THE APPLICATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA AS BIOFERTILIZER FOR CEREAL CROPS (WHEAT, RICE AND MAIZE) OF DISTRICT SWAT, KHYBER PAKHTUNKHWA, PAKISTAN

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DOCTOR OF PHILOSOPHY
IN
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By

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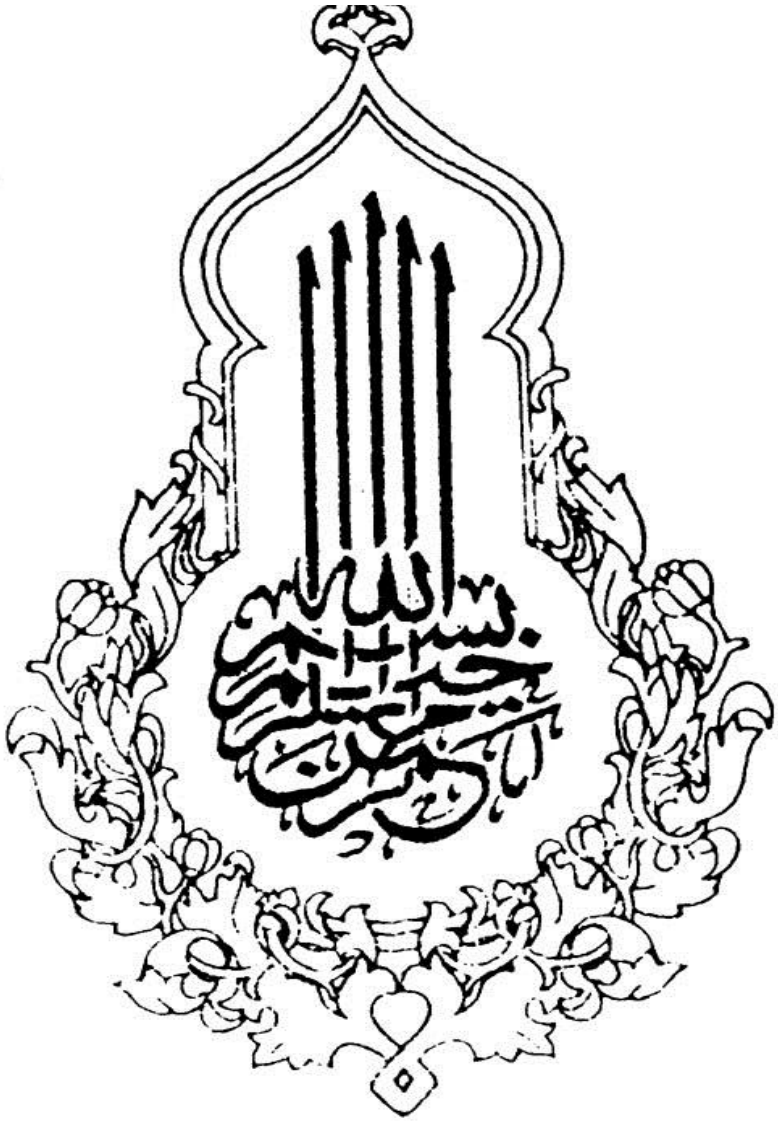
In The Name Of Almighty Allah, The Most Merciful And Mighty



CERTIFICATE

This thesis titled "The Application of Plant Growth Promoting Rhizobacteria as Biofertilizer for Cereal Crops (Wheat, Rice and Maize) of District Swat, Khyber Pakhtunkhwa, Pakistan" submitted by Mr. Midrarullah is hereby approved and recommended as partial fulfillment for the award of Degree of Doctor of Philosophy in Biotechnology.

Internal Examiner/ Supervisor: _____

External Examiner: _____

Director _____
Centre of Biotechnology and Microbiology

Dated: **/ / 2013**

DEDICATION

They fed me when I was hungry, gave strength when weak,
Protected me when in danger, taught me to walk on
my feet, nursed me when hurt, encouraged
when dejected and taught me to live
honorable in this world.
I dedicate this effort
of mine to my

PARENTS

from the depth of my heart.

May God bless them and give me a chance to serve them better
"Amin"

Midrarullah

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Midrarullah

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LIST OF ABBREVIATIONS

ARA	Acetylene Reduction Assay
ARIN	Agriculture Research Institute (North), Mingora, Swat
BNF	Biological Nitrogen Fixation
CFU	Colony Forming Unit
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribose nucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
FID	Flame Ionization Detector
GA	Gibberellic acid
g	Gram
IAA	Indoleacetic acid
Kb	Kilo base pair
LB	Luria Bertani
mL	Milli Liter
µg	Microgram
µL	Micro Liter
NFM	Nitrogen Free Medium
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
rpm	Revolutions per minute
RAPD	Random Amplification of Polymorphic DNA

Abstract

The present study was conducted to isolate and characterize Plant Growth Promoting Rhizobacteria (PGPR) from the rhizosphere of cereal crops at Swat and to assess their impact on plant growth when used as inoculants. A total of 18 bacterial strains were isolated from roots and rhizosphere of cereal crops. On the basis of colony and cell morphology, 4 strains were identified as *Azospirillum*, 11 as *Pseudomonas* strains and three strains remained un-identified. With the exception of 3 strains, all isolates showed IAA production in pure culture. Three bacterial strains (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) were used to inoculate two varieties of wheat (Inqilab 91 and Fakhre Sarhad), two varieties of rice (Fakhre Malakand and JP 5) and one variety of maize (Pahari) at two experimental sites in Swat (ARIN Mingora and Udigram). Among the bacterial strains tested in the present study, *Azospirillum brasilense* strain R1 was more effective in plant growth promotion than other strains for both wheat and rice varieties. *Azospirillum lipoferum* strain RSWT1 showed more positive response than other strains on the yield and growth of maize variety Pahari. The plant height of wheat variety Inqilab 91 was significantly increase up to 18.5 % with *Azospirillum brasilense* strain R1 as compared to non-inoculated control ones. The increase in plant height with *Azospirillum lipoferum* strain RSWT1 was 14.7 % and with *Pseudomonas* Ky1 9.6 %. The number of grains/spike, root and shoot weight and biological yield of the plants inoculated with *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* Ky1 were significantly greater as compared to control treatment. In case of wheat variety Fakhre Sarhad, the inoculation strains also showed positive effects on the growth and yield. At ARIN Mingora, Swat,

inoculation of rice variety Fakre Malakand with *Azospirillum brasilense* strain R1 increased the straw weight by 16.6 %, grain weight by 22.7 % over control. Inoculation of rice variety JP 5 with *Azospirillum brasilense* strain R1 showed 19 % increase in the straw weight and 39.5 % increase in the grain weight. At this experimental site, inoculation with *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 increased grain weight by 4.8 – 13.5 % and 17.3 –18.5 % respectively of the rice varieties Fakre Malakand and JP5. At Udigram, Swat, inoculation of rice variety Fakre Malakand with *Azospirillum brasilense* strain R1 increased the straw weight by 14.2 % and grain weight by 22 % over control. In the rice variety JP 5, any significant beneficial effect of inoculation with *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 was not observed whereas inoculation with *Azospirillum brasilense* strain R1 showed positive results of 15.5 % and 27.4 % increase over control in straw weight and grain weight respectively. The rice variety JP 5 was more responsive to the inoculated strains than rice variety Fakre Malakand. In case Of maize variety Pahari, plant height was significantly increase up to 8.82 % with *Azospirillum lipoferum* strain RSWT1 and with *Azospirillum brasilense* strain R1 up to 6.52% as compared to non-inoculated control ones. The number of ears/plant, number of grains/ear, number of leaves/plant and stem thickness and 1000 grain weight were significantly affected by bacterial inoculation.

Chapter 1

Introduction and Review of Literature

Pakistan is located between 23° - 37° Northern latitude and 62° - 75° Eastern longitude in the North East of Indo-Pak Sub-continent. Agriculture is the backbone of Pakistan economy. Majority of the population (about 68 %) is involved directly or indirectly in agriculture through farming, processing, production and distribution of agriculture commodities. Pakistan has a total geographical area of 79.61 million hectares, out of which 22.10 million hectares are under cultivation. The total land area of Khyber Pakhtunkhwa is 10.2 million hectare, out of which nearly 10 % are under cultivation. The important cereal crops of Khyber Pakhtunkhwa are wheat, rice and maize etc. Pakistan is gifted with two crop seasons i.e. Kharif and Rabi. Kharif crops are cultivated during April- June and harvested from October – December. The important crops of Kharif are rice, maize, sugarcane, cotton, mung and bajra. The cultivation of second growing season "Rabi" starts from October to December and is harvested during April - May. The important "Rabi" crops are wheat, gram, barley, mustard, rapeseed and tobacco [1].

Wheat *(Triticum aestivum* L.) is the leading staple food grain all over the world and one of the most important crops of Pakistan. It belongs to family Poaceae. The contribution of wheat to the value added agriculture is 14.4% and about 3 % to Gross Domestic Products (GDP). Pakistan has an area of 9046 thousand hectares under wheat

cultivation. The production of wheat in Pakistan is about 24032.9 thousand tons/annum with national yield of about 2657 Kg/ha. In Khyber Pakhtunkhwa, the total yield of wheat is 769.5 tons [2].

Rice is an important Kharif crop of Pakistan, ranking second to wheat as a staple food. Rice is a monocotyledonous plant of the genus *Oryza* L, sub family Oryzoideae, of the family Poaceae (Graminae). Rice is cultivated between 36^{0} East South – 55^{0} East North and grow from sea level to an altitude of 2,500 M or even higher. About 92 % of total rice is produced and consumed in Asia [3]. Rice has gradually moved to occupy a pre dominant position in the agriculture economy of Pakistan and is the country's third largest crop in term of area [4]. The contribution of rice to the value added agriculture is 4.9 % and 1.0 % of GDP. Rice is cultivated on an area of 2571 thousand hectares with annual production of 6160 thousand tones [1].

In Khyber Pakhtunkhwa, rice is grown under two different agro-climate conditions i.e. the plain and the upper mountainous valleys. Most of the cultivated area (81%) out of a total of 64719 hectares is situated in the cooler, high altitude areas of Malakand, Hazara Division and adjacent tribal areas of Khyber Pakhtunkhwa. In Swat during 2007 – 2008 rice was grown in an area of approximately 7349 hectares [5].

Maize is an important Kharif crop and belongs to the family Poaceae. It occupies third position in world production of cereal next to wheat and rice. It has short life cycle and can be grown twice a year for grain and fodder purposes as spring and summer crop. In Pakistan, the total area under maize cultivation is about 1044 thousands hectares with

2907 thousand tones yield per annum and 2784 Kg per hectare average yield. In Pakistan, particularly in Khyber Pakhtunkhwa, much effort have been made to increase cereal crops yield per unit area but the total production is still far behind to other cereal crops producing countries of the world. [2].

In Pakistan, chemical fertilizers are the most expensive input in agriculture production. The application of balance fertilizer contributes to increase yield of different crops from 30-60 %. It has been estimated that 1 Kg of chemical fertilizer nutrient produces eight Kg of cereal (wheat, rice and maize). Pakistani soil is deficient in nitrogen (N), phosphorus (P) and potassium (K). These are essential nutrients for plant growth. The utilization of land for a single crop results in depleting soil fertility because land is intensively cultivated and using only essential plant nutrients. The future crops are threatened from essential nutrients when the soil goes without being replenished.

The increase in the yield of cereal crops will be largely depend upon availability of essential nutrients like nitrogen and phosphorus in the soil which can be supplied as chemical fertilizers. However, the increased use of chemical N fertilizers is not affordable by most farmers and their excessive use often results in environmental pollution [6-8]. Therefore microbe-based technologies are becoming popular which can provide nutrients and plant-growth promoting compounds in the rhizosphere of plants. In the last few decades, the advancement in the field of agriculture biotechnology has unlocked new avenues and made possible the application of soil microorganisms for improving the crop production. Plant growth promoting rhizobacteria (PGPR) play significant role in

agricultural environment and are being utilized for sustainable agriculture production. Various mechanisms utilized by the microbes for promotion of plant growth include fixation of nitrogen in the soil, phytohormones production, phosphate solubilization and synthesis of chemicals and enzymes for biocontrol of minor pathogens [9, 10].

1.1. Soil bacteria and rhizosphere colonization

A variety of beneficial bacteria is attached to roots and proliferates on root or found in close association with root and rhizosphere of plants. This attachment of bacteria to the root is known as root colonization [9]. The soil around the root and under the influence of roots is called rhizosphere soil. Complex interaction takes place between the roots and soil microorganisms at this site or space. The soil bounded by plant roots and often a few mm extended area are included in this space [11]. The plant root epidermal layer can also be included in this area [12]. The successful colonization in the plant rhizosphere and its persistence are essential for the positive effects of PGPR on plant growth and development [13, 14]. In the application of microorganisms for useful purposes like phyto-stimulation, phyto-remediation, biocontrol and biofertilizer etc. root colonization in the rhizospheric region is an important step [15].

Various parameters that influence root colonization are root exudates, bacterial strain, living and non-living components of the ecosystem [16]. Other physiological properties might also be important in colonization process of the root system, such as attachment to the root cortex and penetration of the root tissues [17].

The effect of two associated PGPR on root colonization of lupin and pea was explained by Wiehe et al. [18]. They stated that the two strains intensively colonized the root tip and rhizoplane of lupin than the rhizoplane of pea. The fast growth of the root tip of pea may be the possible reason of sparse colonization and may be due to the limited availability of nitrogen in the rhizosphere of pea [19]. The lack of consistency in colonization may be related to changes in exudation pattern of the root during the life cycle of the plant, leading to lower numbers of *Pseudomonas* in the later phase of plant growth [20].

The effect of abiotic factors such as pH and phosphorus on root colonization has been studied by Baushe et al. [21] and Chabot et al. [22]. The former reported the influence of plant aromatic chemical compounds and pH of seed surface on the growth and colonization of cotton plant by PGPR, while the latter described the effect of phosphorus on root colonization by phosphate-solubilzing *R. leguminosarum* biovar *phaseoli* and on growth promotion of maize. The effect of temperature on root colonization have been studied by various authors and the results indicated that rhizobacteria were able to colonize roots at lower soil temperatures (5 °C) more effectively than at higher temperatures (25 °C) [16].

Weger et al. [23] reported that motility of soil microorganisms play important role in root colonization. They stated that the mutant strains have limited ability to colonize potato root because they lack flagella. A similar approach by Bashan and Holguin [24] led to the comparison of a non-motile *Azospirillum brasilense* mutant with the parental

strain. The results indicated that the wild-type was more effective in colonizing roots near the area of inoculation compared to the mutant. The role of chemosensory pathways and flagellar motility has been reviewed by Blair [25].

The bacteria present in the root region that thrives on root exudates and lysate proliferate well in the rhizosphere. The density of bacterial population in the rhizosphere region is 100 folds higher in bulk soil. The microcolonies of different bacterial strains cover upto 15% of the root surface [14, 26]. In the rhizosphere of rice a variety of diazotropic bacteria have been isolated [27-29]. The previous studies revealed that the seed and root exudates attract the rhizobacteria by chemotaxis and this may be the initial process in root and seed colonization [30-32]. The crop shows significant genotypic variation which support useful activities of soil microorganisms for the growth of plant [33]. In the absence of pathogenic microorganisms, many rhizobacteria show similar properties and promote plant growth significantly [34, 35].

1.2. Plant Growth Promoting Rhizobacteria

The free living, symbiotic and endophytic bacteria that colonize root and rhizosphere and have directly or indirectly positive effect on plant growth and development are called plant growth promoting rhizobacteria (PGPR). They directly enhance plant growth by facilitating acquisition of resources or modulating levels of plant hormones (auxin, cytokinin and gibberellin) or indirectly by acting as biocontrol agent or by reducing the effects of pathogenic agents on the growth of plant. They can be classified into two categories on the basis of their association with their host plant. They

may be extracellular (ePGPR) or intracellular (iPGPR) [36]. The extracellular PGPR include *Azotobacter, Agrobacterium, Arthrobacterium, Azospirillum, Serratia, Burkholderia, Bacillus, Chromobacterium, Pseudomonas* and *Flavobacterium,* [37]. Endophytic bacteria (*Azorhizobium, Allorhizobium, Mesorhizobium, Bradyrhizobium* and *Rhizobium* etc.) and *Frankia* species are included in Intracellular PGPR. They form symbiotic association with higher plants and fix atmospheric nitrogen [38].

PGPR play a key role in agriculture environment and are being utilized for sustainable agriculture production. They have successfully promoted the growth of many crops like wheat, rice, pea, lentil, soybean and canola by producing plant hormones, antibiotics, siderophores, fungal and bacterial antagonistic substances [10, 39-42].

1.3. Relationships of PGPR with their host plants

PGPR establish a close association with their host plant and improve the availability of nutrients to the host and thus facilitate their growth and development. The relationship between the host plant and PGPR depends on how and where the PGPR colonize the root and rhizosphere of the host plant. Some rhizobacteria show specificity towards their host. Some are general root colonizer while others are found endophytically in plant tissues [43, 44]. The interaction between soil microorganism and plant occurs in the aerial part of plant “phyllosphere”, plant internal transport system “endosphere” and soil surrounding the root “rhizospheres”. A number of endophytic bacteria associated with plants have been reported to promote plant growth and development [45]. PGPR are found attached to plant surface in many rhizospheric relationships [46, 47].

The excretory products like amino acids and sugar in the rhizosphere provide nutrient and energy for bacterial growth and results in greater increase in bacterial population. A large number of bacterial population are present in the rhizospheric region but only small portion of the total root surface (7-15%) occupied in un uniform form by microbial cells [47, 48].

1.4. Positive effects of PGPR on the plant

PGPR have positive effects on plant. They promote plant growth by increasing rate of germination, root proliferation, yield, chlorophyll content, concentration of nitrogen and magnesium, root and shoot weight, delay leaf senescence and make them resistant to drought. Another major benefit of PGPR use is disease resistance conferred to the plant, known as biocontrol [9, 49-52]. PGPR promote the circulation of nutrients, leading to higher crop production. It has been reported that inoculation with *Azospirillum brasilense,* for example, promote uptake of NO_3^-, K^+, and $H_2PO_4^-$ in rice, corn, sorghum, wheat and setaria and results in higher crop yield [53,54]. The uptake of water and nutrient by the plant is improved due to increase in root surface area and root length [47, 55-57].

The commonly reported responses of plant to the inoculations of PGPR are increases in root weight. The increase in root surface area and root length are also contributed to PGPR inoculations. The inoculation of maize with *Azospirillum brasilense* induces root hairs proliferation and dramatically increases the root surface area [39, 47, 52, 57-60].

Moreover, increase in total dry weight of plant, nitrogen contents in root and grains, rate of germination, number and weight of grain, number of spike, early heading and flowering, leaf size and plant height have been reported in rice, wheat, sorghum and maize [28, 50, 51, 61-63].

It has been reported that the yield, growth and development of non-leguminous crops like wheat, sugar beet, canola, potato and radish have been significantly affected by plant growth promoting rhizobacteria [64-65]. PGPR improve water and nutrient uptake by the plant and contribute to enhance plant growth. In different climatic region and soil type *Azospirillum* have showed promising growth promoting and high yield capacity in agriculturally important cereal crops [58].

A number of PGPR have been found in colonized form in the root and rhizosphere of important cereal crops like wheat, rice and maize [66]. Shoot weight of rice increased significantly due to inoculation with different PGPR strains [67]. Some *Azospirillum* strains inoculated to rice, wheat and maize have shown 10-30% increase in grain and forage yield [68]. PGPR increased the seed emergence, plant weight and yield [69].

Okon and Labandera-Gonzalez [70] conducted a field experiment and showed an increase of 50-60% in the yield of wheat and upto 40% increase in the yield of maize was recorded in response to *Azospirillum* inoculation. Tran Van et al. [71] conducted a field experiment on rice and reported that inoculation of *Burkholderia vietnamiensis* results in an increase of 13-22% in root and shoot weight.

PGPR confer water stress in peppers and tomatoes [72]. Several species of *Bacillus* and Fluorescent *Pseudomonas* were reported for the production of growth metabolites resulting in the improvement of plant growth [73]. Kilian et al [74] studied the mode of action of *Bacillus subtilis* strain, F2B24 against plant pathogen and reported the importance of this bacterium in plant vitality.

Cakmakci et al [75] reported positive response of wheat, maize, cucumber, potato and plea due to PGPR colonization. Increase in nitrogen fixation, nodulation, nutrient uptake and significant increase in the yield of soya bean due to inoculation of PGPR was also documented by Zhang et al. [76]. Sekan and Kandavel [77] worked on the application of PGPR as co-culture in biotization (Metabolic response of plant to microbial inoculation). They reported that inoculation of bacteria enhances biotic and abiotic stress in plants and induce physiological changes in propagules. The co-culturing of plantlets with PGPR results in the production of more biomass and secondary metabolites, e.g. co-culturing of *Pseudomonas* Spp. with plantlets of *Origanum vulgare* L. results increase in the production of chlorophyll and phenolics contents as compared to control treatment [78].

1.5. Mode of Action of PGPR

PGPR promote plant growth by direct and indirect mechanisms [39, 79]. PGPR directly promote plant growth by secreting chemical substances or by facilitating nutrient uptake from the soil. They provide plant with resources and nutrients that they require e.g. fixation of nitrogen, phosphorus and iron. Most of the agricultural lands have

deficiency of one or more of these compounds and thus plant growth is suboptimal. PGPR promote plant growth and development indirectly by removing phytotoxic materials, removal of plant pathogen, production of antibiotics and by chelation of iron. They also promote plant growth by synthesizing extracellular enzymes which hydrolyses cell wall of fungi and develop mycorrhizal association [39, 80]. In broad sense, the bacteria present in the rhizosphere and fixing nitrogen are included in PGPR. Irrespective of the plant growth promoting mechanisms, colonization of the rhizosphere around the root, rhizoplane and root tissue by PGPR is compulsory [39, 81].

Constacurta et al. [82] and Dobbelaere et al. [83] worked on phytohormones (IAA) produce by *Azospirillum brasilense* and explained the mechanism of growth promotion activities of PGPR. Similarly the work of Glick and co-workers [57, 84-87] demonstrated the role of PGPR at genetic level and worked on 1- aminocyclopropane -1-carboxylate deaminase production. Various mechanisms like fixation of nitrogen, solubilization of phosphate, synthesis of plant hormones, chemicals and enzymes for biocontrol of minor pathogens are utilized by the microbes for the promotion of plant growth.

1.5.1. Nitrogen fixation by soil microorganisms

The formation of ammonia (NH_3) from atmospheric nitrogen by the action of microorganisms using a complex system of enzyme like nitrogenase is involved in Biological Nitrogen Fixation (BNF).

Biological nitrogen fixation is becoming more important for not only reducing energy cost but also for sustainable agriculture production [88, 89]. Several diazotropic bacteria from the rhizosphere of cereal crops have been isolated [90].

In the last few decades a variety of *Azospirillum* species were found in close association with root and rhizosphere of important cereal crops [91]. Members of this *Azospirillum* have the ability to fix atmospheric nitrogen into nitrogenous compound and promote growth of plant [92]. A mixed inoculum of *Staphylococcous* and *Azospirillum* promoted the fixation of nitrogen by *Azospirillum* [92]. Combined inoculation of *Azospirillum brasilense* with *Pseudomonas striata* promoted nitrogen and phosphorus uptake by sorghum and caused significant increase in grain yield [93]. Oliveira et al. [94] demonstrated significant increase in shoot dry weight, nodulation and acetylene reduction by co-inoculation of clover with a mixture of *Rhizobium* and *Azospirillum brasilense* Sp7. It has been reported that soil microorganism associated with wetland rice, sugar cane and forage grasses fix about 10-50% of the atmospheric nitrogen [81, 91, 95-97].

Biological nitrogen fixation is carried out by some prokaryotic microbes (diazaotrophic bacteria) in free living state or in symbiotic associations with the plants. Nitrogen-fixing bacteria like *Azospirillum, Azoarcus, Burkholderia, Enterobacter*, *Gluconacetobacter* and *Herbaspirillum* are promising nitrogen fixer and are found in association with the roots of important cereal crops [98-104]. The analysis of nitrogen fixing ability of microoranism in the soil by using 15N showed that most of the nitrogen fixed remains within the root of rhizosphere environment [67].

1.5.2. Phytohormones Production

PGPR promote plant growth by producing hormones or other compounds that regulate plant developmental mechanisms [105]. The important hormones like auxin and ethylene are produced by many bacterial species [106-108]. The production of other phytohormones like Gibberellins and Cytokinins by the bacterial strains has been reported [109-110]. Synthesis of auxin in pure culture by a number of microorganisms has been reported [106, 111]. The rhizosphere isolates produced auxins more efficiently that non-rhizosphere soil isolates [111]. Indole-acetic-acid (IAA) is the principal naturally occurring auxin and is involved in cell division, cell enlargement, root development, phototropism and apical dominance [105]. A typical auxin effect is the formation of lateral roots [112].

Gutierrez-Manero et al. [113] reported the production of phytohormones by two species of Bacillus (*Bacillus licheniformis* and *Bacillus pumils*). Gibberellins and cytokinins both stimulate shoot development [105].

Cytokinins represent another class of phytohormones produced by microorganisms [114]. There are fewer studies on cytokinins synthesis by microorganisms than on microbial biosynthesis of auxins [105]. About 90% of bacterial strains in the rhizosphere of various crop plants demonstrated the ability to produce cytokinin-like compounds [115]. Similarly Kampert and Strzelczyk [116] showed that rhizospheric bacteria of pine seedlings secreted cytokinin-like substances in the growth medium. Cytokinin production by *Azospirillum brasilense, Azotobacter chroococcum* and

Azotobacter vinelandii has been reported [117-119]. Timmusk et al. [120] reported the production of cytokinins by a free-living soil bacterium *Pseudomonas polymyxa* using Immuno Affinity Chromatography (IAC). Salamone et al. [121] have reported higher production of the cytokinins dihydroxyzeatin (DHZR), zeatin riboside (ZR) and isopentenyl adenosine (IPA) by a wild type strain *Pseudomonas fluorescens* G20-18 compared to two mutants, CNT1 and CNT2.

Ethylene, which is a common plant growth inhibitor, has shown the ability to promote plant growth at low level in several plants [122]. Ethylene inhibits the root and shoots elongation and improves organ abscission and senescence at high levels [123]. Several species of PGPR are rich source of enzymes like cyclopropane, carboxalate (ACC) and deaminase [108] and lower ethylene level in stressed or developing plants by cleaving the plant ethylene precursor ACC. Ethylene is also required in sufficient amount for breaking seed dormancy before germination. The high concentration of ethylene after germination inhibits root elongation [124].

1.5.3. Siderophore Production

Plant requires iron for their active growth and development. Iron is generally insoluble in soil solution. Plant root absorbs iron in reduced ferrous ion (Fe^{2+}) form. The aerated soil commonly contains ferric (Fe^{3+}), which precipitate easily in iron oxide forms. Plant secretes organic compounds like phytosiderophores and chelators which bind with Fe^{3+}. Fe^{3+} is received by the root surface from chelators and reduced it to Fe^{2+} and then absorbs it immediately (i.e. 'Strategy I' plant). The grasses also secrete

phytosiderophores (i.e. 'Strategy II' plant). These phytosideophores were absorbed by the plant across the plasmalemma [125]. Production of siderophores by some bacteria species and its absorption in the form of bacterial Fe^{3+} siderophore complexes by a number of plant species have been reported [126, 127].

Siderophores are iron binding low molecular weight molecules. In low iron condition, they are synthesized by many microorganisms. Microbial siderophores increase the iron availability in the rhizospheric region and then stimulate plant growth [69]. Marschner and Romheld [128] reported that rhizobacteria colonizing the root synthesizes siderophores, which act as source of iron for the plant. Many plants like cotton, cucumber, oats, peanut, sorghum and sunflower have demonstrated the ability to utilize radiolabelled microbial siderophores as a source of iron [129-131]. Microbial siderophores promote the growth of cucumber significantly by increasing the chlorophyll content and biomass of plant [132]. The microorganisms living in close association with plant species or within plant tissues facilitate active uptake of microbial siderophores by the plant [130].

1.5.4. Phosphate Solubilization

Phosphate is an important mineral nutrient required for plant growth and development. Soil phosphate is present in insoluble form. Plant can utilize soil phosphate in soluble form i.e. monobasic and diabasic ions [9, 40, 133].

Phosphate solubilizing bacteria are commonly present in the rhizosphere [134-136]. They produce acidic materials which stabilize the inorganic phosphate or mineralize the organic phosphate and thus increase the quantity of soluble phosphorus for the plant in the soil [137]. These bacteria are referred to as phosphobacteria [9, 115, 138]. The PGPR increases nutrients availability to the host plant by converting insoluble phosphate to soluble form in the rhizospheric region [113-115].

Meunchang et al. [136] isolated 62 P-solubilizing bacteria strains from paddy field and suggested the potential of these strains as biofertilizer for rice. Kucey et al. [139] reviewed microbial-mediated increases in plant-available phosphorus. Cattelan et al. [61] performed an *in vitro* screening of 116 isolates obtained from soil for various PGPR traits including the ability of bacteria to solubilize phosphorus. Their study indicated that isolates, which have the ability to produce ACC deaminase or siderophores or those able to solubilize phosphorus, might increase early soybean growth in nonsterile soil.

1.5.5. PGPR as biocontrol of plant pathogens

PGPR present in the rhizosphere have the ability to act as biocontrol of plant pathogens. They secrete iron binding siderophores, synthesize antibiotics and supply it to the plant and thus protect the plant from fungal pathogens [140-142]. They also synthesize enzymes like chitinase, protease or lipase and β-1,3-glucanase which have the ability to break cells of fungi. They also provide metabolites like hydrogen cyanide which show antifungal activities and competition with plant pathogen [142, 143]. They niches on the root surface and acts as biocontrol agent [124, 144-146].

The availability of a large number of biocontrol PGPR play important role in controlling fungal pathogen and showed significant effects on plant growth. It has been reported that 105-106 Colony Forming Units (CFU) in one gram of root in case of *Pseudomonas* is the required level of colonization, which protects the plant from the pathogenic effect of *G. tritici* or *Pythium* spp.

Many researchers have reported that rhizobacteria show rapid growth and competition for carbon and energy source than fungal pathogen and thus provide a base for biological control [140-145]. Although many reports have shown that entire rhizobacterial populations can cause fungistasis in rhizosphere soil [147-150]. Recent investigations have pointed out induced systemic resistance, antibiosis and pathogen-antagonist interaction as three main mechanisms [147, 150-152].

Productions of antibiotic compounds which are not related to siderophores have been reported from a number of *Pseudomonas* strains. In vitro, these antibiotics have the ability to inhibit pathogenic bacteria, fungi, pathogenicity of higher organisms and in some cases higher organisms [150-152].

PGPR activate the plant for better protection against pathogens and restrict the pathogenic activity of soil microorganisms through Induced Systemic Resistance mechanisms (ISR) [155-156]. Induced Systemic Resistance mechanisms have been reported in about 15 plant species since its discovery [157]. PGPR also increase the plant growth by Induced Systemic Resistance [39, 158-159]. Rhizobacteria, which are non-pathogenic also stimulate inducible defense mechanisms in the plant. The inducible

defense mechanisms include synthesis of pathogenesis related proteins, production of antimicrobial phytoalexins and re-enforcement of plant cell wall [160, 161].

Members of actinomycetes like *Micromonospora* sp., *Streptosporangium* sp., *Streptomyces* spp. and *Thermobifida* spp. can be used as biocontrol agent against pathogenic fungi [162]. The pine root disease in *Pinus taeda* caused by *Armillaria* and *Fusaium* can be controlled biologically by Streptomycetes [163]. Actinobacteria can be used as biocontrol agent for tomato againt *Rhizotonia solani* and *Pseudomonas solanacearum* [164] and for banana against *Colletotrichum musae* [165].

1.5.6. Phytoremediation of heavy metals by PGPR

PGPR can be utilized for the phytoremediation of heavy metals. Metal pollution is caused by industrialization and agricultural activities. Ochiai [166] reported that contamination of soil with heavy metals caused functional blocking of molecules, displacement of essential components in biomolecules and modification of structure and function of protein/ enzymes. Heavy metals also inhibit biochemical processes like photosynthesis and respiration resulting reduce growth [167].

The solubility of metals is low in the soil and plant roots are unable to absorb metals from the soil. The availability of metals for the plants are closely related to the properties of soil and the metabolites (organic acid, siderophores and phytohormones) released by rhizobacteria [168]. Rhizobacteria alter the bioavailability of plant and directly affect plant growth dynamics. Plant growth promoting rhizobacteria also play indirect role in chelation, acidification, immobilization, or precipitation of heavy metals

in the soil. They play pivotal role in phytoremediation of metal containing soils. The addition of metal tolerant microbes to nutrient deficient contaminated sites enhances the availability of basic plant nutrients and detoxifies heavy metal contaminated soil [169].

This association between plants and microbes improve phytoremediation and can act as decontaminators. They maintain texture, structure and fertility of soil. PGPR also helps in phytostimulation and rhizovolatilization. They degrade the contaminant and transform pollutants into volatile compounds that are released into the atmosphere. They have the ability to render heavy metals inactive by immobilizing, mobilizing or transforming heavy metals [170]. They alter the solubility, availability and transport of heavy metals by reducing soil pH and by releasing chelators [171].

1.6. PGPR as biofertilizer of wheat production

A number of PGPR have been found in colonized form in the root and rhizosphere of wheat. The growth and yield of wheat crop significantly increases due to inoculation of plant growth promoting rhizobacteria. The germination of seed and development of seedling is affected by inoculation with *Azotobacter, Azospirillum*, *Bacillus* and *Pseudomonas*. The yield of wheat increased upto 30 % with *Azotobacter* and upto 43 % with *Bacillus* inoculation. The increase in plant height, biomass, leaf size, root length, nutrient uptake and tissue nitrogen content was also reported due to *Azospirillum* inoculation. An increase in the root biomass and shoot elongation was also reported with *Pseudomonas* inoculation [172-174].

Dobberlaere et al [175] inoculated spring wheat with *Azospirillum brasilense* and reported better seed germination, early flowering, maturation and significant increase in

the total dry weight (shoot + root) of wheat plant. Khalid et al. [176] reported that the strain of PGPR, genotype of wheat plant and environmental condition play important role in the positive response of wheat to rhizobacteria inoculation. Barbieri and Galli [177] inoculated wheat with *Azospirillum brasilense* and reported significant increase in the length and number of lateral roots. Similarly an increase in the surface area of root due to *Azospirillum brasilense* inoculation was reported by Kucey and Janzen [178].

Phosphate solubilizing bacteria facilitate the availability of phosphorus and improve phosphorus absorption by roots and thus increase yield of wheat significantly under phosphorus deficient condition. Researchers have reported an increase of 31.4 % in the dry shoot weight and 30.7 % in the absorption rate of phosphorus as compared to non-inoculated control treatment [179]. An increase of 11.4-14.7 % in seed yield was observed when wheat was inoculated with *Bacillus cereus* A47 [180]. Chen et al. [181] reported an increase of 6.3 - 15 % in the production of wheat due to bacterial inoculation. Similarly an increase (6-8%) in the emergence rate of spring wheat when inoculated with *Pseudomonas chlovoraphis* 2E3 strain was reported by Kropp et al. [182]. Javed and Arshad [183] reported production of IAA by 38 strains of growth promoting bacteria. They inoculated seed of two wheat types (inglab and lu-2bs) in optimal condition of farm and reported significant increase in the number of tillering, straw weight, thousand grain yield and an increase of 3.5 % in the yield of Inglab and 28 % in the yield of Lu-2bs.

Salamone [184] reported that the growth promotion of wheat plant was resulted due to cytokinin hormones produce by the inoculated *Pseudomonas fluerescens* strain C-

20-18. The negative effects of salinity on wheat can be reduced by using gfp-tagged *Azospirillum lipoferum*. The height and dry weight of leaves and roots was also increased. The absorption rate of water increased due to active growth of root and thus significantly increases yield of wheat [185]. When wheat was inoculated with external polysaccharide producing bacteria, the rate of sodium absorption reduced and plant yield increased. These bacteria induce 149 - 522 % increase in root dry weight and 85 - 281 % increase in shoots [186].

Those bacteria which contain ACC deaminase enzyme improve root length, root weight, straw weight, seed weight, number of tillers and absorption of nitrogen, phosphorus and potassium in plant as compared to non-inoculated control treatment [187].

The combine inoculation of PGPR strains showed significant improvement in phosphorus acquisition and growth of wheat. The co-inoculation of PGPR with phosphate solubilizing bacteria reduces the need of phosphorus application upto 50 % without affecting yield of corn [188]. The dual inoculation improves the yield of grain upto 20 % without phosphorus fertilizer and 30-40 % with phosphorus fertilizer [189].

1.7. PGPR as biofertilizer of rice production

The root and aerial parts of rice plant is colonizing by a variety of beneficial plant growth promoting bacteria [190]. They enable the roots to absorb water and nutrients actively from the soil by changing the morphology of root and also by increasing their biomass [109, 137]. They can be used as potential biofertilizers and recently the interest

of scientist and researchers in rice associated beneficial rhizobacteria has increased [43, 191]. The biological nitrogen fixation is considered to be one of the main reason of beneficial effects of PGPR [192, 193].

Production of phytohormones by these bacteria results in root development, which facilitate active uptake of water and nutrients from the soil [56]. The yield of rice crop increases when inoculated with nitrogen fixing and phosphate solubilizing rhizosphere associated *Bacillus* species [194]. Beneficial effects of phytohormones produced by different PGPR strains have been documented [141]. The indigenous strains of PGPR have shown more positive response increasing the production rate than non-inoculated control treatment [195, 196]. Rice inoculation with indigenous strains of *Azospirillum* led to significant increase in plant height after 40-75 days of transplantation [197]. An increase in the grain yield of rice and barley due to *Bacillus megaterium* inoculation was reported by Khan et al. [198].

The inoculation of rice with *Azospirillum brasilense* showed positive increase in the grain yield from 15-20% [199]. The inoculation of rice in the field with PGPR like *Azotobacter* sp, *Bacillus* sp, *Enterobacter* sp. and *Xanthobacter* sp results in an increase in the grain yield, total dry matter yield and nitrogen accumulation by 6-24%. The positive response of rice to PGPR was achieved because of the increase in leaf area, root length and chlorophyll contents [200].

Tran Van et al. [71] conducted an outdoor pot and field trials and inoculated rice with *Bukholderia vietnamiensis* and reported that when rice plants were inoculated and

transplanted at day 24, increases of shoot weight (33%), root weight (55%), leaf surface (30%) end grain yield 13-22 % and significant increase in grain weight was observed.

Two strains of nitrogen fixing *Pseudomonas* K1 and Ky1 has been isolated from kallar grass of Pakistan. They were initially identified as *Azospirillum* and *Zoogloea* [99, 201]. These strains have been re-identified recently as *Pseudomonas* strains by using 16S rRNA sequence analysis and could be useful as rice inoculant [202].

An increase of 42-64 % in the growth of rice plant due to *Burkholderia brasilensis* and *Burkholderia vietnamiensis* inoculations under gnotobiotic conditions were reported by Baldani et al. [203].

Meuchang et al. [136] reported the nitrogen fixing efficiency, production of Indole Acetic Acid (IAA) and phosphate solubilization ability of indigenous PGPR isolated from the paddy field. The nitrogen fixing bacteria were 56, Indole acetic acid producing bacteria were 59 and phosphate solubilizing bacteria were 62. Their results suggested the utilization of beneficial strains of PGPR for the production of biofertilizer for rice crop.

Govindarajan et al. [204] reported the production indole acetic acid (IAA) and reduction of acetylene to ethylene by 13 bacterial strains isolated from rice in pure culture. They observed an increase of 9.5 - 23.6% in the yield of inoculated crop over the un-inoculated control treatment.

Tariq et al. [205] reported that PGPR application to rice helps in maintaining the Zn concentration in the soil and alleviate the symptoms caused due to Zn deficiency. They reported an increase of 23% in the total biomass, 65% in the grain yield and significant effect on the harvest index and concentration of Zn in the grain.

The rice variety, cultural condition and inoculant strains play important role in the positive response of inoculated strains on growth and yield of rice. A large number of nitrogen fixing bacterial population (about 80 %) associated with wet land rice belongs to *Pseudomonas diazotrophicus*. They have the ability of active growth in flooded condition when H_2 and CO_2 evolved from the rice field [206-207]. Colonization of rice root by *Azorhizobium caulinodans* has been reported. They showed significant nitrogenase activity [208].

A variety of rhizobacteria associated with rice were reported. They include *Alcaligenes*, *Azospirillum*, *Clostridium*, *Enterobacter* and *Pseudomonas* [209]. They also explained the nitrogen fixing quality of *Klebsiella oxytoca* associated with rice plant. An increase of 6% in the nitrogen content of plant and soil and a significant incorporation of $^{15}N_2$ by the bacterial strain was observed. The unidentified diazotrophs associated with rice crop contribute upto 30-40 kg nitrogen/ha [210]. Fujii et al. [29] used *Klebsiella oxytoca* and *Enterobacter cloacae* as inoculant for rice crop and described their nitrogen fixing ability in the presence of ammonia.

Substantial number of studies were conducted by International Rice Research Institute (IRRI), Philippines and reported that most of the rice nutrients requirements (20-25 %) is provided by the rhizobacteria of soil [211-214].

1.8. PGPR as biofertilizer of maize production

PGPR have been reported to colonize the roots and aerial parts of maize [216]. They play pivotal role in the biofertilization of maize. They significantly promote the growth and yield of maize crop by improving the absorption of important nutrients like N, P and K by the plants. The diazotrophs belonging to Azotobacteraceae and Entero-bacteraceae are stimulated by maize for N_2 fixation. Studied have shown that PGPR inoculation stimulate absorption of plant nutrient like N, P, K, Fe, Zn, Mg and Cu etc and caused increase in root length, root weight, leaf area, plant dry weight and plant height [216-218].

PGPR have been found in close association with maize and commercially applied as biofertilizer for increasing maize production. Many studies have shown that *Azospirillum* inoculation stimulate root development by producing phytohormones. In Brazil, commercial inoculant of *Azospirillum brasilense* for maize is available [219]. Members of the gammaproteobacteria and betaproteobacteria and *Burkholderia* spp. have been isolated from the rhizosphere of maize in Brazil [220]. Roesch et al [221] reported that Kleb-siella are also commonly associated with maize. Eighty one strains of PGPR were reported for the production of phytohormones like IAA [222].The inoculation of maize with *Azospirillum* strains have shown 10-30 % increase in forage and grain yield

[68]. Inoculations with phosphate solubilizing bacteria have shown 14.4 % increase in the yield of maize. An increase of 68.4 % in root and 42.6 % in shoot of maize seedling was observed due to PGPR inoculation [175]. Inoculation with *Azotobacter* increases the grain yield of maize upto 17.6 % as compared to non-inoculated control treatment [223]. The inoculation of maize with *Rhodotorula* and *Azotobacter* along with half of the recommended NPK dose showed significant effect on growth promotion than single recommended full dose of NPK [224]. Lin et al. [225] reported that rhizobacteria promote root growth and thus enhances nutrients and water uptake by the plant from the soil and results increase in the yield of maize crop. Abd El-Gawad et al. [226] reported that bacterial inoculation promote plant growth by increasing ear weight, length and diameter, grains number/row and grain weight.

The combine application of PGPRs with NPK and half of the recommended dose of sulfur can provide good quality and quantity of maize and also helps in maintaining the environment clean [227-228]. Chabot et al. [22] described the effect of phosphorus on root colonization by phosphate-solubilizing *R. leguminosarum biovar phaseoli* and on growth promotion of maize.

The inoculation of maize with *Azospirillum brasilense* induces root hairs proliferation and dramatically increases the root surface area. In field experiment *Azospirillum* strains resulted in more visible increase in shoot development of maize than *Pseudomonas* strains [47]. Okon and Labandera-Gonzalez [70] conducted a field experiment and showed an increase of 50-60% in the yield of wheat and upto 40% increase in the yield of maize due to *Azospirillum* inoculation.

Researchers showed that bacterial inoculation have the potential to increase the growth of maize and wheat by producing phytohormones which is responsible for root elongation, seed germination and also stimulate expansion of leaf. Production of auxin as secondary metabolites by more than 80% of microorganism found in the rhizosphere of maize have been reported [229-232].

Plant height due to bacterial inoculation either single, double or triple inoculation are significantly different from control treatment. The triple inoculation of *Azospirillum, Azotobacter and Pseudomonas* strain 168 showed 197.4 cm height and *Azospirillum, Azotobacter and Pseudomonas* strain 41 showed 194.7 cm height respectively [233]. The inoculation of corn seed with PGPR results in 42.6 % increase in the dry weight of plant [234]. The combine inoculation of corn with *Azotobacter* and *Pseudomonas* resulted in 19.8 % increase in corn yield [235]. The combine application of *Azotobacter chroococcum* and *Azospirillum brasilense* resulted significant increase in maize grain yield [236]. The triple inoculation of *Azospirillum lipoferum, Azotobacter chroococcum* and *Pseudomonas fluorescent* strain P21 resulted in 68-70 % increase in the yield of variety SC-704 [233].

The single or combine application of *Bradyrhizobium japonicum* and *Azospirillum brasilense* promote seed germination and growth of seedling in maize [237]. The root and shoot dry weight was higher in co- inoculation than single inoculation or control treatments. An increase in the yield of maize due to single or co-inoculation with

Azospirillum lipoferum, Azotobacter vinelandii, Bacillus subtilis and *Klebsiella planticola* were also reported by Cvijanovic et al. [238].

1.9. Genetic diversity among the microbial population

There are distinct variation in the structure of microbial community present in the bulk non-rhizosphere soil and rhizosphere soil [239]. Genetic and functional diversity exist among soil microorganisms. It is difficult to relate taxonomic and genetic diversity to function of microorganisms because sometime taxonomically distinct microorganism share related functions [240].

A vast diversity in microbial population was reported in the previous studies [241-242]. There are four thousand different bacterial "genomic units" in one gram of soil on the basis of DNA-DNA reassociation [243]. In the rhizosphere of rice five thousand species of bacteria were reported [244-245].

The genetic diversity in microbial community provides evidence of genetic exchange in the bacterial population [246-247]. Extensive reservoirs of genetic diversity are present. However, it can be exploited with the help of applied molecular genetics. Improvement in our knowledge regarding ecology and population dynamics of microorganisms is important because they harbor potentially useful strains of plant growth promoting bacteria. The diverse phenotypic and genotypic variations among the bacterial communities makes its analysis further complicated [248]. It has been proposed that common laboratory techniques cannot be applied for the culture of most of the bacteria (about 90 %) which can be studied under the microscope [244, 249-252]. The

remaining 1% bacteria, which are culturable represent the entire bacterial population [253]. However, phenotypic and genotypic diversity in 99% of bacterial population from the reported 1% exist. The investigated microorganisms represent the minority of the total bacterial population [254].

Several different methods for documentation of genetic information are used. Previously, traditional methods used for distinguishing microbial strains were based on morphology, physiology and biochemical features [255]. Other methods are Guanine pus Cytosine (G+C), reassociation of nucleic acid, extraction of the total DNA, DNA hybridization and DNA microarrays , Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Restriction Fragment Length Polymorphism (RFLP), Terminal Restriction Fragment Length Polymorphism (T-RFLP), Single conformation polymorphism (SSCP) and Amplified Rhibosomal DNA Restriction Analysis (ARDRA) [343, 254, 256-260]. The conventional techniques used for the cultivation represent less that 1% of the microbial diversity of beneficial species [261]. Molecular diversity which are based on 16S rRNA gene analusis like PCR Amplification of 16S rDNA, Terminal Restriction Fragment Length Polymorphism (T-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE) and Amplified Rhibosomal DNA Restriction Analysis (ARDRA) are common techniques used for studying microbial communities in diverse environmental conditions, which include soil [262], marine [263] and rhizosphere ecosystems [264].

These techniques are culture independents and when applied on endophytic bacteria showed limited success because of the disturbance of mitochondrial 18S rDNA and chloroplast 16S rDNA. Chelius and Triplett [265] designed two bacterial primers for directly amplifying bacterial sequences directly from maize root with the help of PCR. They were designed to excluded the disturbances of mitochondrial and chloroplast DNA.

The Denaturing gradient gel electrophoresis (DGGE) and Phospholipid fatty acid analysis (PLFA) were utilized for assessing the impact of management techniques in agriculture like no-tillage and conventional tillage and influence of the environment like precipitation of four different soils were assessed. The clustering pattern for four different soil was common, therefore, the information obtained from both of these techniques (Denaturing gradient gel electrophoresis and Phospholipid fatty acid analysis) were complemented each other [266].

Siciliano et al. [267] used DNA hybridization and Denaturing Gradient Gel Electrophoresis for the assessment of structural and functional diversity in bacterial community. They used bacterial gene probes having catalytic properties like alkB, ndoB, xylE in their experiments. Polycyclic Aromatic Hydrocarbons (PAHs) were used to contaminate the soil during phytoremediation trials. They stated that microbial community can be affected by the plant. They further added that the functioning of microbial community, that aid in degradation effect, have significant influence in the rhizospheric region as well as in adjoining bulk soil containing microbial communities.

Sun et al. [268] conducted a field experiment and used 16S rDNA cloning, comparison of sequence homology and Amplified Ribosomal DNA Restriction Analysis (ARDRA) for studying diversity in endophytic bacteria of rice. They selected two bacterial primers (799f – 1492r) to exclude interference of mitochondrial and chloroplast DNA of rice effectively. Amplification of 16S rDNA sequence of bacteria directly from the root tissue of rice was carried out specifically by these bacterial PCR primers. In the endophytic library of 16S rDNA, fifty two Operational Taxonomic Units (OTUs) were reported among 195 clones. The identification was based on Amplified Rhibosomal DNA Restriction Analysis (ARDRA) banding profiles similarity. In sequence analysis different groups of bacteria in the 16S rDNA were observed which belongs to alpha, beta, gamma, delta and epsilon subclasses of the proteobacteria, Cytophaga/ Flexibacter/ Bacteroides (CFB) phylum, low G+C gram positive bacteria, Acidobacter, Deinococcus- Thermus and Archaea.

The analyses of particular microbial strain were carried out by polymerase chain reaction and identification of natural microbial diversity [269-271]. A modification of PCR referred to as RAPD analysis has been developed [272]. A single oligonucleotide is used as primer in this method to amplify genomic DNA sequence. This oligonucleotide may not be complementary to the specific DNA sequence in the genome. The genome has a number of different annealing sites, which allow amplification. These sites depend on length, GC contents and nucleotide sequence of primer. These polymorphic fragments of DNA, namely RADP (Randomly Amplified Polymorphic DNA), can be applied as genetic marker [273-274]. The different strains of bacterial species *Lactococcus lactis*

[275], *Styphallo coccus* and *Streptococcus pygenes* [272] and also *Azospirillum* sp. [276] can be differentiated by these techniques. A variety of biotechnological methods can be utilized for the analysis of structural and functional diversity in bacterial communities. Each method has a different end point and explains a broader, more complicated picture of variation in the soil microorganisms.

1.10 Objectives

Keeping in view the importance of cereal crops and the potential of rhizospheric PGPR strains, a systematic research was launched at National Institute for Biotechnology and Genetic Engineering, Faisalabad and Agriculture Research Institute (N) Mingora, Swat with the following objectives.

- Isolation of PGPR from the rhizosphere of wheat, rice and maize roots.

- Identification and characterization of the bacterial isolates to study bacterial diversity in plant rhizosphere grown in cold climate.

- Testing of the selected bacterial strains as inoculants for wheat, rice and maize grown at Agriculture Research Institute (N) Mingora, Swat and Udigram Swat.

Chapter 2

Materials and Methods

2.1 Collection and analysis of soil

Six soil samples from the experimental fields at Swat were collected. The samples were collected from 0-30 cm depth. The analysis of the soil samples (physical and chemical) were carried out at Pakistan Agriculture Research Institute, Tarnab, Peshawar. Soil texture was determined by hydrometer method, pH was calculated by using pH meter in 1:1 soil water suspension [277]. Kjeldahl method of Bremmer and Mulvaney [278] was used to determine total nitrogen in soil samples. AB-DTPA or Mehlic No.3 extractable P was determined in samples on the basis of pH of soil samples. That in case of low pH (7 and below 7), Mehlic-3 extractant was used while for pH greater than 7 AB-DTPA extractant was used. The K was determined by flame photometer using the AB-DTPA extracting solution and using the required standard solution. Soil organic matter was determined by using standardized solution of $FeSO_4$ and $K_2Cr_2O_7$ as given by Nelson and Sommer, [279]. Lime was determined by acid neutralization method [280]. The data of the analysis is given in the Table 1.

2.2 Cereal crops and bacterial isolation

Roots of cereal crops (wheat, rice and maize) along with the rhizosphere soil from the plants grown in the fields were collected at Udigram and Agriculture Research Institute (ARI) Mingora, Swat.

In order to collect rhizosphere soil samples, the root system was carefully uprooted along with a good amount of non-rhizosphere soil and placed in polythene bags and brought to the Laboratory. The rhizosphere soil was separated by gently shaking and removing the non-rhizosphere soil. The soil attached to root system is known as rhizosphere soil. The samples were stored at 4^{o}C and used for further studies. One gram of roots along with adhering soil was grounded well with the help of a pestle and mortar. Serial dilutions (10X) were made and 100 µL aliquots from 10^{-3}–10^{-5} dilutions were spread on LB plates (Maniatis et al. [281]; Append. I). Semi solid NFM (Okon et al. [282]; Append. II) was incubated with 100 µL of these serial dilutions. The inoculated plates and NFM vials were incubated for 24-72 hrs at 30^{o}C.

Morphologically different colonies appearing on the growth medium were selected for further purifications. Isolated colonies were streaked on fresh plates with LB medium to get single-cell colonies. Bacterial growth obtained in NFM medium was streaked on NFM agar plates and incubated at 30^{o}C for 24-72 hrs. Single colonies were further streaked on fresh plates to get pure colonies. Single colonies appearing on agar plates were transferred to a drop of sterilized water on the glass slide and observed under light microscope (Nikon Japan).The bacterial cultures obtained were grown at 30^{o}C for 24 hrs and preserved in glycerol (20%) at -20^{o}C.

2.3 Identification and characterization of bacterial isolates

The morphological and physiological characteristics of bacterial strains including cell morphology, formation of pigments on nutrient agar medium, motility and growth at 30 ^{o}C on NFM were studied for identification and characterization of bacterial isolates.

The cultural characteristics of the purified bacterial strains were studied using light microscope. All the strains were grown on Luria- Bertani (LB) agar medium [281]. They were incubated for 24- 48 hours at 30 C°. The purified cultures were maintained on LB agar slants. The colonies of bacterial culture were observed for colour, shape, size and motility. Single colony of each strain was suspended in 0.85% saline. A drop of this suspension was put on a glass slide, covered with cover slip and observed under light microscope. The bacterial strains were tentatively identified on the basis of cell morphology i.e size, shape and motility.

2.4 DNA isolation from bacterial cultures

Bacterial cells were grown in LB broth for 24 hours at 30 ^{o}C. They were centrifuged at 13000 rpm for five minutes. The cell pellets obtained from centrifugation of 1.5 mL cultures were washed with TE buffer (10 mM Tris.Cl; 1mM EDTA, pH 8) and then dissolved in 200 µL of TE. The genomic DNA was isolated by using Genomic Purification Kits (Fermentas, Lithuania). The lysis solution (400 µL) and sample (200 µL) was mixed and inoculated for five minutes at 65 ^{0}C. The solution was then emulsified by inversion after adding chloroform (600 µL). Centrifugation was carried out for two minutes at 10,000 rpm. 720 µL water (nuclease free) was mixed with 80 µL of supplied 10X concentrated solution in order to prepare precipitation solution. The upper portion of the solution was transfer to a fresh tube. Precipitation solution of 800 µL was added and mixed for 1-3 minutes at room temperature. The prepared mixture was then centrifuged for 2 minutes at 10,000 rpm and supernatant was completely removed. 300 µL cold ethanol was added to 100 µL 1.2 M NaCl solutions to dissolve DNA pellet. The

DNA was left to precipitate (10 min at - 20^{o}C) and spined down at 10,000 rpm for 3-4 min. Ethanol was removed from the mixture and 70% cold ethanol was used to wash the pellet. The DNA was used in PCR as template after dissolving in 100 µL water (nuclease free).

2.5 Amplification of DNA by using a random primer in PCR for differentiation of bacterial strains

Each reaction mixture (100 µL) contained 0.5 µL Taq DNA polymerase (5U/µL; Fermentas, Lithuania), 10µL Taq buffer, 10µL dNTPs (final concentration 200 µM each), 1 µL (100 ng/µL) of primer (AAGGCGGCAG), 1µL of template DNA and the volume of the reaction mixture was made to 100 µL with sterilized distilled water. Thirty-five rounds of temperature cycling (94 for 1 min, 38 ^{o}C for 1 minute and 72 ^{o}C for 2 minutes) were followed by incubation at 72 ^{o}C for 7 minutes in a Perkin Elmer GenAmp PCR System 2400.

2.6 PCR amplification of partial *nifH*

For amplification of partial *nifH*, 1 µL of template DNA was added to the PCR reaction mixture (50µL), one µL of each *nif*H primer i.e. PolF (TGCGAYCCSAARGCBGACTC) and PolR (ATSGCCATCATYTCRCCGGA) were used for the amplification of *nif*H coding sequence. Each reaction mixture (50 µL) contained 0.2 µL Taq DNA polymerase (5U/µL; Fermentas), 5µL Taq buffer, 5µ uL dNTPs (final concentration 200 µM each), 1 µL of each *nif*H primer (100 ng/µL) of primer, 1µL of template DNA and the volume of the reaction mixture was made to 50 µL with sterilized distilled water. Thirty-five rounds of temperature cycling (94 for 1 min, 55

^{o}C for 2 minutes and 72 ^{o}C for 3 minutes) were followed by incubation at 72 ^{o}C for 7 minutes in a Perkin Elmer GenAmp PCR System 2400.

2.7 Determination of IAA production:

Culture of the bacterial isolates were grown in conical flasks containing 50mL Nitrogen-free medium [282] supplemented with 100 mg/L L-tryptophan and NH_4Cl 1g/L at 30 ^{o}C. Centrifugation of the culture at stationary phase was carried out at 10,000 rpm for 15 minutes. 1N HCl was used to adjust pH to 2.8. Equal volume of ethyl acetate were used for the extraction of auxins in the acidified culture medium [109], evaporated to dryness and re-suspended in one mL of ethanol. HPLC (Varian Pro star) were used to analyze the sample using C-18 column and UV detector. In this reaction, methanol: acetic acid: water in ratio of 30:1:70 v/v/v were used as mobile phase at the rate of 0.6 mL min^{-1} [283]. Pure indole-3-acetic acid was used as standard for the reaction. The retention time and peak area were compared by using a computer software (Varian) in order to identify and quantify IAA of the samples.

2.8 Acetylene reduction assay (ARA):

Acetylene reduction assay was used to determine the nitrogen fixing ability of bacterial isolates. In this technique acetylene is reduced to ethylene by the ability of nitrogenase. 5 ml of nitrogen free medium (NFM) in semi-solid condition was prepared and autoclaved in 10 ml vials. NFM vials were inoculated with 100 µL of bacterial culture grown in LB Broth. Inoculation was carried out for 48-72 hours at 30 ± 2 ^{o}C. Sterilized suba seals were used to replace the caps of vials under aseptic condition. 0.1 atm acetylene was used to replace 0.1 atm of air by air tight syringe. Incubation of vials

was then carried out for 24 hours at 30± 2 ^{o}C. After incubation, gas sample (100 µL) from each vial was analyzed on gas chromatography (FRACTOVAP Series 2150; CARLO ERBA STRUMENTAZIONE; Column Porapak N, L 1M, ID 2mm). The temperature of the column and injector were maintained at 64 ^{o}C and 175 ^{o}C respectively. Nitrogen was used as a carrier gas (pressure 0.6 kg 1 cm^3). The peaks of unchanged acetylene and ethylene produced were detected on flame ionization detector maintained at 175 ^{o}C and recorded on a recorder (Perkin Elmer) set at potential difference of 1 ml and a chart speed of 5m M/min. Ethylene 0.5/ 70 ml was used as standard.

The specific activity of bacterial cultures was measured by a method described by Lowery et al [284]. Specific activity was expressed in n moles ethylene produced/mg protein / h and was calculated using the following formula:

$$\text{n moles } C_2H_4 \text{ /mg protein/h} = \frac{PV \times \text{Total Pk.ht. (Sample)} \times \text{Total vol. of vial (10ml)} \times \text{vol. injected (sample)}}{RT \times \text{Pk. Ht. (std)} \times \text{vol. Injected} \times \text{mg protein} \times H}$$

Where;

P	=	Atmospheric Pressure (1 atm)
V	=	Volume of Standar C_2H_4 injected (22.4 L)
R	=	Gas constant (0.08206 atm / ^{o}C/ mol)
T	=	Absolute temp (303 ^{o}K)
Pk.ht	=	Peak height (mm) at 1 attenuation
Total Vol.	=	Total volume of the gas phase (ml)
Vol. Inj	=	Volume of gas phase injected (ml)
H	=	Time of incubation (hour)

Conc. Of standard= 7.1 x 10^{-3}ppm

Pk.ht of standard at 1 attenuation = 148 mm

2.9 Inoculation of wheat in the field experiment

Field experiment was carried out during wheat growing season in 2009-2010 at Udigram, Swat. The pot size was kept 14 m^2 each and twelve rows with 3 cm distance. Randomized complete block design was used in the current investigation. Four treatments (T1- *Azospirillum brasilense* strain R1, T2- *Azospirillum lipoferum* strain RSWT, T3- *Pseudomonas* Ky1 and T4- Control) were allotted to main plot. Inoculation was carried out before sowing. Seeds of locally available common wheat varieties Inqilab 91 and Fakhre sarhad were mixed with selected bacterial suspension and the field was sprayed with this mixture respectively. The seeds were sown at the seed rate of 120 Kg/ha. The applied level of Nitrogen and Phosphorus were 120 and 60 Kg/ha respectively. Data was recorded keeping in view different growth parameters. The plant height was calculated by randomly selecting five tillers/ plot and measuring the height of plant from ground to top of spike. The duration between dates of sowing to the change in the colour from green to yellowish of 70-80% plants in each plot was counted for physical maturity.

The days from date of sowing till loss of green colour from glumes and grains were counted to record harvest maturity. The number of grains of five spikes in each plot was counted for calculating the number of grains per spike. The grain weight per spike was calculated by removing the grains from each spike which was randomly taken from five plants in each plot. They were weighed and then average was taken. The thousand grain yield was calculated from threshed clean grains of each sub plot having five samples. The weight of the grains was then calculated with the help of electric balance. In each plot, eight central rows were selected for calculation of biological yield. They were

harvested, bundled, dried and weighed. The biological yield was then converted into Kg per hector. The statistical calculations were carried out by using MSTAT C program and LSD tests.

2.10. Inoculation of rice grown in the lab in Falcon tubes:

Bacterial strains were grown in 100 mL of LB liquid medium in a water bath (25 °C; 150 rpm) for overnight in order to inoculate the plants. Centrifugation of the cell suspension was carried out at 10,000 rpm for 10 minutes. The pelleted cell suspension were washed with distilled water and suspended in 100 mL of distilled water.

The selected bacterial strains (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky 1) were used as inoculum for the rice variety JP 5. Two strains i.e *Azospirillum lipoferum* RSWT1 and *Pseudomonas* Ky1 were obtained from culture collection, Plant Microbiology Division (PMD), National Institute for Biotechnology and Genetic Engineering (NIBGE) and have been purified from rice roots collected from Swat and Kallar grass, respectively. The inoculated strain *Azospirillum brasilense* R1 was purified from rice roots collected from Swat (present study). The seeds were obtained from Agriculture Research Institute North (ARIN), Mingora, Swat. Sodium hypochlorite was used for 5 min in order to carry out surface sterilization of the seeds. The seeds were then washed with sterilized water. The seeds were sown in sterilized sand in 50 mL Falcon tubes and kept in the growth room ($254 \mu Em^{-2}S^{-1}$ and $25^{o}C$). In each tube one seed was sown. One mL of the inoculum was used to inoculate each seedling two days after germination. One mL of Nitrogen-free

Hoagland solution (1/2 strength) was added twice a week as nutrient source. Seedlings were harvested after 4 week of growth.

For measuring the root area and root length, the plant roots were washed in water. The roots of each plant were removed separately and spread on a transparent polyethylene sheet. The sheet with roots was put on the desktop scanner which scanned the roots and created a computer image of the roots.

The root area and root length were measured on the P-IV IBM computer and scanner by using root image analysis programme. This programme has been prepared by Research Foundation programme, Washington State University, USA. In this programme the roots are scanned with the desktop scanner and computer image is created and analysed.

2.11. Inoculation of rice in pot experiment

Seeds of the selected rice varities JP 5 and Fakhre Malakand were cultivated on 1st June 2010. The soil in the pots was properly puddle and leveled thoroughly. Transplantation of the seedling was done on 1st July 2010.

Selected bacterial strains (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky 1) were tested as inoculants for rice varieties Fakhre Malakand and JP 5, grown in pots (33 X 29 cm). The pots were filled with 10 Kg of soil collected from NIBGE fields. Three seedlings were transplanted in each pot. In order to inoculate the seedling, bacterial culture were grown for over night in LB medium at 30 ^{o}C, centrifuged (10,000 rpm) for 10 minutes to get cell pellets which

were then re-suspended in sterilized water. At the time of transplantation, the roots of the seedlings were kept in cell suspension for 30 minutes and then transplanted.

2.12. Raising of rice nursery and inoculation of rice in the field experiment

A fertile piece of land that has easy access to the water channel and convenient drainage system was selected to raise the rice nursery. The land was prepared by ploughed with tractor three times and then irrigated. Eradication of the weeds was carried out through ploughing and planking. During this process the water remained in the field. Seeds of the selected rice variety JP 5 were cultivated on 21 May 2010 at Agriculture Research Institute North (ARIN) Mingora and at Udigram Swat.

In the early stages of growth the water was drained out daily at night. Afterward, the depth of water was kept 2-4 cm to suppress weeds. After 30 days i.e on 20 June 2010, the nursery was transplanted to the fields. During transplantation the water depth was kept 2 cm in the field. Bacterial inoculums were prepared and maintained as mentioned above and 50 mL of the inoculum of each bacterial strain (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) was added to 2 L water and roots of the nursery seedling was inoculated for 1 hr. After inoculation of the rice seedlings, the remaining bacterial suspension was distributed equally in their respective beds. The seedling was brought and distributed through out the field in their respective beds in the form of small bundles. The right number of seedling was detached from the bundles and inserted in the soil not shallower than 1.5 cm and not deeper than 3 cm. Two seedlings per hill at 20 x 20 cm distance were planted. The missing hills were replaced about 10 days after transplantation. Randomized complete

block design was used in the present study with four treatments and four replicates. The plot size was 3 x 3 m at Agriculture Research Institute (N), Mingora and 3 x 5 m at Udigram, Swat. The number of rows at ARIN, Swat was 14 x15 with 210 plants per plot and at Udigram was 12x22 with 330 plants per plot. After one week of transplantation, recommended chemical fertilizers (120 kg/ha N, 60 kg/ha P and 40 kg/ha K) were used.

In order to estimate the bacterial populations, the rhizospheric soil samples were collected 4 week after transplantation. Serial dilutions (10^{-1} to 10^{-6}) of the rhizosphere soil samples were prepared in sterilized water. Aliquots (100 µL) from serial dilutions were spread on LB agar plates and incubated at 30^{o}C for 24 hrs. Following the standard method, only those plates were counted that contain 30- 300 colonies.

The plants were harvest from 30 – 35 days after flowering. Initially, 5 plants from each plot (i.e a total of 20 plants from each treatment) were up-rooted carefully to take out whole root system. Roots were washed carefully to remove adhering soil and kept at 55 ^{o}C for three days to estimate root dry weight. Similarly straw weight and grain weight of all the randomly selected plants was taken.

To record the total grain weight and straw weight from each plot, the grains were separated from the straw and their fresh weight was recorded. In order to carry out dry weight study of grains and straw, 5 kg of grains were dried in oven at 55 ^{o}C for three days. After drying the plant material, dry weight was measured and the difference between fresh weight and dry weight was calculated. Keeping in view the loss per kg, the total dry grain weight of each treatment was calculated. The same methodology was applied for calculating the dry straw weight and total dry weight of the plants per plot and

per treatment. Statistical calculations were carried out by using MSTAT C program and LSD tests.

2.13. Inoculation of maize in the field experiment:

Field experiment was carried out during maize growing season in 2011-2012 in the field of Udigram Swat. A fertile piece of land that has easy access to the water channel and convenient drainage system was selected for the experiment. The land was prepared by ploughing with tractor. During seed bed preparation, the fertilizer doze of 120 Kg/ha N and 60 Kg/ha P was applied. The pot size was kept 15 m^2 each and four rows with 75 cm distance. Randomized complete block design was used in the current investigation. Four treatments (T1- *Azospirillum brasilense* strain RSWT1, T2- *Azospirillum lipoferum* strain R1, T3- *Pseudomonas* Ky1 and T4- Control) were allotted to main plot. The effect of the inoculated strains on different growth parameters of maize crop was observed. Meter rod was used to calculate plant height by randomly selected 12 plants in eac plot and then average were taken. The number of ears in 12 randomly selected plants per plot was counted and average was taken to calculate the number of ear/plant. Twelve ears in each plot were counted and then average was taken to calculate the average number of grain per ear. The number of leaves per plant was calculated by randomly selecting 12 plants from each treatment and then average was taken. The stem thickness (girth) of plant in three central rows of each treatment was measured and then average was taken. The thousand grain yield was calculated by randomly selecting 12 ears from each treatment and recording their weight with the help of electric balance. The ears from

three central rows were threshed and the grain yield per plot was calculated and then converted into Kg/ha by the following formula;

$$\text{Grain yield} = \frac{\text{Grain yield in three central rows}}{\text{No of rows harvested x Row to Row length x Row length}} \times 10000\ m^2$$

The stimulatory effects of the inoculated bacterial strains on biological yield were calculated on three central rows of each treatment at their maturity and were harvested. They were sun dried, weighed with the help of spring balance and then converted into Kg/ha by the following formula;

$$\text{Biological Yield (Kg/ ha)} = \frac{\text{Biological yield of three central rows}}{\text{No of rows harvested x Row to Row length x Row length}} \times 10000\ m^2$$

Chapter 3

Results

3.1 Soil collection and analysis

Six soil samples from the experimental fields at Swat were collected. Out of six samples, three were collected from Agricultural Research Institute Mingora, Swat and three from Udigram, Swat. Analysis of the soil samples (physical and chemical) were conducted at Pakistan Agricultural Research Institute, Tarnab, Peshawar. Two textural classes i.e. sandy loam and silt loam were observed among the soil collected from both sites. The data of the analysis is given in the Table 1**.**

3.2 Isolation of bacteria from rice roots and rhizosphere

A total of 18 bacterial isolates were obtained from the roots and rhizosphere of rice collected from Agricultural Research Institute (North) Mingora and Udigram Swat. Out of 18 bacterial strains, 10 were isolated from ARIN, Swat and 8 from Udigram, Swat. In addition to these, two bacterial strains (*Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) were collected from Plant Microbiology Division, NIBGE, Faisalabad.

3.3 Identification and characterization of bacterial isolates

The characterizations (morphological and physiological) of isolated bacterial strains were carried out on the basis of colony morphology (colour, shape and size), cell shape and motility. The cultural characteristics of the purified bacterial strains were

studied using light microscope. Eleven isolates (MRSWT3, MRSWT4, MRSWT6, R1-1, R1-2, R3-1, R3-2, R4-1, R5-2, R6-1 and R6-2) were tentatively identified as *Pseudomonas* strains and four isolates (R1, R2, R3, R5) as *Azospirillum* strains (Table 2). Most of the *Pseudomonas* strains have thin, rod-shaped and motile cells. They formed white or off white colonies on LB agar plates. The *Azospirillum* strains have rod-shaped cells and showed spiral motility. They formed pinkish colonies on LB agar plates.

Three strains, R2-1, R4-2, MRSWT5 could not be identified. The white colonies of strain R2-1 were very hard and were submerged in the agar plates. The colony morphology of this strain was totally different from all others. The strain R4-2 formed fluffy colonies. The cells were rod shaped and slightly motile. The colonies of isolate MRSWT5 were also yellowish and fluffy. The cells showed morphological characteristics similar to those of strain R4-2.

3.4 Use of random primer in PCR to differentiate bacterial isolates

To confirm that bacterial isolates purified from the roots and rhizosphere of rice are different strains and not the re-isolates of the same strain, random primer was used in PCR to differentiate eight randomly selected *Pseudomonas* strains by comparison of the amplified DNA banding patterns. All the four strains showed different banding pattern of the PCR product (Fig 1 and 2). On agarose gels, PCR products of each strain showed several major and minor DNA bands. The unique banding pattern of each strain confirmed that RI-2, R3-1, R4-1, R5-2, MRSWT3, MRSWT4, MRSWT6 and R6-2 are different strains and not the re-isolates of the same strain.

3.5. Detection of *nif*H in bacterial isolates

The nitrogen-fixing ability of the bacterial isolates was identified by using *nif*H primers in PCR to confirm presence of nitrogen-fixing genes or structural genes of nitrogenase enzyme (Fig-3). PCR product of expected size (360bp) was obtained from DNA template of only two bacterial isolates (*Azospirillum* R1 and *Azospirillum* R3). This amplification of partial *nif*H confirmed presence of structural genes of nitrogenase enzyme which is involved in conversion of atmospheric nitrogen into fixed (ammonium) form usable by microbes and plants.

3.6. Phytohormones Production (IAA):

The pure cultures of bacterial strains were tested for detection and quantification of IAA in nitrogen-free medium supplemented with 100 mg/L L- and NH_4Cl 1g/L. The results are given in Table 3.

Out of 20 strains tested, 17 produced IAA. Production of IAA was higher in R4-1 strain with 45.6 µg/mL, followed by R2 with 45.5 µg/mL and MRSWT6 with 35.8 µg/mL of IAA. *Pseudomonas* Ky1 also produced higher amount (32.5 µg/mL) of IAA. The value of IAA production by the isolates R4-2 and R6-2 were comparatively low (4.5 µg/ mL and 5.6 µg/mL respectively) while the isolates MRSTW3, R3-1 and R5-2 did not show any IAA production in pure culture.

3.7. Nitrogenase activity (Acetylene reduction assay):

The nitrogen fixing ability of soil microorganism was determined by acetylene reduction assay. This technique support associative nitrogen fixation by soil

microorganisms. The nitrogenase activity of bacterial isolates is expressed in n moles C_2H_4 produced/Mg protein/hr and is presented in Table 3. Out of the total bacterial strains tested for ARA, 14 strains were ARA positive while the rest 6 strains were ARA negative. The overall nitrogenase activity varied between 21.5 n moles C_2H_4/mg protein/hr to 890.65 n moles C_2H_4/mg protein/hr. Highest specific activity was observed in case of isolate R4-1 which was found to be 890.65 n moles C_2H_4/mg protein/hr followed by bacterial isolate R2 and R3-2 with 641.98 n moles C_2H_4/mg protein/hr and 370.23 n moles C_2H_4/mg protein/hr respectively .The lowest specific activity was observed for bacterial isolate R5 which was found to produce only 21.5 n moles C_2H_4/mg protein/hr.

Table 1: Analysis of the soil samples collected from Swat

Localities	Clay %	Silt %	Sand %	Texture Classes	Total Soluble salt %	pH	Nitrogen (N) ppm	Phosphorus (P) ppm	Potassium (K) ppm	Organic Matter (OM)%	Lime stone ($CaCO_3$)
ARIN-1*	10.2	41	48.8	Sandyloam	0.069	7.3	26.25	3.89	152	0.71	4.0
ARIN-2	9.0	38	52	-	0.150	7.7	24.5	3.86	98	0.27	7.34
ARIN-3	19.4	68	17.6	Silt loam	0.144	8.5	23.5	17.0	144	1.53	7.0
Udigram1	19.4	63	12.2	-	0.096	8.1	21.6	11	108	1.35	6.25
Udigram2*	17.1	55	14.4	-	0.132	7.4	23.7	20	88	1.4	12.0
Udigram3	10.1	38	47.3	Sandyloam	0.058	7.2	22.8	14.3	141	0.69	3.41

* Soil samples were collected from the experimental sites where inocula were tested in plots.

Table 2: Isolation of bacterial strains from cereal crops.

Sr/No	Code	Characters of the Isolates	Tentative Identification	Locality
01	R1	Pinkish colonies on LB agar plates, rod shaped cells, spiral motility	*Azospirillum*	ARIN Swat
02	R2	Pinkish colonies on LB agar plates, rod shaped cells, spiral motility	*Azospirillum*	ARIN Swat
03	MRSWT3	Medium size rods, slightly motile, colony yellowish fluffy.	*Pseudomonas*	ARIN Swat
04	MRSWT4	Rod shaped cells, slightly motile, off-white colonies.	*Pseudomonas*	Udigram Swat
05	MRSWT5	Whitish or off white colonies on LB, long rod shaped cells joined together, motile.	Un-identified	ARIN Swat
06	MRSWT6	Whitish yellow colonies, long rod shaped.	*Pseudomonas*	Udigram Swat
07	R3	Pinkish colonies on LB agar plates, rod shaped cells, spiral motility	*Azospirillum*	Udigram Swat
08	R5	Pinkish colonies on LB agar plates, rod shaped cells, spiral motility	*Azospirillum*	Udigram Swat
09	R1-1	Whitish yellow colonies, short rods, slightly motile.	*Pseudomonas*	ARIN Swat
10	R1-2	Medium size, straight thin rods, motile, whitish colonies.	*Pseudomonas*	Udigram Swat
11	R2-1	Very hard, white colonies, submerged in the agar plates	*Un- identified*	ARIN Swat
12	R3-1	Whitish colony, long thin rod, slightly motile.	*Pseudomonas*	Udigram Swat
13	R3-2	Very short rods , slightly motile	*Pseudomonas*	Udigram Swat
14	R4-1	Similar to R1-2	*Pseudomonas*	ARIN Swatr
15	R4-2	Light yellow colonies, round or slightly rod shaped cells.	Un-identified	ARIN Swat
16	R5-2	White colonies, cells short rod shaped or slightly curved, motile	*Pseudomonas*	ARIN Swat
17	R6-1	Medium size rods, slightly motile, colony yellowish.	*Pseudomonas*	ARIN Swat
18	R6-2	White yellowish colonies, motile, spreading but not fluffy, short motile rods.	*Pseudomonas*	Udigram Swat

Table 3: Characterization of bacterial isolates for phytohormone production and nitrogenase activity.

Sr.No	Code	IAA(production) µg/mL	Specific activity (n moles C_2H_4 produced/ mg/ protein/hr)
01	R1	16.5	143.68
02	R2	45.5	641.98
03	MRSWT3	-ve	270.36
04	MRSWT4	13.5	nd
05	MRSWT5	7.2	nd
06	MRSWT6	35.8	320.17
07	R3	5.7	nd
08	R5	27.2	21.5
09	R1-1	8.6	70.89
10	R1-2	12.4	1010
11	R2-1	17.5	30.45
12	R3-1	-ve	nd
13	R3-2	8.5	370.23
14	R4-1	45.6	890.65
15	R4-2	4.5	nd
16	R5-2	-ve	nd
17	R6-1	17.5	80.23
18	R6-2	5.6	312.45
19	RSWT 1	6.8	24.8
20	Ky1	32.5	175.78

nd= Not detected

Table 4: Total viable bacterial counts in the rhizosphere of inoculated rice varieties.

Treatment	ARIN Mingora		Udigram Swat	
	CFU/ g soil (X 10^5)		CFU/ g soil (X 10^5)	
	Fakhre Malakand	JP 5	Fakhre Malakand	JP 5
T1- Control	1.03	1.12	1.43	1.32
T2- RSWT1	2.20	1.95	1.67	1.52
T3- R1	2.35	2.11	2.13	2.90
T4-Ky 1	1.83	1.97	2.17	1.95

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*

R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

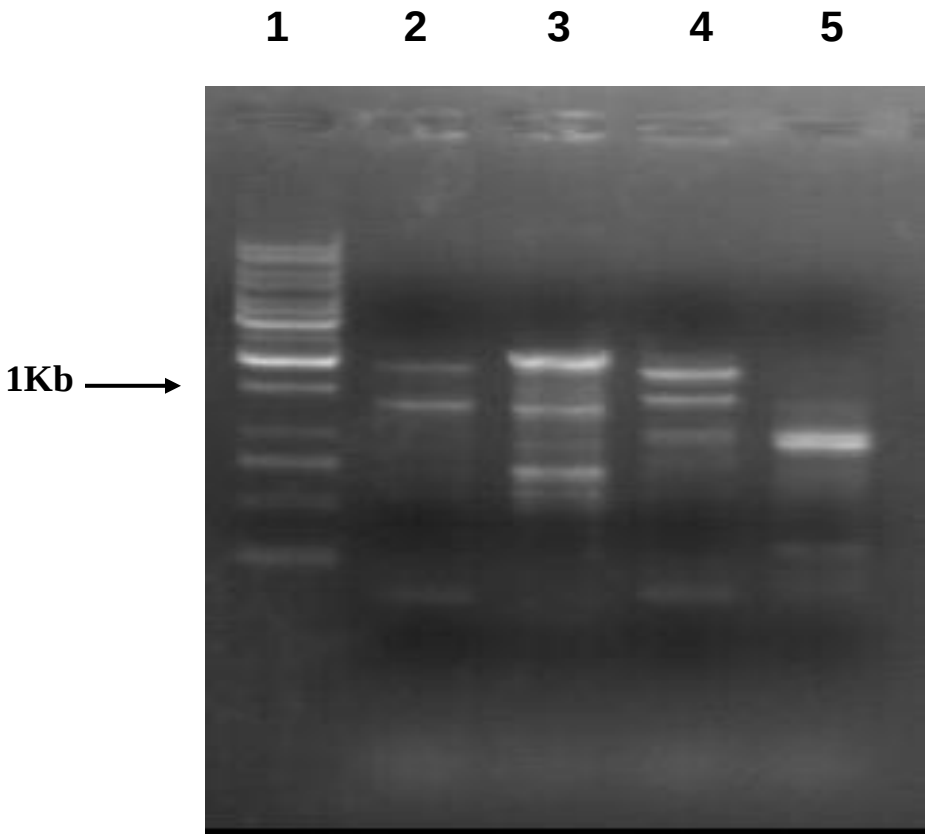

Fig 1: Differentiation of *Pseudomonas* strains by using a random primer in PCR.

Lane 1- 1Kb size marker (Fermentas)
Lane 2- *Pseudomonas* RI-2
Lane3- *Pseudomonas* R3-1
Lane4- *Pseudomonas* R4-1
Lane 5- *Pseudomonas* R5-2

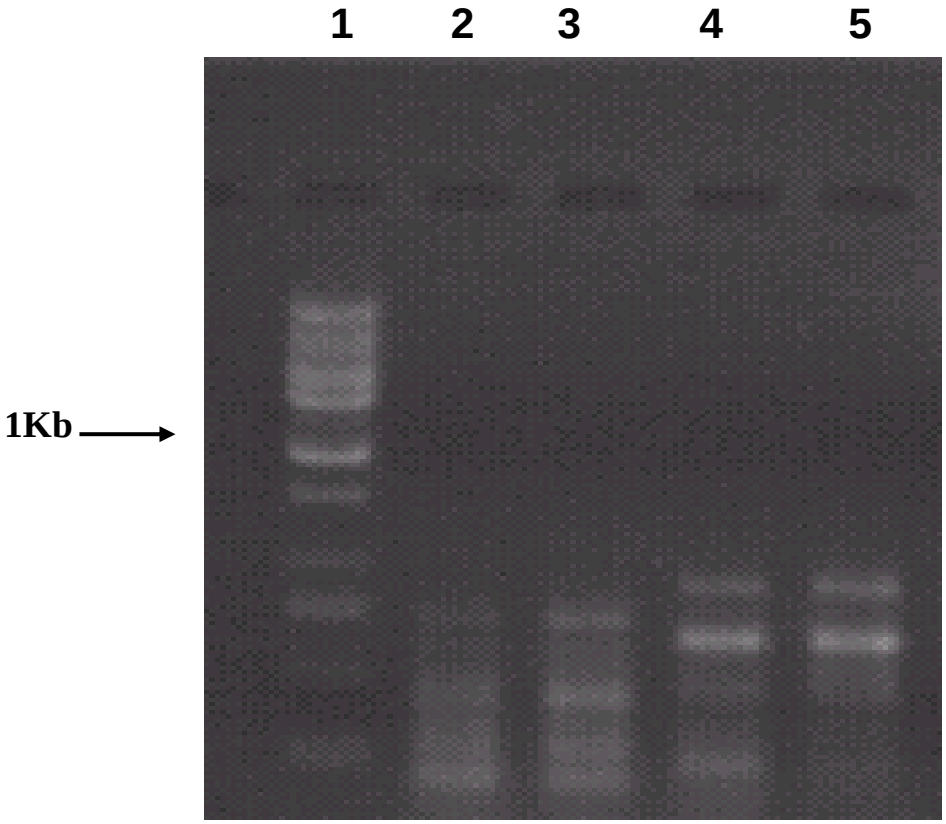

Fig 2: Differentiation of *Pseudomonas* strains by using a random primer in PCR

Lane 1- 1Kb size marker (Fermentas)
Lane 2- *Pseudomonas* MRSWT3
Lane3- *Pseudomonas* MRSWT4
Lane4- *Pseudomonas* MRSTW6
Lane 5- *Pseudomonas* R6-2

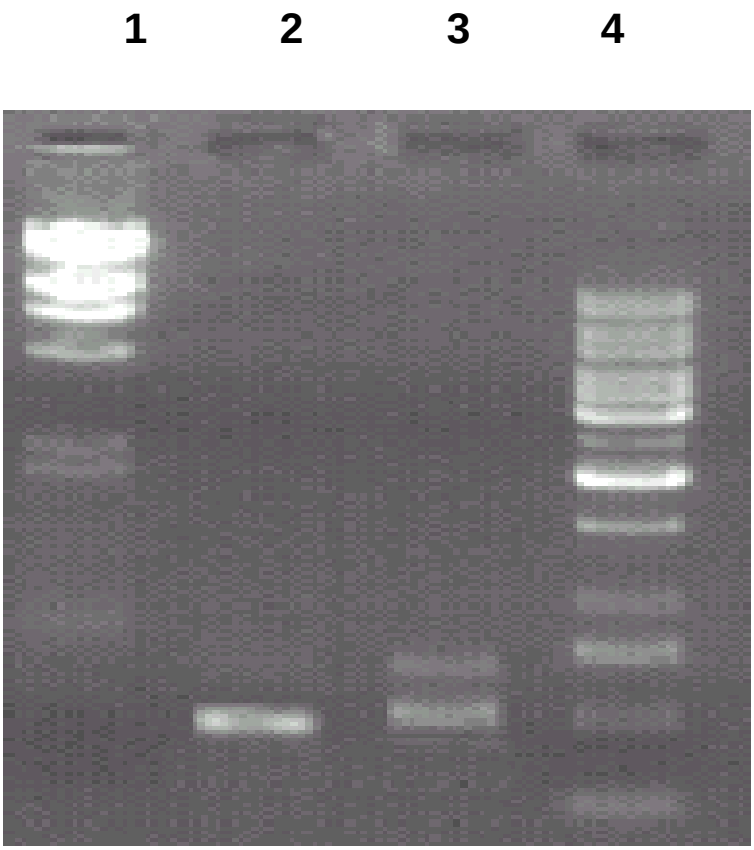

Fig.3- PCR amplification of partial *nif*H from *Azospirillum*

Lane1- **λ/Hind 111 marker (Fermentas)**
Lane2- ***Azospirillum* R1**
Lane3- ***Azospirillum* R3**
Lane4- **1Kb size marker (Fermentas)**

3.8. Effect of inoculated strains on the growth of wheat in the field:

To study the effect of inoculated bacterial strains on the yield and vigor of wheat plant, two wheat varieties (Inqilab 91 and Fakhre Sarhad) were grown at Udigram Swat. The bacterial strains used as inoculant were *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky 1.

3.8.1. Effect of inoculated strains on the growth of wheat variety Inqilab 91:

Effects of the bacterial inoculation was studied on plant length, root and shoot weight, number of spikes/m^2, number of grains/spike, thousand grain yield, biological yield and physiological and harvest maturity. After 70 days of sowing, the inoculated strains showed significant effect of fresh and dry weight of root and shoots. The root and shoot weight of the plants inoculated with *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* Ky1 were significant ($P > 0.05$) greater as compared to control treatment. Maximum root and shoot fresh weight 3.125 g and 6.647 g respectively was observed for the treatment in which *Azospirillum brasilense* strain R1 was used as inoculant. This is followed by *Azospirillum lipoferum* strain RSWT1 with root fresh weight of 3.086 g and shoots fresh weight of 5.436 g. The treatment with *Pseudomonas* Ky1 showed root and shoot fresh weight of 2.885 g and 4.823 g respectively. The increase in root fresh weight was 24.2 % with *Azospirillum brasilense* strain R1, 23.3 % with *Azospirillum lipoferum* strain RSWT1and 17.9 % with *Pseudomonas* Ky1 as compared to control treatment. The increase in shoot fresh weight was 36 % with *Azospirillum brasilense* strain R1, 21.7 % with *Azospirillum lipoferum*

strain RSWT1and 11.8 % with *Pseudomonas* Ky1 as compared to control treatment (Table 5).

The inoculated strains also showed significant increase in root and shoot dry weight. The increase in root dry weight was 32.3 % with *Azospirillum brasilense* strain R1, 28 % with *Azospirillum lipoferum* strain RSWT1and 18.9 % with *Pseudomonas* Ky1 as compared to control treatment. The increase in shoot dry weight was 37.9 % with *Azospirillum brasilense* strain R1, 25.2 % with *Azospirillum lipoferum* strain RSWT1and 14.4 % with *Pseudomonas* Ky1 as compared to control treatment (Fig. 5).

Maximum plant length was observed in the treatment of *Azospirillum brasilense* strain R1. All inoculation caused significant ($P > 0.05$) increases in plant height over all treatments except non-inoculated control. The plant height was significantly ($P > 0.05$) increased up to 18.5 % with *Azospirillum brasilense* strain R1 as compared to non-inoculated controls. The increase in plant height with *Azospirillum lipoferum* strain RSWT1 was 14.7 % and 9.6 % with *Pseudomonas* Ky1. Taller plants (92 cm) were measured for the treatments of *Azospirillum brasilense* strain R1 followed by *Azospirillum lipoferum* RSWT1 (88 cm) and *Pseudomonas* Ky1 (83 cm) while shorter plants (75 cm) were measured for non-inoculated control ones (Fig. 4).

Bacterial inoculation caused the significant ($P > 0.05$) increase in number of grains/ spike over all treatments except non inoculated control ones. Highest number of grains (45) was counted for *Azospirillum brasilense* strain R1 treated plants, whereas lowest number of grains (33) was counted for non-inoculated control plants (Table 5).

In the experiment conducted at Udigram Swat, inoculation of wheat variety Inqilab 91 with *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* Ky1 showed significant increase in number of spikes/m^2. This increase was 22.3 % with *Azospirillum brasilense* strain R1, 16.9 % with *Azospirillum lipoferum* strain RSWT1 and 9.2 % with *Pseudomonas* Ky1 as compared to control treatment (Table 5).

The thousand grain weight of wheat variety Inqilab 91 was influenced by bacterial inoculation (Fig.6). Heavier grains (39.42 g) were recorded for treatment with *Azospirillum brasilense* strain R1 while lighter grains (34.63 g) were noted for controls. Higher biological yield (11658 kg/ha) was produced when treatment was done with *Azospirillum brasilense* strain R1 followed by *Pseudomonas* Ky1. *Azospirillum lipoferum* strain RSWT1 treated plants showed biological yield of 10947 kg/ha while lower biological yield 9313 kg/ha was noted for non-inoculated controls (Fig. 7). Similarly, the effect of inoculation with *Azospirillum brasilense* strain R1 and *Azospirillum lipoferum* strain RSWT1 on number of tillers were significantly higher ($P > 0.05$) as compared to control treatment. Inoculation with *Pseudomonas* Ky1 showed non significance effect on number of tillers/plant (Fig. 8).The data reveled that physiological and harvest maturity days were effected by bacterial inoculation. The inoculated strains took higher number of days to physiological and harvest maturity than non-inoculated. The treatment with *Azospirillum brasilense* strain R1 took higher number of days (147 and 163) to physiological and harvest maturity whereas lower number of days (127 and 145) to physiological and harvest maturity respectively were recorded for non-inoculated control one (Fig 9).

Table 5: Effect of inoculated strains on different parameters of wheat variety Inqilab 91grown at Udigram, Swat

S/N	Variety/ Treatment	Plant Length (cm)	After 90 days of sowing				No of Tillers	No of Spikes/m^2	No of Grains/ spike	Thousand grain yield (g)
			Root Fresh wt (g)	Root Dry wt (g)	Shoot Fresh wt (g)	Shoot Dry wt (g)				
01	Inq-91-T1U	75 (±3.6)	2.367 (±2.3)	1.328 (±6.21)	4.253 (±2.33)	2.428 (±3.11)	2.45 (±4.67)	216 (±3.56)	33 (±2.36)	34.63 (±2.11)
02	Inq-91-T2U	92 (±6.2)	3.125 (±1.6)	1.963 (±2.43)	6.647 (±1.25)	3.675 (±2.47)	3.82 (±3.28)	278 (±4.26)	45 (±1.48)	39.42 (±2.43)
03	Inq-91-T3U	88 (±7.2)	3.086 (±2.45)	1.849 (±3.11)	5.436 (±2.43)	3.246 (±1.76)	3.75 (±1.37)	260 (±2.34)	41 (±1.76)	37.24 (±1.27)
04	Inq-91-T4U	83 (±4.0)	2.885 (±3.47)	1.638 (±1.37)	4.823 (±1.39)	2.836 (±3.21)	2.86 (±1.83)	238 (±2.61)	40 (±1.78)	37.21 (±3.39)

T1 – Control (non- inoculated) T2- *Azospirillum brasilense* R1 T3- *Azospirillum lipoferum* RSWT1

T4 – *Pseudomonas* Ky1 U – Udigram Swat Inq-91- Wheat variety Inqilab 91;

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Note: The values are average of 5 plants and the values in brackets represent Standard Deviation

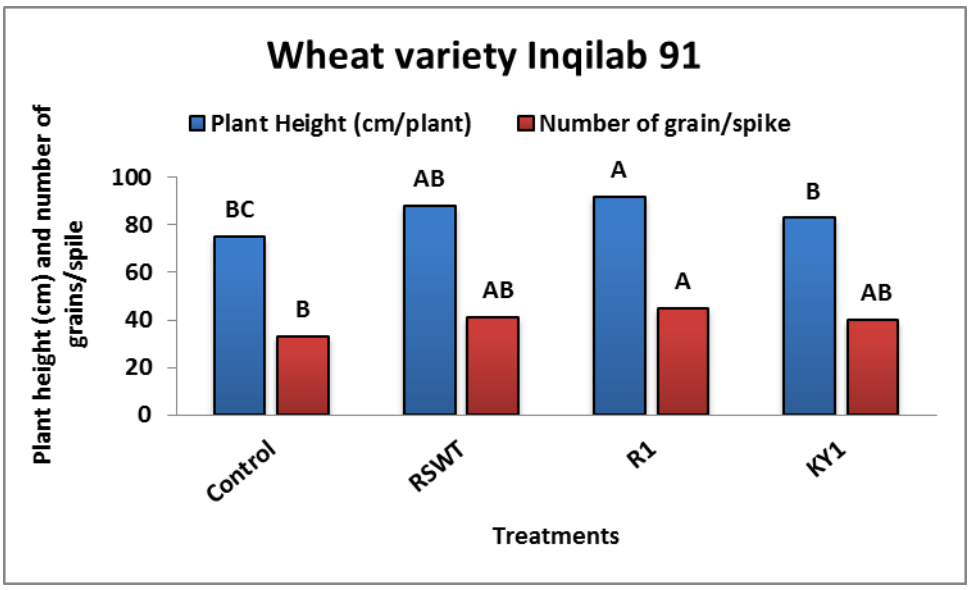

Figure 4: The effect of bacterial inoculation on plant length and Number of Grains

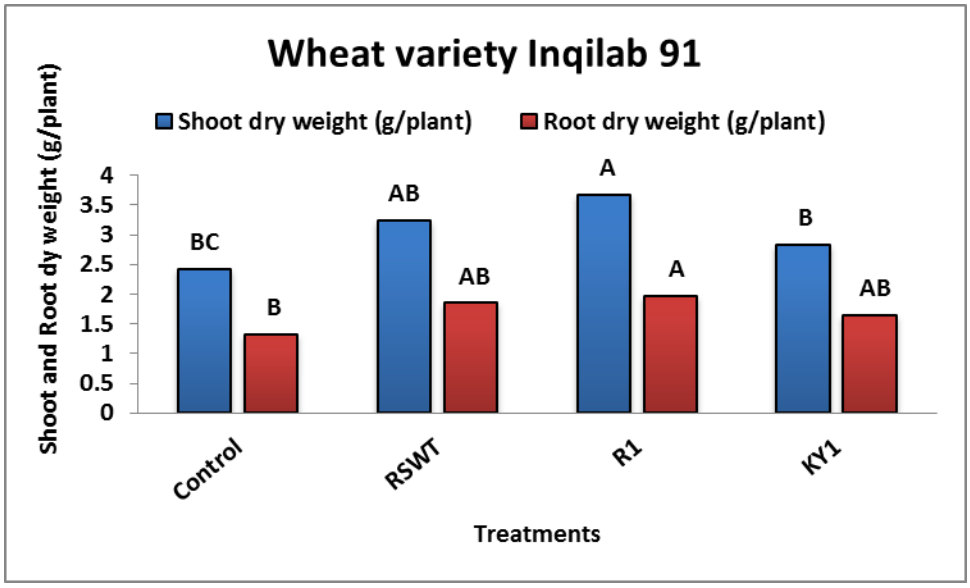

Figure 5: The effect of bacterial inoculation on root and shoot dry weight

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 12 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

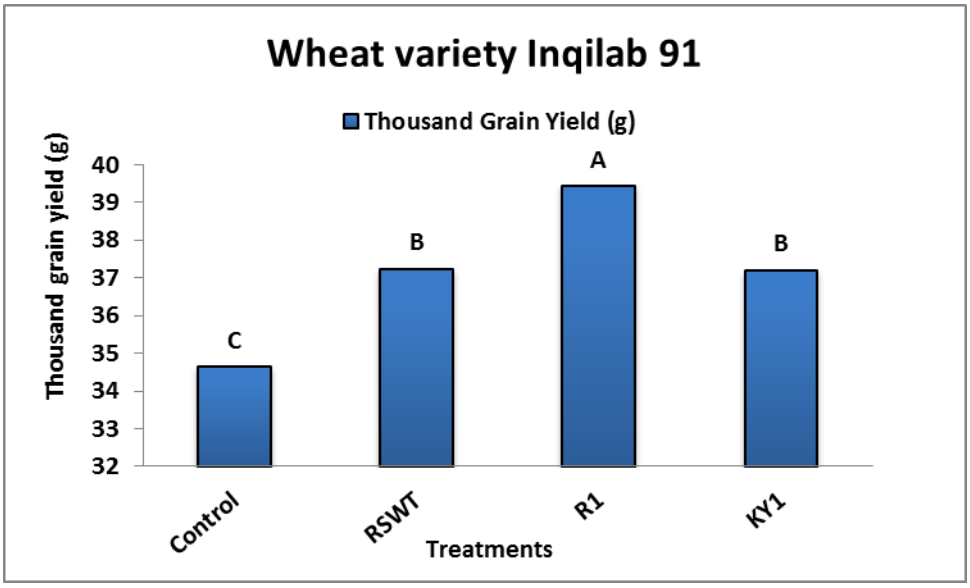

Figure 6: The effect of of bacterial inoculation thousand Grain Yield (g)

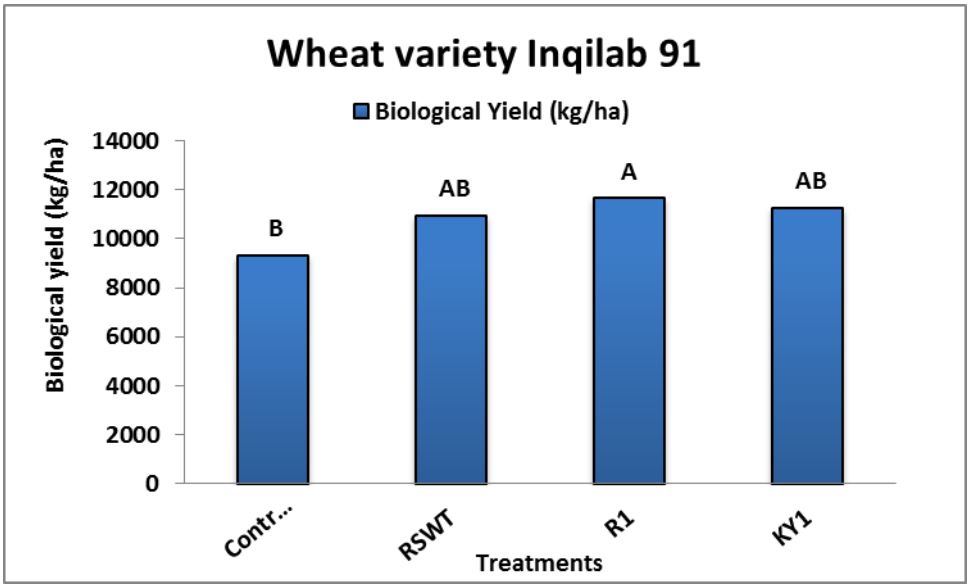

Figure 7: The effect of bacterial inoculation on Biological Yield (Kg/ha) of Inqilab 91

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 12 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

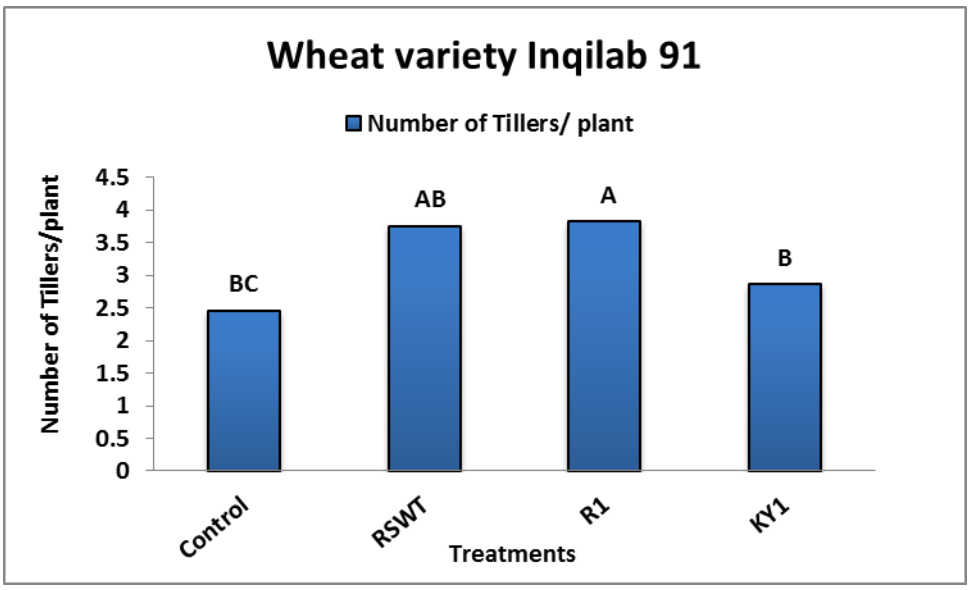

Figure 8: The effect of of bacterial inoculation on Number of Tillers/ plant

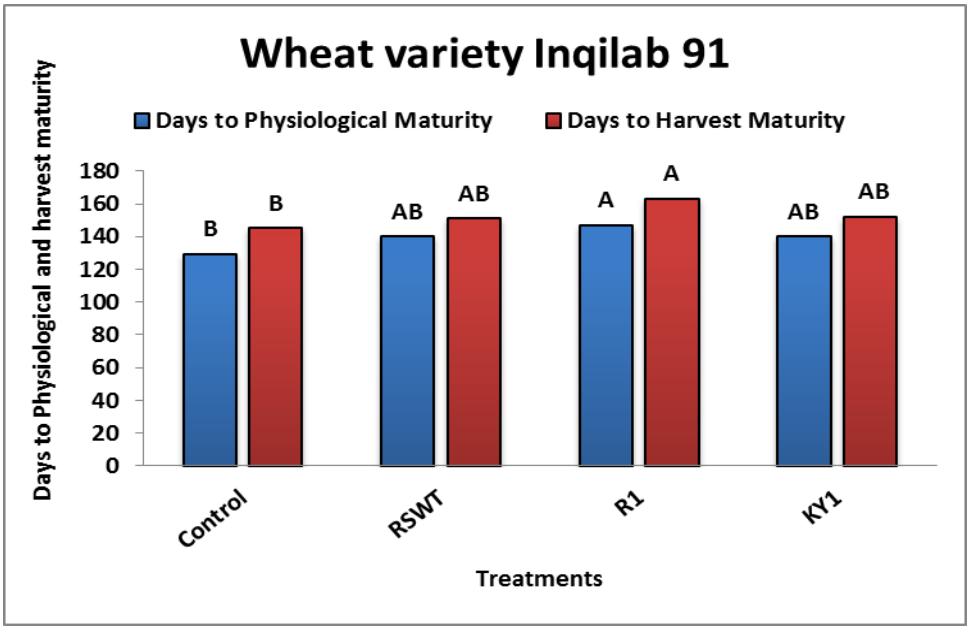

Figure 9: The effect of of bacterial inoculation on Physiological and Harvest Maturity

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 12 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

3.8.2. Effect of inoculated strains on the growth of wheat variety Fakhre Sarhad:

The present investigation showed significant positive effects of bacterial inoculation on different growth parameters like plant length, root and shoot weight, number of spikes/m^2, number of grains/spike, thousand grain yield, biological yield and physiological and harvest maturity. After 70 days of sowing, the inoculated strains showed significant effect of fresh and dry weight of root and shoots. The root and shoot weight of the plants inoculated with *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* Ky1 were significantly ($P > 0.05$) greater as compared to control treatment. Maximum root and shoot fresh weight 3.115 g and 6.328 g respectively was observed for the treatment in which *Azospirillum brasilense* strain R1 was used as inoculant. This is followed by *Azospirillum lipoferum* strain RSWT1 with root weight of 2.689 g and shoots weight of 5.988 g. The treatment with *Pseudomonas* Ky1 showed root and shoot fresh weight of 2.562 g and 4.427 g respectively. The increase in root fresh weight was 14.5 % with *Azospirillum brasilense* strain R1. The inoculated bacterial strains *Azospirillum lipoferum* strain RSWT1and *Pseudomonas* Ky1 did not show significant effects on root fresh weight as compared to control treatment. The increase in shoot fresh weight was 36.86 % with *Azospirillum brasilense* strain R1, 33.28 % with *Azospirillum lipoferum* strain RSWT1and 9.75 % with *Pseudomonas* Ky1 as compared to control treatment (Table 6).

The inoculation with *Azospirillum brasilense strain* R1 showed 34.6 % increase in the shoot dry weight and 21.8 % increase in root dry weight. The bacterial strain

Azospirillum lipoferum RSWT1 increase the shoot dry weight upto 28.9 %. However its effect on root dry weight was not significant (Fig.11).

The plant height was significantly increase up to 12 % with *Azospirillum lipoferum* strain RSWT1 as compared to non-inoculated control ones. The increase in plant height with *Azospirillum brasilense* strain R1 was 7.5 %. The effect of inoculation with *Pseudomonas* Ky1 was not significant as compared to control treatment. Higher plants (83 cm) were measured for treatment with *Azospirillum lipoferum* RSWT1 followed by *Azospirillum brasilense strain* R1 (79 cm). The lower height of the plants (72 cm and 73 cm) was measured for *Pseudomonas* Ky1 and non-inoculated control ones respectively (Fig. 10).

In wheat variety Fakhre Sarhad the inoculated bacterial strains caused significant increase in number of grains/ spike over all treatments except non inoculated control ones. Highest number of grains (40) was counted for treatment with *Azospirillum brasilense* strain R1 whereas lowest number of grains (32) was counted for non-inoculated control plants (Fig.11).

In the experiment conducted at Udigram Swat, inoculation of wheat variety Fakhre Sarhad with *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* Ky1 showed significant increase in number of spikes/m^2. This increase was 16.4 % with *Azospirillum brasilense* strain R1, 13.19 % with *Azospirillum lipoferum* strain RSWT1 and 6.42 % with *Pseudomonas* Ky1 as compared to control treatment (Table 6).

The effect of bacterial inoculation on thousand grain yield is given in Table 6. Heavier 1000 grains (37.79 g) were recorded for treatment with *Azospirillum brasilense* strain R1 while lighter 1000 grains yield (32.57 g) was noted for control non-inoculated ones. The treatment with *Azospirillum lipoferum* strain RSWT1showed grains weight of 36.34 g and *Pseudomonas* Ky1 showed 33.59 g.

Biological yield data collected from whole plots indicated that the effect of inoculations was significant. Higher biological yield (10524 kg/ha) was produced when treatment was done with *Azospirillum brasilense* strain R1 followed by treatment with *Azospirillum lipoferum* strain RSWT1 (10345 kg/ha). The treatment with *Pseudomonas* Ky1 showed biological yield of 9247 kg/ha while lower biological yield 8485 kg/ha was noted for non-inoculated control treatments (Fig. 13). Similarly, the inoculation of *Azospirillum brasilense strain R1* and *Azospirillum lipoferum* strain RSWT1 showed positive effects on number of tillers/plant than those of non-inoculated ones. Inoculation with *Pseudomonas* Ky1 showed no significance effect on number of tillers/plant (Fig. 14).

The field experiment showed that the physiological and harvest maturity days were effected by bacterial inoculation. In case of wheat variety Fakhre Sarhad, the non-inoculated control treatment took higher number of days to physiological and harvest maturity than inoculated ones. The treatment with *Azospirillum brasilense* strain RSWT1 took lower number of days (130 and 144) to physiological and harvest maturity whereas higher number of days (137 and 157) to physiological and harvest maturity respectively was recorded for non-inoculated control ones (Fig 15).

Table 6: Effect of inoculated strains on different parameters of wheat variety Fakhre Sarhad grown at Udigram, Swat

S/N	Variety/ Treatment	Plant Length (cm)	After 90 days of sowing				No of Tillers	No of Spikes/m^2	No of Grains/ spike	Thousand grain yield (g)
			Root Fresh wt (g)	Root Dry wt (g)	Shoot Fresh wt (g)	Shoot Dry wt (g)				
05	FS-T1-U	73 (±7.6)	2.674 (±1.28)	1.437 (±3.26)	3.995 (±4.53)	2.265 (±2.35)	2.14 (±2.47)	204 (±1.74)	32 (±2.34)	32.57 (±1.61)
06	FS-T2-U	79 (±5.6)	3.115 (±3.47)	1.838 (±1.57)	6.328 (±3.52)	3.467 (±3.22)	3.48 (±3.11)	244 (±2.37)	40 (±3.21)	37.79 (±2.38)
07	FS-T3-U	83 (±7.9)	2.689 (±4.59)	1.426 (±2.97)	5.988 (±4.18)	3.189 (±3.18)	3.42 (±3.12)	235 (±2.39)	38 (±4.17)	36.34 (±3.17)
08	FS-T4-U	72 (±5.7)	2.562 (±3.21)	1.483 (±2.41)	4.427 (±4.63)	2.426 (±2.56)	2.36 (±2.15)	218 (±3.47)	35 (±1.67)	33.59 (±1.53)

T1 – Control (non- inoculated) T2- *Azospirillum brasilense* R1 T3- *Azospirillum lipoferum* RSWT1
T4 – *Pseudomonas* Ky1 U – Udigram Swat FM- Wheat variety Fakhre Sarhad.

Note: The values are average of 5 plants and the values in brackets represent Standard Deviation

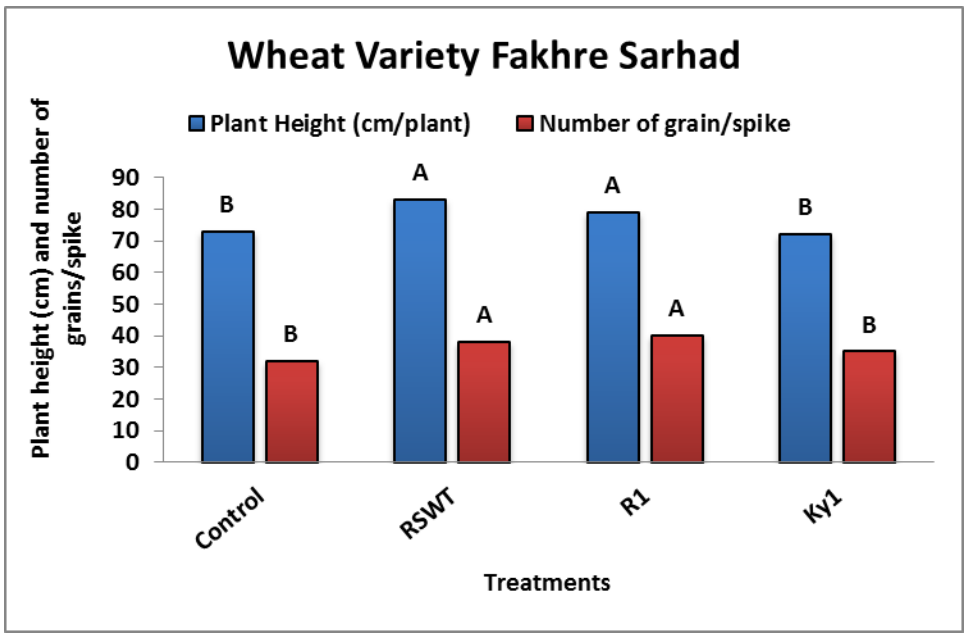

Figure 10: The effect of bacterial inoculation on plant length and Number of Grains

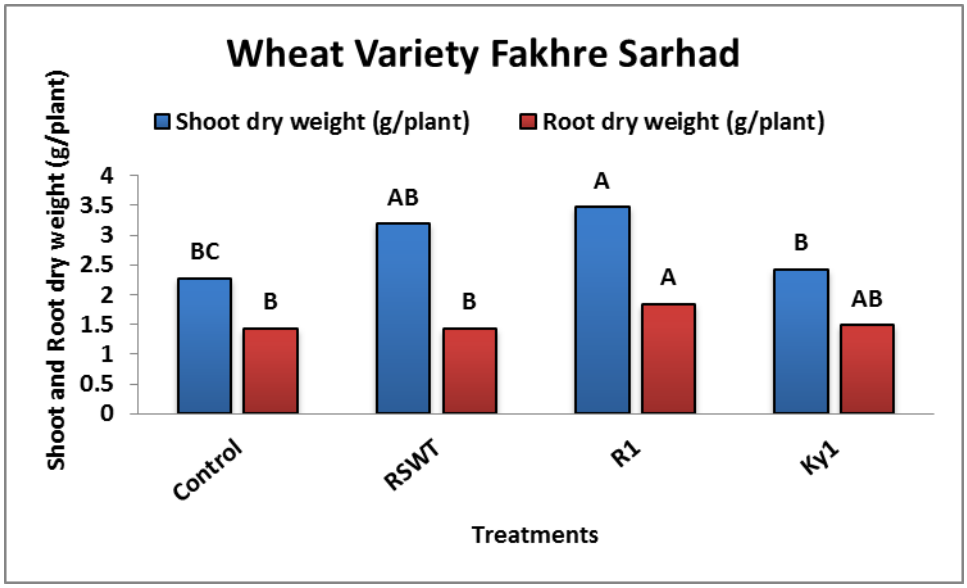

Figure 11: The effect of bacterial inoculation on root and shoot dry weight

Control: Non-inoculated **RSWT:** *Azospirillum lipoferum*

R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

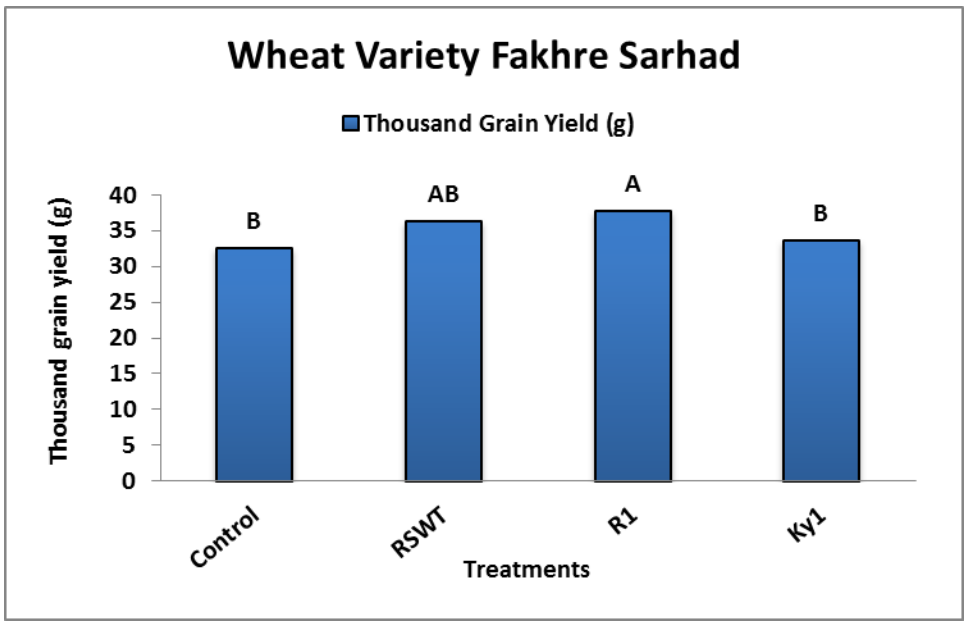

Figure 12: The effect of of bacterial inoculation on thousand Grain Yield (g)

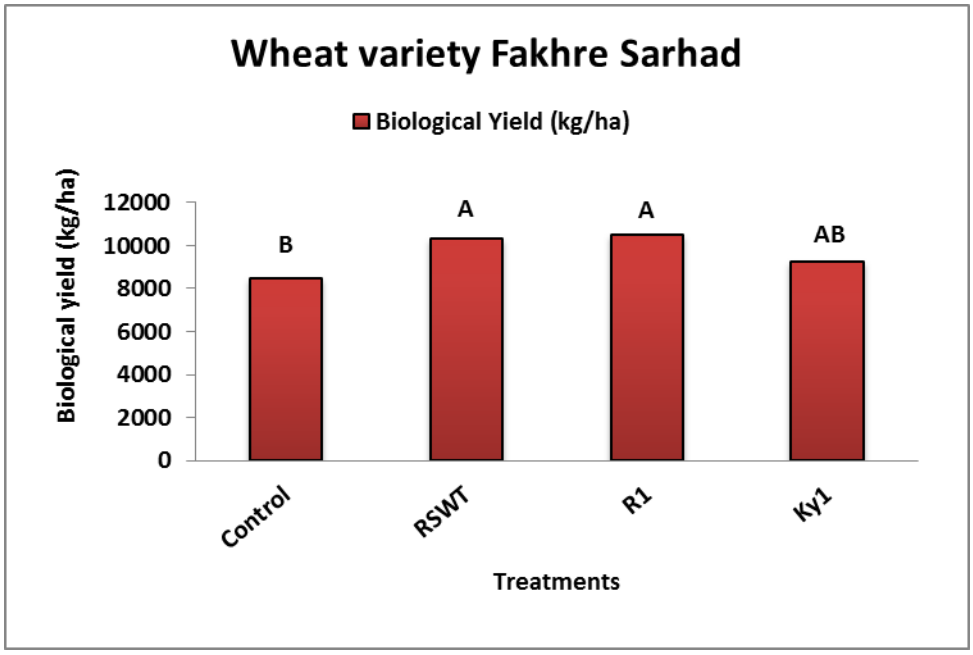

Figure 13: The effect of bacterial inoculation on Biological Yield (Kg/ha)

Control : Non-inoculated **RSWT : *Azospirillum lipoferum***
R1 : *Azospirillum brasilense* **Ky1 : *Pseudomonas***

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

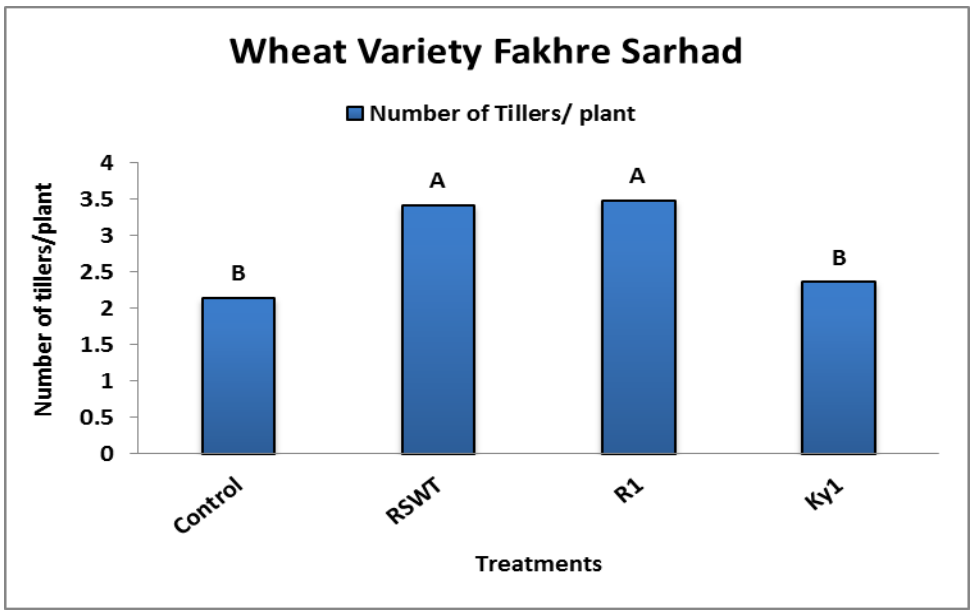

Figure 14: The effect of bacterial inoculation on Number of Tillers/ plant

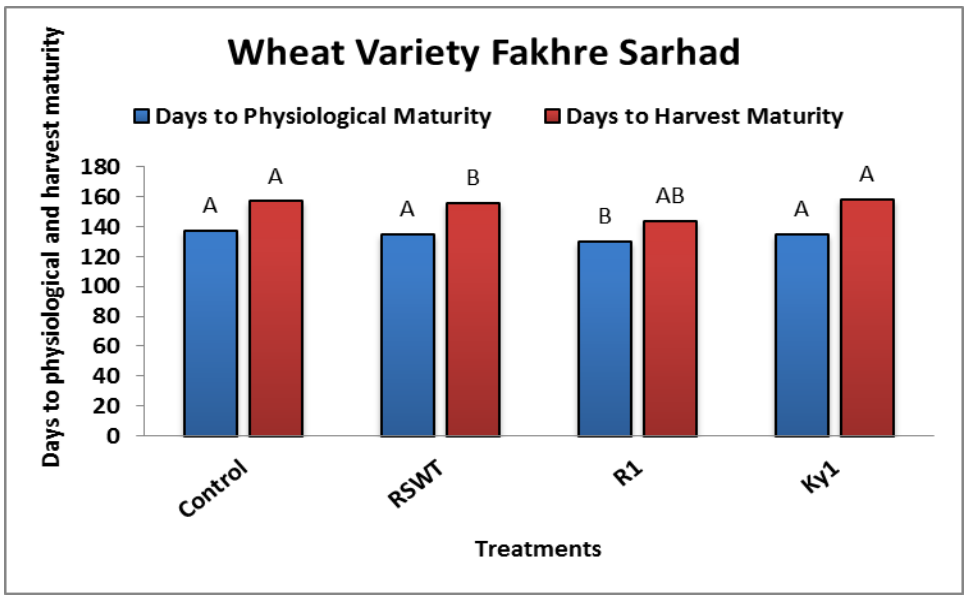

Figure 15: The effect of bacterial inoculation on Physiological and Harvest Maturity

Control: Non-inoculated **RSWT1 :** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

3.9. Effect of inoculated strains on the growth and yield of rice

3.9.1. Inoculation of rice with bacterial strains in Falcon tubes

The rice varieties Fakhre Malakand and JP 5 were grown and inoculated with bacterial strains in Falcon tubes to study their effects on growth and development. The bacterial strains used as inoculants were *Azospirillum lipoferum* strain RSWT1, *Azospirillum brasilense* strain R1 and *Pseudomonas* strain Ky1. Effect of the bacterial inoculation was studied on root length, root area, root weight and shoot weight. All the inoculated strains showed positive effect on plant growth of both the rice varieties as compared to non-inoculated control (Figure 16 and 17).

In rice variety Fakhre Malakand, the root area was not significantly different in inoculated and non-inoculated treatments, while the root length showed positive response to bacterial inoculation than control treatment. Among the strains tested, the *Azospirillum brasilense* strains R1 showed best results. The plant inoculated with *Azospirillum brasilense* strain R1 showed maximum increase in root weight while maximum increase in shoot weight of plants was observed due to *Pseudomonas* strain Ky1 inoculation.

In the rice variety JP 5, statistically significant improvement in the root area was not observed in any inoculated treatments compared to non-inoculated control. In all the inoculated treatments, improvements in root length, root weight and shoot weight were observed as compared to control treatments. The only exception was noted in root weight of plants inoculated with *Pseudomonas* strain Ky1 where the difference between root

weight of control plants and the plants inoculated with *Pseudomonas* Ky1 was not statistically different.

3.9.2 Inoculation of rice in Pot experiment

Seeds of the selected rice varities Fakhre Malakand and JP 5 were also cultivated in pots at NIBGE, Faisalabad. The pot experiment failed most probably due to high day temperatures compared to Swat. Due to death of plants the pot experiment was abandoned.

3.9.3. Effect of inoculated strains on different growth parameters of the field grown rice plants

A field experiment was conducted with rice varieties Fakhre Malakand and JP 5 at Agricultural Research Institute (N) Mingora and Udigram Swat (Figures 50 - 57). Soil analysis of the experimental sites at Agricultural Research Institute (North) Mingora, Swat indicated that the soil was sandy loam (pH 7.3) while the soil at experimental site Udigram was also sandy loam (pH 7.4). Three bacterial strains *Azospirillum lipoferum* strain MRSWT1, *Azospirillum brasilense* strain R1 and *Pseudomonas* strain Ky1 were used as inoculants. Roots of one month old rice seedlings were kept in the bacterial suspensions for 1 hour before transplanting (Fig. 51). Four treatments with four replicates in randomized complete block design were studied.

Rhizospheric soil samples were collected one month after transplantation to study total bacterial population. Bacterial population was studied through total viable counts on LB agar plates. The soil samples collected from both the experimental fields showed

bacterial population in the range of 10^5 cells/ g of soil (Table 3). In general the bacterial population detected in the soil of Treatment 3 (*Azospirillum brasilense* R1) on LB plates was higher than all others. Maximum bacterial population (2.90×10^5 cells per g of soil) was detected in the rhizosphere of rice variety JP 5 grown at Udigram Swat.

Whole rice plants (20 plants form each treatment) from both experimental sites i.e Agricultural Research Institute (North) Mingora, Swat and Udigram, Swat were collected to study the effect of bacterial inoculants on various growth parameters including root weight (Table 7 and 8).

In the experiment conducted at Agricultural Research Institute (N) Mingora, Swat, maximum plant length of rice variety Fakhre Malakand was observed in the treatment in which *Azospirillum brasilense* strain R1 was used as inoculant, while maximum plant length of rice variety JP 5 was noted in non-inoculated treatments. In the rice variety JP 5, inoculation with *Azospirillum brasilense* strain R1 resulted in improved growth of plants which was evident in increased root weight, shoot weight and grain weight as compared to all other treatments including control. In the rice variety Fakhre Malakand also the same strain performed more efficiently as compared to other bacterial inoculants tested in the present study. Inoculation with this strain (*Azospirillum brasilense* strain R1) resulted in significant increase in fresh and dry weight of root and shoot. However maximum increase in grain weights (fresh and dry weight), number of grains and tillers of this variety was observed in plants inoculated with *Pseudomonas* strain Ky1.

The same three bacterial strains (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) were also tested as inoculants for the two rice varieties at Udigram, Swat. Maximum plant height of rice variety JP 5 was noted in the treatment in which *Pseudomonas* strain Ky1 was used as inoculant. In this variety maximum beneficial effect on most growth parameters (root fresh weight, shoot fresh weigh, shoot dry weight, grain fresh, dry weight and number of grains) was observed in plants inoculated with *Azospirillum lipoferum* strain RSWT1.

In the rice variety Fakhre Malakand grown at Udigram, Swat, the inoculant *Azospirillum brasilense* strain R1 showed excellent growth promotion which is evident in fresh and dry weight of shoots and grains. However, maximum plant length was observed in non-inoculated treatments and maximum beneficial effects on plant roots were observed in plants inoculated with *Azospirillum lipoferum* strain RSWT1.

3.9.4. Effect of bacterial inoculation on growth of rice in the field experiment

Yield data collected from whole plots indicated that bacterial inoculation of rice varieties Fakhre Malakand and JP 5 at both experimental sites (i.e ARIN, Swat and Udigram, Swat) showed more yields than non-inoculated control. The effect of the inoculated strains was positive on fresh and dry weight of both rice varieties.

3.9.5. Effect of bacterial inoculation on growth of rice variety Fakhre Malakand

In the experiment conducted at Agricultural Research Institute (N) Mingora, Swat, inoculation of rice variety Fakhre Malakand with *Azospirillum brasilense* strain R1 significantly increased the fresh and dry weight of straw, grain and total plant weight (Fig

24-26). This increase was 20.3 % in fresh straw weight, 16.6 % in dry straw weight (Fig 24), 28 % in fresh grain weight, 22.7% in dry grain weight (Fig 25), 20.3 % in total plant fresh weight and 19.8 % in total plant dry weight (Fig 26) over the non-inoculated control.

The inoculation of rice variety Fakhre Malakand with *Pseudomonas* strain Ky1 also showed significant increase in the straw weight by 13.4 %, dry grain weight by 17.3 % and total plant dry weight by 14.7 %, (Figure 24-26). Inoculation of *Azospirillum lipoferum* strain RSWT1 showed no significant effect on the total plant dry matter, however an increase of 4.8 % in the dry grain weight of rice variety Fakhre Malakand was observed.

At the experimental site Udigram, Swat, maximum growth promotion of rice variety Fakhre Malakand was observed in the treatment in which *Azospirillum brasilense* strain R1 was used as inoculant. The increase in fresh straw weight, fresh grain weight and total fresh weight was 14.3 %, 22 %, 16.8%, respectively, over non-inoculated control (Fig 30-32). The values for dry weight increase were 14.2 % for straw weight, 22 % for grain weight and 17 % for total plant weight. Improvement in the growth of plants inoculated with two other bacterial strains (*Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) over non-inoculated control treatment was observed but it was not comparable to that obtained from the treatment in which *Azospirillum brasilense* strain R1 was used as inoculant.

3.9.6. Effect of bacterial inoculation on growth of rice variety JP 5

At the experimental site (ARIN) the *Azospirillum brasilense* strain R1 showed maximum growth promotion of rice variety JP 5 as compared to other inoculants used in

this study (Fig 24-29, 56). Inoculation with this strain resulted in 19 % increase in fresh straw weight, 39.3 % increase in fresh grain weight and 29 % increase in the total plant fresh weight over non-inoculated control. The increase in the dry straw weight, dry grain weigh, dry total plant weight were 19 %, 39.5 %, 30.8 % respectively. Inoculation with *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 showed significant increase (18.5 % and 13.57 % respectively) in the dry grain weight of rice variety JP 5.

At Udigram Swat, similar growth promotion activities of *Azospirillum brasilense* strain R1 was observed on rice variety JP 5. Inoculation with this strain resulted in 15.9 % increase in fresh straw weight, 27.5 % increase in fresh grain weight and 19.5 % increase in total fresh plant weight over the non-inoculated control. The values for dry weight were 15.5 % increase in straw weight, 27.4 % increase in grain weight and 16 % increase total plant weight over non-inoculated control. In the same variety any significant beneficial effect of inoculation with *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 was not observed on straw weight, grain weight and total plant weight.

Table 7: Effect of inoculated strains on different parameters of rice plants grown at ARIN, Swat (Whole plant data)

S/N	Variety	Plant Length (cm)	Root Fresh wt (g)	Root Dry wt (g)	Shoot Fresh wt (g)	Shoot Dry wt (g)	Grain Fresh wt (g)	Grain Dry wt (g)	No Of Grains (g)	No of sterile Grains	No of Tillers	No of Panicles	Fresh Wt (g) of Panicles	Dry wt (g)of Panicle
01	JP-5-T1R	**133.7 (±3.5)**	**16.2 (±419)**	**8.5 (±2.1)**	**73.0 (±4.7)**	**38.4 (±1.8)**	**41.3 (±11.3)**	**27.9 (±7.1)**	**1366.6 (±440)**	**119.8 (±114)**	**10.4 (±1.7)**	**9.6 (±2.1)**	**1.2 (±0.5)**	**0.8 (±0.2)**
02	JP-5-T2R	**130.3 (±2.7)**	**20.8 (±3.8)**	**10.1 (±1.7)**	**92.0 (±12.9)**	**56.0 (±11.1)**	**47.4 (±11.5)**	**39.3 (±11.7)**	**1563.2 (±24.1)**	**19.6 (±20.6)**	**13.6 (±3.3)**	**13.2 (±2.7)**	**1.9 (±0.6)**	**1.2 (±0.5)**
03	JP-5-T3R	**130.1 (±2.2)**	**30.2 (±5.6)**	**15.7 (±2.8)**	**94.8 (±20.1)**	**63.5 (±13.6)**	**48.2 (±12.4)**	**39.8 (±10.1)**	**1993.4 (±350)**	**21 (±21.9)**	**14 (±1.8)**	**13.2 (±2.7)**	**2.4 (±0.8)**	**1.6 (±0.7)**
04	JP-5-T4R	**128.2 (±7.7)**	**27.9 (±10.1)**	**13.9 (±5.1)**	**92.0 (±78.6)**	**59.2 (±52.1)**	**47.0 (±18.3)**	**37.1 (±15.4)**	**1517.6 (±685)**	**22.4 (±18.6)**	**14.6 (±5.0)**	**13.8 (±5.3)**	**2.0 (±1.2)**	**1.3 (±0.6)**
05	FM-T1-R	**107.1 (±2.9)**	**8.5 (±0.8)**	**4.7 (±0.7)**	**72.0 (±9.9)**	**40.5 (±3.1)**	**42.7 (±5.7)**	**29.6 (±2.8)**	**1442 (±45.2)**	**133.2 (±46.5)**	**10.4 (±1.4)**	**9.6 (±1.5)**	**2.3 (±0.4)**	**1.5 (±0.3)**
06	FM-T2-R	**112.9 (±7.6)**	**10.4 (±2.0)**	**5.5 (±1.1)**	**85.9 (±13.3)**	**53.3 (±9.7)**	**49.8 (±5.2)**	**40.6 (±4.8)**	**1633.6 (±85.7)**	**31.2 (±45.5)**	**14.4 (±0.8)**	**13.8 (±0.8)**	**2.4 (±0.4)**	**1.6 (±0.4)**
07	FM-T3-R	**117.0 (±14.5)**	**27.1 (±1.6)**	**13.1 (±4.4)**	**106.9 (±15.4)**	**72.3 (±15.5)**	**56.1 (±11.3)**	**48.4 (±10.6)**	**1958 (±370)**	**19.4 (±9.8)**	**13.4 (±2.8)**	**12.4 (±2.8)**	**3.5 (±2.2)**	**1.9 (±1.2)**
08	FM-T4-R	**105.0 (±11.3)**	**16.3 (±9.1)**	**8.3 (±5.1)**	**114.0 (±54.1)**	**60.3 (±22.5)**	**67.8 (±27.5)**	**58.2 (±23.5)**	**2241.2 (±947)**	**19.8 (±27.5)**	**17.6 (±9.4)**	**16 (±1.0)**	**3.4 (±0.5)**	**2.4 (±0.8)**

T1 – Control (non- inoculated) T2- *Azospirillum lipoferum* RSWT1 T3- *Azospirillum brasilense* R1
T4 – *Pseudomonas* Ky1 R – Agriculture Research Institution (N) Mingora Swat JP 5- Rice variety JP 5
FM- Rice variety Fakhre Malakand.

Note: The values are average of 20 plants and the values in brackets represents Standard Deviation

Table 8: Effect of inoculated strains on different parameters of rice plant grown at Udigram, Swat (Whole plant data)

S/N	Variety	Plant Length (cm)	Root Fresh wt (g)	Root Dry wt (g)	Shoot Fresh wt (g)	Shoot Dry wt (g)	Grain Fresh wt (g)	Grain Dry wt (g)	No Of Grains	No of sterile Grains	No of Tillers	No of Panicles	Fresh Wt (g) of Panicle	Dry wt of (g) Panicle
09	JP-5-T1-U	127.4 (±3.6)	14.3 (±1.2)	7.2 (±0.7)	140.9 (±7.2)	99.2 (±32.8)	44.9 (±3.2)	31.0 (±4.7)	1210.4 (±318.6)	121.4 (±75.0)	15.8 (±1.6)	12.2 (±1.7)	1.7 (±0.2)	1.1 (±0.1)
10	JP-5-T2-U	129.6 (±5.2)	29.2 (±3.4)	16.8 (±3.8)	135.6 (±6.7)	94.4 (±13.6)	73.4 (±14.3)	66.8 (±13.8)	2327 (±337.4)	26.8 (±25.7)	17.8 (±1.4)	17.2 (±1.3)	2.1 (±0.5)	1.5 (±0.4)
11	JP-5-T3-U	126.4 (±6.2)	24.7 (±8.2)	16.9 (±7.2)	89 (±33.5)	65.0 (±24.7)	50.9 (±15.8)	45.2 (±15.8)	1851.6 (±615)	10 (±10)	16 (±2.2)	14.8 (±1.8)	1.8 (±0.7)	1.2 (±0.6)
12	JP-5-T4U	135.4 (±6.5)	21.6 (±14.4)	15.5 (±11.3)	108.6 (±71.4)	88.1 (±57.0)	67.3 (±22.4)	58.8 (±20.8)	2168 (±680.3)	8.4 (±38.7)	16.2 (±3.9)	15 (±3.4)	2.7 (±0.7)	1.8 (±0.6)
13	FM-T1-U	132.5 (±4.0)	19.9 (±7.6)	10.4 (±4.0)	109.5 (±26.2)	56.9 (±18.5)	67.3 (±30.0)	59.6 (±28.1)	2328 (±873.2)	23 (±13.2)	18.4 (±7.2)	16.6 (±5.2)	2.7 (±0.8)	2.0 (±0.6)
14	FM-T2-U	105.3 (±5.3)	25.5 (±4.7)	17.5 (4.1)	127.5 (±21.8)	101.9 (±19.7)	67.8 (±11.9)	60.6 (±10.6)	2679.2 (±416.7)	12.4 (±8.5)	17.6 (±3.8)	16.2 (±2.2)	2.3 (±0.4)	1.8 (±0.4)
15	FM-T3-U	110.3 (±6.8)	22.6 (±7.9)	16.2 (±7.6)	137.0 (±30.6)	112.6 (±22.7)	69.8 (±16.7)	72.4 (±12.5)	2221.4 (±543.3)	9 (±8.8)	16.6 (±5.9)	15.4 (±4.9)	2.3 (±0.9)	1.6 (±0.4)
16	FM-T4-U	114.5 (±7.5)	20.9 (±5.6)	14.3 (±5.7)	95.9 (±25.3)	67.2 (±28.6)	50.7 (±20.0)	44.5 (±19.8)	1786.4 (±446.2)	8.4 (±6.1)	14.4 (±1.6)	13.4 (±0.8)	2.2 (±0.4)	1.4 (±0.4)

T1 – Control (non- inoculated) T2- *Azospirillum lipoferum* RSWT1 T3- *Azospirillum brasilense* R1 T4 – *Pseudomonas* Ky1 U- Udigram, Swat, JP 5- Rice variety JP 5 FM- Rice variety Fakhre Malakand.

Note: The values are average of 20 plants and the values in brackets represents Standard Deviation

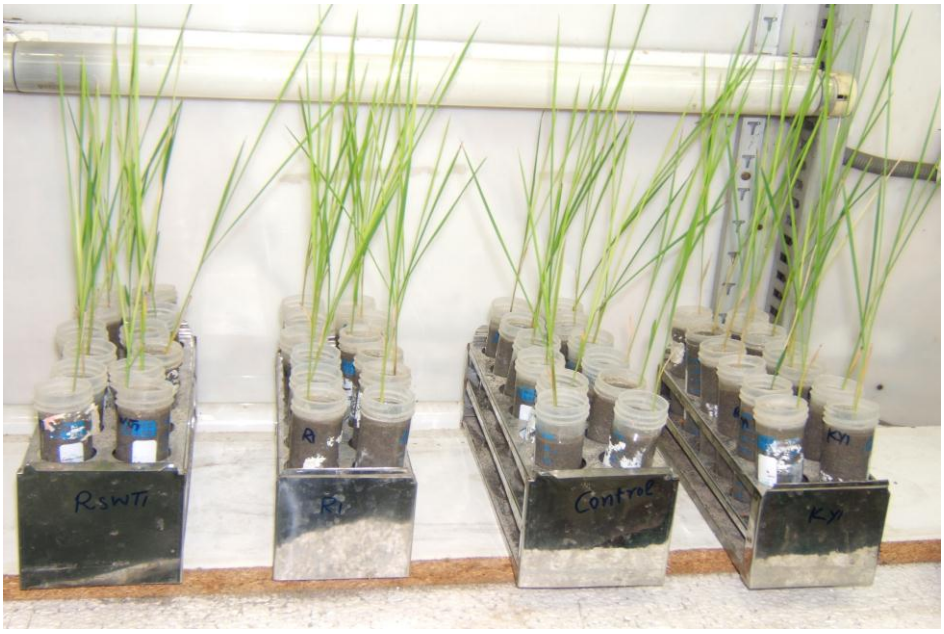

Fig 16: Inoculation of rice variety JP 5 in Control-Temperature Room

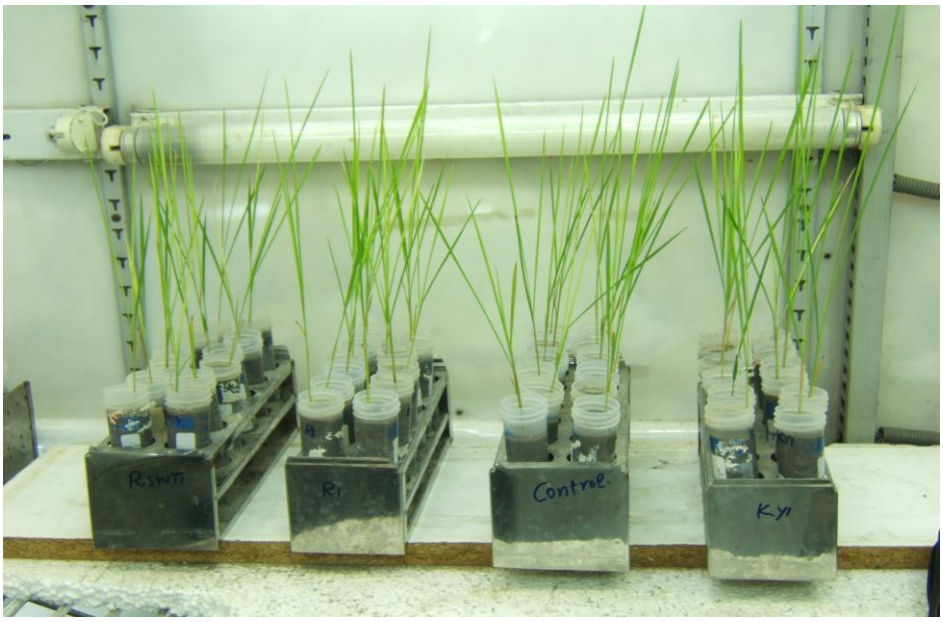

Fig 17: Inoculation of rice variety Fakhre Malakand in Control-Temperature Room

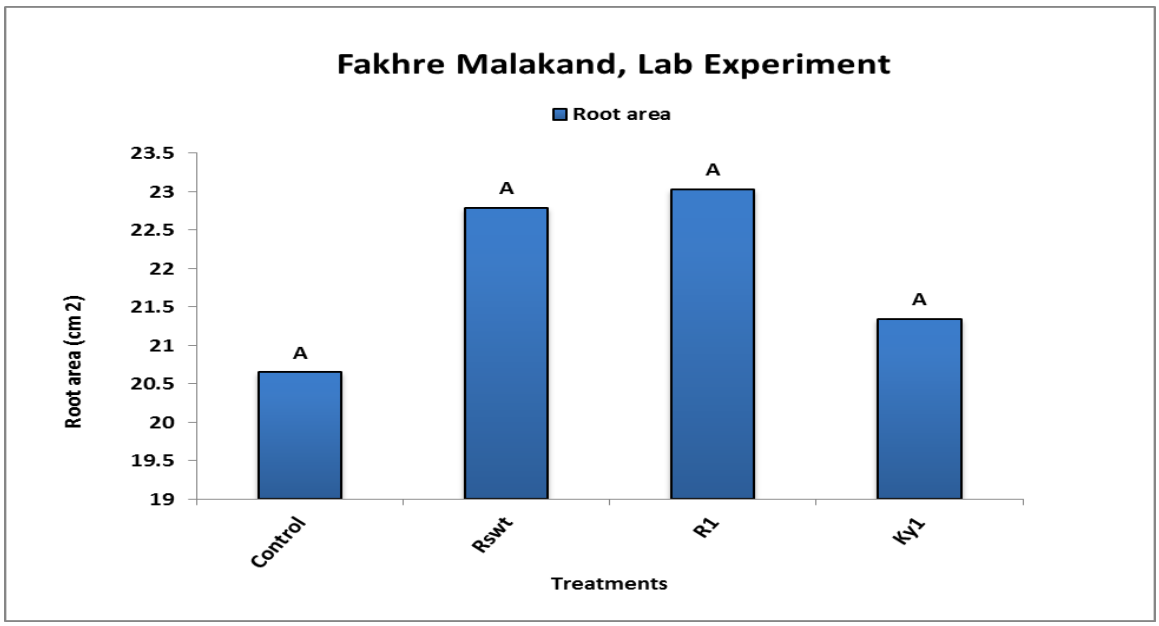

Fig 18: Effect of the bacterial inoculation on the root area of rice variety Fakhre Malakand

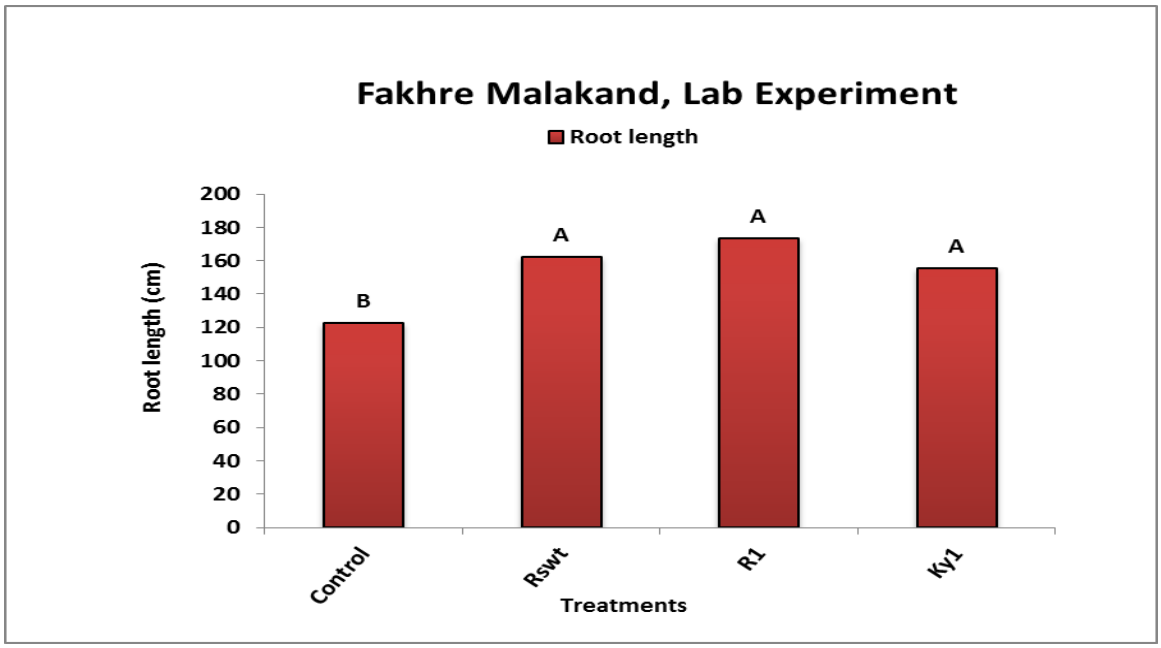

Fig 19: Effect of the bacterial inoculation on the root length of rice variety Fakhre Malakand

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 12 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

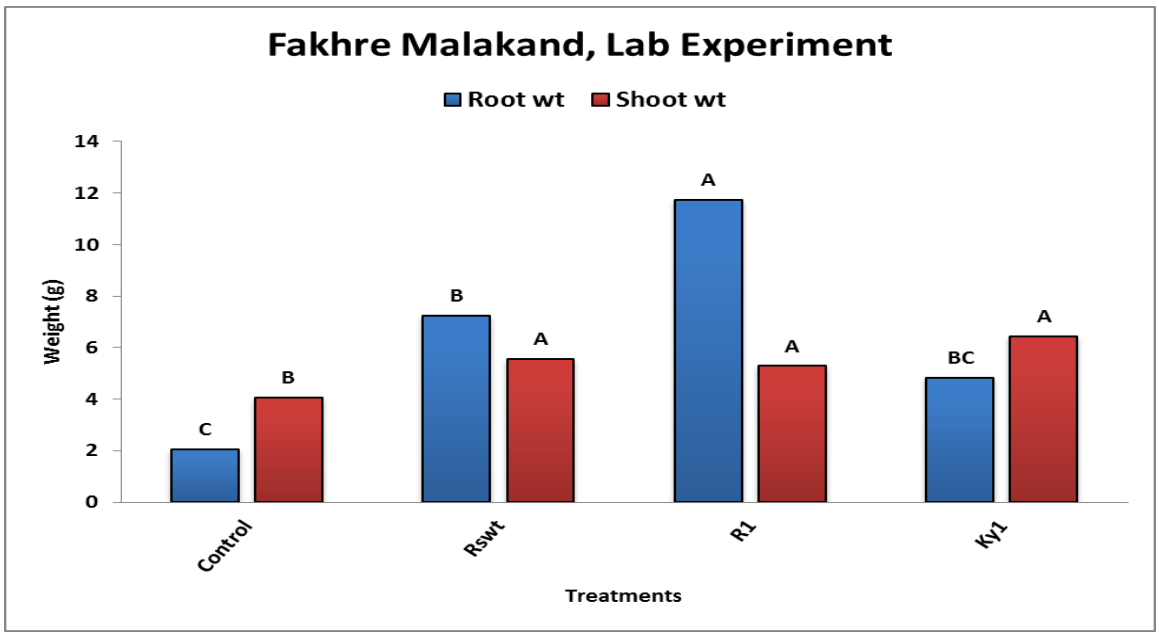

Fig 20: Effect of the bacterial inoculation on root and shoot weight of rice variety Fakhre Malakand.

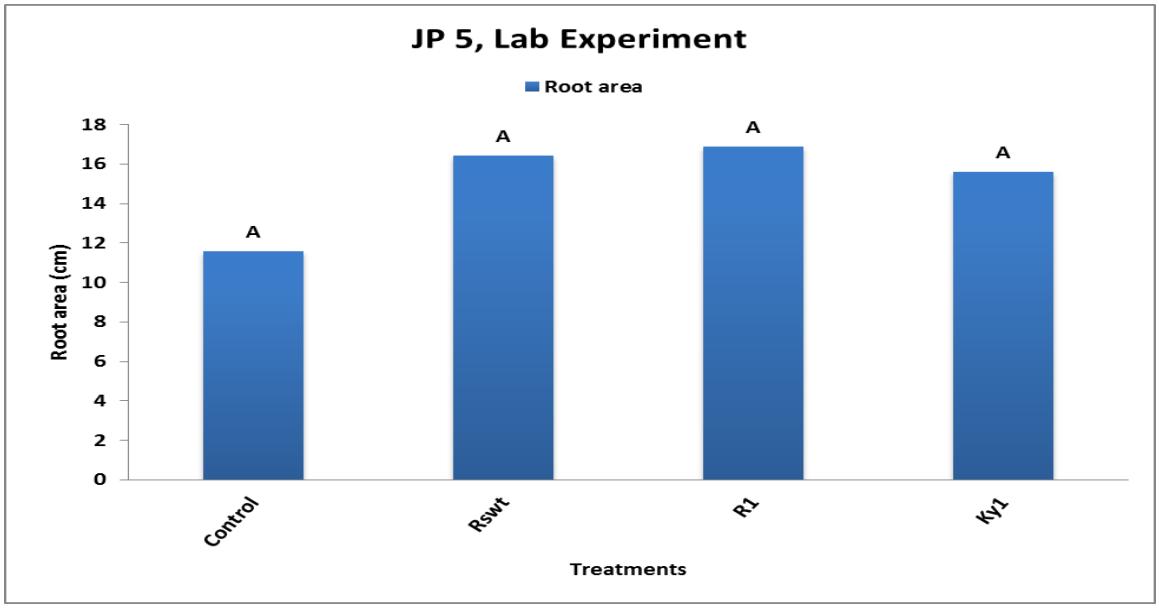

Fig 21: Effect of the bacterial inoculation on the root area of rice variety JP 5.

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 12 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

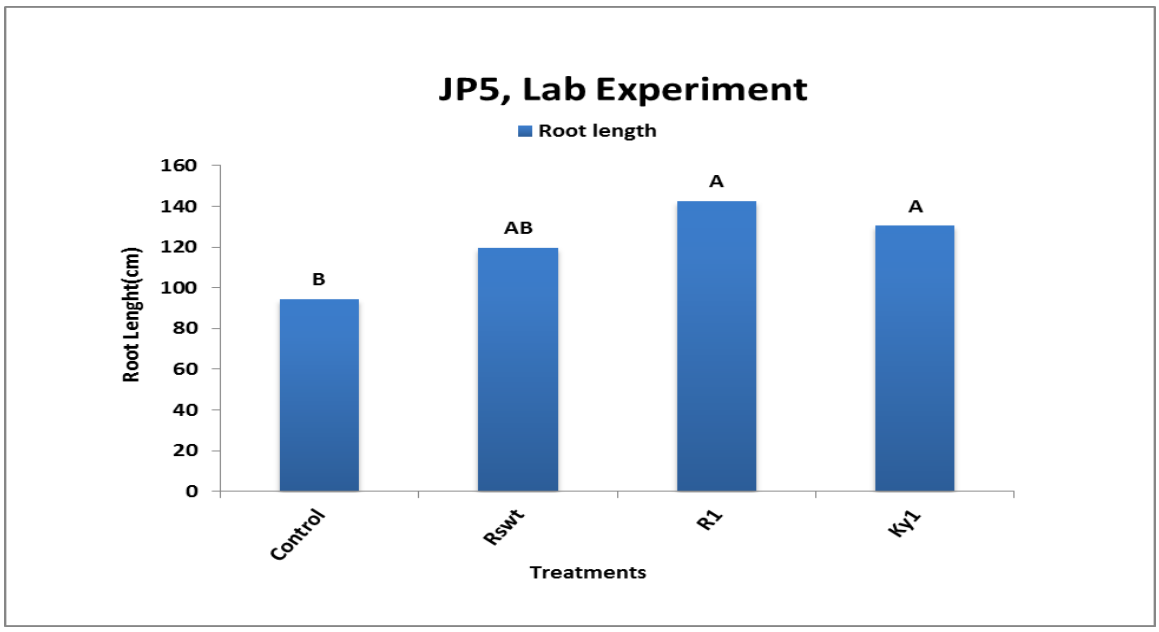

Fig 22: Effect of the bacterial inoculation on the root length of rice variety JP 5.

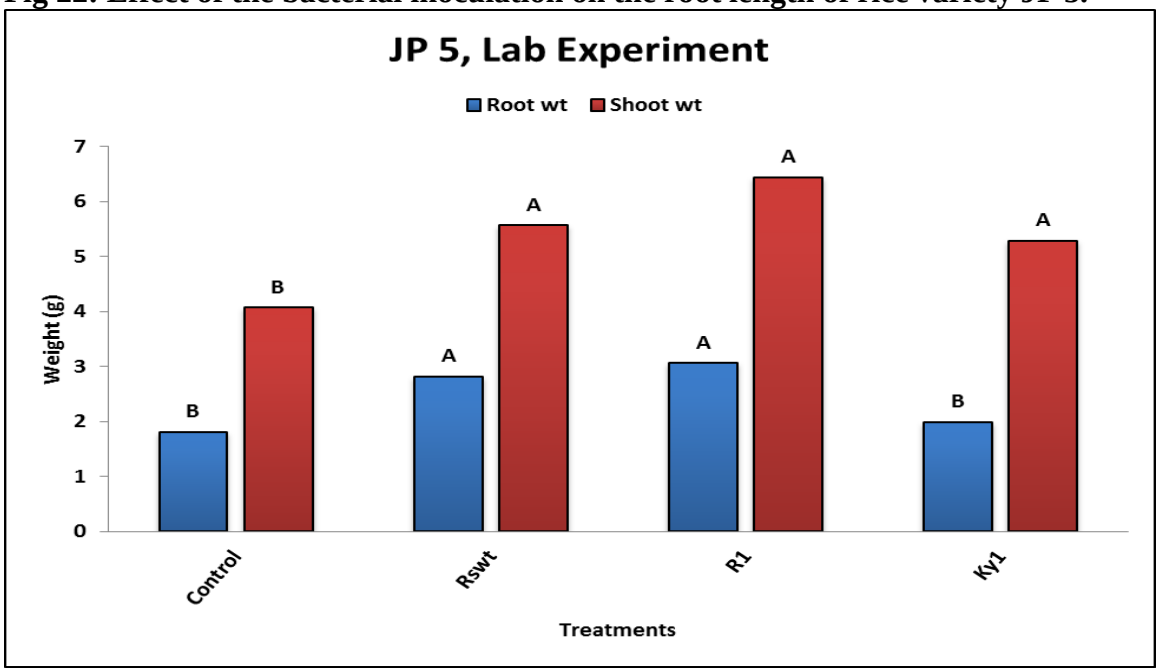

Fig 23: Effect of the bacterial inoculation on the root weight and shoot weight of rice variety JP 5.

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 12 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

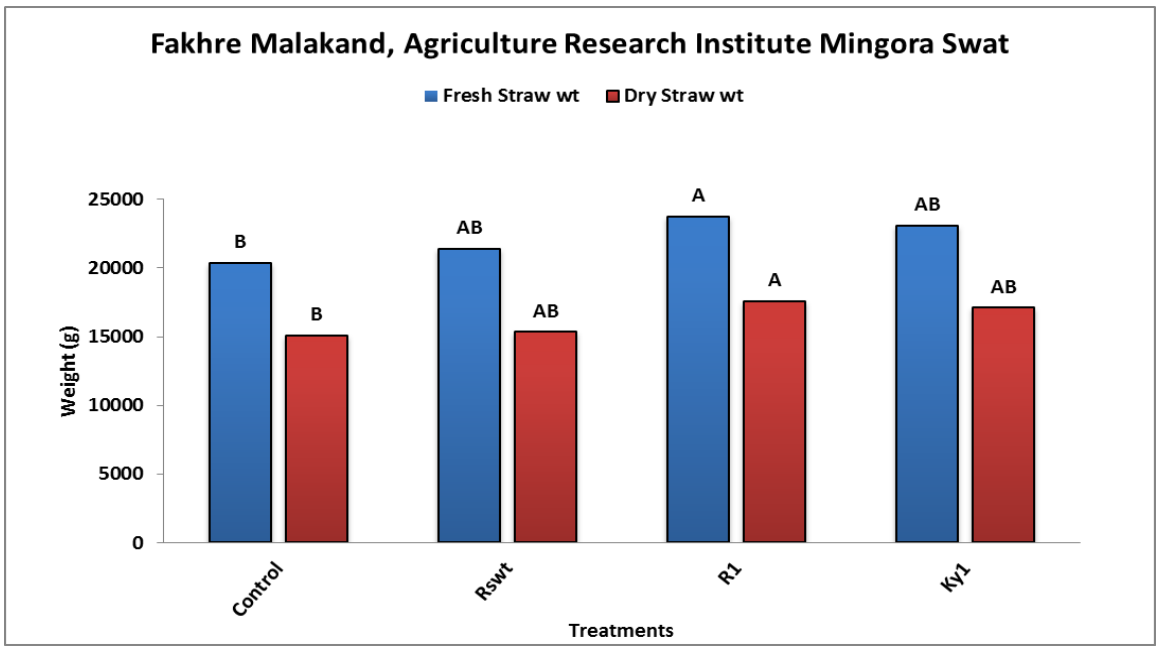

Figure 24: **Effect of inoculated strains on straw weight of rice variety Fakhre Malakand**

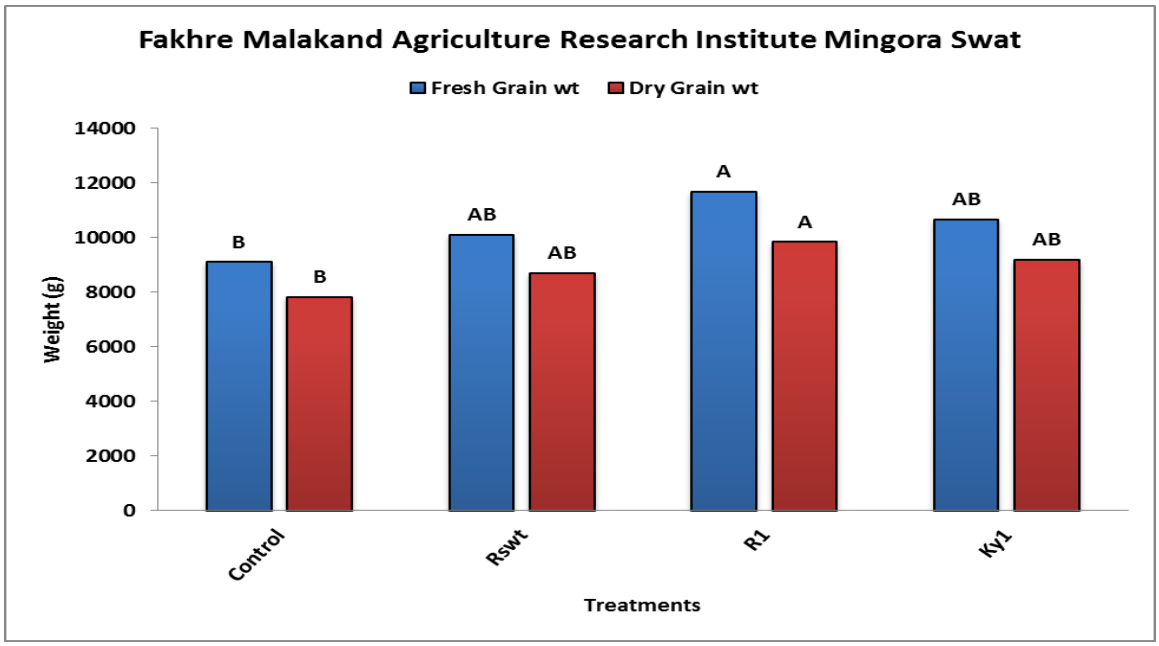

Figure 25: **Effect of inoculated strains on grain weight of rice variety Fakhre Malakand**

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 12 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

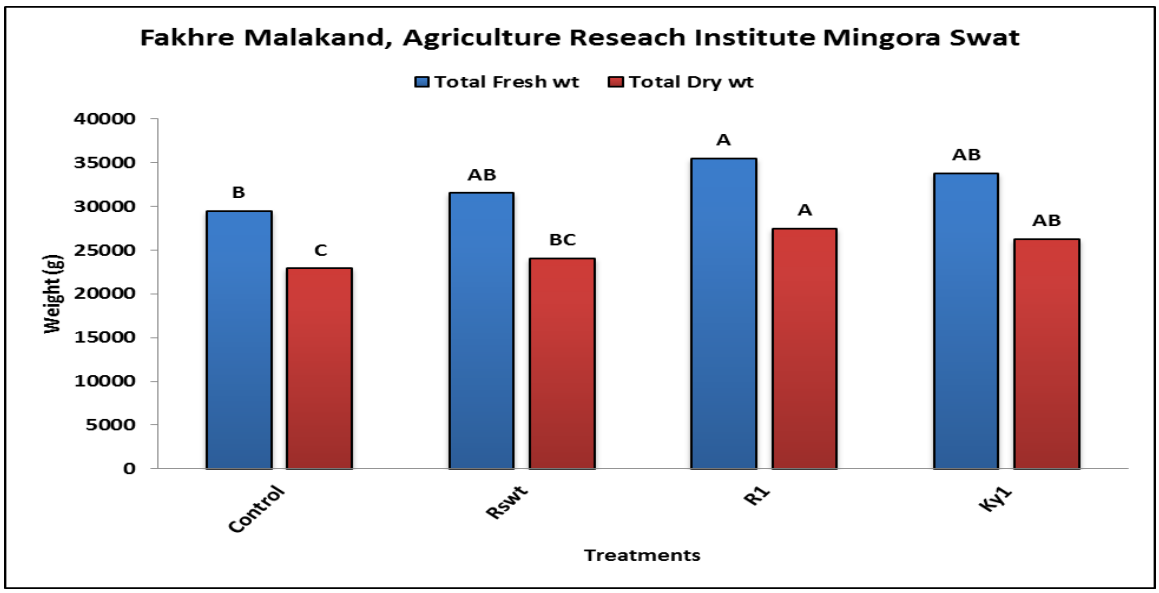

Figure 26: Effect of inoculated strains on total plant weight (Straw+Grain) of rice variety Fakhre Malakand

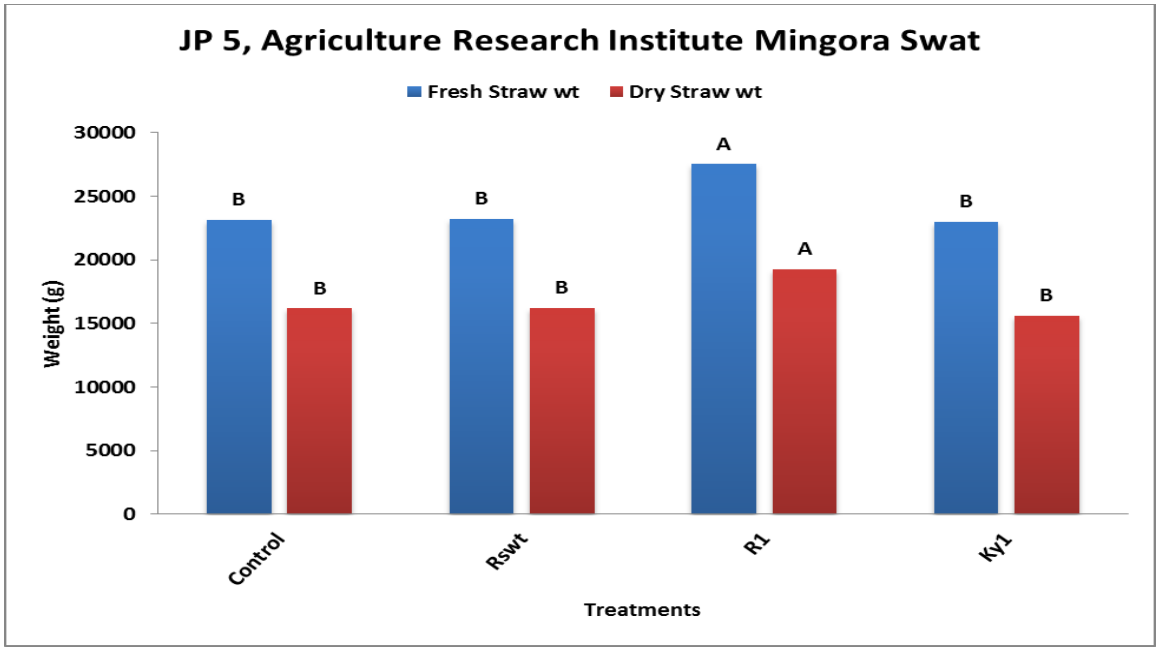

Figure 27: Effect of inoculated strains on straw weight of rice variety JP 5

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*

R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

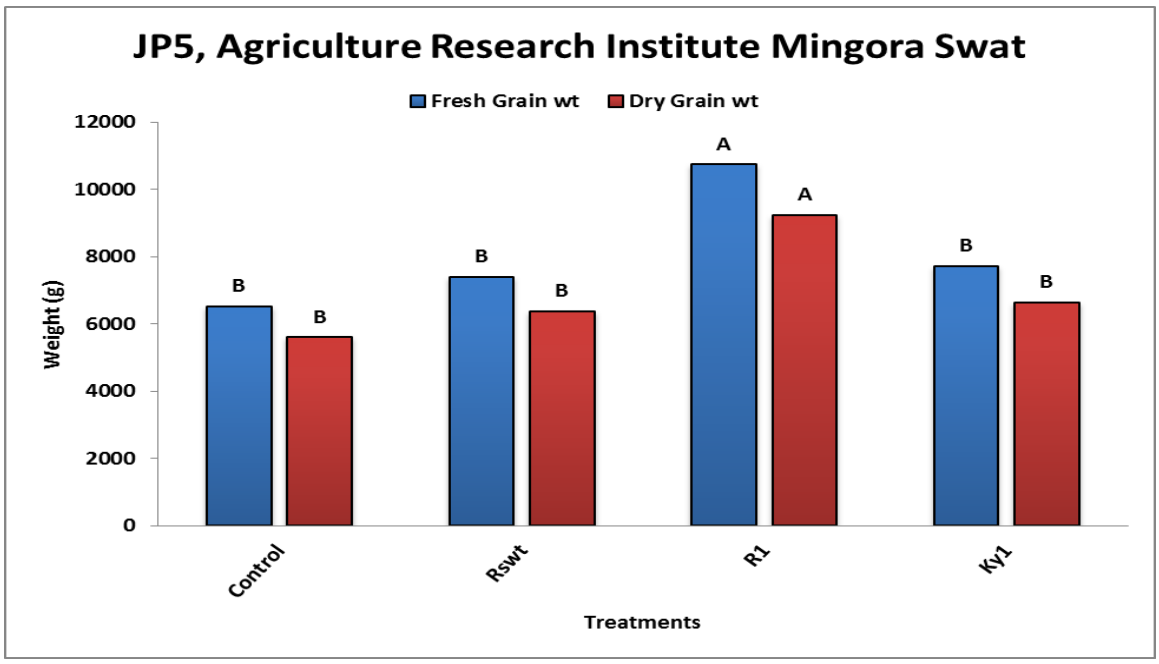

Figure 28: Effect of inoculated strains on grain weight of rice variety JP 5

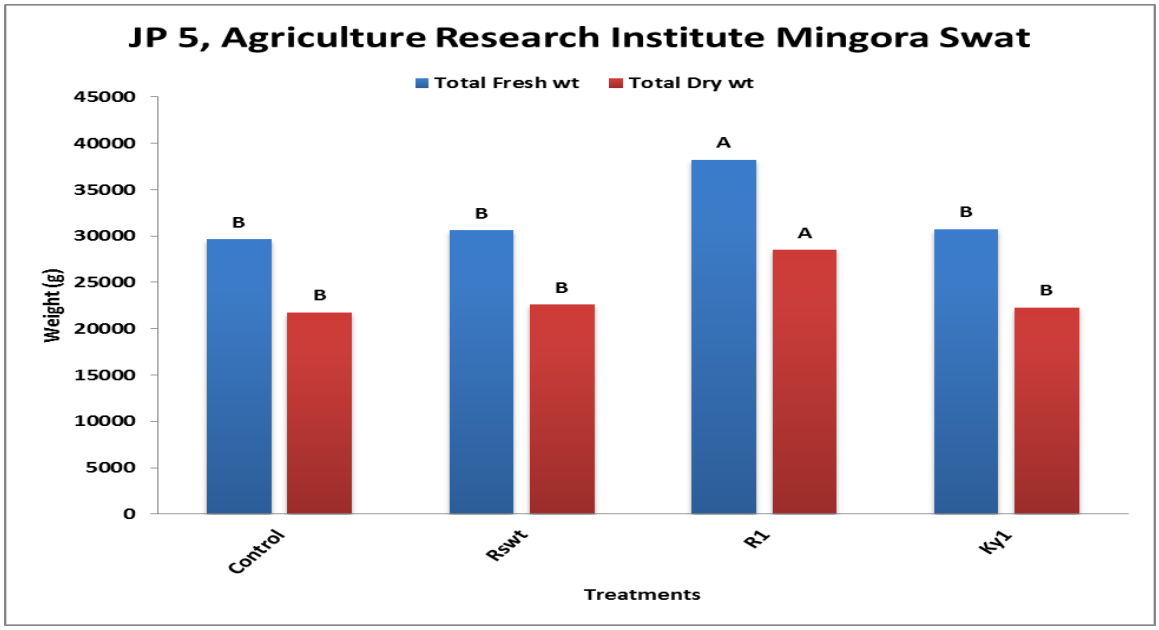

Figure 29: Effect of inoculated strains on total plant weight (Straw + Grain) of rice variety JP 5

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

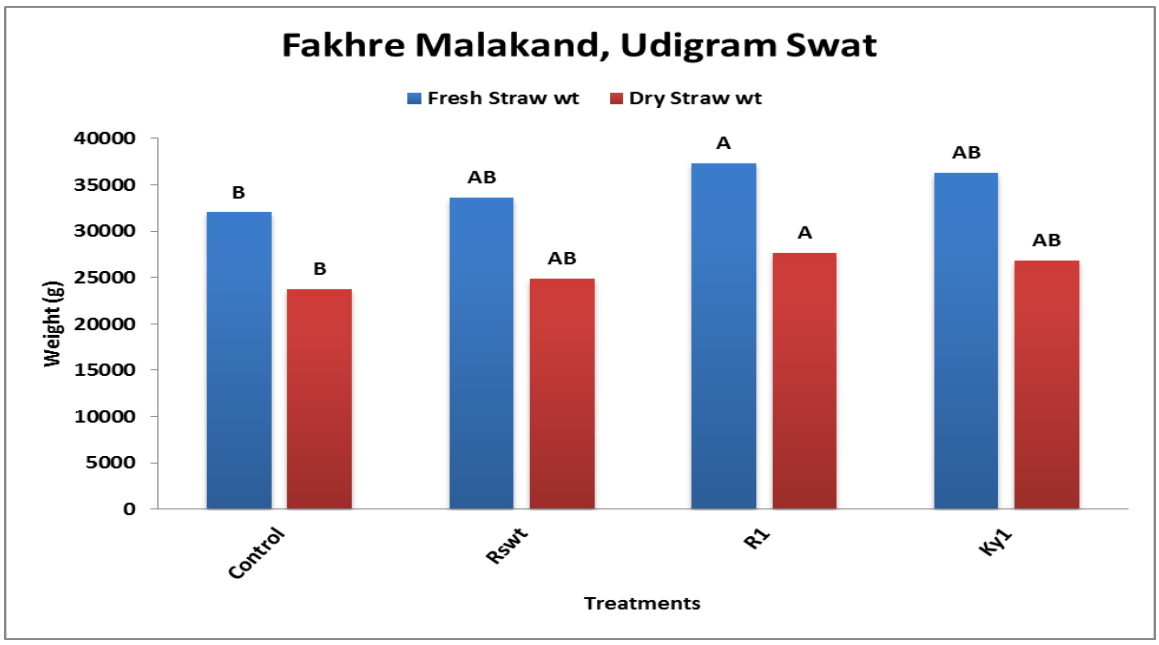

Figure 30: Effect of inoculated strains of straw weight of rice variety Fakhre Malakand

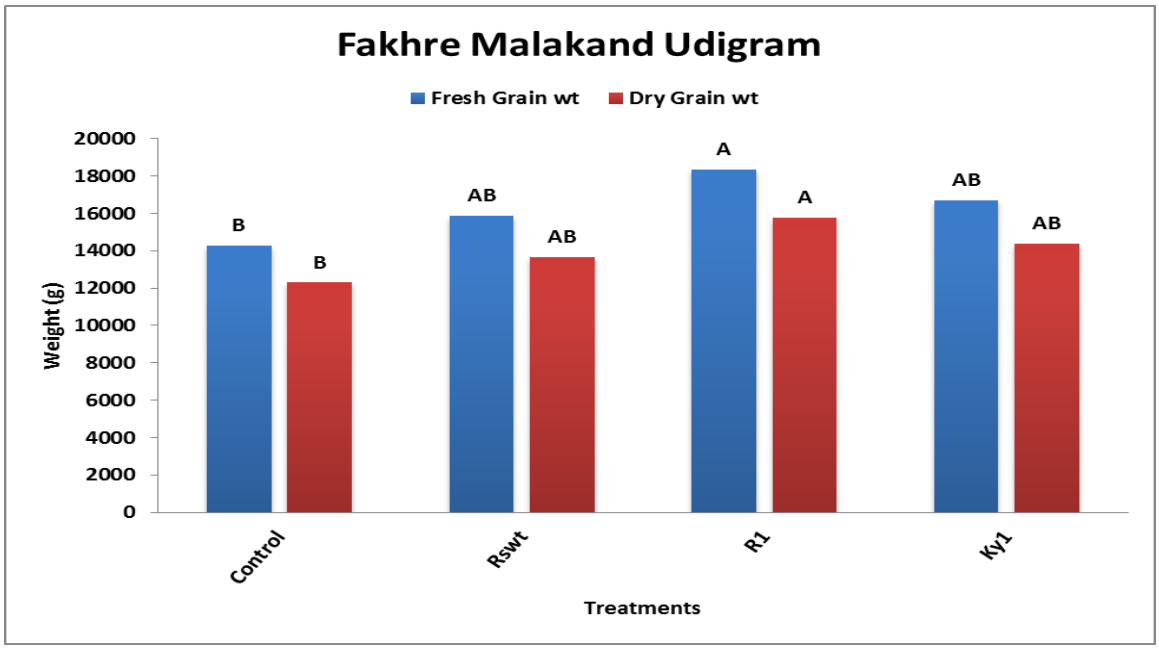

Figure 31: Effect of inoculated strains of grain weight of rice variety Fakhre Malakand

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

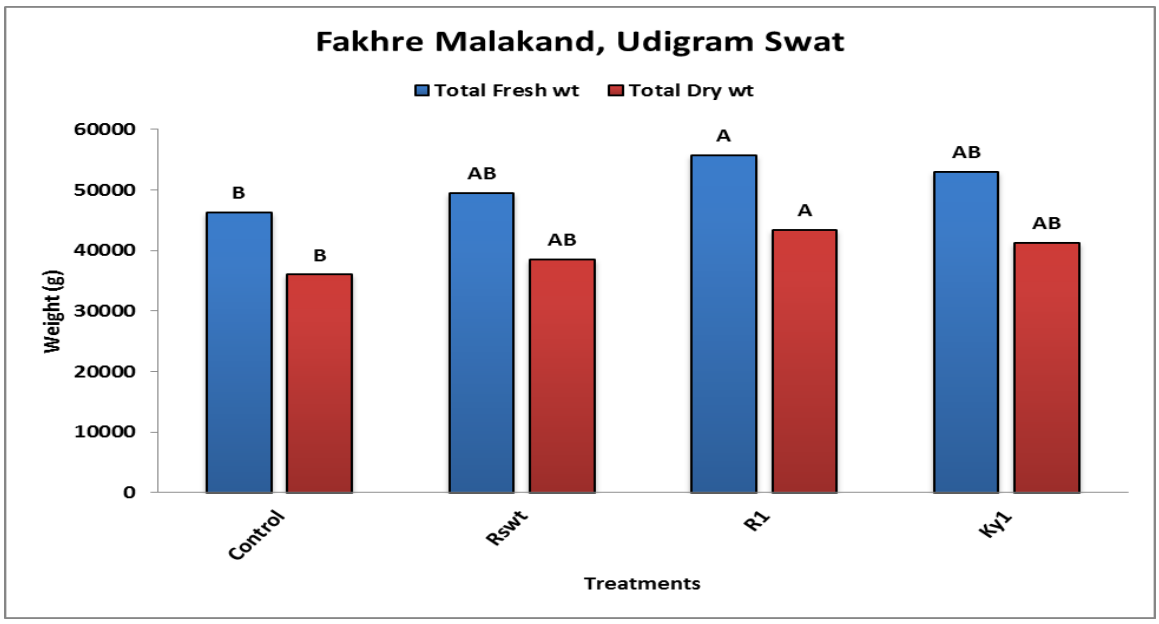

Figure 32: **Effect of inoculated strains of total weight (straw + grain) of rice variety Fakhre Malakand**

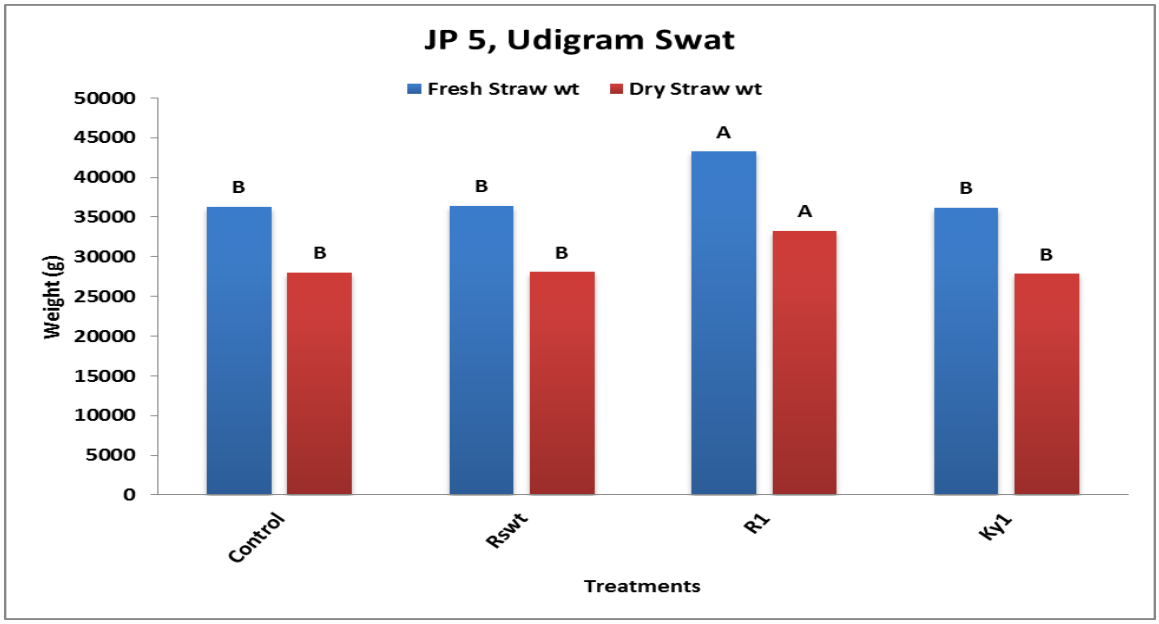

Figure 33: **Effect of inoculated strains of straw weight of rice variety JP 5**

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

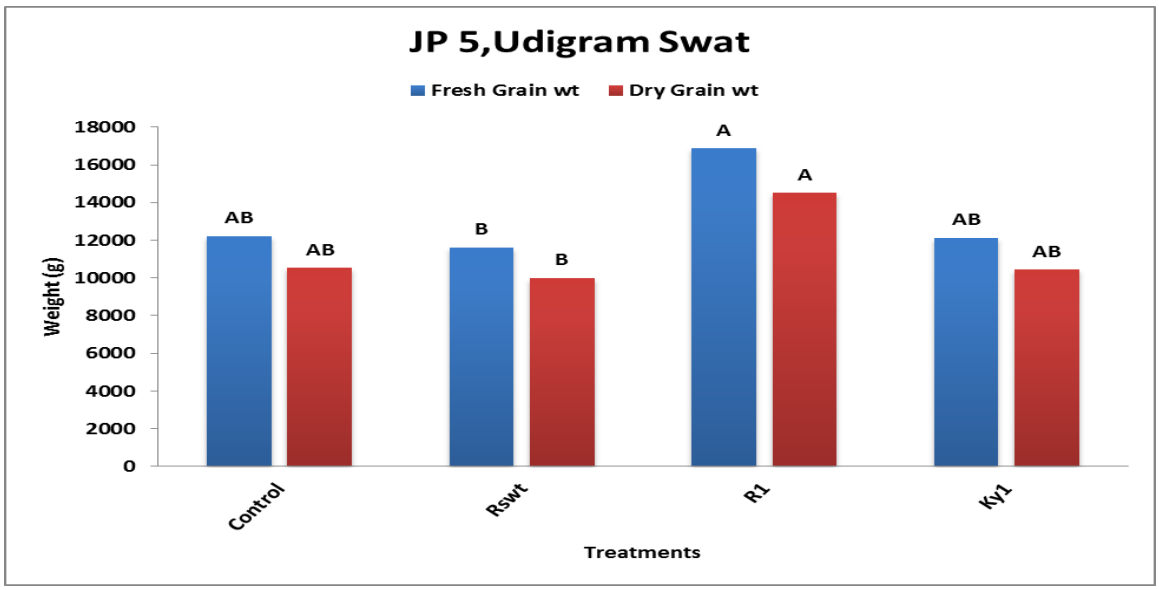

Figure 34: Effect of inoculated strains of grain weight of rice variety JP 5

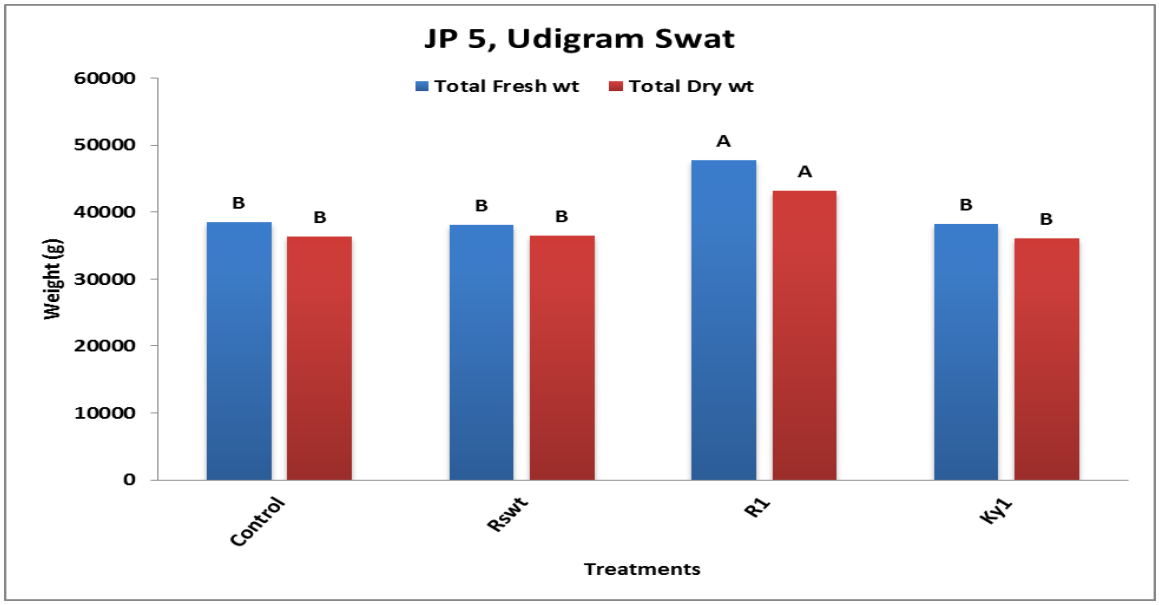

Figure 35: Effect of inoculated strains of total weight (straw + grain) of rice variety JP 5

Control: Non-inoculated **RSWT1:** *Azospirillum lipoferum*
R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

3.8. Effect of inoculated strains on the growth of maize in the field:

The present results clearly indicated that plant growth promoting rhizobacteria significantly promote plant growth and productivity when compared to non-treatment control. Inoculation with *Azospirillum brasilense* strain R1 increased fresh root weigh (50%) and fresh shoot weight (37.3%) of seedling after one week of inoculation. The increase in the dry root weight and dry shoot weight of seedling was 56% and 39% respectively. *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 also showed significant increase (30.5 % and 24.2 % respectively) in the root fresh weight and 42% and 37 % in root dry weight respectively. The increase in shoot dry weight due to *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* Ky1 was 25 % and 3 % respectively as compared to control treatments (Table 9).

Inoculation with *Azospirillum brasilense* strain R1 result increase in number of ear/plant (45.9%), ear length (23.7%) and ear weight (17.25%). The increase with *Azospirillum lipoferum* strain RSWT1 was 35%, 27% and 19.4% in number of ear/plant, ear length and ear weight respectively. *Pseudomonas* strain Ky1 also showed significant increase (20.4 % and 10%) in the number of ear/plant and ear length; however the comb weight was not significantly affected by this bacterial strain (Table 9).

Higher plants (150 cm) were measured for treatment with *Azospirillum lipoferum* strain RSWT1 followed by *Azospirillum brasilense* strain R1 (147 cm) and *Pseudomonas* Ky1 (141 cm) while lower height of the plants (138 cm) was measured for non-inoculated control ones. The results showed that plant height was significantly increase up to 8.82%

with *Azospirillum lipoferum* strain RSWT1 and with *Azospirillum brasilense* strain R1 up to 6.52% as compared to non-inoculated control ones (Figure 36).

The results showed that number of leaves per plant was significantly affected by bacterial inoculation. Maximum number (13.56) of leaves per plant was noted in case of *Azospirillum lipoferum* strain RSWT1. This is followed by *Azospirillum brasilense* strain R1 with 13.52 number of leaves/plant. Minimum number of leaves per plant of 11.67 and 11.66 was recorded treatment with *Pseudomonas* Ky1 and non-inoculated control treatment (Figure 38). Positive response of bacterial inoculation on stem thickness (girth) was also recorded. Maximum stem girth (5.36 cm) and ear length (24.23 cm) was noted in case of *Azospirillum lipoferum* strain RSWT1. This is followed by *Azospirillum brasilense* strain R1 with stem girth of 5.24 cm and ear length of 23.16 cm. Minimum stem thickness of 5.06 cm and 5.02 cm and ear length of 19.65 cm and 17.67 cm was recorded treatment with *Pseudomonas* Ky1 and non-inoculated control treatment respectively (Figure 37 and 40).

The inoculation caused the highest significant increase in number of grains/ear over all treatments except non inoculated control ones. Maximum number (368) of grains per ear was noted in case of *Azospirillum lipoferum* strain RSWT1. This is followed by *Azospirillum brasilense* strain R1 and *Pseudomonas* strain Ky1 with 345 and 310 numbers of grains per ear respectively. Minimum number of grain (290) per ear was recorded for non-inoculated control treatment. The increase in number of grain/ear was 21.2 % for treatment with *Azospirillum lipoferum* strain RSWT1 followed by

Azospirillum brasilense strain R1 (15.9 %) and *Pseudomonas* Ky1 (6.45 %) as compared to non-inoculated control ones (Figure 41).

The plant inoculated with *Azospirillum lipoferum* strain RSWT1 showed maximum increase upto 11.78 % in thousand grain yield. This is followed by *Azospirillum brasilense* strain R1 with 9.68 % increase in thousand grain yield as compared to control treatment. The effect of inoculation with *Pseudomonas* Ky1 on thousand grain yield was not significant (Figure 42).

Biological yield data collected from whole plots indicated that the effect of inoculations was significant. Higher biological yield (9210 kg/ha) was produced when treatment was done with *Azospirillum lipoferum* strain RSWT1 followed by treatment with *Azospirillum brasilense* strain R1 (8960 Kg/ha). The treatment with *Pseudomonas* Ky1 showed biological yield of 7496 kg/ha while lower biological yield (7360 kg/ha) was noted for non-inoculated control treatments (Figure 43).

Table 9: Effect of inoculated strains on different parameters of Maize plant grown at Udigram, Swat

S/N	Variety	After one week on inoculation				Plant Length (cm)	Stem Girth (cm)	No of leaves/plant	No of Ears/plant
		Root fresh wt (g)	Root dry wt (g)	Shoot fresh wt (g)	Shoot dry wt (g)				
1	MZ-P-T1-U	2.211 (±3.24)	1.02 (±2.45)	9.63 (±1.67)	4.98 (±3.25)	138 (±2.47)	5.02 (±3.27)	11.66 (±3.14)	1.87 (±2.33)
2	MZ-P -T2-U	3.163 (±2.16)	1.78 (±3.86)	12.21 (±2.48)	6.69 (±3.89)	150 (±3.52)	5.36 (±4.21)	13.56 (±1.63)	2.89 (±7.48)
3	MZ-P -T3-U	4.562 (±3.67)	2.35 (±2.34)	15.38 (±)	8.24 (±)	147 (±)	5.24 (±3.69)	13.52 (±1.39)	3.46 (±2.45)
4	MZ-P -T4U	2.936 (±1.87)	1.63 (±2.69)	11.67 (±3.29)	5.13 (±3.17)	141 (±3.46)	5.06 (±5.21)	11.67 (±2.44)	2.35 (±3.27)

S/N	Variety	Comb length (cm)	Comb diameter (cm)	Rows of grain/comb	No of grain/row	No of grains /comb	Comb weight (g)	Grain wt/comb (g)	Thousand grain yield (g)
1	MZ-P-T1-U	17.67 (±3.11)	3.26 (±3.11)	12.24 (±3.44)	23.69 (±3.14)	290 (±5.46)	193.26 (±1.87)	97.32 (±3.15)	328.47 (±3.28)
2	MZ-P -T2-U	24.23 (±2.37)	4.23 (±3.17)	14.12 (±2.37)	26.06 (±4.13)	368 (±4.83)	239.73 (±2.37)	133.67 (±3.21)	372.34 (±3.51)
3	MZ-P -T3-U	23.16 (±1.85)	4.13 (±2.41)	13.47 (±1.98)	25.61 (±2.13)	345 (±5.17)	233.56 (±2.31)	126.34 (±4.27)	363.67 (±2.77)
4	MZ-P -T4U	19.65 (±2.33)	3.97 (±2.17)	12.95 (±3.71)	23.94 (±3.47)	310 (±3.47)	201.47 (±2.15)	103.39 (±2.85)	334.12 (±3.41)

T1 – Control (non- inoculated) T2- *Azospirillum lipoferum* RSWT1 T3- *Azospirillum brasilense*; R1
T4 – *Pseudomonas* Ky1 U- Udigram, Swat, MZ-P – Maize variety Pahari

Note: The values are average of 12 plants and the values in brackets represent Standard Deviation

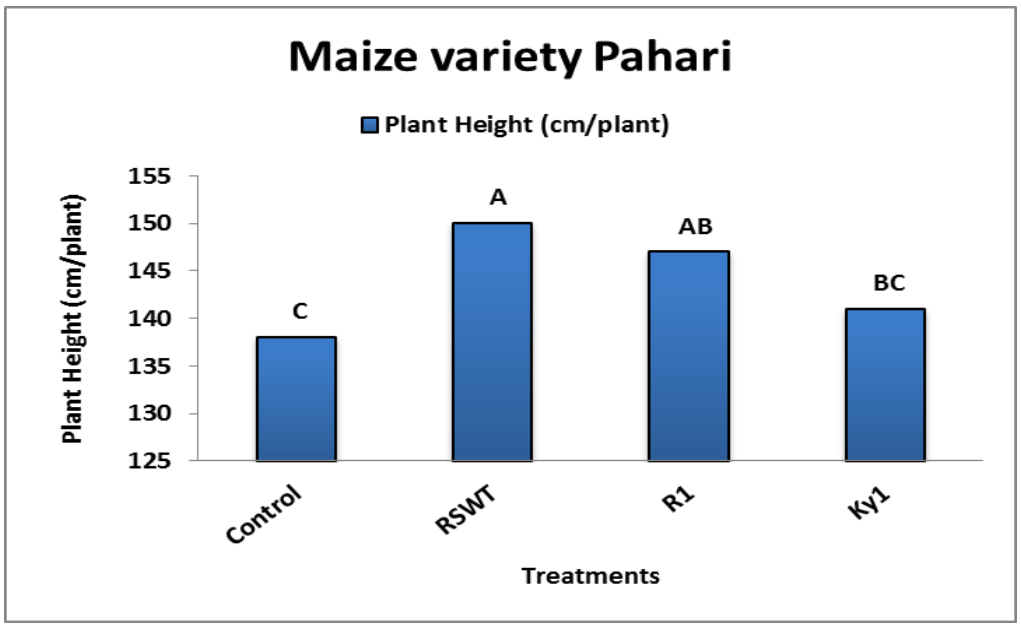

Figure 36: The effect of bacterial inoculation on plant height (cm/plant)

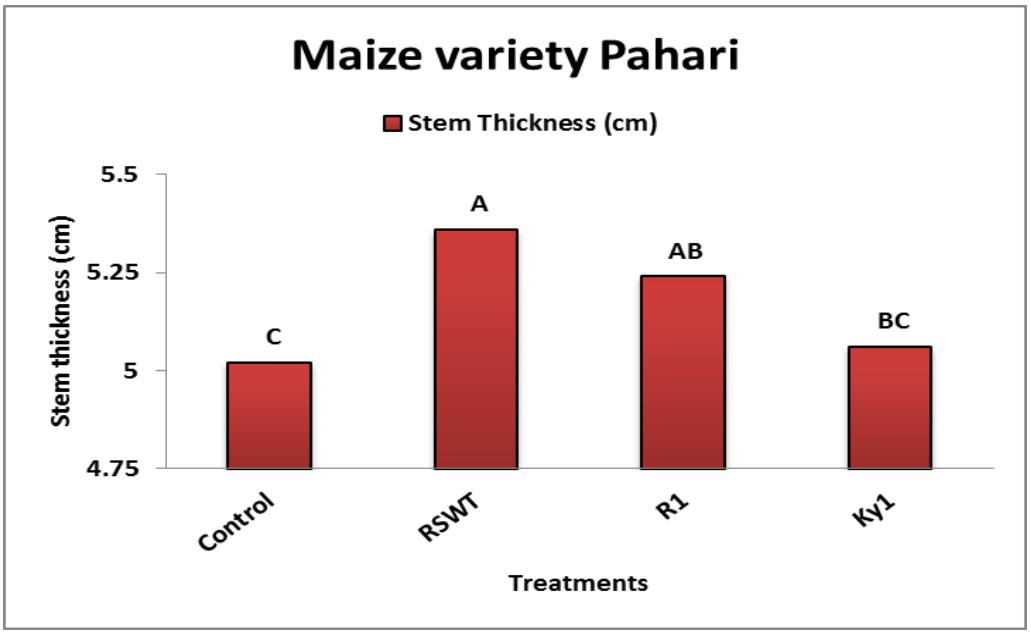

Figure 37: The effect of bacterial inoculation on stem thickness

Control: Non-inoculated **RSWT:** *Azospirillum lipoferum*

R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

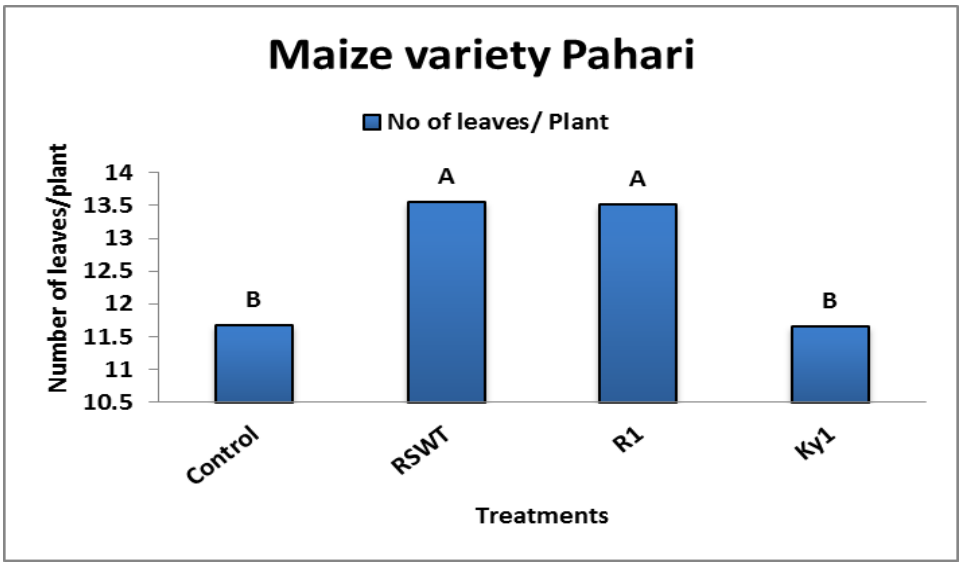

Figure 38: The effect of bacterial inoculation on number of leaves/ plant

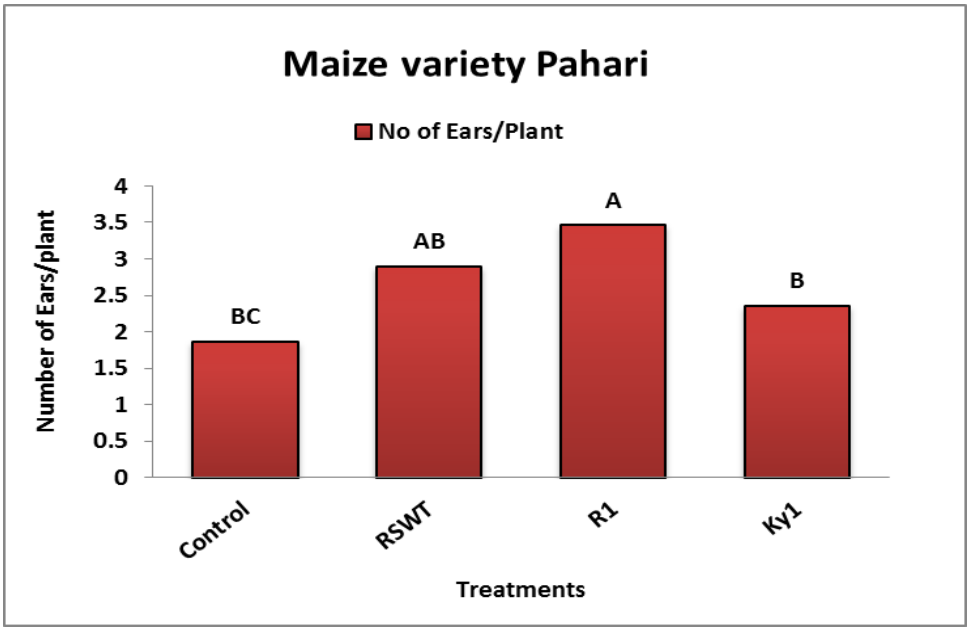

Figure 39: The effect of bacterial inoculation on number of ears/plant

Control: Non-inoculated **RSWT:** *Azospirillum lipoferum*

R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

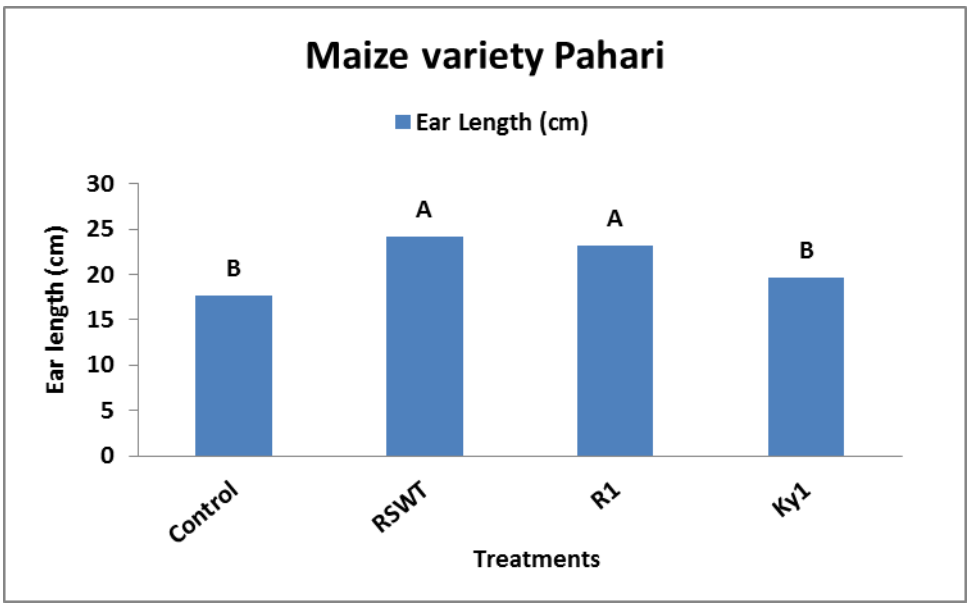

Figure 40: The effect of bacterial inoculation on ear length

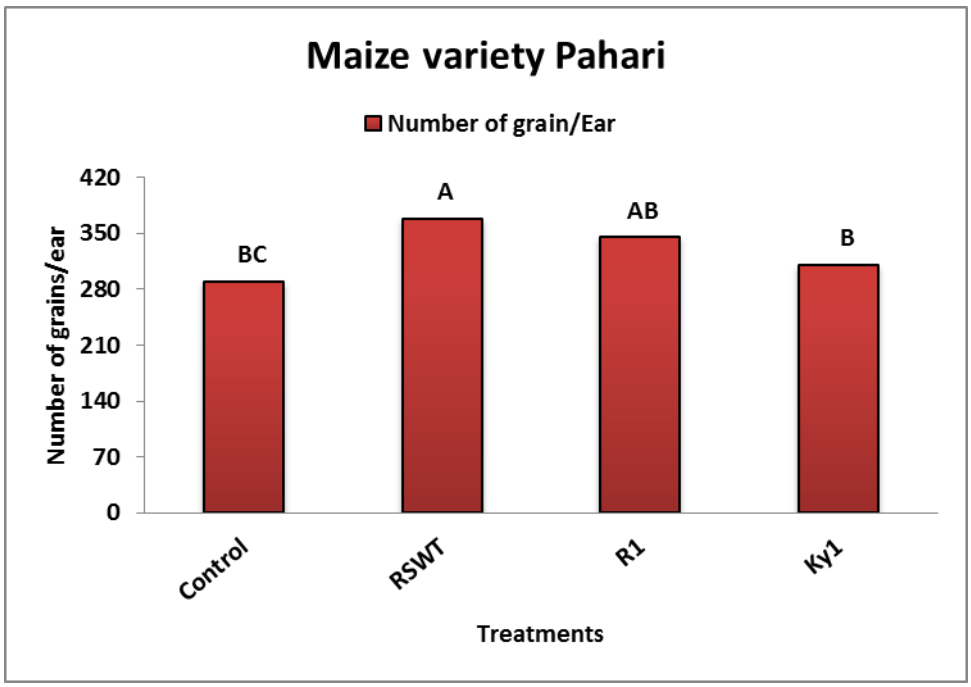

Figure 41: The effect of bacterial inoculation on number of grains/ear

Control: Non-inoculated **RSWT:** *Azospirillum lipoferum*

R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

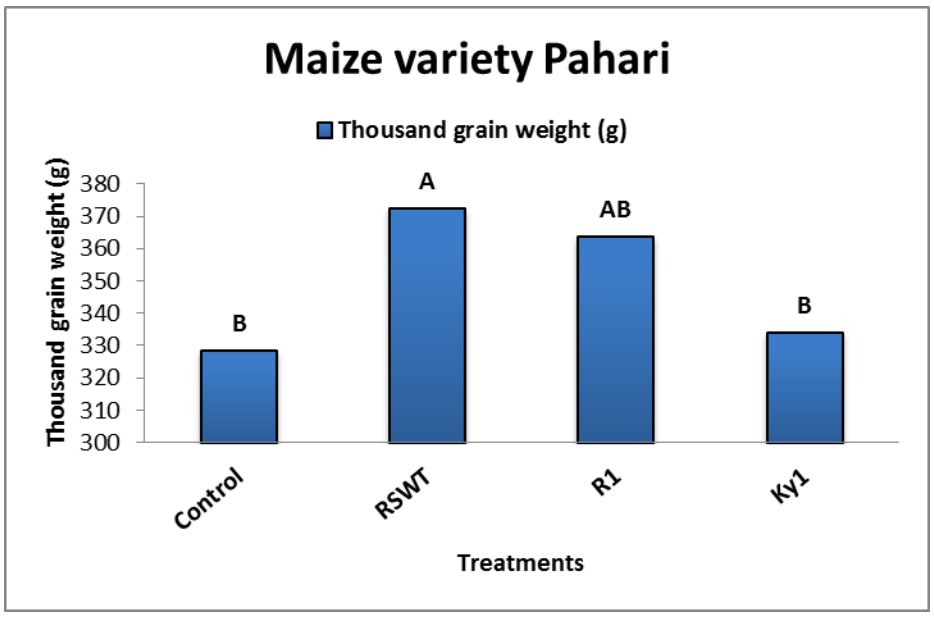

Figure 42: The effect of bacterial inoculation on thousand grain weight

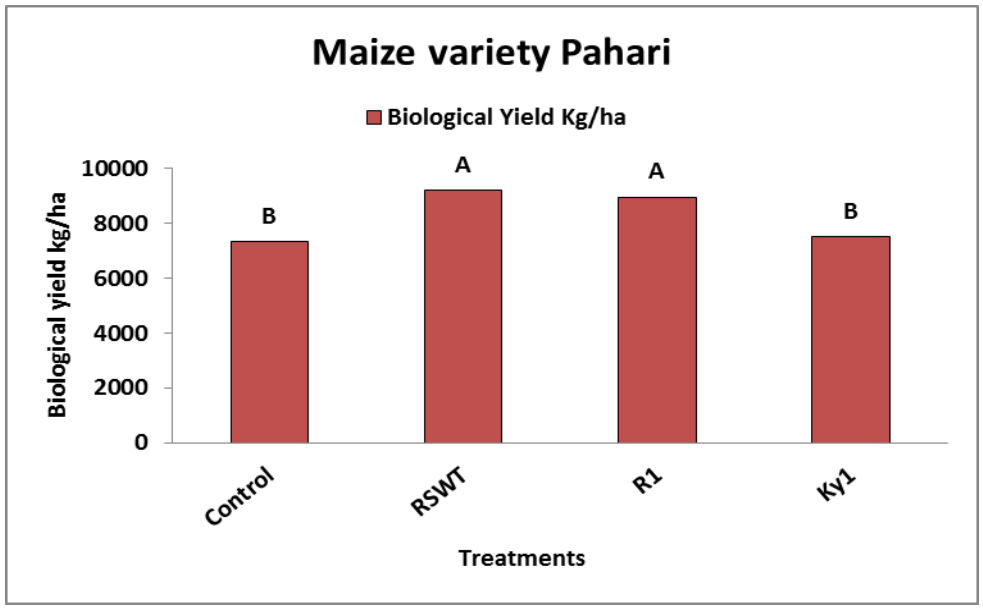

Figure 43: The effect of bacterial inoculation on Biological yield Kg/ha

Control: Non-inoculated **RSWT:** *Azospirillum lipoferum*

R1 : *Azospirillum brasilense* **Ky1 :** *Pseudomonas*

Note: The values are an average of 4 replicates. Different letters given above the bars in the graphs show that values are different at 5 % level of significance.

Fig 44: Preparation of land for field experiment of wheat plant

Fig 45: A view of the cultivated wheat seedlings

Field Experiment at Udigram Swat

Fig 46: A view of the cultivated wheat plots

Fig 47: View of the wheat plant at maturity stage

Field Experiment at Udigram Swat

Fig 48: A view showing significant effect of *Azospirillum brasilense* R1 on wheat yield

Fig 49: Harvesting of wheat

Field Experiment at Udigram Swat

Fig 50: Preparation of land for field experiment

Fig 51: Inoculation of rice seedlings with bacterial strains

Field Experiment at Agriculture Research Institute (N) Mingora Swat

Fig 52: Transplantation of rice from nurseries to field

Fig 53: A view of the cultivated rice plots

Field Experiment at Agriculture Research Institute (N) Mingora Swat

Fig 54: A view showing significant difference of inoculation on rice plants

Fig 55: View of the rice plant at maturity stage

Field Experiment at Agriculture Research Institute (N) Mingora Swat

Fig 56: A view showing significant effect of *Azospirillum brasilense* R1 on rice yield

Fig 57: Harvesting of rice

Field Experiment at Agriculture Research Institute (N) Mingora Swat

Fig 58: A view of the cultivated maize plots

Fig 59: A view showing significant effect of *Azospirillum* spp on maize

Field Experiment at Udigram Swat

Fig 60: View of the maize plant at pre- maturity stage

Fig 61: View of the maize plant at maturity stage

Field Experiment at Udigram Swat

Chapter 4

Discussion

Pakistan is basically an agricultural country and its economy is mainly based on agricultural products. The total cereal crops production of the country is lower than other developing countries of the world [1]. Pakistani soil is deficient in nitrogen (N), phosphorus (P) and potassium (K) which are essential nutrients for plant growth. The utilization of land for a single crop results in depleting soil fertility because land is intensively cultivated and only essential plant nutrients are used. The future crops are threatened from essential nutrients when the soil goes without being replenished. The increase in the yield of cereal crops is largely dependent upon availability of essential nutrients like nitrogen and phosphorus in the soil, which can be supplied as chemical fertilizers. However, the increased use of chemical N fertilizers being so costly is not affordable and their excessive use often results in environmental pollution [6-8]. Therefore microbe-based technologies are becoming popular which can provide nutrients and plant-growth promoting compounds in the rhizosphere of plants.

The root, rhizosphere and aerial parts of cereal crops are colonized by a number of beneficial bacterial strains [190]. They can be used as potential biofertilizer for cereal crops [43,191]. The successful colonization and persistence of beneficial bacteria in the rhizosphere is essential for their growth promotion activities [13, 15]. The positive effect of PGPR on plant growth and development is also affected by the number and strains of these bacteria in the rhizosphere [285-287].

In the present study, 18 bacterial isolates were obtained from the roots and rhizosphere of cereal crops collected from Agricultural Research Institute (North) Mingora and Udigram Swat. Out of the sample of these 18 bacterial strains, 10 were isolated from ARIN Swat and 8 from Udigram Swat. Tentative identification of the isolates on the basis of colony and cell morphology indicated that they belong mostly to bacterial genera *Azospirillum* and *Pseudomonas*. The isolates showed resemblances in morphological characteristics with *Azospirillum* and *Pseudomonas* [288-291]. The physiological and morphological characteristics of isolated bacterial strains were carried out and on the basis of colony morphology, cell shape and motility 11 isolates (MRSWT3, MRSWT4, MRSWT6, R1-1, R1-2, R3-1, R3-2, R4-1, R5-2, and R6-1, R6-2) were tentatively identified as *Pseudomonas* strains and the isolates R1, R2, R3, and R5 as *Azospirillum brasilense* strains. Focusing their physical features, it was found that most of the *Pseudomonas* strains formed white or off-white colonies and the cells were thin, rod shaped and motile. The *Azospirillum* strains had pinkish colonies on LB agar plates and had the cells with rod shape and spiral motility. Similarly, *Azospirillum* strains were isolated and maintained in semi-solid NFM medium using the selective medium for *Azospirilla* [282]. In the N-free growth medium all *Azospirillum* strains showed excellent growth and the characteristic of spiral motility. In the rhizosphere of cereal crops a variety of diazotropic bacteria had been isolated [27-29]

The comparative analysis of the results showed that the population of *Pseudomonas* in the rhizosphere of cereal crops was more than *Azospirillum*. These results were consistent with earlier studies of Barraquio et al. [206] and Watanable et al.

[207]. They reported that 80 % of the nitrogen fixing bacteria population were associated with wet land rice belonged to *Pseudomonas diazotrophicus.* They pointed out that these bacteria have the ability of active growth in flooded condition when H and CO_2 evolved from rice field.

Three isolates R2-1, R4-2 and MRSWT5 could not be identified. The strain R2-1 formed very hard white colonies that were submerged in the agar plates. The colony morphology of this strain was totally different from all others. The isolate R4-2 and MRSWT5 formed yellowish fluffy colonies and the cells were motile rods. These strains require further DNA based studies for proper identification.

It was suggested that the bacterial isolates purified from the roots and rhizosphere of cereal crops were different strains and that they were not the re-isolates of the same strain. In order to confirm this suggestion, random primer was used in PCR to differentiate four randomly selected *Pseudomonas* strains by comparison of the amplified DNA banding patterns. It was found that all the four strains showed different banding pattern of the PCR product. On agarose gels, PCR products of each strain showed several major and minor DNA bands. The unique banding pattern of each strain confirmed that RI-2, R3-1, R4-1, R5-2, MRSWT3, MRSWT4, MRSWT6 and R6-2 were different strains and not the re-isolates of the same strain. Such differentiation of the isolates is important to avoid duplications in detailed studies.

Acetylene reduction activity and the presence of *nifH* gene were also studied in the bacterial isolates to detect nitrogen-fixing ability of the isolates. Partial *nifH* gene was

also amplified by PCR using template DNA from bacterial isolates. PCR product of expected size (360 bp) with this particular set of primers were detected only in two *Azspirillum* strains (*Azospirillum* R1 and *Azospirillum* R3) showing the presence of structural genes for nitrogenase enzyme. Thus the strains (*Azospirillum* R1 and *Azospirillum* R3) were found as nitrogen-fixers and good candidates for inoculum production. These finding are in line with the results of Paulina et al. [292] who reported that diazotrophy is a common property among plant root associated bacteria. Isolation of nitrogen-fixing bacterial strains from the rhizosphere of several plant species has been reported [206,207,293] and at least five species of *Pseudomonas* have been included in the list of diazotrophs [294].

Phytohormone (IAA) production by the bacterial isolates in pure cultures was detected and quantified by High Pressure Liquid Chromatography (HPLC). HPLC analysis of these strains revealed their ability of phytohormone production. The production of phytohormones may influence the growth and branching intensity of the root [295,296]. The bacterial isolates were tested for IAA production in Nitrogen-free medium (NFM) supplemented with 100 mg/L L-tryptophan and NH_4Cl 1g/L. Tryptophan is a precursor in IAA synthesis. Out of 21 strains tested, 18 produced phytohormone (IAA), which is the principal naturally occurring auxin. Production of IAA was higher in R4-1 strain with 45.6 µg/mL, followed by R1 with 45.5 µg/mL and MRSWT6 with 35.8 µg/mL of IAA respectively. *Pseudomonas* Ky1 also produced higher amount (32.5µg/mL) of IAA. Production of plant growth hormones by rhizobacteria by *Pseudomonas* strain was documented [115,283,297]. The *Azospirillum* strain R4 also

produced appreciable quantity of IAA (27.2 µg/mL). The *Azospirillu*m strains R1 and RSWT1 produced 16.5 µg/mL and 6.8 µg/mL of IAA respectively. Production of IAA by the *Azospirillum* was reported by Zimmer et al. [298] in the late stationary phase when tryptophan was present in the medium and under aerobic condition. The value of IAA production by the nitrogen fixing isolates R4-2 and R3 were comparatively low (4.5µg/mL and 5.2µg/mL respectively). The production of phytohormones like auxins, cytokinins and gibberellins in chemically defined medium by *Azospirillum* and *Pseudomonas* strains has been reported [299-301]. The strains producing high amounts of IAA have a great potential for use as PGPR for large scale production of inoculum for cereal crops.

A number of research papers have been published, mostly reporting growth enhancement of cereal crops due to PGPRs inoculation [75,141,195-198, 302].

The present results clearly indicated that bacterial inoculation significantly promoted growth and yield of wheat plant when compared to non-treatment controls. All inoculation caused significantly increases in plant height of wheat variety Inqilab 91 over all treatment except non-inoculated control. Results were almost consistent with previous work available in literature [91]. The results showed that plant height was significantly increased up to 18.5 % with *Azospirillum brasilense* strain R1 as compared to non-inoculated controls. The increase in plant height with *Azospirillum lipoferum* strain RSWT1 was 14.7 % and 9.6 % with *Pseudomonas* Ky1. These finding were in line with that of Polyanskaya et al. [303], who assessed the effect of inoculation with PGPRs on wheat crops and observed positive increase in plant length and weight. Inoculation

caused significant increase in number of grains/spike over all treatments except non inoculated controls. Similar results were also obtained by Boddey and Dobereiner [91] who pointed out that *Azospirillum brasilense* strain Sp-245 significantly increased the grain number and yield. Regarding the effects of bacterial inoculation on root weight, data showed that root and shoot weight of the plants inoculated with *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* Ky1 were significantly greater than control treatments. The increase in root dry weight was 32.3 % with *Azospirillum brasilense* strain R1, 28 % with *Azospirillum lipoferum* strain RSWT1and 18.9 % with *Pseudomonas* Ky1 as compared to control treatment. The increase in shoot dry weight was 37.9 % with *Azospirillum brasilense* strain R1, 25.2 % with *Azospirillum lipoferum* strain RSWT1and 14.4 % with *Pseudomonas* Ky1 as compared to control treatment. These findings showed resemblances with that of Dobbelaere et al. [175]. They reported positive response of bacterial inoculation on germination rate and significant increase in root and shoot dry weight. Barbieri and Galli [177] inoculated wheat with *Azospirillum brasilense* and reported significant increase in the length and number of lateral roots. Similarly an increase in the surface area of root due to *Azospirillum brasilense* inoculation was reported by Kucey and Janzen [178].

It was found that bacterial inoculation caused significant increase in number of grains/spike over all treatments except non inoculated control ones. Highest number of grains (45) was counted for *Azospirillum brasilense* strain R1 treated plants, whereas lowest number of grains (33) was counted for non-inoculated control plants. In the experiment conducted at Udigram Swat, inoculation of wheat variety Inqilab 91 with

Azospirillum brasilense strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* Ky1 showed significant increase in number of spikes/m^2. This increase was 22.3 % with *Azospirillum brasilense* strain R1, 16.9 % with *Azospirillum lipoferum* strain RSWT1 and 9.2 % with *Pseudomonas* Ky1 as compared to control treatment. The increase in total dry weight of plant, nitrogen contents in root and grains, rate of germinations, number and weight of grain, number of spike, early heading and flowering, leaf size and plant height due to bacterial inoculation had been reported in rice, wheat, sorghum and maize [28, 50-51, 61-63].

The thousand grain weight was influenced by bacterial inoculation. Heavier grains (39.42 g) were recorded for treatment with *Azospirillum brasilense* strain R1 while lighter grains (34.63 g) was noted for controls. The increase in thousand grain weight was 12.1 % with *Azospirillum brasilense* strain R1, 7 % with *Azospirillum lipoferum* strain RSWT1 and 6.9 % with *Pseudomonas* Ky1. Xia et al [180] reported an increase of 11.4-14.7 % in seed yield of wheat when inoculated with *Bacillus cereus* A47. Chen et al. [181] reported an increase of 6.3 to 15 % in the production of wheat due to bacterial inoculation. Similarly an increase (6-8%) in the emergence rate of spring wheat inoculated with *Pseudomonas chlovoraphis* 2E3 strain was reported by Kropp et al. [182]. Javed and Arshad [183] reported production of IAA by 38 strains of growth promoting bacteria. They inoculated seed of two wheat types (inglab and lu-2bs) in optimal condition of farm and reported significant increase in the number of tillering, straw weight, thousand grain yield and an increase of 3.5 % in the yield of Inglab and 28 % in the yield of Lu-2bs.

Biological yield data collected from whole plots indicated that the effect of inoculations was significant. Higher biological yield of wheat variety Inqilab 91 was produced when treated with *Azospirillum brasilense* strain RSWT1 followed by the treatment with *Pseudomonas* Ky1. The treatment with *Azospirillum lipoferum* strain R1 produced higher biological yield while lower biological yield was noted for non-inoculated controls. The influence of PGPRs on the yield and growth of cultivated plant was also shown by Okon and Labandera-Gonzalez [70]. They reported that the local isolates of *Azospirillum* under field condition increased yield of wheat by 15-30%. Dobbelaere et al. [175] also reported consistent positive effects upto 26 % increase in yield of wheat due to *Azospirillum brasilense* inoculation. Saubident et al. [54], inoculated wheat crop with *Azospirillum* and reported higher biomass, grain yield, protein content and plant nitrogen content. They concluded that nitrogen uptake is the possible mechanism responsible for the promotion of plant growth.

The results showed that all the three selected PGPRs (*Azospirillum brasilense* strain RSWT1, *Azospirillum lipoferum* strain R1, *Pseudomonas* Ky1 had significant effects on growth, development and yield of wheat variety Inqilab 91 in natural environmental condition.

Similar to the previous studies, the present investigation also showed significant effects of bacterial inoculation on wheat variety Fakhre Sarhad. Positive effects were found on different growth parameters of wheat variety Fakhre Sarhad when compared to non-inoculated control. The plant height was significantly increased up to 12 % with *Azospirillum lipoferum* strain RSWT1 as compared to non-inoculated control ones. The

increase in plant height with *Azospirillum brasilense* strain R1 was 7.5 %. The effect of inoculation with *Pseudomonas* Ky1 was not significant. Higher plants (83 cm) were measured for treatment with *Azospirillum lipoferum* strain RSWT1 followed by *Azospirillum brasilense* strain R1 (79 cm). The lower height of the plants (72 cm and 73 cm) was measured for *Pseudomonas* Ky1 and non-inoculated control ones. These finding were in line with the results of Polyanskaya et al. [303], who observed positive increase in plant length and weight of wheat crop due to inoculation of PGPRS.

In wheat variety Fakhre Sarhad the inoculation caused the highest significant increase in number of grains/spike over all treatments except non-inoculated control ones. Highest number of grains (40) was counted for treatment with inoculation with *Azospirillum brasilense* strain R1 whereas lowest number of grains (32) was counted for non-inoculated control plants. The increase in number of grain/spike was 20 % with *Azospirillum brasilense* strain R1, 15.78 % with *Azospirillum lipoferum* strain RSWT1 and 8.57 % with *Pseudomonas* Ky1. The bacterial inoculation showed positive effects on root and shoot weight of the plants. The inoculation with *Azospirillum brasilense* strain R1 showed 34% increase in the shoot dry weight and 21% increase in root dry weight. The bacterial strain *Azospirillum lipoferum* strain RSWT1 increased the shoot dry weight upto 28%. However its effect on root dry weight was not significant. Dobbelaere et al. [175] also reported better germination and significant increase in root and shoot dry weight due to bacterial inoculation.

The thousand grain yield of wheat variety Fakhre Sarhad was also significantly affected by the inoculated bacteria strains. Heavier 1000 grains (37.79 g) were recorded

for treatment with *Azospirillum brasilense* strain R1 while lighter 1000 grains yield (32.57 g) was noted for control non-inoculated ones. The treatment with *Azospirillum lipoferum* strain RSWT1showed grains weight of 36.34 g and *Pseudomonas* Ky1 showed 33.59 g. This increase was 13.8 % with *Azospirillum brasilense* strain R1, 8.37 % with *Azospirillum lipoferum* strain RSWT1 and 3.03 % with *Pseudomonas* Ky1. Similar results were also obtained by Boddey and Dobereiner [91] who pointed out that *Azospirillum brasilense* strain Sp-245 significantly increased grains number and yield, during their study.

Biological yield data collected from whole plots of wheat variety Fakhre Sarhad indicated that the effect of inoculations was significant. Higher biological yield (10524 kg/ha) was produced when treatment was done with *Azospirillum brasilense* strain R1 followed by treatment with *Azospirillum lipoferum* strain RSWT1 (10345 kg/ha). The treatment with *Pseudomonas* Ky1 showed biological yield of 9247 kg/ha while lower biological yield 8485 kg/ha was noted for non-inoculated control treatments. Similarly, the inoculated strains *Azospirillum brasilense* R1 and *Azospirillum lipoferum* RSWT1 showed significant effect on number of tillers/plant than those of non-inoculated ones. The increase in number of tillers/plant was 38.5 % with *Azospirillum brasilense* strain R1 and 37.4 % with *Azospirillum lipoferum* strain RSWT1. Inoculation with *Pseudomonas* Ky1 showed no significance effects on number of tillers/plant. Okon and Labandera-Gonzalez [70] reported the effect of local isolates of *Azospirillum* on wheat crop in the field and reported 15-30 % increase in the yield and an increase of 50- 60 % in the yield when fertilized. Dobberlaere et al. [175] also reported consistent positive effects upto 26

% increase of yield. Saubident et al. [54] inoculated wheat crop with *Azospirillum* and reported higher biomass, grain yield, protein content and plant nitrogen content. They concluded that nitrogen uptake is the possible mechanism responsible for the promotion of plant growth in this case.

The analysis of the field experiments showed that physiological and harvest maturity days were significantly affected by bacterial inoculation. Higher number of days to physiological and harvest maturity was observed in non-inoculated control treatment than inoculated ones. On the other hand, the treatment with *Azospirillum brasilense* strain RSWT1 taken lower number of days (130 and 144) to physiological and harvest maturity whereas higher number of days (137 and 157) to physiological and harvest maturity respectively was recorded for non-inoculated control ones. Similar increase (6-8%) in the emergence rate of spring wheat when inoculated with *Pseudomonas chlovoraphis* 2E3 strain was also reported by Kropp et al [182].

There was a good agreement between the results obtained from both wheat varieties (Inqilab 91 and Fakhre Sarhad) in all characters due to bacterial inoculation excepts days to physiological and harvest maturity. It was shown that the effects of PGPR strains may be highly specific with respect to wheat variety. The wheat variety and bacterial strains play important role in the beneficial effects on the growth and yield of wheat (319).

In the light of the above results it is recommended that new techniques may be developed for the utilization of PGPRs (especially *Azospirillum*) in order to reduce utilization of chemical fertilizers in agriculture practices for preventing environmental pollution and to maximize crop yield.

To study the effects of the bacterial strain on the growth and development of rice plant, both the rice varieties Fakhre Malakand and JP 5 were grown and inoculated in Falcon tubes. This system of growing seedlings in Falcon tubes is very useful for efficient and rapid screening of inoculants. The bacterial strains used as inoculants were *Azospirillum lipoferum* strain RSWT1, *Azospirillum brasilense* strain R1 *and Pseudomonas* strain Ky1. Beneficial effects of these strains were evident from the increase in the root area, root length, root weight, and shoot weight of the inoculated plants as compared to the non-inoculated control plants.

In the rice variety Fakhre Malakand, the root weight increased significantly while the root area was not increased due to bacterial inoculation. Inoculation with *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 increased the root weight but the increase was less than the root weight of the plant inoculated with *Azospirillum brasilense* strain R1. Root length and shoot weight of the plants inoculated with *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 were significantly greater as compared to control. In the rice variety JP5 positive effect of the inoculations was also obtained on both the root area and root length though the root area of the inoculated plants was not significantly different from control treatment. Maximum increase in the root weight and shoot weight was recorded for the plants inoculated with *Azospirillum brasilense* strain R1. A similar response was observed by Okon [67] who reported significant increase in the root length, shoot weight of rice plant inoculated with PGPRs and also by Richardson [302], Tanimoto [112] and Cakmakci et al. [75]. They explained that PGPR inoculation to rice

effectively increase the surface area of roots and root weight. Positive effects of PGPR on root length, root weight, root volume, root area, shoot weight, panicle emergence index and higher Zn mobilization efficiency as compared to the un-inoculated control was also reported by Tariq et al. [205].

Seeds of the selected rice varieties Fakhre Malakand and JP 5 were also cultivated in pots at NIBGE, Faisalabad. The pot experiment failed most probably due to high day temperatures and ultimately due to the death of the plants the pot experiment was abandoned. Both the rice varieties Fakhre Makakand and JP 5 are coarse-grain varieties and have been developed specifically for cold climate. These rice varieties are mostly grown in the hilly areas like Malakand, Hazara and other adjacent areas of Khyber Pakhtunkhwa.

To study the effect of the bacterial inoculants on rice grown in cold climate, field experiments were conducted at Agricultural Research Institute (N) Mingora and Udigram, Swat. Three bacterial strains (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) were used to inoculate rice varieties Fakhre Malakand and JP 5. For estimation of bacterial populations colonizing rhizospheric soil, samples were collected four week after the transplantation and total bacterial population was studied through total viable counts on LB agar plates. The soil samples collected from both the experimental fields showed bacterial population in the range of 10^5 cells/ g of soil. In general the bacterial population detected in the soil of Treatment 3 (*Azospirillum brasilense* strain R1) on LB plates was higher than all others. Maximum bacterial population (2.9×10^5 cells per g of soil) was detected in the

rhizosphere of rice variety JP 5 grown at Udigram Swat. Rhizobacteria survive in association with rice roots due to availability of root exudates while rice aerenchymatous tissues may be implicated in oxygen supply as has been proposed for vesicular arbuscular fungi [304-307].

From both experimental sites whole rice plants (20 plants from each treatment) were collected to study the effect of bacterial inoculants on various growth parameters. This was especially required to study effects of bacterial inoculation on root weight because it was not possible to uproot all plants from the plots.

In the experiment conducted at Agricultural Research Institute (N) Mingora, Swat, maximum plant length of rice variety Fakhre Malakand was observed in the treatment in which *Azospirillum brasilense* strain R1 was used as inoculant while maximum plant length of rice variety JP 5 was noted in non-inoculated treatments. In the rice variety Fakhre Malakand, *Azospirillum brasilense* strain R1 performed more efficiently as compared to other bacterial inoculants tested in the present study. Inoculation with this strain (*Azospirillum brasilense* strain R1) resulted in a maximum increase in root weight and shoot weight. However maximum increase in grain weight of this variety was observed in plants inoculated with *Pseudomonas* strain Ky1. In the rice variety JP 5, inoculation with *Azospirillum brasilense* strain R1 resulted in improved growth of plants which was evident in increased root weight, shoot weight and grain weight as compared to all other treatments including control.

The same three bacterial strains (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) were also tested as inoculants for the two rice varieties Fakhre Malakand and JP 5 at Udigram, Swat. In the rice variety Fakhre Malakand, maximum beneficial effects on plant roots were observed in plants inoculated with *Azospirillum lipoferum* strain RSWT1 while maximum fresh and dry weight of shoots and grains was noted in plants inoculated with *Azospirillum brasilense* strain R1.In the rice variety JP 5, maximum plant height was noted in the treatment in which *Pseudomonas* strain Ky1 was used as inoculants. In this variety maximum beneficial effects on most growth parameters (root fresh weight, shoot fresh weigh, shoot dry weight, grain fresh and dry weight) were observed in plants inoculated with *Azospirillum lipoferum* strain RSWT1.

All the bacterial strains used in the present study were nitrogen fixers and phytohormone producers. Therefore, the relative contribution of their mechanisms in growth promotion is unclear. Similar remarks have been made by other researchers [39, 81,159]. Results of the same nature were also observed by Tran Van et al [71], who inoculated rice in out door pot and field trials with *Bukholderia vietnamiensis* and reported that when rice plants were inoculated and transplanted at day 24, significant increase of shoot weight, root weight, leaf surface was observed.

Complete yield data collected from whole plots indicated that the inoculation of bacterial strains in both experimental fields resulted in more yields than non inoculated ones. The effect of the inoculated strains was positive on both fresh and dry weight of both rice varieties. In the experiment conducted at Agricultural Research Institute (N)

Mingora, Swat, inoculation of rice variety Fakhre Malakand with *Azospirillum brasilense* strain R1 significantly increased the fresh and dry weight of straw, grain and total plant (straw + grain) weight. This increase was 16.6 % in dry straw weight, 22.7% in dry grain weight and 19.8 % in total plant dry weight over the non-inoculated control. The inoculation of rice variety Fakhre Malakand with *Pseudomonas* strain Ky1 also showed significant increase in the straw weight by 13.4 %, total dry grain weight by 17.3 % and total plant dry weight by 14.7 %. Inoculation of *Azospirillum lipoferum* strain RSWT1 showed no significant effect on the total plant dry matter. However, an increase of 4.8 % in the total dry grain weight of rice variety Fakhre Malakand was resulted.

At this experimental site (ARIN), the same *Azospirillum brasilense* strain R1 showed the maximum growth promotion of rice variety JP 5 as compared to other inoculants used in this study. Inoculation with this strain resulted in 19 % increase in fresh straw weight, 39.3 % increase in fresh grain weight and 29 % increase in the total fresh weight over non-inoculated control. The increase in the dry straw weight, dry grain weigh, dry total weight were 19 %, 39.5 %, 30.8 %, respectively. *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 also showed significant increase (18.5 % and 13.5 % respectively) in the total grain yield. The results are parallel to the finding of Okon and Hadar [68] who reported 10 - 30% increase in grain and forage yield due to the inoculation of *Azospirillum* strains to rice.

The field experiment at Udigram, Swat showed significant effect of the inoculated strains on different growth parameters. The straw weight, grain weight and total plant weight of all the inoculated treatments was significantly higher than control. However,

Azospirillum brasilense strain R1 was found to be the best inoculant as maximum increase in straw, grain and total plant weight was obtained in plants inoculated with this strain. The results were similar to those observed by Omar et al [199], who reported that inoculation of rice with *Azospirillum brasilense* increase yield by 15- 20%. Similarly significant effects of this species of *Azospirillum* were also documented in different reports [308-310]. These results are also similar to those observed by Alam et al [200], who reported that inoculation of rice in the field with PGPR like *Azotobacter* sp, *Enterobacter* sp, *Bacillus* sp. and *Xanthobacter* sp results in an increase in the grain yield, total dry matter yield and nitrogen accumulation by 6-24%. They stated that the positive response of rice to PGPR was achieved because of the increase in leaf area, root length and chlorophyll contents [200]. The finding of Tariq el al. [205] is also in consistent with these results. They reported that PGPR application to rice helps in maintaining the Zn concentration in the soil, alleviate the symptoms caused due to Zn deficiency. They reported an increase of 23% in the total biomass, 65% in the grain yield and significant effect on the harvest index and concentration of Zn in the grain. The strain *Azospirillum brasilense* R1 in both experimental fields showed statistically more significant increase in the total plant weight, grain weight and straw weight. This PGPR species has been reported to increase the number of tillers and height of rice plant [311].

The present data obtained from the field experiment were also endorsed by the previous studies on PGPRs used as inoculants for other rice varieties, corn, sugar beet and tomatoes. The use of PGPR in rice [75, 302] sugar beet and barley [312] and tomatoes [313] promote growth, development and yield. The floral and foliar application

of *Bacillus* OSU-142 decreased shot-hole disease in apricot and resulted significant increase in PNE contents, growth and yield [314, 315]. Positive effects of PGPR inoculations on various growth parameters of rice, soybean have been reported using different PGPR alone or as co-inoculants with rhizobial strains [75, 76, 81]. The growth promoting mechanism of *Azospirillum* spp., *Pseudomonas* spp. and other rhizobacteria is yet to be explained [159]. It has been reported that production of plant growth regulators like auxin, gibberellins and cytokinins may be the possible mechanism of these rhizobacteria for the promotion of growth and yield of these plants [53, 109, 196, 316].

The comparative analysis of the results showed that rice variety JP 5 in both experimental sites was more responsive to the inoculated strains (e.g *Azospirillum brasilense* strain R1) than rice variety Fakhre Malakand. It was shown that the effects of PGPR strains may be highly specific with respect to plant variety. Host variations in the interaction with beneficial plant-associated microbes are also considered to be an important factor [317,318]. The rice variety, bacterial strain, culture condition play pivotal role in the establishment of bacterial association for beneficial effects on the growth and yield of rice and varies with respect to these factors [319].

Negative effects of the inoculated strains on certain growth parameters were also observed. In the whole plant study, the height of inoculated rice variety JP 5 plants at ARIN was less than non-inoculated control. Similarly the shoot weight of the inoculated rice variety JP5 at Udigram, Swat was comparatively less than non-inoculated treatment. Negative effects of bacterial inoculations on legumes and other plant species due to over production of growth hormones, production of antibiotics and competition with

Rhizobium for attachment sites on root surfaces have also been reported previously [320].

Comparatively analysis of the lab experiment, single plant data and whole plot data showed variation. In the single plant study at ARIN, Swat the strain *Pseudomonas* Ky1 showed more positive response on shoot weight and grain weight of rice variety Fakhre Malakand than other inoculated strains. Similarly at Udigram, Swat, *Azospirillum* lipoferum RSWT1 showed best results on certain growth parameters like plant height, shoot weight and grain weight in case of rice variety JP 5 than other inoculated strains. On the contrary, in the field experiments the strain *Azospirillum brasilense* R1 showed positive response on straw weight, grain weight and total weight than other treatment. In general there was a good agreement between the results obtained from both experimental fields of ARIN, Swat and Udigram, Swat. Such variation between single plants and whole plot analysis proved that on the basis of single plant analysis or small scale cultivation, the exact picture about the effectiveness of a particular strain or comparing different inoculations cannot be drawn.

The present results clearly indicate that the plant growth promoting rhizobacteria significantly promoted growth and productivity of maize plant when compared to non-treatment control. The stimulatory effects of rhizobacteria inoculation have been reported by other studies both in laboratory and field experiments [321]. Okon and Labandera-Gonzalez [70] reported that local isolates of *Azospirillum* increases maize yield from 15 to 25 % and with fertilization yield increased up to 40 %. All inoculation caused significantly increases in plant height over all treatment except non-inoculated control. Inoculation with *Azospirillum brasilense* strain R1 increased fresh root weigh (50%) and

fresh shoot weight (37.3%) of seedling after one week of inoculation. The increase in the dry root weight and dry shoot weight of seedling was 56% and 39% respectively. *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 also showed significant increase (30.5 % and 24.2 % respectively) in the root fresh weight and 42% and 37 % in root dry weight respectively. The increase in shoot dry weight due to *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1 was 25 % and 3 % respectively as compared to control treatment. The results are parallel to the finding of Dobbelaere [229] who reported an increase of 68.4 % in root and 42.6 % in shoot of maize seedling due to PGPR inoculation.

Inoculation with *Azospirillum brasilense* strain R1resulted an increase in number of ear/plant (45.9%), ear length (23.7%) and ear weight (17.25%). The increase with *Azospirillum lipoferum* strain RSWT1 was 35%, 27% and 19.4% in number of ear/plant, ear length and ear weight. *Pseudomonas* strain Ky1 also showed significant increase (20.4 % and 10%) in the number of ear/plant and ear length. However the comb weight was not significantly affected by this bacterial strain. These findings are in line with those of Saubident et al. [54], who reported significant increase in dry matter yield of maize due to *Azospirillum* inoculation in the field experiment. Higher plants (150 cm) were measured for treatment with *Azospirillum lipoferum* strain RSWT1 followed by *Azospirillum brasilense* strain R1 (147 cm) and *Pseudomonas* Ky1 (141 cm) while lower height of the plants (138 cm) was measured for non-inoculated control ones. The results showed that plant height was significantly increased up to 8.82% with *Azospirillum lipoferum* strain RSWT1 and with *Azospirillum brasilense* strain R1 up to 6.52% as compared to non-inoculated control ones. These results are in agreement with the

findings of Jacoud et al. [56], who stated that plant height of maize was significantly increased due to inoculation of *Azospirillum lipoferum* CRT1.

The results showed that number of leaves per plant was significantly affected by bacterial inoculation. Maximum number (13.56) of leaves per plant was noted in case of *Azospirillum lipoferum* strain RSWT1. This was followed by *Azospirillum brasilense* strain R1 with 13.52 number of leaves per plant. Minimum number of leaves per plant of 11.67 and 11.66 was recorded for treatment with *Pseudomonas* Ky1 and non-inoculated control treatment. Positive response of bacterial inoculation on stem thickness was also recorded. Maximum stem girth (5.36 cm) and ear length (24.23 cm) was noted in case of *Azospirillum lipoferum* strain RSWT1. This is followed by *Azospirillum brasilense* strain R1 with stem girth of 5.24 cm and ear length of 23.16 cm. Minimum stem thickness of 5.06 cm and 5.02 cm and ear length of 19.65 cm and 17.67 cm was recorded for treatment with *Pseudomonas* Ky1 and non-inoculated control treatment respectively. Similar results were also reported by Kokalis-Burelle et al. [321], who reported statistically significant increase in plant growth in two growing years in stem diameter, stem area, number of leaves and yield due to bacterial inoculation.

The inoculation caused the highest significant increase in number of grains/ear over all treatments except non inoculated control ones. Maximum number (368) of grains per ear was noted in case of *Azospirillum lipoferum* strain RSWT1. This was followed by *Azospirillum brasilense* strain R1 and *Pseudomonas* strain Ky1 with 345 and 310 numbers of grains per ear respectively. Minimum number of grain (290) per ear was recorded for non-inoculated control treatment. Similar results were also obtained by

Boddey and Dobereiner [91] who pointed out that *Azospirillum brasilense* strain Sp-245 significantly increases the grains number and yield.

Biological yield data collected from whole plots indicated that the effect of inoculations was significant. Higher biological yield (9210 kg/ha) was produced when treatment was done with *Azospirillum lipoferum* strain RSWT1 followed by treatment with *Azospirillum brasilense* strain R1 (8960 Kg/ha). The treatment with *Pseudomonas* Ky1 showed biological yield of 7496 kg/ha while lower biological yield (7360 kg/ha) was noted for non-inoculated control treatments. These results are in agreement with that of Okon & Labandera-Gonzalez [70], who studied the effect of local isolates of *Azospirillum* on wheat crop in the field and reported 15-30 % increase in the yield and an increase of 50- 60 % in the yield when fertilized.

The results showed that all the three selected PGPRs (*Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1, *Pseudomonas* Ky1 had promising positive effects on different growth parameters of maize grown under natural condition.

In Pakistan, this is the first field experiment to evaluate the beneficial effects of rhizobacteria inoculation on growth, development and yield of wheat variety Inqilab 91 and Fakhre Sarhad, rice varieties JP 5 and Fakhre Malakand and Maize variety Pahari. In the present study both the *Azospirillum* strains used as inoculants were isolated from rice grown in the same area. It has been reported that indigenous strains of PGPR show more positive response in increasing the production rate than non-inoculated control treatment [195, 196]. Rice inoculation with indigenous strains of *Azospirillum* led to significant

increase in plant height after 40-75 days of transplantation [197]. An increase of 42-64% in the growth of rice plant due to *Burkholderia brasilensis* and *Burkholderia vietnamiensis* inoculations under gnotobiotic conditions were reported by Baldani et al. [203].

Conclusion

In the present study, a total of 18 bacterial strains were isolated from roots and rhizosphere of cereal crops. On the basis of colony and cell morphology, 4 strains were identified as *Azospirillum*, 11 as *Pseudomonas* strains and three strains remained un-identified. With the exception of 3 strains, all isolates showed IAA production in pure culture. The results showed positive impact of Plant Growth Promoting Rhizobacteria (PGPR) on the growth and yield of cereal crops when used as inoculants. Among the strains tested in the present study, *Azospirillum brasilense* strain R1 was more effective in plant growth promotion than other strains for both wheat and rice varieties. *Azospirillum lipoferum* strain RSWT1 showed more positive response than other strains on the yield and growth of maize variety Pahari. The present study suggests that that beneficial strains of PGPR like *Azospirillum brasilense* strain R1, *Azospirillum lipoferum* strain RSWT1 and *Pseudomonas* strain Ky1) can be used as biofertilizer for cereal crops. It is also recommended that new techniques may be developed for the utilization of PGPRs (especially *Azospirillum*) in order to reduce utilization of chemical fertilizers in agriculture practices for preventing environmental pollution and for maximum crop yield. Additional field studies in Swat area are required to confirm the beneficial role of bacterial inocula on growth, development and yield of economically important cereal crops and also locally isolated PGPR may be tested on other wheat, rice and maize varieties grown in the area. This would help in developing a biofertilizer (inoculant) for use in agriculture in the future.

Chapter 4

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Appendix I

LB MEDIA

(Maniatis et al. 1982)

Tryptone	10 g/ L
Yeat extract	5 g/ L
NaCl	5 g/ L
Agar	18 g/ L
H_2O	1000 mL
pH	7.5

Appendix II

NITROGEN FREE MEDIUM (NFM)

Nitrogen Free Medium (Okon et al. 1977) consist of the following components (L^{-1})

Carbon Source	
DL- malic acid	5.0 g
Buffer	
K_2HPO_4	0.5 g
Macronutrients	
$MgSO_4.7H_2O$	0.2 g
$CaCl_2.2H_2O$	0.02 g
NaCl	0.1 g
Fe.EDTA solution (1.64%)	4 ml
Minor element solution	
The minor element solution contains:	
$CuSO_4.5H_2O$	0.4 g
$ZnSO_4.7H_2O$	0.12 g
H_2BO_4	1.4 g
$Na_2MoO_4.2H_2O$	1.0 g
$MnSO_4.H_2O$	1.5 g
H_2O	1000 ml
Vitamin solution	1 ml
The vitamin solution contains	
Biotin	10 mg
Pyridoxol-HCl	20 mg
H_2O	100 ml
Indicator	
Bromothymol blue 0.5%	
Solution in 0.2N KOH	2 ml
pH	7.0

In the preparation of semi-solid media 0.2 % (2 g/L) agar was used while for agar plates 1.5 % (1.5 g/L) agar and 0.02 g yeast extract was used.

HOAGLAND NUTRIENT SOLUTION

(Hoagland and Arnon 1950)

1M Ca $(NO_3)_2$	10 mL/ L
1 M KNO_3	10 mL/L
1 M $MgSO_4.7H_2O$	4 mL/L
Fe.EDTA 1.64%	2 ml/L
Micronutrients	2 ml/L
1 M $CaCl_2$	10 ml/L
1 M KCl	10 ml/L

Hoagland Micronutrients Trace Elements

Boric Acid	0.31 g/ L
$MnSO_4$	1.115 g/ L
$ZnSO_4.7H_2O$	0.430 g/ L
$Na_2MoO_4.2H_2O$	0.1225 g/ L
$CuSO_4.5H_2O$	0.00125 g/ L
KI	0.0375 g/ L
$CaCl_2$	0.00125 g/ L
Total volume	500 mL