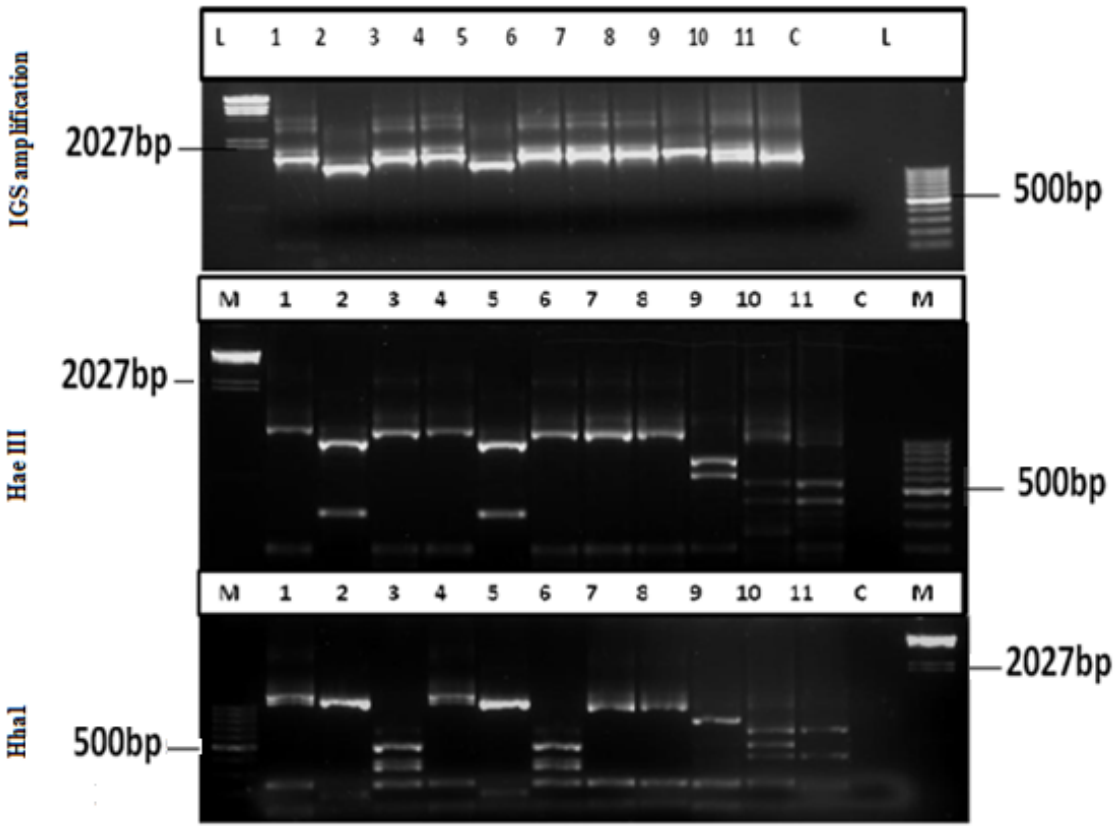
3.2. Molecular characterization

Intergenic region was amplified successfully as shown in Fig. 3.13 on agarose gel electrophoresis and RFLP analysis categorized the similar and different genera of isolates. Among twelve endonuclease restriction enzymes, only two (Hae III and Hha1) were observed with clear cutting pattern as shown in Fig. 3.13 of agarose gel electrophoresis. The RFLP analysis of amplified IGS region showed that Hae III cleaved the IGS regions of same genera (*Enterobacteriaceae*) but different species into equal segments. While Hha 1 cut the IGS region of individual species discretely. The RFLP analysis was helpful to predict the diversity among Zn solubilizing isolates which was confirmed by further molecular characterization.

The 16S *rRNA* and *gyrB* gene analysis of the strains FA-2 and FA-4 revealed their identities as *Serratia liquefaciens* and *Serratia marcescens* respectively. The 16S *rRNA*, *gyrA* and *B* gene analysis of FA-3 was confirmed as *Bacillus thuringiensis.* The strain FA-9 was identified as *Pseudomonas aeruginosa* by 16S *rRNA* gene analysis but this marker was incapable to identify strain FA-11. However, the *gyrB* gene analysis show the identification of both strains FA-9 and FA-11 with *P. aeruginosa* and *Enterobacter* sp. The sequences of 16S *rRNA* and *gyrB* gene have been submitted to National Center for Biotechnology Information (NCBI) GENBANK database (Table 3.7). The agarose gel electrophoresis of *gyrB* is given in Fig 3.14.

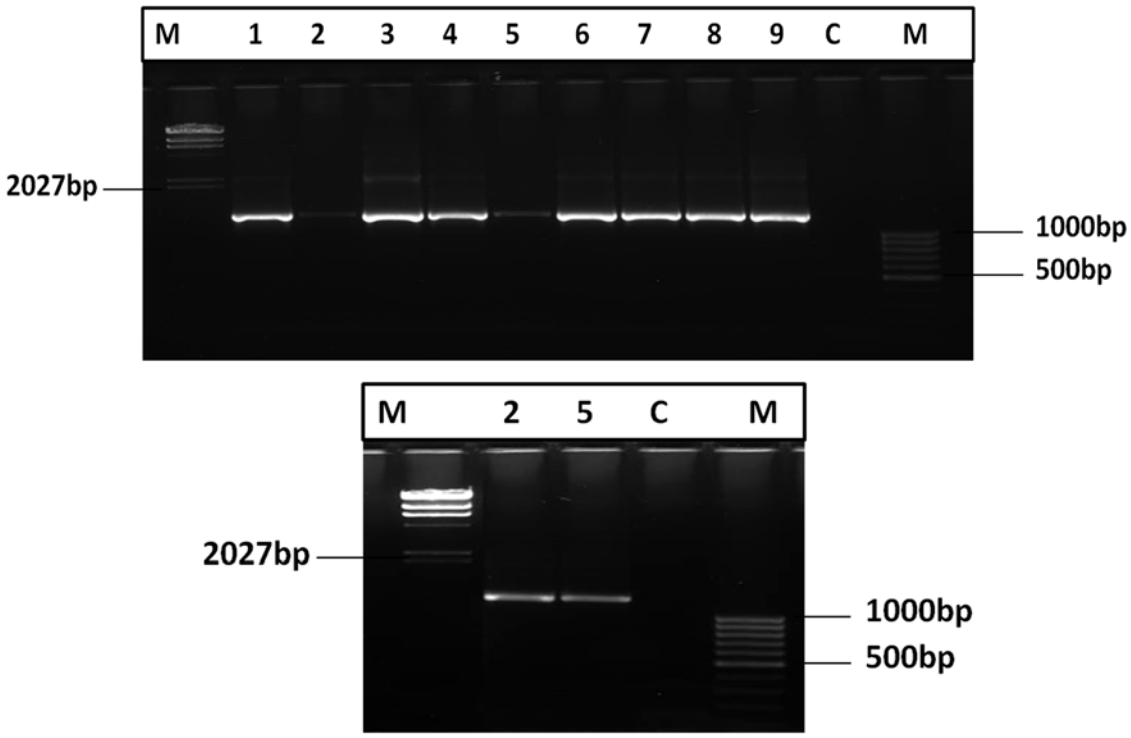
All the Zn solubilizing PGPR were found positive for ACC-deamminase activity by *acdS* gene amplification which confirmed the results from plate assay. The *nif* gene amplification proved all strains to have N-fixing ability except the strain *B. thuringiensis* (Table 3.3).

PqqC gene was amplified successfully and the identity of the Zn solubilizing PGPR was confirmed as shown in Fig. 3.15. The gene sequences have been submitted to National Center for Biotechnology Information (NCBI) GENBANK database.



Lane No.	Strains	Lane No.	Strains	Lane No.	Strains
1	*S. liquefaciens* FA-2	4	*S. liquefaciens* FA-2	7	*Enterobacter* sp. FA-11
2	*B. thuringiensis* FA-3	5	*B. thuringiensis* FA-3	8	*Enterobacter* sp. FA-11
3	*S. marcescens* FA-4	6	*S. marcescens* FA-4	9	*P. aeruginosa* FA-9
C	Control	L / M	Ladder / Marker	10,11	Reference strains

Fig. 3.13 IGS amplification and RFLP analysis by endonuclease enzymes (Hae III (BsuR1) and Hha I



Lane No.	Strains	Lane No.	Strains	Lane No.	Strains
1	*S. liquefaciens* FA-2	4	*S. liquefaciens* FA-2	7	*Enterobacter* sp. FA-11
2	*B. thuringiensis* FA-3	5	*B. thuringiensis* FA-3	8	*Enterobacter* sp. FA-11
3	*S. marcescens* FA-4	6	*S. marcescens* FA-4	9	*P. aeruginosa* FA-9
C	Control	M	Marker		

Fig 3.14 *gyrB* gene amplification of ZSB

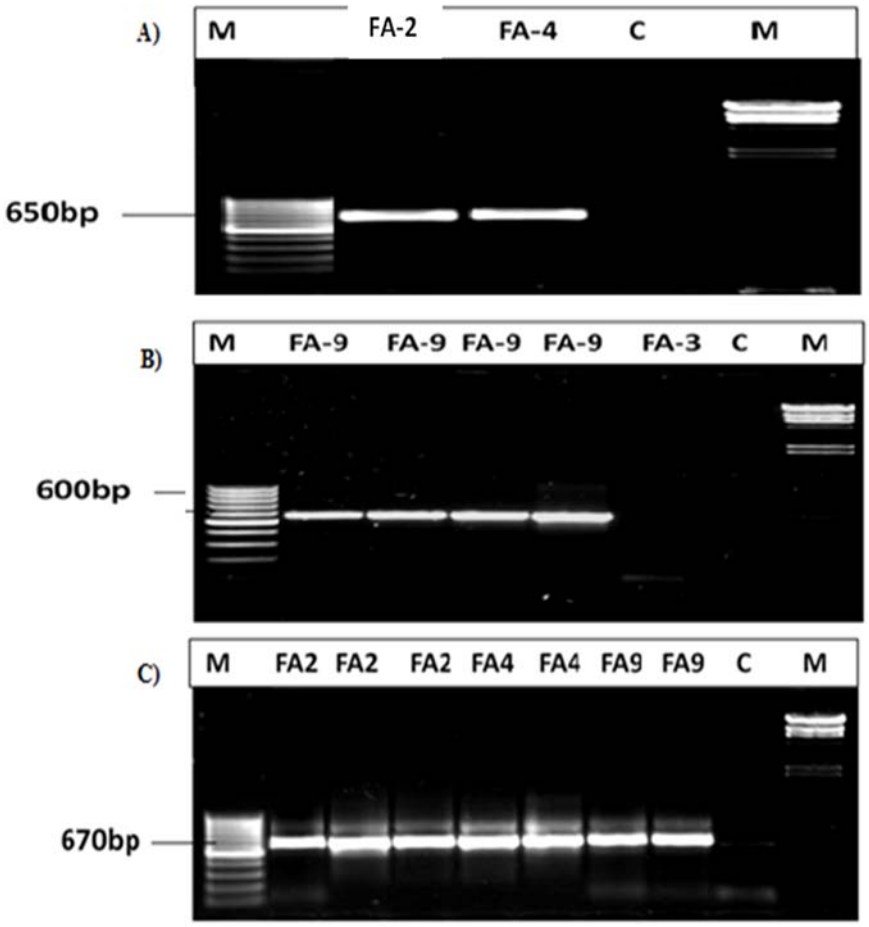


Fig. 3.15 *PqqC* gene amplification of ZSB by using various set of primers (A) *PqqC*_Sm (B) *PqqC*_Pa (C) *PqqC*_Gen

Table 3.7 *16S rRNA*, *gyrB* and *PqqC* gene sequences of ZSB submitted to GENBANK at NCBI

Serial no.	**Accession no**			**PGPR**
	16S rRNA	*gyrB*	*PqqC*	
1	KC935383	KJ813005	submitted	*S. liquefaciens* FA-2
2	KC935384	KJ813006	submitted	*B. thuringiensis* FA-3
3	KC935385	KJ813007	submitted	*S. marcescens* FA-4
4	KC935386	KJ813008	submitted	*P. aeruginosa* FA-9
5	N.D	KJ813009	submitted	*Enterobacter* sp. FA-11

N.D = Not detected

3.3. Field evaluation of Zn solubilizing PGPR on wheat

Experiment I

3.3.1. Effect of Zn solubilizing PGPR on wheat grown at AARI, Faisalabad

Data pertaining to the days to headings, tillers plant^{-1} and yield as affected by different treatments are given in Table 3.8. Data for days to headings is found to be statistically significant. Maximum days to anthesis (106) were recorded with absolute control while minimum days (104) were observed with treatments FA-2, FA-4 and Zn fertilizer respectively. Grains spike^{-1} were significantly higher (consortium = 59, FA-2 = 59, FA-4 = 58, FA-3 = 56) as compare to other treatments (fertilizer = 55, negative Zn mobilizer strain = 52). The lowest number of grains per spike (50) was recorded from control where no fertilizer / inoculants were applied.

Data showing number of tillers plant^{-1} is presented in Table 3.8 indicated that different treatments of Zn mobilizing PGPR were found to be highly significant. Highest number of tillers plant^{-1} (14) was obtained where consortia of three strains (FA-2 + FA-3 + FA-4) was applied while treatments FA-2 and FA-3 was observed with similar number (13) of tillers plant^{-1}. Lower number of tillers was recorded for Zn fertilizer treatment (12) and minimum number of tillers plant^{-1} (11) was attained from control where neither fertilizer nor inoculum was applied.

Relative study of the means confirmed that, maximum grain yield (8.5 t ha^{-1}) was recorded in plots where consortia (FA-2 + FA-3 + FA-4) was applied. This treatment showed 9% increase in yield as compared to Zn fertilizer treatment (Fig. 3.16). It was statistically at par with FA-2 (8.0 t ha^{-1}), FA-3 (8.0 t ha^{-1}), FA-4 (8.0 t ha^{-1}) and Zn fertilizer treatment (7.8 t ha^{-1}). The lowest value was noticed with non zinc mobilizer strain treatment (7.2 t ha^{-1}) followed by control having yield 7.0 t ha^{-1}. It was evident from the results that Zn mobilizing rhizobacterial application significantly increased the grain yield of wheat as illustrated (Table 3.8) (Fig. 3.16).

Among wheat cultivars, variations were observed for growth and yield parameters. The cultivars Chakwal-50 and Lasani-08 showed statistically at par values for tillers plant^{-1}, grain yield and biomass followed by the cultivars SH-2002 and Inqlab-91 (Table 3.8).

3.4. Zinc analysis of wheat grain grown at AARI, Faisalabad

Grain Zn concentration affected by different treatment is given in Table 3.9. The significant increase in the Zn concentration 64, 79, 81 and 64% was found over control in wheat genotypes Chakwal-50, Inqlab-91, Lasani-08 and SH-2002 respectively treated with consortium (FA-2, FA-3 and FA-4). Variable effect was observed by the individual bacterial inocula with all four wheat varieties. Data revealed that the consortium contributed more to grain Zn concentration compared with individual bacterial strains (Fig. 3.17). Less increase in grain Zn concentration 6.5, 7.0, 15.2 and 12.5% was noticed over control by Zn fertilizer treatment with genotypes Chakwal-50, Inqlab-91, Lasani-08 and SH-2002 in that order (Table 3.9).

Table 3.8 Growth and yield parameters of wheat crop inoculated with ZSB at Ayub Agriculture Research Institute (AARI), Faisalabad

Experiment 1

Treatments **average over varieties**	Days to headings (No.)	Grains spike^{-1} (No.)	Tiller plant^{-1} (No.)	Grain yield (t ha^{-1})	Biomass (t ha^{-1})
Control	106 ^{B}	50 ^{C}	11 ^{D}	7.0 ^{C}	9.5 ^{B}
S. liquefaciens FA-2	104 ^{A}	59 ^{A}	13 ^{B}	8.0 ^{B}	10.9 ^{A}
B. thuringiensis FA-3	105 ^{A}	56 ^{B}	13 ^{B}	7.8 ^{B}	10.7 ^{A}
S. marcescens FA-4	104 ^{A}	58 ^{A}	12 ^{C}	8.0 ^{B}	10.2 ^{AB}
Consortium*	105 ^{A}	59 ^{A}	14 ^{A}	8.5 ^{A}	10.9 ^{A}
Zinc fertilizer	104 ^{A}	55 ^{B}	12 ^{C}	7.8 ^{B}	10.7 ^{A}
Non Zn mobilizing strain FA-1	106 ^{B}	52 ^{C}	11 ^{D}	7.2 ^{C}	10.5 ^{B}
Varieties **average over strains**					
Chakwal-50	104 ^{B}	56.1 ^{AB}	13.3 ^{A}	8.1 ^{A}	10.7 ^{A}
Inqlab 91	105 ^{A}	55.2 ^{BC}	11.9 ^{B}	6.9 ^{C}	10.1 ^{B}
Lasani-08	104 ^{B}	53.9 ^{C}	12.6 ^{A}	8.3 ^{A}	10.7 ^{A}
SH-2002	104 ^{B}	57.1 ^{A}	11.5 ^{B}	7.7 ^{B}	10.5 ^{AB}
Treatments (*P*)	<0.001	<0.001	<0.001	<0.001	0.001
Varieties (*P*)	0.005	0.002	<0.001	<0.001	0.054
Treatments x Varieties (*P*)	0.841	<0.001	0.170	0.007	0.086

Mean values (n=3); Mean values with different letters in the same column differ significantly at $P<0.05$ based on LSD

* *Serratia liquefaciens* FA-2 + *Bacillus thuringiensis* FA-3 + *Serratia marcescens* FA-4

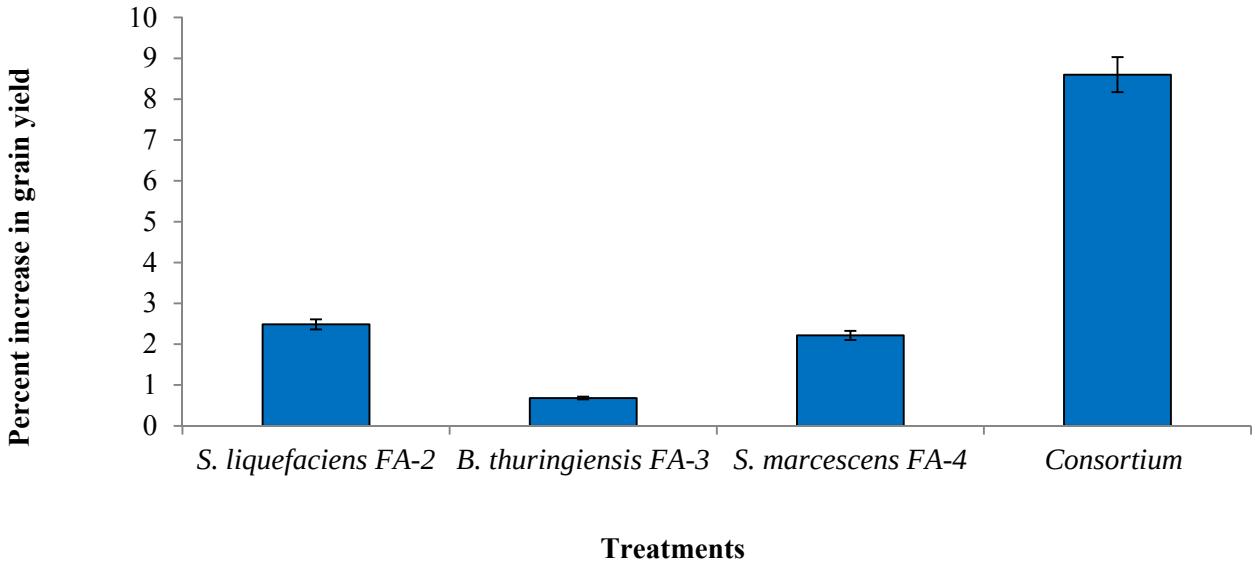


Fig. 3.16 Percent increase in grain yield over chemical fertilizer at Ayub Agriculture Research Institute (AARI) inoculated with ZSB (Experiment I)

Table 3.9 Grain Zn concentration of four wheat genotypes inoculated with ZSB at Ayub Agriculture Research Institute (AARI), Faisalabad

Experiment I

Treatments	Zn contents (mg kg^{-1})			
	Chakwal-50	Inqlab 91	Lasani-08	SH-2002
Control	33.0 ^{D}	28.6 ^{D}	27.6 ^{D}	32.6 ^{E}
S. liquefaciens FA-2	47.3 ^{B}	33.6 ^{BC}	35.0 ^{BC}	44.0 ^{BC}
B. thuringiensis FA-3	41.0 ^{C}	34.9 ^{BC}	35.2 ^{BC}	40.1 ^{CD}
S. marcescens FA-4	48.2 ^{B}	37.5 ^{B}	38.6 ^{B}	46.0 ^{B}
Consortium *	54.1 ^{A}	51.4 ^{A}	49.9 ^{A}	53.6 ^{A}
Zinc fertilizer	35.1 ^{D}	30.6 ^{CD}	31.8 ^{BCD}	36.7 ^{DE}
Non Zn mobilizing strain FA-1	34.1 ^{D}	31.6 ^{CD}	31.3 ^{CD}	34.0 ^{E}
LSD	4.9	4.68	7.24	5.37

Mean values (n = 3); Mean values with different letters in the same column differ significantly at $P<0.05$ based on LSD

* *Serratia liquefaciens* FA-2 + *Bacillus thuringiensis* FA-3 + *Serratia marcescens* FA-4

3.5. Root colonization assay

Three Zn solubilizing strains were selected for field trials based upon *in vivo* plant growth promoting activity. Characterization for root colonization ability showed that the strain FA-2 (log_{10} 7.66 CFU mL^{-1}) exhibited maximum colony forming unit (CFU) while FA-4 (log_{10} 6.90 CFU mL^{-1}) and FA-3 (log_{10} 5.88 CFU mL^{-1}) were also positive in rhizosphere at germination. Mean values of strain FA-2, FA-3 and FA-4 showed significant variation among each other for endophytic colonization at germination. Our results revealed that strain FA-4 possessed high root colonization ability, i.e. log_{10} 4.38 CFU mL^{-1} followed by the FA-2 strain at harvesting. Strain FA-3 showed low root colonization ability compared with the other strains (Table 3.10).

3.6. Percent (%) survivability

Characterization for root colonization ability showed that the strain FA-4 exhibited maximum value of survivability (72.9 %) followed by FA-2 (69 %) and FA-3 (63 %) in rhizosphere. All three strains showed significant variation for endophytic survivability. Our results revealed that strain FA-2 had highest survivability (99 %) followed by the strain FA-3 (97 %) and strain FA-2 (81 %) (Fig. 3.17).

Experiment II

3.7. Effect of Zn solubilizing PGPR on wheat grown at BARI, Chakwal (Rain fed zone) and CRS, Multan (Irrigated zone)

3.7.1. Effect of Zn solubilizing rhizobacteria on growth and development

Data pertaining to growth and development show clearly that the flag leaf area significantly increased due to the application of Zn solubilizing PGPR although location and varietal differences also exist significantly as shown in Table 3.11. Highest value (18 cm^2) for flag leaf area was observed with inoculation of Zn solubilizing strains FA-2 and FA-4 followed by consortium of the strains and Zn chemical fertilizer as shown in Table 3.11. Among four varieties, SH-2002 and Inqilab-91 exhibited maximum leaf area (18 cm^2) followed by Chakwal-50 and Lasani-08 respectively (Table 3.12).

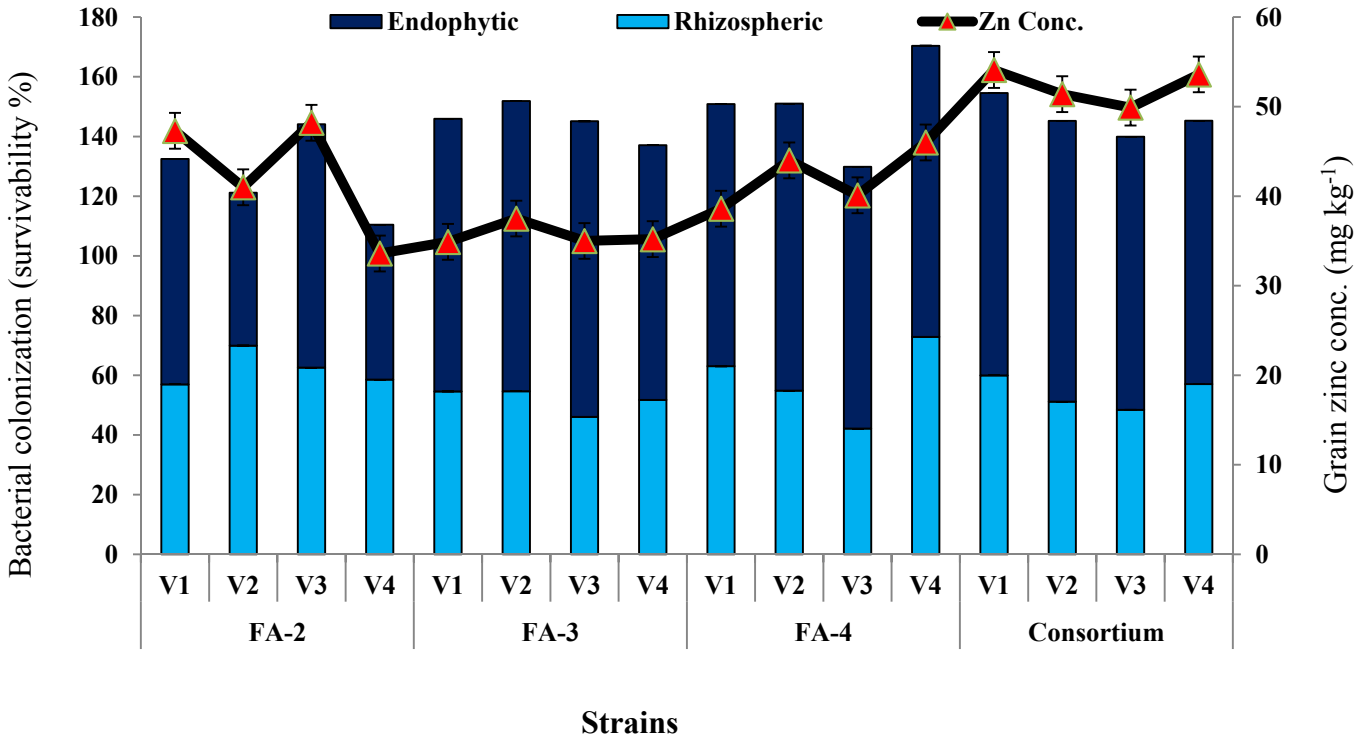


Fig. 3.17 Percent (%) survivability (rhizospheric and endophytic colonization) of ZSB and grain zinc concentration (mg kg^{-1}) of wheat after inoculation at AARI (Experiment I); Data are mean values of three replicates; *S. liquefaciens* FA-2, *B. thuringiensis* FA-3, *S. marcescens* FA-4; Consortia = *S. liquefaciens* FA-2 + *B. thuringiensis* FA-3 + *S. marcescens* FA-4

Table 3.10 Colonization of inoculated ZSB on the roots of wheat at AARI, Faisalabad. **Experiment I**.

Strains	Root colonization (log_{10} CFU mL^{-1})			
	At germination		At harvesting	
	Rhizosphere	Endophytic	Rhizosphere	Endophytic
S. liquefaciens FA-2	7.66 ^{A}	3.59 ^{B}	4.36 ^{B}	2.7 ^{c}
B. thuringiensis FA-3	5.88 ^{C}	3.58 ^{C}	3.50 ^{C}	3.03 ^{B}
S. marcescens FA-4	6.90 ^{B}	3.78 ^{A}	4.38 ^{A}	3.32 ^{A}
LSD	0.025	0.004	0.038	0.171
S.E	0.006	0.001	0.009	0.043

Mean values (n=4); Mean values with different letters in the same column differ significantly at $P<0.05$ based on LSD; CFU = colony-forming units; S.E = standard error.

Table 3.11 Growth and development of various wheat cultivars as affected by inoculation of ZSB under rain fed and irrigated conditions

Experiment II

	Days to headings (No.)	**Flag leaf area** (cm^2)	**Plant height** (cm)	**Spike length** (cm)	**Dry straw weight** (kg ha^{-1})
Treatments	<0.001	0.016	<0.001	<0.001	<0.001
Varieties	0.868	<0.001	<0.001	<0.001	0.037
Locations	<0.001	<0.001	<0.001	<0.001	<0.001
T x V	0.959	0.969	<0.001	0.372	0.044
T x L	0.003	0.919	<0.001	0.002	0.003
V x L	0.587	0.087	<0.001	0.234	0.341
T x V x L	0.986	0.97	<0.001	0.995	0.013
S.E	1.165	3.148	2.307	0.6963	302.3

Statistical analysis (n = 4) showing significance of treatments with 0.001 very highly significant; 0.01 highly significant; 0.05 significant; <0.05 non significant.

Days to heading were significantly affected by the Zn solubilizing PGPR treatment while location and treatments with locations also showed significant differences for days to headings. No varietal differences were observed for days to headings as shown in Table 3.12.

The data for spike length showed significant variation with ZSB treatments as compared to control while ZSB treatments at both location showed significant variations as shown in Table 3.9. Spike length measurement was higher (11 cm) with Zn solubilizing strain FA-2 followed by FA-3, FA-4 and consortium (10 cm) of strains as mentioned in Table 3.13.

Relative study of the means for dry straw weight, confirmed that maximum value was recorded in the plots with consortium of strains (9.73 t ha^{-1}), strain FA-4 (9.48 t ha^{-1}) and strain FA-2 (9.40 t ha^{-1}) followed by chemical Zn fertilizer Table 3.12.

3.7.2. Effect of Zn solubilizing rhizobacteria on yield and yield components

Yield and yield components of various wheat cultivars as affected by inoculation of Zn solubilizing PGPR under rain fed and irrigated conditions are shown in Table 3.13. The data for spike length indicated the significant affect of the PGPR on all four wheat genotypes and both locations. Grains $spike^{-1}$ was significantly higher (42) with strain FA-4 and FA-3 followed by FA-2 and consortium (40) as compared to Fertilizer treatment and control as mentioned in Table 3.14.

Zn solubilizing PGPR showed very highly significant affect on the thousand grain weight of the wheat genotypes at both locations as shown in Table 3.14. Thousand seed weight was higher with FA-4 (44 g) followed by FA-2 and consortium (43 g) as compared to control, chemical fertilizer and negative control (41 g).

Table 3.12 Growth and development parameters of wheat (*Triticum aestivum* L.) inoculated with ZSB under rain-fed and irrigated conditions (Experiment II)

Treatments (T) **average over V×L**	Flag leaf area (cm^2)	Days to headings (No.)	Spike length (cm)	Dry straw weight ($t\ ha^{-1}$)
Control	15	121	9	8.32
S. grimesii FA-2	18	121	11	9.40
B. thuringiensis FA-3	17	121	10	8.88
S. marcescens FA-4	18	120	10	9.48
Consortium	16	120	10	9.73
Zinc fertilizer	16	121	9	9.27
Non Zn mobilizing strain	15	121	9	8.56
LSD	2.2	0.7	0.5	0.58
S.E	1.1	0.3	0.2	0.29
Varieties (V) **average over T×L**				
Chakwal-50	15	121	9	9.24
Inqlab-91	18	121	10	8.84
Lasani-08	14	121	9	8.98
SH-2002	18	121	11	9.32
LSD	0.6	1.3	0.4	0.34
S.E	0.3	0.6	0.2	0.15
Locations (L) **average over T×V**				
Rain fed zone	10	133	9	3.90
Irrigated zone	23	109	10	14.28
LSD	1.2	0.5	0.3	0.31
S.E	0.6	0.2	0.3	0.2

Mean values (n = 4), LSD = Least significant difference, S.E = Standard error

No= number

Table 3.13 Yield and yield components of various wheat cultivars as affected by inoculation of ZSB under rain fed and irrigated conditions (Experiment II)

	Grains spikes^{-1} (No)	Thousand seed weight (g)	Tillars plant^{-1} (No)	Grain yield (t ha^{-1})
Treatments (**T**)	<0.001	<0.001	<0.001	<0.001
Varieties (**V**)	0.009	<0.001	0.031	0.473
Locations (**L**)	<0.001	0.003	0.431	<0.001
T x **V**	0.004	<0.001	0.494	0.207
T x **L**	<0.001	<0.001	<0.001	0.001
V x **L**	0.053	0.022	0.935	0.119
T x **V** x **L**	0.007	0.005	0.391	0.05
S.E	745.7	1.138	1.192	478.6

Statistical analysis (n = 4) showing significance of treatments with 0.001 very highly significant; 0.01 highly significant; 0.05 significant; <0.05 non significant

No= number

Data showing number of tillers plant^{-1} is presented in Table 3.14 indicated that different treatments of Zn mobilizing PGPR were found to be highly significant with wheat genotypes and at both locations. Highest number of tillers plant^{-1} (9) was obtained where all three strains and consortia of three strains (FA-2 + FA-3 + FA-4) was applied. Lower number of tillers was recorded for Zn fertilizer treatment (8) followed by control (7) (Table 3.14).

It was evident from the results that Zn mobilizing rhizobacterial application significantly increased the grain yield of wheat as illustrated in Table 3.14 and Fig 3.18. Comparative study of the means confirmed that, maximum grain yield (4.36 t ha^{-1}) was recorded in plots with strain FA-4 followed by consortia (4.25 t ha^{-1}), FA-2 (4.21 t ha^{-1}) and FA-3 (4.08 t ha^{-1}) respectively as described in Table 3.14. The lower value was noticed with chemical fertilizer (3.74 t ha^{-1}) followed by non zinc mobilizer strain treatment (3.70 t ha^{-1}) and control having yield 3.66 t ha^{-1}.

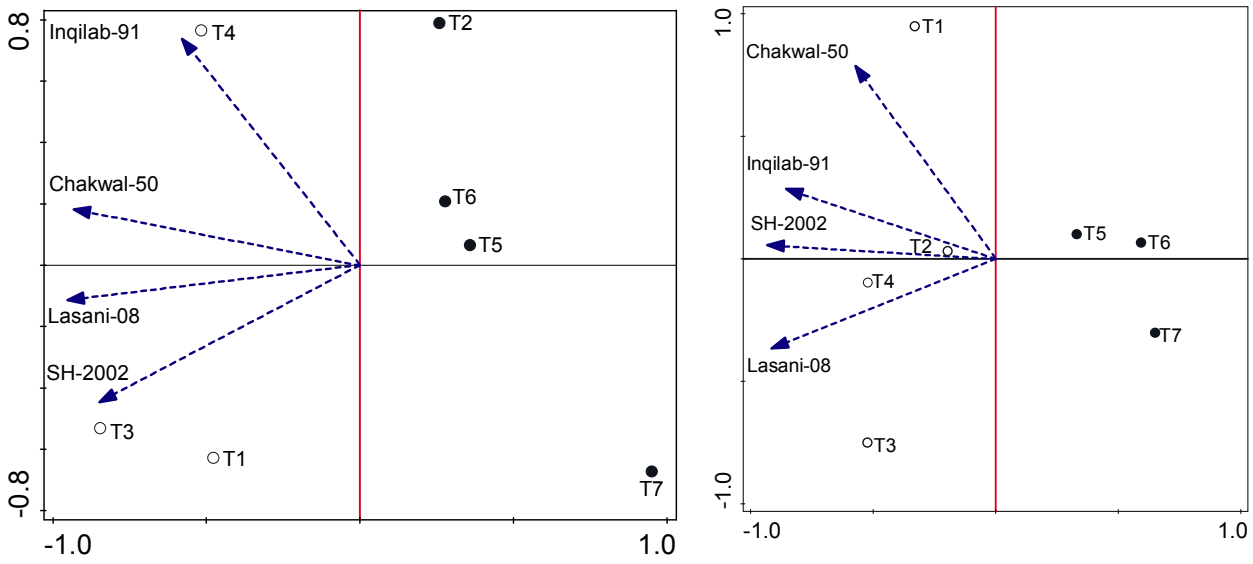
Characterization for root colonization ability at diverse climatic conditions showed that Zn solubilizing strains significantly affected the wheat genotypes in rhizosphere while no affect was observed with locations (Table 3.15). Contrary to rhizosphere root colonization, the endophytic rhizobacteria significantly affected both locations and all four wheat genotypes as illustrated in Table 3.15. Among Zn solubilizing PGPR, FA-3 showed higher rhizospheric colonization (7.6 $\log_{10}$ CFU mL^{-1}) followed by FA-2 (7.5 $\log_{10}$ CFU mL^{-1}) and FA-4 (6.8 $\log_{10}$ CFU mL^{-1}) while FA-4 indicated higher value (3.0 $\log_{10}$ CFU mL^{-1}) for endophytic colonization followed by FA-2 (2.2 $\log_{10}$ CFU mL^{-1}) and FA-3 (2.1 $\log_{10}$ CFU mL^{-1}) respectively. Our results revealed that there is no significant variation between both location for rhizospheric colonization while endophytic population significantly differ at both locations as illustrated in Table 3.16. Among various wheat genotypes, SH-2002 showed maximum rhizospheric colonization (7.5 $\log_{10}$ CFU mL^{-1}) followed by Lasani-08 (7.4 $\log_{10}$ CFU mL^{-1}) and Inqilab-91 (7.3 $\log_{10}$ CFU mL^{-1}) while Lasani-08 exhibited higher endophytic colonization (3.0 $\log_{10}$ CFU mL^{-1}) followed by Chakwal-50 (2.8 $\log_{10}$ CFU mL^{-1}). Two wheat genotypes, SH-2002 and Inqilab-91 showed at par value (2.0 $\log_{10}$ CFU mL^{-1}).

Table 3.14 Yield and yield components of various wheat cultivars as affected by inoculation of ZSB under rain fed and irrigated conditions (Experiment II)

Treatments (T) **average over V×L**	Grains spikes^{-1} (No.)	1000 seed weight (g)	Tillars plant^{-1} (No.)	Grain yield (t ha^{-1})
Control	35	41	7	3.66
S. grimesii FA-2	40	43	9	4.21
B. thuringiensis FA-3	42	42	9	4.08
S. marcescens FA-4	42	44	9	4.36
Consortium	40	43	9	4.25
Zinc fertilizer	37	41	8	3.74
Non Zn mobilizing strain	37	41	8	3.70
LSD	2.0	0.8	0.8	0.37
S.E	0.8	0.4	0.4	0.19
Varieties (V) **average over T×L**				
Chakwal-50	39	43	9	4.09
Inqlab-91	38	41	8	3.93
Lasani-08	38	41	9	3.90
SH-2002	40	43	8	4.07
LSD	2	0.6	1	0.32
S.E	1	0.3	0.2	0.14
Locations (L) **average over V×T**				
Rain fed zone	25	43	8	2.42
Irrigated zone	52	42	9	5.58
LSD	1.9	0.4	1.6	0.15
S.E	0.6	0.2	1	0.08

Mean values (n = 4), LSD = Least significant difference, S.E = Standard error

No= number



Rain fed fields

Irrigated fields

Fig. 3.18 CANOCO principal component analysis visualizing the distance between treatments (circles) and showing patterns of grain yield of different wheat cultivars (arrows) in response to various treatments under irrigated and rain fed conditions (Experiment II).

Table 3.15 Root colonization of various wheat cultivars as affected by inoculation of ZSB under rain fed and irrigated conditions (Experiment II)

	Root colonization (log_{10} CFU mL^{-1})	
	Rhizospheric	Endophytic
Strains (**S**)	<0.001	<0.001
Varieties (**V**)	0.049	<0.001
Locations (**L**)	0.485	<0.001
S x **V**	<0.001	<0.001
S x **L**	0.324	0.002
V x **L**	0.116	0.911
S x **V** x **L**	0.567	0.028
S.E	0.3168	0.141

Statistical analysis (n = 4) showing significance of treatments with 0.001 very highly significant; 0.01 highly significant; 0.05 significant; <0.05 non significant

Table 3.16 Root colonization of wheat inoculated with ZSB under rain-fed and irrigated conditions (Experiment II)

	Root colonization (log_{10} CFU mL^{-1})	
Strains (S) **average over V×L**	Rhizospheric	Endophytic
S. grimesii FA-2	7.5	2.2
B. thuringiensis FA-3	7.6	2.1
S. marcescens FA-4	6.8	3.0
LSD	0.2	0.1
S.E	0.1	0.1
Varieties (V) **average over S×L**		
Chakwal-50	7.0	2.8
Inqlab 91	7.3	2.0
Lasani-08	7.4	3.0
SH-2002	7.5	2.0
LSD	0.3	0.3
S.E	0.1	0.1
Locations (L) **average over V×S**		
Rain fed zone	7.3	2.4
Irrigated zone	7.3	2.5
LSD	0.2	0.02
S.E	0.1	0.01

Mean values (n = 4), LSD = Least significant difference, S.E = Standard error

3.8. Percent (%) survivability

All three strains showed significant variation for rhizospheric and endophytic survivability as shown in Fig. 3.19. Characterization for root colonization ability showed that the strain FA-4 exhibited maximum value of survivability (47.1%) followed by FA-2 (45.3%) and FA-3 (38.2%) in rhizosphere. Our results revealed that strain FA-2 had highest survivability (90.9%) followed by the strain FA-3 (90.5%) and strain FA-4 (83.3%) for endophytic colonization.

3.9. Zinc concentration analysis of wheat grain

Grain and straw Zn concentration of wheat affected by different Zn solubilizing PGPR under rain fed and irrigated conditions is given in Table 4. The grain Zn concentration was significantly increased over control with consortium of strains (90%) followed by FA-2 (79%), FA-4 (76%), FA-3 (55%) and chemical Zn fertilizer (45%). Data revealed that the consortium contributed more to grain Zn concentration compared with individual bacterial strains (Fig. 3.20).

3.10. Zn translocation efficiency

Zn translocation efficiency as affected by Zn solubilizing PGPR under rain fed and irrigated conditions is illustrated in Fig. 3.21. Statistically analysis revealed that Zn solubilizing PGPR significantly translocated more Zn towards grain over control/Fertilizer. The maximum value or Zn translocation efficiency was recorded with consortium of the strains followed by individual PGPR.

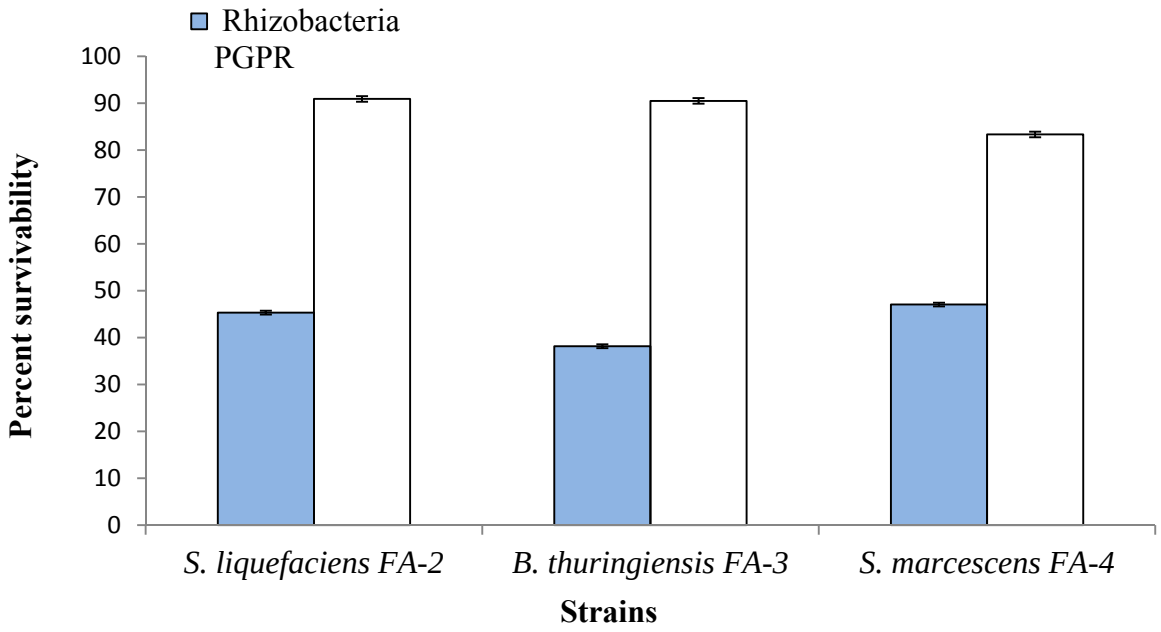


Fig. 3.19 Percent survivability of rhizospheric and endophytic ZSB under rain fed and irrigated conditions (Experiment II).

Data are mean values of three replicates; Error bars indicate the standard error

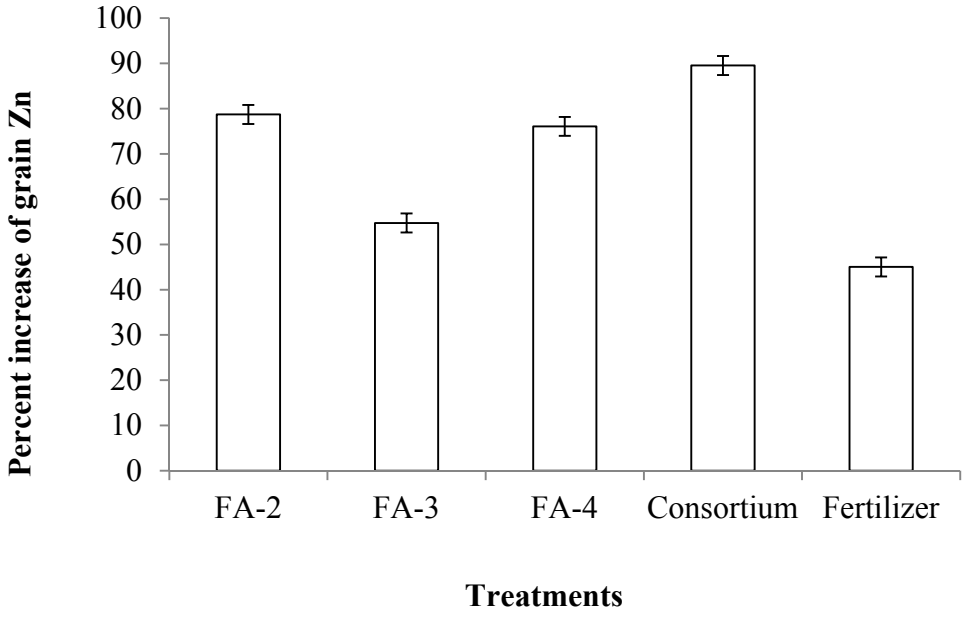


Fig. 3.20 Percent increase in grain Zn concentration over control after inoculation with different treatments under rain fed and irrigated conditions (Experiment II).

Data are mean values of three replicates; Error bars indicate the standard error.

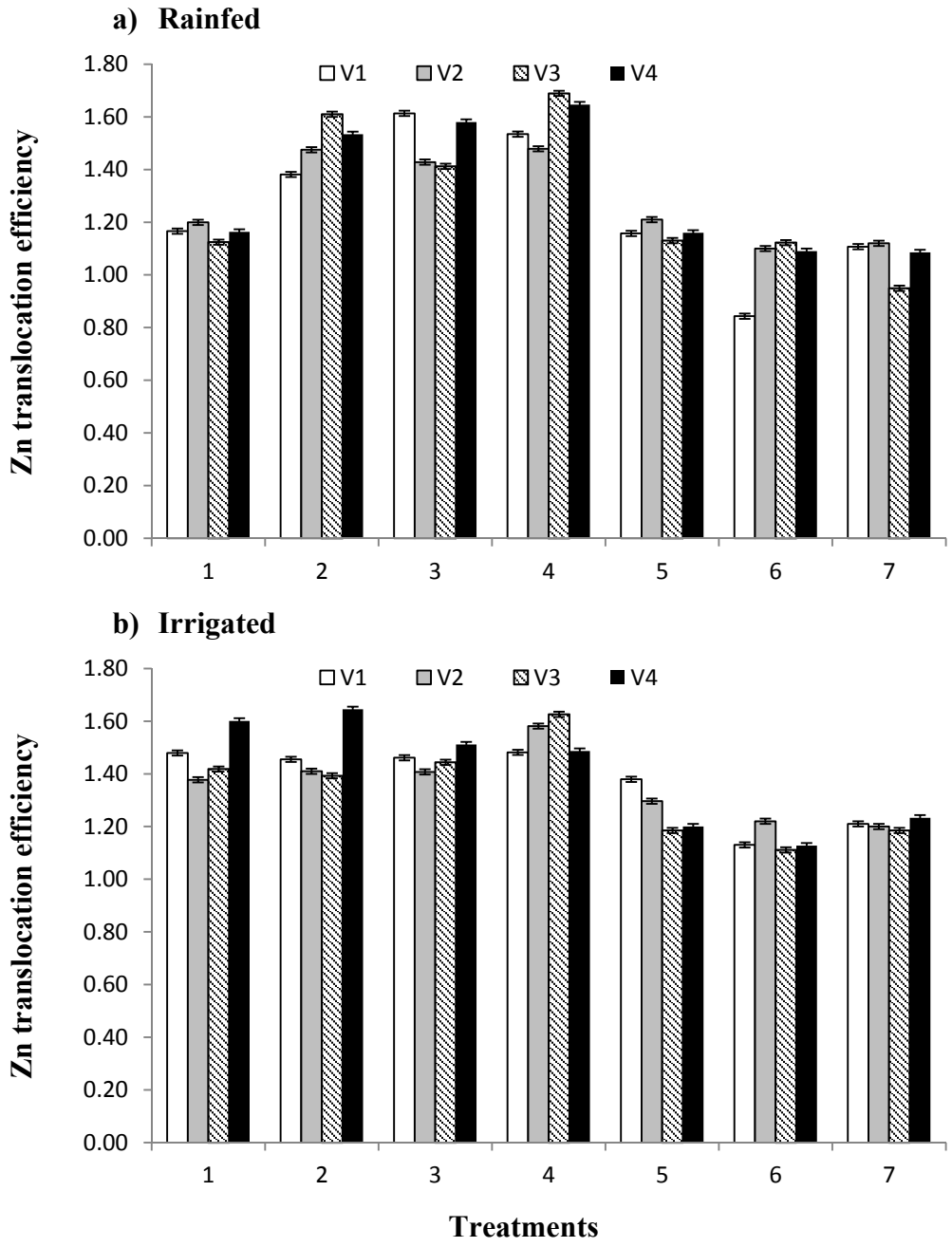


Fig. 3.21 Zn translocation efficiency in wheat with various treatments under rain fed and irrigated conditions (Experiment II)

1 = *S. liquefaciens* FA-2, 2 = *B. thuringiensis* FA-3, 3 = *S. marcescens* FA-4, FA-4 = Consortium (FA-1 + FA-2 + FA-3), 5 = Chemical Zinc fertilizer, 6 = Non Zn mobilizer strain, 7 = Control.

Data are mean values of three replicates; Error bars indicate the standard error.

Chapter 4
Discussion

4. Discussion

Developing world is facing Zn deficiency mainly due to the growth of crop plants on Zn deficient soils (Hafeez *et al.,* 2013). There are different interventions to eliminate the Zn deficiency in wheat. One such intercession is the exploitation of PGPR. Through the help of these microbes, the concentration of numerous minerals can be increased in edible tissues simultaneously with increasing yield and plant growth (Welch and Graham 2004; Cakmak *et al.,* 2010; Mäder *et al.,* 2011; Rana *et al.,* 2012; Hafeez *et al.,* 2013; Ramesh *et al.,* 2014). PGPR may induce plant growth promotion through different direct or indirect modes of action (Mitter *et al.,* 2013; Ahmad and Kibret, 2014). The proposed study is about rigorous selection of Zn solubilizer and use of these Zn solubilizing rhizobacteria to overcome Zn deficiency in wheat. For this purpose, we selected potential Zn solubilizing rhizobacteria after intensive qualitative and quantitative screening of wheat associated rhizobacteria. The population or presence of Zn solubilizer from culture collection and fresh isolates of wheat rhizosphere was 4.6 and 4%, respectively. Molecular characterization of the Zn solubilizing PGPR showed that the strains are belonging to various taxa. Moreover, these strains significantly increased wheat yield as well as Zn content of grains under rain fed and irrigated field conditions.

The plate assay results showed good potential of Zn solubilizing rhizobacteria to solubilize the insoluble Zn ores namely zinc carbonate ($ZnCO_3$), zinc oxide (ZnO) and zinc phosphate ($Zn_3(PO_4)_2$) as compared to zinc sulphide (ZnS) ore which was not solubilized. The results revealed that the ZSB has ability to solubilize Zn until 18th day after inoculation. As the plate assay has some limitations, it is not considered as relatively authentic procedure to assess the solubilization or mineralization ability of bacteria. Therefore, the rhizobacteria showing good potential of Zn solubilization on agar plate were further tested in a broth assay supplemented with the three insoluble Zn compounds (zinc carbonate, zinc oxide and zinc sulphide). The strain *S. marcescens* FA-4 and *P. aeruginosa* FA-9 solubilized $ZnCO_3$ compound distinctly over strain *B. thuringiensis* FA-3, *S. liquefaciens* FA-2 and *Enterobacter* sp. FA-11 while the strains *P. aeruginosa* FA-9

and *B. thuringiensis* FA-3 solubilized the ZnO appreciably over *S. marcescens* FA-4 and *S. liquefaciens* FA-2 and *Enterobacter* sp. FA-11. Similar studies have been performed by Saravanan *et al.* (2003) in which *Bacillus* sp. and *Pseudomonas* sp. were screened on zinc carbonate and zinc oxide with varying solubilization potential. Nutrients mobilization potential by *Pseudomonas* sp. and *Enterobacter* sp. has been well documented previously (Saharan and Nehra, 2011; Bhattacharyya and Jha, 2012). Zn solubilizing strains solubilized zinc carbonate, zinc oxide and zinc phosphate. These findings are in agreement with the previous reports (Di Simine *et al.,* 1998; Saravanan *et al.,* 2007b; Sharma *et al.,* 2014). Furthermore, solubilization of Zn was found higher with zinc carbonate and zinc oxide as compared to zinc phosphate. This is in contrast to the finding of Ramesh *et al.,* (2014), who reported that MDSR7 and MDSR14 efficiently solubilized zinc phosphate than zinc oxide and zinc carbonate. Our results are however, in accordance with Saravanan *et al.* (2007a), who indicated that *Gluconacetobacter diazotrophicus* PAL5 more effectively solubilized zinc oxide than zinc carbonate and zinc phosphate. However, Zn solubilization was not observed with zinc sulphide ore on plate assay as described earlier (Amalraj *et al.,* 2012). The solubilization of zinc sulphide by the rhizobacteria is dependent on the potential to oxidize sulphide ions (Fowler and Crundwell, 1998). Less or lack of the ability to oxidize sulphide ions might be the conceivable explanation for its slight or no solubilization in broth and plate assays. Solubilization of complex metal such as Zn enhances the bioavailability of minerals in soil by chelation or acid leaching. A range of mechanisms has been reported together with proton excretion, production of organic acids and various chelating metabolites (Hafeez *et al.,* 2013; Sharma *et al.,* 2014). Solubilization of the zinc carbonate and zinc oxide may possibly be due to excretion of various types of organic acids e.g. gluconic acids or its derivatives (2 and 2, 5 – keto-gluconic acids) by ZSB. In our study, ZSB were found to produce gluconic acid which is possibly the solubilizing factor of insoluble compounds. Gluconates are produced by fungi and bacteria belonging to *Pseudomonas* and other genera as an outcome of exterior oxidative cycle efficient on glucose and aldose sugars (Rajkumar and Freitas, 2008; Hafeez *et al.,* 2013). This may also be the probable reason of zinc ores solubilization in the present study. The Zn solubilization ability in quantitative assay was found to be inconsistent with qualitative assay. Nutrients

mobilization potential by *Pseudomonas* sp. and *Enterobacter* sp. has been well documented (Saharan and Nehra, 2011; Bhattacharyya and Jha, 2012). So, mild solubilization of ZnS in liquid broth might be due to the production of a range of organic acids by ZSB. Moreover, a correlation between plate assay and AAS quantification show, that plate assay underestimate the potential of rhizobacteria for Zn solubilization.

The microorganisms with the potential to solubilize P could enhance the availability of P and ultimately crop yield (Gull *et al.,* 2004; Zhao *et al.,* 2011). All the strains solubilized tri-calcium phosphate and zinc phosphate efficiently but FA-11 showed mild activity with tri-calcium phosphate and no solubilization activity with zinc phosphate on the agar plate. The solubilization or mineralization of phosphate might be due to synthesis of a variety of different phosphatases, catalyzing the hydrolysis of phosphoric esters (Glick, 2012). The solubilization of various inorganic P compounds might be due to the release of different organic compounds (Scervino *et al.,* 2010). Among organic acids, gluconic acid is supposed to play a role in the solubilization or mineralization mechanism of insoluble compounds (Hafeez *et al.,* 2013). The ZSB used in this study appreciably produced gluconic acid. These outcomes are in agreement with the previous reports (Fasim *et al.,* 2002; Sarvanan *et al.,* 2007a; Wani *et al.,* 2007). Bacterial strains promote the growth of cereals by their ability to fix N_2 (Shah *et al.,* 2000) and in this way provide the plant with essential nutrients. The genes for nitrogen fixation, named *nif*H are found in both symbiotic and free living bacteria (Kim and Rees, 1994). In this study, all strains except *Bacillus thuringiensis* FA-3, showed nitrogen fixing ability through *nif*H gene amplification. All the strains produced IAA in the course of tryptophan dependent pathway, which could be useful while interaction with plants as plants exudates have tryptophan that may help the IAA production potential of the bacteria. These results are in agreement with earlier reports in which IAA producing rhizobacteria were isolated and have beneficial impact on plant growth promotion (Ali *et al.,* 2013).

Exo-ploysaccharide activity is an important attribute for plant growth promotion as it provide protection from environmental intimidation as desiccation. It also contributes to bacterial aggregation, surface attachment, and plant–microbe symbiosis (Laus *et al.,* 2005). All Zn solubilizing PGPR produced the exo-polysaccharide (EPS) *in vitro* which significantly enhanced the attachment of bacteria to roots of plants for colonization. Soil environment is quite complex consisting of numerous biotic and abiotic factors, which greatly influence the traits of particular strains in the rhizosphere environment. It is well documented that the stress factors prevailing in plant rhizosphere have great impact on plant and change the metabolites of rhizosphere leading to alteration in soil microbial communities and their interaction with the host plant (Compant *et al.,* 2010). All strains were positive for EPS activity with varying potential while FA-11 showed highest activity. Similar results are significantly mentioned by Naveed *et al.* (2014) in which the *Enterobacter* spp. FD17 was found to have EPS producing ability. ACC deaminase activity of PGPR help plants to withstand stress (biotic or abiotic) by lowering the level of the stress hormone ethylene through the activity of enzyme ACC deaminase, which hydrolyzes ACC into α-ketobutyrate and ammonia instead of ethylene (Arshad *et al.,* 2007). In our study, biochemical assay and PCR amplification of *acdS* gene for ACC deaminase activity reflected that the Zn solubilizing strains are positive for this trait. Therefore, ZSB can also tolerate stress and in this way alleviate plants from abiotic stress. Fluorescent *Pseudomonas* has been characterized for ACC deaminase activity by plate assay and PCR amplification of *acdS* gene in previous studies (Ali *et al.,* 2013). Presently, a wide range of bacterial genera exhibiting ACC deaminase activity have been identified such as *Achromobacter, Alcaligenes, Azospirillum, Burkholderia, Enterobacter, Pseudomonas* and *Serratia* etc. (Zahir *et al.,* 2009; Kang *et al.,* 2010).

Growth curve analysis of ZSB with various Zn compounds showed exponential phase which was consistent with zone diameter and AAS quantification of solubilized Zn while the possible reason behind growth inhibition of ZSB in the increasing level of ZnO salts. However FA-4 showed growth unlikely to this possibility. Previously, high level tolerance to heavy metals by rhizobacteria is reported (Raju *et al.,* 1999). In this study,

ZSB showed Zn tolerance ranging from 100 to 300 ppm of Zn. *P. aeruginosa* FA-9 exhibited tolerance up to 300 ppm of ZnO. Comparable results have been described earlier by Bhat and Vyas (2014), in which *Pseudomonas sp.* a-1-8 (T-2) showed heavy metal tolerance up to 400 ppm of Zn while varying level of resistance have been reported among the PGPR such as *Bacillus* and *Pseudomonas* by Niranjan *et al.* (2004).

Siderophores are recognized for making iron (Fe) available to the plant and the production of siderophores by microorganism can bind iron with high specificity and affinity, making the iron unavailable for other microorganisms, and thereby limiting their growth. This strategy may certainly be involved in the biological control of plant diseases. Competition for iron by siderophore production has long been recognized as an important antagonistic trait found in many of the bacterial biocontrol agents against plant pathogens (Gull and Hafeez, 2009; Naureen *et al.,* 2009). Therefore, siderophore-producing Gram-negative bacteria such as *Pseudomonas* and *Enterobacter* genera are promising biological control agents (Dutta, 2006; Tian *et al.,* 2009). In our study, all strains expressed siderophore production and antifungal actions against various species of *Fusarium* particularly strains FA-9 and FA-3. The strain FA-9, FA-3 and FA-4 showed more antifungal zone as compared to all other strain. The *Pseudomonads* are reported with strong antifungal activity against *Pyricularia oryzae* and *Rhizoctonia solani* mainly through the production of antifungal metabolites (Gull *et al*., 2004; Vardhan *et al.,* 2009). A positive correlation between siderophore production and antifungal activity of the ZSB was observed. Most of the bacterial strains exploited as biopesticides belong to the genera *Agrobacterium, Bacillus and Pseudomonas* (Fravel, 2005). In this study, two ZSB (*B. thuringiensis* and *P. aeruginosa*) showed hemolytic activity. A correlation was found between hemolysis and growth inhibition of phytopathogenic fungi by these ZSB. Our results are in accordance with Monteiro *et al.* (2005) in which antifungal and hemolytic (surfactant) activities are shown to have positive correlation. Moreover, it is well documented that surfactins have biocontrol traits *in vitro* and could be involved in antimicrobial/ antifungal activities in the rhizosphere (Bais *et al.,* 2004).

For the identification and characterization of *Pseudomonas* sp. via PCR-based methods, various targets such as 16S rRNA (Relman *et al.,* 1992) and *gyrB* (Qin *et al.,* 2003) have been reported. Targeting the *gyrB* gene has been designed and appraised as a very sensitive and precise assay when tested with *P. aeruginosa* strains and species of pseudomonads closely related to *P. aeruginosa* (Lavenir *et al.,* 2007). Zn mobilizing potential among various bacterial taxa has been indicated in previous studies (Di Simine *et al.,* 1998; Fasim *et al.,* 2002; Saravanan *et al.,* 2007; Rajkumar and Freitas 2008; He *et al.,* 2010). Zn mobilizing rhizobacteria FA-2, FA-4, FA-3, FA-9 and FA-11 were identified as *S. liquefaciens*, *S. marcescens*, *B. thuringiensis*, *P. aeruginosa* and *Enterobacter* sp. on the basis of *16S rRNA* and *gyrB* gene sequencing. It has been recognized that Zn mobilizing abilities appear to be recurrent among different bacterial taxa (Di Simine *et al.,* 1998; Saravanan *et al.,* 2007; Rajkumar and Freitas, 2008). In addition to Zn mobilizing ability of *S. liquefaciens* FA-2 and *S. marcescens* FA-4, their human pathogenicity cannot be ignored and needs further toxicological studies to allow field application. The genus *Bacillus* is the center of attention for researchers as they are omnipresent in nature and have numerous traits concerning plant growth promotion (Kohler *et al.,* 2007; Ramírez and Kloepper, 2010; Zhao *et al.,* 2011). However, it is difficult to differentiate the *B. cereus* group (*B. anthracis, B. thuringiensis* and *B. cereus*) from one another via 16S rDNA sequence analysis due to their indistinguishable features (La Duc *et al.,* 2004). The *Bacillus* sp. strain FA-3 was further characterized by *gyrA* and *gyrB* gene analysis and based on that is 99% similar to *B. thuringiensis.*

Zn solubilizing rhizobacteria significantly influenced the growth, yield and Zn concentration of wheat grain over un-inoculated control and Zn fertilizer. These PGP activities are associated with their ability to ensure high availability of free zinc to the plants either through mineralization or solubilization of bound zinc with other compounds in the form of carbonates, bicarbonates and hydroxyl carbonates. This is confirmed with an increase in soil enzyme activities representing rhizobacterial climax and a turn down in the rhizosphere pH upon bacterial inoculation as reported (Neumann and Romheld, 2002; Oburger *et al.,* 2009). The considerable increase in wheat growth, yield and Zn contents by ZSB inoculation under rain fed and irrigated conditions is due to

the drought tolerance feature of PGPR as reported earlier (Marulanda *et al.,* 2007). Various direct and indirect mechanisms of PGPR have been explored behind the stress tolerance and plant growth promotion (Glick, 1995) as mentioned above in detail.

Root colonization by bacteria is a basic step to initiate the positive plant microbe interaction, which indirectly depends on the characteristics of bacteria as well as host plant. Root exudates secreted by plants are rich in nutrition for PGPR and facilitate their colonization (Bhattacharjee *et al.,* 2012). An efficient rhizobacteria must be competent to colonize roots to establish itself in the rhizosphere at population density enough to exert the beneficial effects. Inoculation of the combination of three strains showed enhanced effect as compared to individual rhizobacterial inoculation in our study. Similar results have been reported earlier (Rosas *et al.,* 2009; Yadegari *et al.,* 2010; Mäder *et al.,* 2011; Rana *et al.,* 2012; Ramesh *et al.,* 2014). It was noticed that an increase in percent survivability appreciably enhanced Zn concentration in wheat grain. Moreover, percent survivability for endophytic PGPR was higher as compared to rhizospheric zone in this study. This is also in agreement with previous studies in which *B. thuringiensis* was reported efficient for endophytic colonization (Praca *et al.,* 2012). PGPR that colonizes root is best as biocontrol agent application against soil-borne diseases, thus improving plant growth (El-Mehalawy *et al.,* 2004). Evaluation of root colonization by Zn mobilizer strains showed that rhizospheric bacterial population declines significantly at harvesting stage as compared to germination but the endophytic population showed no significant decrease under rain fed and irrigated fields. It is well documented in a previous study that bacterial strains having ability to produce EPS increased the accumulation of sunflower root rhizospheric soil under water stress as compared to well watered fields.

Zn translocation efficiency was significantly higher with the ZSB inoculation over chemical fertilizer and control at both locations such as rain fed and irrigated fields. Similar work has done previously on rice inoculated with Zn solubilizing PGPR (Tariq *et al.,* 2007). The increase in Zn concentration and yield of wheat could be attributed to the Zn mobilization potential of ZSB from the wheat rhizosphere. As the micronutrient Zn is

required in 1.24 ppm of soil concentration (Srivastava and Gangwar, 1999) but the DTP-Zn concentration of the native soils was found 0.3 - 0.5 ppm which is very low.

All the four cultivars showed varied rate of increase in grain Zn concentration over Zn fertilizer after inoculation with PGPR. The crop cultivar is an additional factor as exemplified during a study where inoculation of wheat with *Pseudomonas* strains promoted plant growth differently depending upon the wheat genotypes in saline soil (Egamberdieva, 2010). Our data suggested that cultivars should be selected based on their capacity to interact with PGPR and consortium of Zn solubilizers could be optimized to introduce and maintain a suitable population of bacteria in the soil for maximum benefit to wheat crop. This could lead to inoculate a best combination of PGPR in suitable crop cultivar for Zn biofortification of wheat. With the introduction and inoculation of potential and best performing Zn mobilizers, the farmer's community can get maximum profit from their limited resources.

4.1 Conclusion

We report the potential for using Zn solubilizers as a strategy to enhance yield and Zn contents of wheat grain. The Zn solubilizing PGPR, *S. marcescens, S. liquefaciens and B. thuringiensis* has the ability to promote plant growth by various mechanisms such as nutrients mineralization, organic acid and siderophore production, nitrogen fixation, ACC-deaminase activity, EPS production and antifungal traits while the ZSB, *P.aeruginosa* has the capacity to solubilize and tolerate elevated level of Zn. Hence, *P.aeruginosa* could be used for Zn phytoremediation. The colonizing ability and enhanced Zn translocation in wheat grains by ZSB proved these strains as efficient candidates for utilization as biofertilizers. The consortium of Zn solubilizers could be optimized to introduce and maintain a suitable density of bacteria in the soil for maximum benefit to wheat crop. Co-inoculation of PGPR in wheat crop for Zn biofortification can be an economical source to overcome Zn deficiency in the diet to save poor nations in the world. All the four cultivars showed varied rate of increase in grain Zn concentration over Zn fertilizer after inoculation with PGPR. The crop cultivar is an additional factor as exemplified during this study. The data suggested that cultivars should be selected based on their capacity to interact with PGPR and commercial field application of these bacterial strains is recommended.

Chapter 5
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Appendixes

Appendix 1
Luria Bertani (LB) medium

Yeast extracts	5 g
NaCl	5 g
Tryptophan	10 g
Agar	15 g
Distilled water	1000 mL

Adjust pH to 7.5 and then autoclave

AppendixII
Bunt and Rovira (Basal medium)

Glucose	10.g
Ammonium sulphate	1.0g
Potassium chloride	0.2g
Dipotassium hydrogen phosphate	0.1g
Magnesium sulphate	0.2g

Adjust pH to 7 and then autoclave

Appendix III
Gram s′ staining

Crystal violet stain

Crystal violet	5g
Ammonium Oxalate	2g
Ethanol	50 mL
Distilled water	200 mL

Iodine Solution

Iodine	1g
Potassium Iodide	2g
Ethanol	25 mL

Distilled water	10 mL

Safranin

Safranin	2.5 g
Ethanol	10 mL
Distilled water	100 mL

Appendix IV
Tryptic soy broth

Enzymatic Digest of Casein	17.0 g
Enzymatic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	2.5 g
Dextrose	2.5 g

Adjust pH to 7.3 and then autoclave

Appendix V
Pikovskaia Medium

$Ca_3 (PO_4)_2$	3.0 g
Sucrose	10.0 g
$(NH_4)_2 SO_4$	0.5 g
NaCl	0.2 g
$MgSO_4 . 7H_2O$	0.1 g
KCl	0.2 g
Yeast extract	0.5 g
Mn SO_4	0.004 g
Fe SO_4 (Fe-EDTA)	0.002 g
$CaCO_3$	0.3g
Agar	15 g
Distilled Water	1000 mL

Adjust pH to 7.0 and then autoclave

Appendix VI
modified DF (Dworkin and Foster)

Glucose	2.0 g
Gluconic acid	2.0 g
Citric acid	2.0 g
KH2PO4	4.0 g
Na2HPO4	6.0 g
MgSO4.7H2O	0.2 g
Micro nutrient solution (CaCl2	200 mg
FeSO4.7H2O	200 mg
H3BO3	15 mg
ZnSO4.7H2O	20 mg
Na2MoO4	10 mg
KI	10 mg
NaBr	10 mg
MnCl2	10 mg
COCl2	5 mg
CuCl2	5 mg
AlCl3	2 mg
NiSO4	2 mg

(Distill water 1000 ml) 10 ml and distill water, 990 ml; supplemented with 3 mM ACC as sole nitrogen source

Appendix VII

Chrom Azorul Sulfate (CAS) Medium

MOPS Buffer

MOPS buffer	0.5 M
Mg SO_4. $7H_2O$	10 g
$CaCl_2$. $2H_2O$	1.0 g

Stock Solution

Solution-I

CAS	0.065 g
Distilled water	50 mL

Solution-II

$FeCl_3$. $6H_2O$	0.135g
Distilled water	500 mL

Solution-III

HDTMA	0.0729 g
Distilled water	40 mL

Mix Solutions

Solution-I	50 mL
Solution-II	10 mL
Solution-III	40 mL

Adjust pH to 7.0 and then autoclave

Minimal medium

Glycerol	30 mL
K_2HPO_4	3 g
KH_2PO_4	0.5 g
NaCl	05 g
MgSO4.7H2O	0.5 g
D-Tryptophan	0.61 g
Distilled water	1000 mL

Adjust pH to 7.0 and then autoclave

Note: Autoclaved medium was amended with filter-sterilized ZnSO4 (0.35 mM) and Mo_7 $(NH_4)6O_{24}.4H_2O$ (0.5 mM).

Appendix XI

10X TBE (Tris Borate EDTA for Gel electrophoresis)

Tris base	108 g
Boric acid	55g
EDTA (0.5M)	40mL
Distilled water	1000mL

Appendix XII

1.2% agarose gel

Agarose	1.2 g
1xTBE	100 mL

Appendix XIII

1% agarose gel

Agarose	1.0 g
1xTBE	100 mL

Appendix XIV

16S *rRNA* gene sequences of *S. liquefaciens* FA-2 (Accession No. KC935383)

GCAGTCGAGCGGTAGCACAGGGAGCTTGCTCCTGGGTGACGAGCGGCGGA
CGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGG
AAACGGTAGCTAATACCGCATAATGTCTACGGACCAAAGTGGGGGACCTT
CGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGG
GTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGT
GAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGGTTCA
GTGTTAATAGCACTGTGCATTGACGTTACTCGCAGAAGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAA
TCCCCGCGCTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTT
GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTG
GAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
TGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAG
CTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT
CAAATGAATTGACGGGGGCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTCGCTA
GAGATAGCTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCACGTAATGGCGGGAACTCAAAG

16S *rRNA* gene sequences of *B. thuringiensis* FA-3 (Accession No. KC935384)

GTCGAGCGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAA
CCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAA
GGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGT

GATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCG
TGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACA
AGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCA
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA
TCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATG
TGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTG
AGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGA
GATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGAC
ACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGT
GCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAA
AACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGG

16S *rRNA* gene sequences of *S. marcescens* FA-4 (Accession No. KC935385)

CAGTCGAGCGGTAGCACAAGGGAGCTTGCTCCCTGGGTGACGAGCGGCGG
ACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCT
TCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGG
GTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGT
GAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTG
AGCTTAATACGTTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAA
TCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTC

GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTG
GAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
TGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAG
CTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT
CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCA
GAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGT
CGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAG

16S *rRNA* gene sequences of *P. aeruginosa* FA-9 (Accession No. KC935386)

GTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGA
GTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGG
CGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCT
CACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGG
CCTACCAAGGCGACGATCCGAAACTGGTCTGAGAGGATGATCAGTCACAC
TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAG
GTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAA
TACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTG
CCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGG
CTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGG
GTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC
ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGC
GATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATG

GATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCTTTGTCC
TTAGTTACCA

***gyrB* gene sequence of** *S. liquefaciens* FA-2 (Accession No. KJ813005)

TGCCGCAGCCCAGCGCGGTGATCAACGTCGCCACTTCCTGCGAAGACAGC
ATCTTGTCGAAGCGCGCCTTCTCTACGTTGAGGATTTTACCTTTCAACGGC
AGGATTGCCTGGTTCTTACGGTTACGTCCCTGCTTGGCAGAGCCGCCCGCT
GAGTCCCCTTCCACCAGGTACAGTTCAGACAGCGCAGGATCACGTTCCTGG
CAGTCCGCCAGTTTGCCCGGCAGGCCGGCCAGATCCAGCGCGCCCTTACG
GCGGGTCATTTCACGCGCTTTACGCGCCGCTTCACGAGCACGCGCTGCGTC
GATGATTTTACCGACGACGATTTTCGCATCGCCCGGGTTTTCCATCAGATA
ATCGACCAGCTTCTCGTTCATCAGCGTTTCAACCGCGGTTTTCACCTCGGA
AGACACCAGCTTGTCTTTGGTCTGAGAAGAGAACTTCGGATCAGGCACCTT
CACCGAAACCACGGCAATCAGACCTTCACGCGCATCGTCGCCGGTGGCGC
TGATTTTGGCCTTTTTGCTGTAGCCTTCTTTATCCATGTAGCTGTTCAGGGT
ACGGGTCATCGCGGTACGGAAACCGACCAGATGGGTACCGCCGTCACGCT
GCGGGATGTTGTTGGTGAAGCAGTAGATGTTTTCCTGGAAACCGTCGTTCC
ACTGCAGCGCCACTTCCACACCGATGTCGTCTTTGACGGTCGAGAAGTAGA
ACACGTTCGGGTGGATCGGGGTTTTGTTCTTGTTCAGATATTCAACAAACG
CCTTGATACCGCCTTCGTAGTGGAAGTGGTCTTCTTTGTCGGTACGTTTGTC
TTTCAGGCGGATAGACACGCCGGAGTTCAGGAAAGAGAGTTCGCGCAGGC
GCTTGGCCAGAATGTCGTACTCAAATTCGGTATTGTTGGTGAAGGTTTCAT
AGCTCGGCCAGAAACGGACCGTGGTGCCGGTCTGATCGGTTTCACCGACC
ACTTTCAGCGGCGACAATGGCTCGCCCATGGCATAGGTTTGCTCATGCACC
TTGCCTTCACGACGGATCACCAGTTCCAGCTTCTCAGACAGGGCGTTAACA
ACGG

***gyrB* gene sequence of** *B. thuringiensis* FA-3 (Accession No. KJ813006)

TTTGTACCGATTGCCGTAATAATGGTACGAACTTCATCATTTGATAAAATC
TTATCTAAGCGCGCCTTTTCCACATTAATAATTTTACCCTTCAGCGGTAAA
ATTGCTTGGAAATGACGATCGCGTCCTTGTTTTGCAGATCCACCCGCAGAG
TCACCCTCTACGATGTAAATTTCACTAATTGCTGGATCTTTCGAAGAGCAA
TCAGCTAATTTACCAGGTAAACTTGAAACTTCTAACGCACTCTTTCGACGT
GTCAATTCACGCGCTTTTTTCGCAGCTACACGTGCACGTGCAGCCATCGTA
CCTTTTTCTACAATTTTTCGCGCTACATTAGGATTTTCTAGTAAGAACTTTT
CAAATGCCTCTGAGAATACAGACTCTGTAATTGTTCTCGCTTCACTATTCC
CAAGTTTTGTCTTCGTTTGTCCTTCAAATTGTGGATTTGGATGCTTGATTGA
TACAATTGCTGTTAAACCTTCACGAACATCCTCACCAGTTAAATTACTGTC
CGCATCTTTTAAAATGCTATTTTTACGACCATAGTCGTTGATTACACGAGTT
AAAGCTGTTTTAAAACCTACCTCATGTGTACCACCTTCATACGTATGGATG
TTATTCGTAAATGAGTAAATATTATTTGTGTATCCTTCGTTATATTGAAGAG
AAACCTCAACCTGAATACCATCTTTTGAACCTTCTACGTACACAGGCTCTT
CATGAATCGGTTGTTTTGAGCGATTTAAATGCTCAACGTATGATTTAATTC
CACCTTCGTAATGGAATTCTTTCTTTTGCTTATGTTCACGTTTATCTTCAATT
GTTAATTTAATATTACGATTTAAAAACGCTAATTCACGCATACGAGTTGCT
AGCGTATCAAAATCGTATACTGTTGTTTCTTGGAAAATTTCCGGATCTGGT
TTAAATCGAGTTATTGTTCCTGTTTGATCGGTGTCACCAATGACTTTTAAAT
CCGCAACCGGAATACCTCTTTCGTATTTTTGGGAATGGATTTTACCTTCAC
GATGTACAAATACTT

***gyrB* gene sequence of** *S. marcescens* FA-4 (Accession No. KJ813007)

ATGCCGCAACCCAGCGCGGTGATCAGCGTCGCCACTTCCTGCGAAGAGAG
CATTTTGTCGAAACGCGCTTTCTCGACGTTGAGGATTTTCCCCTTCAGCGG
CAAGATAGCCTGATTCTTGCGGTTGCGCCCCTGCTTGGCAGAGCCGCCCGC
GGAGTCCCCTTCCACCAGGTACAGTTCGGACAGCGCCGGATCGCGTTCCTG
GCAGTCCGCCAGCTTGCCCGGCAGGCCGGCCAGATCCAGCGCGCCTTTGC
GGCGGGTCATCTCACGCGCTTTACGCGCCGCTTCGCGCGCGCGCCGCGT
CGATGATTTTGCCGACCACGATTTTCGCGTCGCCCGGATTCTCCATCAGGT
AATCCACCAGCTTCTCGTTCATCAGCGTTTCAACCGCGGTTTTCACCTCGG

AAGAGACCAGCTTGTCCTTGGTCTGAGAGGAGAACTTCGGATCCGGCACC
TTCACCGACACCACGGCGATCAGGCCTTCACGCGCGTCATCGCCGGTGGC
GCTGACCTTGGCCTTCTTGCTGTAACCTTCCTTCTCCATGTAGCTGTTCAGC
GTACGGGTCATCGCGGTACGGAAACCGACCAGGTGCGTGCCGCCGTCGCG
CTGCGGAATGTTGTTGGTGAAGCAGTAGATGTTTTCCTGGAAACCGTCGTT
CCACTGCAGCGCCACTTCTACGCCGATGTCGTCTTTCACGGTGGAGAAGTA
GAACACGTTCGGGTGGATCGGGGTTTTGTTCTTGTTCAGGTACTCGACAAA
CGCCTTGATGCCGCCTTCGTAATGGAAGTGGTCTTCACGGTCGGTGCGCTT
GTCCTTCAGGCGAATAGACACGCCGGAGTTGAGGAAGGACAGCTCGCGCA
GGCGCTTGGCCAGAATGTCGTACTCGAATTCGGTCACGTTGGTGAAGGTCT
GGTGGCTCGGCCAGAAACGCACCATGGTACCGGTCTGCTCGGTTTCGCCCA
CCACGTTCAGCGGCGCCTGCGGCTCGCCGTGGCTGTAAGTCTGTTCGTG

***gyrB* gene sequence of** *P. aeruginosa* FA-9 (Accession No. KJ813008)

ATGCCACAGCCCAGGGCGGTGATCAGCGTACCGACCTCCTGGGAGGAGAG
CATCTTGTCGAAGCGCGCCTTTTCGACGTTGAGGATCTTGCCCTTGAGCGG
CAGGATCGCCTGGGTCCGGCGATTGCGGCCCTGCTTGGCGGAACCGCCCG
CGGAGTCACCCTCCACGATGTACAGTTCGGAGAGCGCCGGGTCCTTTTCCT
GGCAATCGGCCAGTTTGCCGGGCAGGCCGGCGATGTCCAGCGCGCCCTTG
CGGCGGGTCATCTCGCGCGCCTTGCGCGCGGCCTCGCGGGCACGGGCGGC
GTCGATCATCTTGCCGACCACGGCCTTGGCTTCGTTGGGATTCTCCAGCAG
GAAGTCGGCGAAGTACTTGCCCATCTCCTGTTCCACCGCAGTCTTCACCTC
GGAGGAGACCAGCTTGTCCTTGGTCTGCGAGCTGAACTTCGGGTCCGGTAC
CTTCACCGAGATGATCGCGGTGAGGCCTTCGCGGGCATCGTCGCCGGTGGT
GGCGATCTTGAACTTCTTCGCCAGGCCTTCGGCCTCGATGTAGTTGTTCAG
GTTACGCGTCAGCGCCGAACGGAAACCGGCCAGGTGGGTGCCGCCGTCAC
GCTGCGGGATGTTGTTGGTGAAGCAGAGCAGGTTCTCGTTGAAGCTGTCGT
TCCACTGCAAGGCGACTTCCACACCCACGCCGTCCTCTTCACGCTGGACGT
TGAAGTGGAATACCTCGTTCACCGCGGTCTTGTTGGTGTTCAGGTACTCGA
CGAAGGCCTTCAGACCGCCTTCGTACTTGAACAGCTCCTCCTTGCCGGTAC
GCTCGTCGCGCAGCAGGATGCCGACGCCGGAGTTGAGGAAGGACAGCTCG

CGGATGCGCTTGGCCAGGATGTCCCAACTGAAGTGGATGTTGCTGAAGGT
CTCCGGGGACGGCTTGAAGTGAACTTCGGTGCCGGAGCCATCGGTCTCGC
CCACTTCGCGCAATGGGAACTGCGGAACGCCCGTGGTGGTAGACCT

***gyrB* gene sequence of** *Enterobacter* FA-11 (Accession No. KJ813009)

GGTGATAAGGGTAGCAACTTCCTGGGAAGAGAGCATTTTGTCGAAGCGCG
CTTTCTCAACGTTGAGGATTTTACCCTTCAACGGCAGAATCGCCTGGTTCTT
ACGGTTACGCCCCTGCTTCGCAGAGCCGCCCGCGGAGTCCCCTTCCACCAG
GTACAGTTCGGAGCGAGCCGGATCGCGTTCCTGGCAATCTGCCAGTTTGCC
CGGCAGGCCAGCCAGATCCAGCGCACCTTTACGGCGAGTCATTTCACGGG
CTTTACGCGCCGCTTCACGCGCACGAGCCGCATCGATAATTTTGCCGACCA
CGATTTTCGCGTCAGACGGGTTTTCCAGCAGGTATTCGCTCAGCAGCTCGT
TCATCTGCTGTTCAACGGCGGTTTTCACCTCGGAAGAAACCAGTTTATCTT
TGGTCTGAGAGGAGAATTTCGGGTCCGGGACTTTAACCGACACCACAGCA
ATCAGGCCTTCACGGGCATCATCACCGGTTGCGCTGACTTTCGCTTTTTTAC
TGTAGCCTTCTTTATCCATGTAGGCGTTCAGCGTACGAGTCATCGCCGTAC
GGAAACCGACAAGGTGTGCGCCACCATCACGCTGCGGAATGTTGTTGGTA
AAGCAGTAGATATTTTCCTGGAAACCATCGTTCCACTGCAGCGCAACTTCG
ACGCCGATACCGTCTTTTTCGGTGGAGAAGTAGAAGATATTCGGGTGGAT
AGGCGTTTTGTTTTTGTTGAGATACTCAACAAATGCCTTGATGCCGCCTTC
GTAGTGGAAATGGTCTTCTTTGCCGTCACGCTTATCGCGCAGACGAATCGA
CACGCCGGAGTTCAGGAATGACAGCTCGCGCAGGCGTTTCGCCAGAATGT
CGTATTCAAATTCAACCACGTTAGAAAACGTCTCGTGGCTCGGCCAGAAA
CGTACGGTGGTACCGGTTTTTTCCGTATCGCCAGTGACGGTCAGCGGAGCC
TGCGGCACGCCGTG

***gyrA* gene sequence of** *B. thuringiensis* FA-3

GTGCTCTTCCGGATGTTCGAGACGGTTTAAAACCGGTTCATAGACGGATTT
TGTATGCAATGAATGATTTAGGCATGACAAGTGACAAGCCTTATAAAAAA
TCCGCGCGTATCGTTGGAGAAGTTATCGGGAAATACCACCCGCACGGTGA

TTCAGCGGTATATGAATCCATGGTCAGAATGGCTCAGGATTTCAACTACCG
TTATATGCTCGTTGACGGTCATGGAAACTTCGGTTCTGTTGACGGAGACTC
AGCGGCGGCCATGCGTTATACAGAAGCAAGAATGTCTAAAATCTCAATGG
AGATTCTTCGTGACATCACAAAAGACACAATCGATTACCAGGATAACTAT
GACGGGTCAGAAAGAGAACCTGTCGTTATGCCTTCAAGGTTCCCGAATCT
GCTCGTGAACGGTGCTGCCGGCATTGCGGTAGGTATGGCAACAAACATTC
CTCCGCACCAGCTGGGAGAAATCATTGACGGTGTACTTGCTGTCAGTGAG
AATCCGGACATTACTATTCCAGAGCTTATGGAAGTCATTCCAGGGCCTGAT
TTCCCGACCGCAGGTCAAATCTTGGGACGCAGCGGTATCCGGAAAGCATA
CGAATCAGGCCGAGGCTCTATCACGATTCGGGCAAAAGCTGAGATCGAAC
AAACATCTTCGGGTAAAGAAAGAATTATCGTTACAGAGTTACCTTACCAA
GTAAATAAGGCGAAATTAATTGAGAAAATTGCTGATCTCGTAAGGGACAA
AAAGATAGAGGGTATCACAGATCTGCGTGATGAGTCAGATCGTACAGGTA
TGAGAATTGTCATTGAAATCAGACGCGATGCCAATGCAAATGTCATCTTAA
ACAATCTGTACAAACAAACTGCTCTACAAACATCTTTTGGCATCAACCTGC
TTGCACTTGTTGATGGCCAGCCGAAAGTTTTAACTCTTAAGCA

***PqqC* gene sequence of strain** *P. aeruginosa* FA-9

ATGAGCCGTGCCGCCATGGATCGCGCCGAATTCGAACGGGCGCTGCGCGA
CAAGGGACGCTACTACCATATCCACCATCCGTTCCATGTCGCGATGTACGA
GGGCCGTGCCAGTCGCGAACAGATCCAGGGCTGGGTGGCGAACCGTTTCT
ACTACCAGGTCAACATCCCGCTGAAGGACGCGGCGATCCTGGCCAACTGT
CCCGACCGCGAGGTCCGCCGCGAGTGGATCCAGCGTATCCTCGACCACGA
CGGCGCCCCCGGCGAGGCGGGCGGCATCGAGGCCTGGCTGCGTCTGGCGG
AAGCGGTGGGCCTGGAGCGCGAGCAGGTGCTGTCCGAGGAGCGGGTGCTG
CCCGGCGTGCGCTTCGCCGTCGACGCCTACGTCAACTTCGCCCGTCGCGCC
AGTTGGCAGGAGGCGGCGAGCAGTTCGCTGACCGAACTCTTCGCCCCGCA
GATCCACCAGTCGCGGCTGGACAGCTGGCCGCGCCACTATCCGTGGATCG
AGGCGGCCGGCTACGAGTATTTCCGCAGTCGCCTGGCCCAGGCCCGGCGC
GACGTCGAGCACGGCCTGCGGATCACCCTGGAGCACTATCGGACCCGCGA
GGCACAGGAACGCATGCTGGATATCCTGCAATTCAAGCTGGACGTGTTGT

GGAGCATGCTCGACGCGATGAGCATGGCCTACGAACTCGAACGCCCGCCG
TACCACACGGTGACCCGCGAGCGGGTCTGGCATCGGGGGCTGGCATCATG
A

***PqqC* gene sequence of strain** *S. liquefaciens* FA-2

TGGATCCCCCGGGCTGCAGCCCAATGTGGAATTCGCCCTTCCCGCGAGCAG
ATCCAGGGCTGGGTGGCCAACCGCTTCTACTACCAGACCAGCATTCCGATC
AAGGATGCGGCGATCATGGCCAACTGCCCGCAGCCGGAAACCCGCCGTAA
ATGGGTGCAGCGCATCCTGGATCACGACGGCTACGGCGGCAGCGAAGGCG
GCATCGAGGCCTGGCTGCGCCTCGGCGAGGCGGTTGGGCTGCAGCGCGAC
GCCCTGCTATCCGAGCAGCGGGTGCTGCCCGGCGTGCGTTTCGCGGTGGAT
GCCTACGTCAACTTCGCGCGCCGCGCCTGTTGGCAGGAAGCGGCCTGCAG
CTCGCTGACCGAACTGTTCGCGCCGCAGATCCATCAGTCGCGCCTCGACAG
CTGGCCGCAGCACTACCCGTGGATCGACGCCGCCGGTTACGACTATTTTCG
CAGCCGCCTCGGCCAGGCCAACCGCGACGTGGAACACGGCCTGGCGCTGG
CGCTGGACTATTGCGATACCGTTGAAAAGCAGCAGCGCATGCTGGAGATC
CTGCAGTTCAAGCTGGACATTCTGTGGAGCATGCTGGACGCCATGAGCAT
GGCCTAAAGGGCGAATTCCACAGTGGATATCAAGCTTATCGATACCGTCG
ACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTA
ATTGCGCGCTTGGCGTAATCATGG

***PqqC* gene sequence of strain** *S. marcescens* FA-4

TAGTGGATCCCCCGGGCTGCAGCCCAATGTGGAATTCGCCCTTCCCGCGAG
CAGATCCAGGGCTGGGTGGCCAACCGCTTCTACTACCAGACCAGCATTCC
GATCAAGGATGCGGCGATCATGGCCAACTGCCCGCAGCCGGAAACCCGCC
GTAAATGGGTGCAGCGCATCCTGGATCACGACGGCTACGGCGGCAGCGAA
GGCGGCATCGAGGCCTGGCTGCGCCTCGGCGAGGCGGTTGGGCTGCAGCG
CGACGCCCTGCTATCCGAGCAGCGGGTGCTGCCCGGCGTGCGTTTCGCGGT
GGATGCCTACGTCAACTTCGCGCGCCGCGCCTGTTGGCAGGAAGCGGCCT
GCAGCTCGCTGACCGAACTGTTCGCGCCGCAGATCCATCAGTCGCGCCTCG
ACAGCTGGCCGCAGCACTACCCGTGGATCGACGCCGCCGGTTACGACTAT

TTTCGCAGCCGCCTCGGCCAGGCCAACCGCGACGTGGAACACGGCCTGGC
GCTGGCGCTGGACTATTGCGATACCGTTGAAAAGCAGCAGCGCATGCTGG
AGATCCTGCAGTTCAAGCTGGACATTCTGTGGAGCATGCTGGACGCCATG
AGCATGGCCTAAAGGGCGAATTCCACAGTGGATATCAAGCTTATCGATAC
CGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGAG
GGTTAATTGCGCGCTTGGCGTAATCATG.

List of Publications

- Hafeez FY, **Abaid-Ullah M** and Hassan MN. 2013. Plant growth promoting rhizobacteria (PGPR) as zinc mobilizers: promising approach for cereals biofortification. In: Bacteria in Agrobiology: Crop Productivity. Dinesh K. Maheshwari, Meenu Saraf and Abhinav Aeron (Eds). Springer Publishers. Germany.

- **Abaid-Ullah, M**, Hassan M.N, Jamil, M, Brader, G, Shah, M.K.N, Sessitcsh, A and Hafeez, F.Y. (2015). Plant Growth Promoting Rhizobacteria: An Alternate Way to Improve Yield and Quality of Wheat (*Triticum aestivum*). *International Journal of Agriculture and Biology*, 17: 51-60

- **Abaid-Ullah, M**, Hassan, M.N, Brader, G, Sessitcsh, A and Hafeez, F.Y. Biofertilizer / biopesticide potentiality of zinc solubilizing rhizobacteria isolated from the wheat rhizosphere grown in arid zone. (Submitted)

- **Abaid-Ullah, M**, Hassan, M.N, Jamil, Shah, M.N.K and Hafeez, F.Y. Efficacy of Zn solubilizing PGPR under rain fed and irrigated field conditions. (Submitted)