Studies on Plant Growth Promoting Rhizobacteria Associated with Walnut (*Juglans regia* L.)

Shakeela Sofi
(2013-463-D)

Division of Plant Pathology

Faculty of Agriculture
Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir

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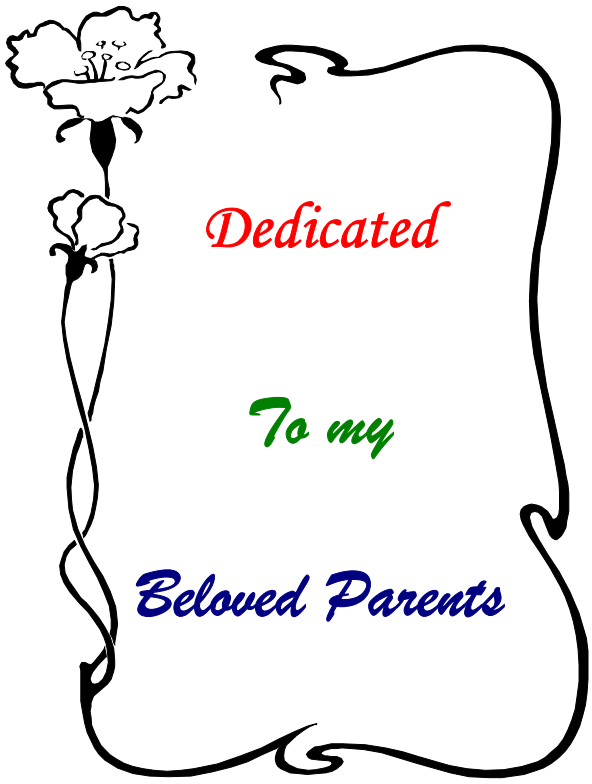
Dedicated
To my
Beloved Parents

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Agriculture, Division of Plant Pathology

Certificate – I

This is to certify that the thesis entitled, **"Studies on Plant Growth Promoting Rhizobacteria Associated with Walnut (*Juglans regia L.*)"** submitted in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology**, to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Shakeela Sofi (Regd. No. 2013-463-D)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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Prof. & Head,
Division of Plant Pathology

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Agriculture, Division of Plant Pathology

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Chairman

Dr. G.H. Dar,
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Associate Professor
Division of Environmental Sciences,
SKUAST-Kashmir

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University of Agricultural Sciences & Technology of Kashmir
Faculty of Agriculture, Division of Plant Pathology

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This is to certify that the thesis entitled, **"Studies on Plant Growth Promoting Rhizobacteria Associated with Walnut (*Juglans regia L.*)"** submitted by **Ms. Shakeela Sofi (Regd. No. 2013-463-D)** to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology** was examined and approved by the Advisory Committee and External Examiner on …….

Chairman
Advisory Committee

External Examiner

Prof. & Head,
Division of Plant Pathology

Dean,
Faculty of Agriculture,
SKUAST-Kashmir

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Agriculture, Division of Plant Pathology

Name of the student : **Shakeela Sofi**

Registration No. : 2013-463-D

Major subject : Plant Pathology

Minor subjects : Soil Science/Environmental Sciences

Major advisor : **Dr. G.H. Dar,**
Prof. & Ex-Head,
Division of Environmental Science,
SKUAST-Kashmir

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are viewed as a novel and potential bioresource for providing substantial benefits to agriculture crops. The use of PGPRs is gaining worldwide acceptance in agriculture and appears to be one of the important future agri-input. The present study was conducted with the aim to isolate rhizobacteria from walnut rhizosphere and also characterize some efficient PGPR. During surveyof four walnut growing districts of Kashmir valley i.e. Shopian, Budgam, Baramulla and Kupwara, surface and rhizosphere soils were collected from 36 sites belonging to 12 blocks. The soils were assessed for physicochemical characteristics and rhizosphere soils for rhizobacterial population. The organic carbon content of all the sites was found in the range of 0.62 to 1.65 per cent. Also, the available nitrogen and phosphorus ranged between 252 to 540 and 17.8 to 28.5 kg/ha, respectively, with maximum value observed at Krewa-manul (Shopian). The highest available potassium (205.7 kg/ha) was found at Warsun (Kupwara). The EC values ranged between 0.1 to 0.6 dS/m. Among 36 sites, soil texture of 13 sites each was silty clay loam and clay loam texture while 8 and 2 sites had loam and silty loam texture, respectively. The rhizobacterial population on NA medium was maximum at Krewa-manul (95.0 $\times 10^5$ cfu/g soil) and minimum at Palhalan (25.0$\times 10^5$ cfu/g soil). Ninety eight morphologically dissimilar bacterial

isolates were selected and characterized on the basis of Gram's staining and colony and cell morphology. Gram positive bacteria were most dominant amongst the isolates. Most colonies were circular in form having entire margins and convex elevation. The isolates were biochemically characterized as per the procedures described in *Bergey's Manual of Determinative Bacteriology.* Morpho-biochemical characterization revealed that 65 isolates tentatively belonged to genus *Bacillus*, 12 to genus *Azotobacter,* 17 to genus *Micrococcus* and 4 isolates to *Pseudomonas.* Amongst the 98 rhizobacterial isolates, isolate WI 65 showed maximum phosphate solubilizing index (3.40) while isolate WI 17 had minimum PSI(0.70). In liquid PVK medium, maximum P-solubilization was recorded for rhizobacterial isolate WI 90 (312 mg/l) while isolate WI 46 solubilized minimum phosphate (37 mg/l). Fifty four isolates produced siderophore with maximum zone size of 16.3mm in isolate WI 36 and a minimum of 3.0 mm zone size in isolates WI 72, WI 83 and WI 86. Quantitatively, isolate WI 12 yielded maximum per cent siderophore units (27.21) while isolate WI 1produced minimum per cent siderophore unit (4.75). Only 58 out of 98 isolates exhibited indole-3-acetic acid producing ability with maximum IAA production of 30µg/ml by isolates WI 36 and WI 41 and minimum (3.94µg/ml) by isolate WI 59.Forty eight isolates were positive for gibberellic acid production with maximum GA production by isolate WI 90 (65.33µg/ml) and minimum by isolate WI 8 (10.0µg/ml). The screening for chitinase activity revealed that only 35.71 per cent bacterial isolates were able to produce chitinase enzyme. The chitinase producing isolates showed average production of 15.71 units chitinase activity/ml with higher activity depicted by isolate WI 63 (30.47 units/ml) and least by isolate WI 18 (8.90 units/ml). CZ:CS was significantly higher for isolate WI 63 (4.05) and least for isolate WI 50 (1.60). Of the 98 isolates, 82 isolates showed HCN production with seven isolates having very high HCN. Similarly, six isolates showed very high ammonia production while 19 isolates exhibited no ammonia production. All isolates were screened for their antifungal activities against five fungal pathogens *viz*., *Dematophora necatrix, Alternaria solani, Fusarium oxysporum*, *Pythium aphanidermatum* and *Phytophthora capsici*. Twenty three isolates showed antagonistic activity against *D. necatrix* with isolate WI 90 inflicting highest growth inhibition of 66% and isolate WI 4 showing least inhibition of 7.6%.Twenty isolates were antagonistic to *A. solani* with maximum inhibition(55.6%) by isolate WI 63 andminimum by isolate WI 7 (8.9%). Only 19 isolates inhibited the growth of *F. oxysporum.* The isolate WI 62 depicted highest antifungal activity (43.8% inhibition) while least activity was shown by isolate WI 2 (10.7% inhibition). Only 21 and 20 isolates were antagonistic to *P. aphanidermatum* and *P. capsici.* Isolate WI 63 showed maximum of 45.5 per cent growth inhibition against *P. aphanidermatum* while isolate WI 49 showed least inhibition(15.4%). Against *P.capsica,* isolate WI 65 exhibited highest growth inhibition (49%) while isolate WI 38 showed minimum growth inhibition (8.8%).On the basis of maximum plant growth promoting traits and high performance score, twelve bacterial isolates were further selected for molecular characterization. The phylogenetic analysis revealed that rhizobacterial isolates

resembled with many reference sequences in global bacterial gene pool and were identified on the basis of maximum sequence similarity. The rhizobacterial isolate WI 90 was identified as *Bacillus licheniformis* strain WI 90. Similarly, isolate WI 62 was identified as *Bacillus tequilensis* strain WI 62, isolates WI 36 as *Bacillus cereus* strain WI 36, and isolates WI 63 and WI 65 as *Bacillus subtilis* strain WI 63 and strain WI 65, respectively. Isolates WI 12,WI 41 and WI 80 were identified as *Micrococcus luteus* strain WI 12, strain WI 41 and strain WI 80, respectively. The isolates WI 60 and WI 30 were identified as *Micrococcus yunnanensis* strain WI 60 and strain WI 30, respectively; and isolates WI 11and WI 91 as *Micrococcus* sp. strain WI11 and strain WI91, respectively. These strains can be exploited in walnut grown in nutritionally poor soils under fragile stressed conditions.

Key words: Walnut, PGPR, *Bacillus*, *Micrococcus*

Signature of Student

Dated: _____

Signature of Major Advisor

Dated: _____

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Shakeela Sofi

Place: Shalimar, Srinagar

Dated:

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Chapter - 1

INTRODUCTION

The Persian walnut (*Juglans regia* L.), also known as English walnut, belongs to family Juglandaceae. It is native from the Balkans in southeast Europe, southwest and central Asia, to the Himalayas and southwest China. Walnut is one of the most important nut crops grown in India, especially in North-western Himalayan belt, expanding to Darjeeling and Sikkim. Jammu and Kashmir state is the major producer of walnut covering an area of 89,339 ha with production of about 2,66,280 metric tonnes (Anonymous, 2016). The J&K state produces 85 per cent of the total walnut produce of the country and assumes a unique distinction, rather monopoly, in the production of export quality walnut. In Kashmir valley Anantnag, Pulwama, Kupwara, Budgam, Baramulla and Srinagar are major walnut growing districts while in Jammu walnuts are grown in Doda followed by Poonch and Udhampur. Walnut trees flourish in temperate climate at an altitudinal range of 900-3500 masl. The tree grows well in cool climates that are free from spring frosts.

The rhizosphere of a plant is a microecological zone in direct proximity with plant roots. It is functionally defined as the particulate matter and microorganisms that cling to the roots after being gently shaken in water (Walker *et al.*, 2003). The rhizosphere is a metabolically busier fast changing competitive environment than the surrounding soil. The plant roots, the component of rhizosphere, can affect the physical environment of rhizosphere. As plants transpire water with more force during day than during night, they change the soil water potential immediately near their roots and so the rhizosphere undergoes more fluctuations than the bulk soil. Movement of organic matter away from the root as well as bacteria colonizing new locations occurs more readily in sandy soils than clay soils. Sand has larger pores between each granule allowing microorganisms and exudates to travel. Therefore, larger the granule size, further

the rhizosphere and microorganisms associated with it will extend into the surrounding soil. Several factors may lower the pH in rhizosphere. Respiration leads to carbon dioxide production and eventually to bicarbonate/carbonic acid generation. In addition to the respiration of roots, the rhizosphere is very rich in carbon due to the respiration of prokaryotes, fungi and small animals living in the rhizosphere. Generally fungi are more abundant in acidic soils than alkaline and bacteria have a very broad pH spectrum for their survival. The effects of pH in rhizosphere is critical in supporting a biologically diverse microbial community.

Plant-derived compounds are responsible for providing the additional carbon that allows the rhizosphere to host a large variety of organisms. Microbial communities include rhizospheric bacteria which are numerous in soil averaging between 10^6 and 10^9 organisms per gram rhizosphere soil; while fungi, both pathogenic and symbiotic, average between 10^5 and 10^6 organisms per gram rhizosphere soil (Clark, 1967). Up to 15 per cent root surface area is covered with rhizosphere-specific microorganisms thereby provide many sites for biological interactions (Berendsen *et al.*, 2012). A range of interactions do occur in the rhizosphere from beneficial symbiotic relationships to detrimental pathogenic interactions (Sylvia *et al.*, 2005). Microorganisms in rhizosphere complete both chemical and physical modifications to the soil profile in and around the rhizosphere that affect plants. They can be either beneficial to the plant (by pathogen suppression) or detrimental (by competition for nutrients). Chemical changes occur as a result of humification of organic matter. The resultant mineralization of various organic compounds (phosphorus, sulfur and nitrogen, for example) provides plants with forms of nutrition that are readily available for uptake. The turnover of microbial populations also results in the release of nutrients. The fixation of atmospheric dinitrogen by both asymbiotic and symbiotic bacteria results in increases to the available nitrogen pool that can be accessed by plants in and near the rhizosphere. Symbiotic mycorrhizae cause an increase in the effective rooting area of plants, thereby provide added nutrient

mining capabilities to the plant. Rhizosphere microbes can also release plant growth regulators.

In rhizosphere, intensive interactions between plants, soil and microflora and fauna occurs in the region that includes plant roots and surrounding soil due to the presence of high energy compounds and C content (Dakora and Phillips, 2002). This accumulation in the rhizosphere corresponds to all the compounds produced by plant roots, most of which are organic in nature derived from photosynthesis and other plant processes (Pinton *et al.*, 2001). Several biochemical signal exchanges take place between microbial communities and their host plants. A wide diversity of bacteria and plant-associated microbes can interact with host plants in a beneficial way either by enhancing their growth or mitigating diseases (Beattie, 2006; Nihorimbere *et al.*, 2011). It is known that the majority of bacteria that promote plant growth are rhizosphere inhabitants which have been designated as plant growth-promoting rhizobacteria.

Rhizobacteria that exert beneficial effects on plant growth and development are referred to as plant growth promoting rhizobacteria (PGPR) (Ashrafuzzaman *et al.,* 2009). PGPR promote plant growth through ability to produce either growth regulators or solubilize mineral phosphates and other nutrients or fix N_2 or antagonism against phytopathogenic microbes by the production of siderophores, antibiotics or cyanide (Sarvanakumar *et al.,* 2007). PGPR may use more than one of above mechanisms to enhance plant growth. Experimental evidence suggests that plant growth stimulation is the net result of multiple mechanisms that may be activated simultaneously (Martinez *et al.*, 2010). PGPR belong to diverse genera, especially of *Azospirillum, Alcaligenes, Arthrobacter, Enterobacter, Erwinia, Acinetobacter, Bacillus, Burkholderia, Flavobacterium, Pseudomonas, Rhizobium* and *Serratia.* All of them are able to exert beneficial effects on plant growth (Tilak *et al.*, 2005). The beneficial microbial interactions with plants have been grouped into three categories. The first category corresponds to microbes that interact direct or indirectly with the plant and are responsible for improving plant nutrients in

rhizosphere. The second category are responsible for stimulating plant growth indirectly by preventing pathogenic growth. The third category involves the microbes that directly influence plant growth by phytohormone production, phosphate solubilization and siderophore/volatile compound production (Podile and Kishore, 2006; Nihorimbere, 2011).

There is a growing market for microbial inoculants across the world which shows annual increase of approximately 10 per cent (Berg, 2009). Other important facts of microbial inoculants when compared with chemical pesticides and fertilizers are their ecofriendly nature, better safety, more target-oriented activity, effectiveness in smaller doses, ability to multiply and sustain for longer periods, quick decomposition, least chances of induced resistance by pathogens/pests and finally and effective in organic/conventional agriculture (Berg, 2009). Worldwide, the rhizosphere of vegetable crops has extensively been studied while the rhizosphere of horticultural crops such as apple and cherry has been explored to a lesser extent (Bashan, 1998). The rhizosphere of nut crops like walnut has not been explored as yet. Since walnut trees exhibit allelopathy, therefore only specific type of microorganisms may be able to thrive under its canopy. Thus, the microorganisms in walnut rhizosphere may apparently be quite different from those obtained from the rhizosphere of other temperate horticultural crops. Perusal of literature has revealed that with the exception of a preliminary report (Dar *et al.*, 2009), no studies on rhizosphere microbes of walnut have been conducted in the Western Himalayas, especially in the Jammu and Kashmir state. In order to explore the diversity existing in walnut rhizosphere especially with respect to plant growth promoting bacteria, the present study was aimed with the following objectives:

1) Isolation and morphological characterization of bacteria from walnut rhizosphere;
2) Screening of isolates for various plant growth promoting attributes;
3) Assessment of efficient species/strains for their antifungal activity against various soil borne pathogens.

Chapter - 2

REVIEW OF LITERATURE

Burgeoning human population has exerted tremendous pressure on limited available cultivable lands to produce more food to meet human requirement. This has forced continuous expansion of food ecosystems into less fertile areas. The increasing use of chemical fertilizers and pesticides have additionally put these soils under severe stress. The use of soil microbes which can fix atmospheric nitrogen, solubilize phosphorus or stimulate plant growth through the synthesis of growth promoting substances and antagonize plant pathogenic fungi has gained importance in modern agriculture in view of the deleterious effect of chemical fertilizers/pesticides on the soil as well as plant and human health (Glick *et al*., 1995). Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by several mechanisms (Cleyet-Marcel *et al*., 2001 and Kloepper, 1994). PGPR belong to diverse genera of *Azospirillum, Alcaligenes, Arthrobacter, Enterobacter, Erwinia, Acinetobacter, Bacillus, Burkholderia, Flavobacterium, Pseudomonas, Rhizobium* and *Serratia.* All of them are able to exert beneficial effects on plant growth (Tilak *et al*., 2005). PGPR have been explored in most of the vegetable crops but less in temperate horticultural crops, especially nut fruit crops. The present chapter briefly reviews the work done in India and abroad on PGPR with thrust on horticultural crops.

2.1 Plant growth promoting rhizobacteria associated with horticultural crops

The roots of horticultural crops are often colonized by many microorganisms both the deleterious and beneficial ones. The rhizobacteria through the process of root colonization survive onto seed or into soil, multiply in spermosphere (the region surrounding the seeds) in response to seed exudates, rich in carbohydrates and amino acids (Kloepper *et al*., 1985); attach to the root surface (Compant *et al*., 2005) and colonize the developing root system in soils

containing indigenous microorganisms (Benizri *et al.*, 2001; Lugtenberg *et al.*, 2001).

Dar *et al.* (2009) during a study on the rhizosphere microbial composition of walnut plants (cv. SKAU-W-0035) found its roots inhabited by bacteria, fungi and actinomycetes with maximum population noticed in the month of June and minimum in December. The predominant microorganisms observed in rhizosphere belonged to the genera *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Aspergillus* and *Penicillium.* Three isolates of predominant bacteria were screened for their plant growth promoting activities. *Azotobacter* (AZB III), *Azospirillum* (AZS II), *Bacillus* (BAC I) and *Pseudomonas* (PS II) were found to be efficient isolates owing to their ability to produce ammonia, IAA and HCN, solubilize phosphorus and antagonize pathogens.

Karakurt *et al.* (2010) evaluated the effects of four strains of PGPR (*Agrobacterium rubi* A-18, *Bacillus subtilis* OSU-142, *Burkholderia gladioli* OSU-7 and *Pseudomonas putida* BA-8) on growth and leaf nutrient content of Starking Delicious, Granny Smith, Starkrimson Delicious, Starkspur Golden Delicious and Golden Delicious apple cultivars grafted on semi-dwarf rootstock MM-106. The application of bacterial strains increased the leaf number and area as well as the number of annual shoots and their diameter. Use of *P. putida* strain BA-8 resulted in production of highest number of annual shoots (52.4) while *B. subtilis* strain OSU-142 resulted in largest leaf area (16.12 cm^2) and highest Mg (0.13%) and Fe (32.7 ppm) contents in leaves. The applications of *A. rubi* strain A-18 decreased the concentration of N, K and Cu and increased the concentration of P and Zn in apple leaves while the use of *B. gladioli* strain OSU-7 resulted in highest Mn content (40.3 ppm).

Mehta *et al.* (2010) studied 13 bacterial isolates from apple rhizosphere and identified *Bacillus circulans* MTCC 8983 as a highly efficient P solubilizing strain. The strain solubilized tricalcium phosphate and produced substantial amount of soluble phosphorus (957.3 mg/l), indole acetic acid (15.13 µg/ml), 57.8

per cent siderophore and showed growth inhibition against *Dematophora necatrix* (46.57%).

Karakurt and Aslantas (2010) conducted a study to determine the effects of bacterial inoculation (*A. rubi* A-18, *B. subtilis* OSU-142, *B. gladioli* OSU-7 and *P. putida* BA-8) on flower thinning, set and fruit properties of apple cultivars Starking Delicious, Granny Smith, Starkrimson Delicious and Starkspur. The suspensions prepared with above bacterial strains were applied to the crown of trees by spraying at initial and full bloom. The fruit set rate showed significant variations within cultivars. Starkspur and Golden Delicious cultivars had highest fruit set with a rate of 12 per cent while Granny Smith cultivar had lowest fruit set of 4 per cent. Bacterial treatments decreased fruit set by 12 per cent (OSU-142) and 33 per cent (A-18 and BA- 8). The bacterial treatments had no significant effect on fruit size, width and height of fruit; but it decreased specific gravity of fruit, stalk thickness, stalk length and stalk hole deepness. The bacterial treatments generally reduced the rate of total soluble solid, total sugar, reduced sugar, ascorbic acid contents, titrable acidity and pH rate.

Peyvandi *et al.* (2010) while growing olive micro shoots, inoculated with or without *Pseudomonas fluorescent* strain P19 or P21, observed that both the strains significantly affected root growth and architecture and found bacterial treatments more efficient than IBA.

Akca and Ercisli (2010) found that the plant growth promoting bacterium *Bacillus* OSU-142 sprayed on 10 years old sweet cherry cv. 0900 Ziraat trees at full bloom, 15 and 30 days after full bloom stages alone or in combination with NPK significantly increased fruit weight, length, diameter, seed weight and soluble solid content (SSC). They suggested that *Bacillus* OSU-142 application has potential to increase fruit quality in sweet cherry cv. 0900 Ziraat.

Karakurt *et al.* (2011) observed that PGPR *B. subtilis* OSU-142, *B. megaterium* M-3, *B. cepacia* OSU-7 and *P. putida* BA-8 alone and in

combinations increased fruit set and plant vegetative growth, as well as affected fruit pomological and chemical characteristics in cherry trees (*Prunus cerasus* cv. Kutahya).

Kapoor *et al.* (2012) isolated 26 strains of *Pseudomonas* from apple and pear rhizosphere in normal and replant sites of Himachal Pradesh, India and found that the Pseudomonad count was more in normal site as compare to replant site which they suggested was the reason for suppression of growth and yield decline in apple and pear at replant sites. They found that fluorescent Pseudomonads had potential to synthesize different secondary metabolites with diverse PGPR activities which enhanced the soil fertility and promoted plant growth. They further screened the isolates for the production of various PGPR activities and proteolytic enzymes production *viz.*, protease, chitinase and glucanase. The strains AN-1-UHF, AN-5-UHF, PN-7-UHF and PN-13-UHF were selected on the basis of their higher PGPR attributes and proteolytic activities. These strains were found to be very efficient in antimicrobial activities.

Arikan *et al.* (2013) found the effects of *Bacillus mycoides* isolate T8 and *B. subtilis* isolate OSU-142 on yield and fruit properties in quince. The foliar applications of isolates T8, OSU-142 and T8+OSU-142 significantly increased yield/tree, number of fruits, fruit weight, fruit width and fruit height as compared to the control. The highest fruit weight was observed due to the application of strain OSU-142+T8. The bacterial applications did not change the rate of soluble dry matter, titratable acidity and pH but enhanced fruit size and firmness, thus revealing that *Bacillus* strains T8 and OSU-142 have great potential in enhancing the fruit yield.

Xuan *et al.* (2014) observed that the inoculation of phosphate solubilizing bacteria (PSB) *Pseudomonas aurantiaca* or *P. fluorescens,* both under natural and controlled conditions, significantly improved height, shoot and root dry weight, phosphorus and nitrogen uptake and net photosynthetic rate of 1-year-old walnut seedlings. Further, the application of these two PSB strains improved soil quality

as revealed by increase in the activities of soil dehydrogenase, neutral phosphatase and urease.

2.2 PGPR as root colonizers

The bacteria capable of colonizing plant root systems and promoting their growth are referred to as PGPR (Kloepper and Schroth, 1978). PGPR associations range in their magnitude of bacterial proximity to the root and intimacy of association. In general, these can be separated into i) extracellular PGPR, those existing in the rhizosphere, rhizoplane or in the spaces between cells of root cortex and ii) intracellular PGPR, those which exist inside root cells, following their colonization on seeds and roots.

Root colonization is the process whereby bacteria survive after inoculation onto seed or into soil, multiply in the spermosphere (the region surrounding seed) in response to seed exudates rich in carbohydrates and amino acids (Kloepper *et al*., 1985), attach to root surface (Compant *et al*., 2005) and colonize the developing root system in soils containing indigenous microorganisms (Benizri *et al*., 2001; Lugtenberg *et al*., 2001). A number of bacterial traits and specific genes are known to contribute to the process of root colonization but till date only a few have been identified. These include motility, chemotaxis to seed and root exuadates, production of pilli and fimbriae, production of specific cell surface components, ability to use specific components of root exudates and protein secretion (Benizri *et al*., 2001; Lugtenberg *et al*., 2001). Lucas *et al.* (2005) investigated the effect of three PGPR on biological nitrogen fixation (BNF), nodulation and growth promotion in soybean (*Glycine max*). The strains Aur 6, Aur 9 and Cell 4 belonged to *Pseudomonas fluorescens*, *Chrysobacterium balustinum* and *Serratia fonticola,* respectively. The results revealed that the inoculation with different PGPR under greenhouse conditions significantly affected various growth parameters such as stem, leaf weight and fresh root weight.

2.3 PGPR as phosphate solubilizers

Phosphate solubilizing microorganisms are the microbes that convert insoluble phosphatic compounds into soluble forms (Prerna-Akhaury *et al.*, 1997; Raju and Reddy, 1999). Important bacterial genera capable of phosphate solubilization are *Bacillus* and *Pseudomonas* (Illmer and Schinner, 1992; Motsara *et al.*, 1995) while *Aspergillus* and *Penicillum* are important phosphate solubilizing fungi (Motsara *et al.*, 1995). Some strains of *Rhizobium* reportedly also solubilize both organic and inorganic phosphate (Abd-Alla, 1994). A high proportion of phosphate solubilizing microorganisms are concentrated in plant rhizosphere (Gaur, 1990).

Pareek and Gaur (1973) studied the extent of solubilization of tricalcium phosphate and rock phosphate by organic acids and found that aliphatic acids were comparatively more effective in phosphate solubilization than phenolic acids. Citric acid and fumaric acid had highest P-solubilizing ability.

Banik and Dev (1983) studied eight bacterial and fungal phosphate solubilizing microbes on $AlPO_4^-$-sucrose agar medium and found that *Bacillus* spp. were more effective in solubilizing tri-calcium phosphate $\{Ca_3(PO_4)_2\}$. All the isolates produced organic acids like oxalic acid, succinic acid, citric acid and 2-ketogluconic acid in detectable amounts. Jones (1998) proposed that organic acids such as malate, citrate and oxalate might be involved in many processes operating in the rhizosphere, including nutrient acquisition, metal detoxification, alleviation of anaerobic stress in roots, mineral weathering and pathogen attraction. Rodriguez and Fraga (1999) found that strains of *Pseudomonas*, *Bacillus* and *Rhizobium* were among the most powerful phosphate solubilizers and the strains of *Bacillus* were able to produce a mixture of lactic acid, isovaleric acid, isobutyric acid and acetic acid.

Goldstein *et al.* (1999) concluded that the resultant acidification of rhizosphere by mineral phosphate solubilizing bacteria may play a role in nutrient

acquisition and other eco-physiological parameters essential for plant survival. They observed that rock phosphate ore may be acidified and efficiently processed by bringing the ore in contact with bacteria or bacterial fermentation products (strong organic acids). Suitable conditions of pH, proton/bicarbonate release (anion/cation exchange), gaseous (O_2/CO_2) exchange and release of root exudates like organic ligands contributed to the changes in the bioavailability of soil inorganic-P in rhizosphere (Hinsinger, 2001).

Vasquez *et al.* (2000) observed that the culture media supplemented with insoluble tricalcium phosphate and incubated with the roots of black and white mangrove became transparent after a few days of incubation. Further, they evaluated the potential of phosphate solubilizing rhizo-microbes, both qualitatively and quantitatively, in the media containing tri-calcium phosphate as sole phosphorus source and found that the concentration of soluble phosphate in liquid culture increased abruptly with time and the pH in culture dropped drastically.

Hwangbo *et al.* (2003) examined the production of 2-ketogluconic acid (2-KGA) and phosphate solubilization by *Enterobacteria intermedium* and concluded that the addition of gluconic acid, $CaCO_3$ and O_2 in culture medium resulted in maximum production of 2-KGA. However, for optimum phosphate solubilization, $CaCO_3$ had adverse effect and thus recommended its exclusion from culture medium.

Vassilev and Vassileva (2003) employed free-immobilized microbes in composting, solid-state fermentation and liquid submerged fermentation and observed that the production of organic acids helped in simultaneous solubilization of rock phosphate. They concluded that these products help the soil plant systems to promote its growth and P- acquisition.

Rashid *et al.* (2004) isolated and screened phosphate solubilizing microorganisms (PSM) on the basis of solubilization index (SI) and drop in pH.

The HPLC analysis confirmed that only a few PSM produced gluconic acid, fumaric, succinic, acetic acid, oxalic acid and citric acid.

Rodriguez *et al.* (2004) observed that *Aspergillus brasilense* and *Aspergillus lipoferum* produced gluconic acid when grown in sparingly soluble calcium phosphate medium when the usual fructose carbon sources were amended with glucose. At the same time, there was reduction in pH of the medium and soluble phosphate was released into the medium.

Sujatha *et al.* (2004) observed that dicalcium phosphate was solubilized more readily than tricalcium phosphate by thermophilic bacteria, actinomycetes and fungi. Further, the degree of phosphate solubilization varied with incubation period and phosphate solubilization decreased considerably under alkaline conditions.

Suliasih and Widawati (2005) observed that the bacterial population in soil ranged from 5.0×10^3-7.5×10^6 and 5.0×10^3-1.5×10^7 cells/g for phosphate solubilizing bacteria (PSB) and nitrogen fixing bacteria (NFB), respectively. The isolated PSB were *Bacillus pantothenticus, B. megaterium, Bacillus* spp.*, Chromobacterium lividum, Enterobacter alvei, E. agglomerans, Flavobacterium breve, Flavobacterium* spp.*, Klebsiella aerogenes, Klebsiella.* spp.*, Pseudomonas* spp.*, Proteus* spp. and NFB were *Azotobacter chroococcum, A. paspalii, Azotobzcter* spp.*, Rhizobium* spp. and *Azospirillum* spp.

Ivanova *et al.* (2006) studied the effect of rock phosphate concentration in liquid medium, its particle size and duration of bioconversion on the solubilization of Tunisian phosphorite by *Erwinia* spp. and *Azotobacter* spp., isolated from soil. They found that lower the concentration of phosphate in nutritive medium greater is the conversion percentage of P_2O_5 into soluble forms due to the production of low molecular mass organic acids.

Khan *et al.* (2006) reported that PSM could play an important role in supplying phosphate to the plants in a more environment-friendly and sustainable

manner. They found the performance of PSM *in situ* was contradictory but the solubilization of phosphatic compounds by naturally abundant PSM was very common under *in vitro* conditions. They concluded that the variability in the performance greatly hampered the large-scale application of PSM in sustainable agriculture.

Naik *et al.* (2008) isolated various strains of phosphate solubilizing *Pseudomonas* and resulted that phosphate solubilizing strains from banana rhizospheric soil of Puducherry (India) showed high variability in utilizing carbon sources. They concluded that these phosphate solubilizing strains play a vital role in plant growth promotion, disease suppression and subsequent enhancement of yield of banana.

Mundra *et al.* (2011) isolated a yeast strain PS4 which was capable of solubilizing insoluble inorganic phosphate from rhizosphere of seabuckthorn *(Hippophae rhamnoides* L.), growing in the Indian Trans-Himalaya. Based on morphological, biochemical, whole cell FAME analysis and molecular characterization, strain PS4 was identified as *Rhodotorula* sp. The strain solubilized $Ca_3(PO_4)_2$ to a great extent than $FePO_4$ and $AlPO_4$. The solubilization of insoluble phosphate was associated with drop in pH of the culture media. The finding suggested that the inoculation of tomato seedling with the strain increased fruit yield roots and shoot length. *Rhodotorula* sp. PS4 with phosphate-solubilizing ability under stress conditions appeared to be attractive for exploring their plant growth-promoting activity towards the development of microbial inoculants in stressed region.

Sharma *et al.* (2012) isolated a number of rhizospheric microorganisms from tea plants in Darjeeling hills and their screening for solubilization of tricalcium phosphate (TCP) revealed that eight isolates were able to solubilize TCP in Pikovskaya's agar, liquid medium. The amount of phosphate solubilized ranged from 40.62±1.1 to 136.73±1.7 mg/l. Phosphate solubilizing activities of these strains were associated with a drop in the pH of medium.

Shankarrao, (2012) studied total 28 phosphate solubilizing bacteria, isolated from rhizospheric soil of neem, mango and jatropha plants. The solubilization index of each isolates was determined on Pikovskaya agar medium. The isolate M (III), M (III) col-2, M(III) col-4, N (b) col-1, N (c) col-2, J(A) and J-C col-2 showed high P solubilization potential having SI =2.11 - 3.35 recorded and quantitatively solubilized 160, 182, 270, 164, 200, 228 and 182 mg/ml P, respectively, after 7 days of incubation. The isolates were identified and characterized for plant growth promoting activities such as production of ammonia, indole acetic acid, cell wall degrading enzyme; cellulase, chitinase and proteolytic enzyme and antagonistic against plant pathogenic fungi and bacteria. The P-solubilization was accompanied by reduction in pH of the medium. The results indicated that rhizospheric soil isa rich source for isolating phosphate solubilizing bacteria which promote plant growth by more than one PGPR trait and have wide application in soil ecology.

Kesaulya *et al.* (2015) isolated bacteria from potato rhizosphere which showed phosphate solubilizing ability. The isolate HB3 (14.24 mg/l) showed phosphate solubilizing ability while isolate HB18 also solubilize phosphate but showed low concentration of soluble phosphate (4.46 mg/l).

2.4 Rhizobacteria as siderophore producers

Iron is an essential growth element for all living organisms as well as a cofactor for a number of enzymes and iron chelating proteins. Under iron limiting conditions, PGPR produce low molecular weight compounds called siderophores to competitively acquire ferric ions (Whipps, 2001). These compounds scavenge iron from the environment and make the mineral available to microbes. Bacterial siderophores differ in their ability to sequester iron but in general they deprive pathogenic fungi of the iron since the fungal siderophores have lower affinity. Sidrophores are iron chelating compounds which have very high affinity for ferric ions. The bacterial iron chelators sequester the limited supply of iron available in rhizosphere thereby making it unavailable to pathogenic fungi so restrict their

growth (Loper and Henkel, 1999). DeVillegas *et al.* (2002) evaluated siderophore production by *Pseudomonas aeruginosa* PSS in a conventional batch system in succinate, glucose and glutamic media and observed that increase of Fe (III) concentration had negative impact on siderophore production, specially above 10 µM.

Sharma and Johri (2003) compared the siderophore production by fluorescent *Pseudomonas* spp. GRP3A, PRS9 and *Pseudomonas chlororaphis* ATCC 9446 in standard succinate and citrate media. Succinate was found better suited for siderophore production.

Rachid and Ahmed (2005) studied the biosynthesis of siderophores in four basal media supplemented with different concentrations of iron and observed that ferric ions increased growth yield and completely repressed siderophores production above 200 g/l, but showed positive effect below 160 g/l. Penicillin and lead elicited siderophore production in presence of excess iron.

Chakraborty *et al.* (2006) isolated *Bacillus megaterium* from the rhizosphere of tea and assessed its ability to promote plant growth and cause disease reduction. They reported that plant growth promotion and disease reduction was due to the production of siderophores.

Mishra and Kumar (2012) studied *in vitro* production of siderophore on different carbon sources by two PGPR strains. Application of selected isolates significantly increase the growth of rice (*Pusa sugandha* III). The strains were identified with 16S rRNA gene sequencing as *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *B. subtilis* was found to 14 times more efficient than *B. amyloliquefaciens* in terms of siderophore production.

Parray *et al.* (2013) isolated bacterial strains from saffron rhizosphere soil during theflowering stage of corms and screened them for siderophore production. Most of isolates were Gram negative rod type *Bacillus subtilis* showed 62 per cent siderophore production.

2.5 Rhizobacteria as indole-3-acetic acid (IAA) and giberellic acid producers

The ability to synthesize phytohormones is widely distributed among plant associated bacteria. IAA is assumed to be the most abundant and widespread auxin that mediates an enormous range of development and growth responses including embryo symmetry establishment, initiation of cell division, promote vascular differentiation, root initiation and apical dominance. Besides its hormonal functions, IAA is involved in the stimulation of ethylene synthesis (Glick, 1994). The roots in plants are most sensitive to the fluctuations in IAA and respond positively to the increasing amounts of exogenous IAA by improving elongation of primary root, formation of lateral and adventitious roots to growth cessation.

Ahmed *et al.* (2005) reported IAA production of 1 to 5 mg/ml by indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan.

Gravel *et al.* (2007) evaluated five bacterial and three fungal isolates for their growth promoting effect on mature healthy tomato plants grown under hydroponic conditions and observed that *Pseudomonas putida* and *Trichoderma atroviride* had IAA producing and degrading activity. When both the strains were grown in presence of increasing concentration of L-tryptophan (upto 0.75mM), the fresh weight of roots and shoots of tomato seedlings showed increase.

Karnwal (2009) tested *Pseudomonas fluorescens* AK1 and *P. aeruginosa* AK2 for their ability to produce IAA in pure culture in presence of L-tryptophan tested at 50, 100, 200 and 500 µg/ml. Their study revealed that in both strains IAA production increased with increase in tryptophan concentration (0.5, 1.2, 4.3 and 9.3 µg/ml and 0.2, 0.7, 3.8 and 8.3 µg/ml, respectively.

Khamna *et al.* (2010) studied IAA production by *Streptomyces species* isolated from some Thai medicinal plant rhizosphere and reported that *Strepromyces* spp. produced IAA in yeast malt extract medium. They found that

Streptomyces CMU-H009, recovered from soil, association with lemongrass was very effective in producing IAA. The culture filtrate of strain CMU-H009 stimulated significant increase in germination and root elongation in maize and cowpea plants.

Kaushal *et al.* (2011) isolated PGPR from cauliflower rhizosphere growing indifferent agro-climatic zones of Himachal Pradesh. Five efficient isolates designated as MK2, MK4, MK5, MK7 and MK9 were selected and characterized after successful experiments under *in vitro* and net house conditions at varying levels of N and P. All the isolates induced IAA production and solubilized phosphorus. The conjoint use of PGPR and N & P fertilizers significantly increased the number of non-wrapper leaves, curd diameter, curd depth and curd weight in cauliflower. Further, PGPR isolates remarkably increased the yield of cauliflower.

Mehta *et al.* (2014) isolated a P-solubilizing bacterial strain (CB7) from apple rhizosphere of Himachal Pradesh (India) and identified it as *Bacillus circulans* on the basis of phenotypic characteristics, biochemical tests, fatty acid methyl esters analysis and16S rRNA gene sequencing. The isolate exhibited plant growth-promoting traits like P-solubilization, auxin production, 1-aminocyclopropane-1-carboxylate deaminase activity, siderophore production, nitrogenase activity and antagonistic activity against *Dematophora necatrix*. P-solubilization activity was associated with the release of organic acids and drop in pH of Pikovskaya's medium. Remarkable increase in seed germination (22.3%), shoot length (15.9%), root length (25.1%), shoot dry weight (52.9%), root dry weight (31.4%), nitrogen (18.7%), potassium (57.7%) and phosphorus (22.2%) contents in shoot biomass over control was observed. Isolate CB7 appeared promising PGPR to be developed as a biofertilizer for enhancing soil fertility and plant growth.

Pant and Aggarwal, (2014) studied and identified IAA producing rhizobacteria from the rhizosphere of *Withania somnifera*. A total ten bacterial

isolates were recovered from rhizospheric soil associated with *W. somnifera*, recognized as *Bacillus* sp. by morphological and biochemical characterization and tested for indole acetic acid production. Out of ten rhizobacterial isolates, six were selected as efficient producers of IAA. The amount of indole acetic acid produced was detected in the different concentration of tryptophan, which lead to the elevation in indole acetic acid production as compared to in absence of tryptophan. The IAA production was further confirmed by TLC. The PGPR showed significant increase in germination, root and shoot length in *W. somnifera*as compared to the untreated control.

Sharma *et al.* (2015) assessed plant growth promoting activity of rhizobacteria isolated from mid hill region of Himachal Pradesh (India) from tomato. Of the 100 isolates, 26 were further screened for PGP traits and antifungal activity. The strain S25 showed significantly higher phosphate solubilization and IAA production. On the basis of *in vitro* screening, 10 most efficient isolates were screened for growth promotion in tomato under net house conditions. The strain S25 induced highest seed germination, root length; root dry weight, shoot length and shoot dry weight in tomato over all the other treatments. The strain was identified as *Bacillus subtilis* and could be used as PGPR inoculant for growth and yield enhancement in tomato.

The optimal cultural parameters for gibberellic acid (GA) production by *Pseudomonas* sp., isolated from wastes of processed olive, was affected by physiological conditions such as incubation period, pH of growth medium and incubation temperature (Karakoc and Aksoz, 2006). The highest level of GA (250.06 mg/l) production was obtained in nutrient broth when the bacterial culture was incubated at 30ºC for 72 h and medium pH 7.0 on a rotatory shaker and in dark conditions.

Perrig *et al.* (2007) evaluated phytohormone in two strains of *Azospirillum* used for inoculant formulation in Argentina. GA_3 production was significantly higher in strain Cd (0.66 µg/ml) than in strain Az 39 (0.30 µg/ml). Inoculation

assays demonstrated variability in growth promotion and yield increase. The two strains possessed the ability to produce and release IAA and GA3.

2.6 Rhizobacteria as hydrogen cyanide (HCN) and ammonia producers

HCN is produced by many rhizobacteria and play a role in biological control of pathogens (Defago and Haas, 1990). Production of HCN by certain strains of fluorescent pseudomonads is reportedly involved in the suppression of soil borne pathogens (Thomshow and Weller, 1995). The cyanide producing strain CHAO of *Pseudomonas* stimulated root hair formation and altered plant physiological activities (Voisard *et al.,* 1989). Four PGPR strains produced HCN and induced systemic resistance in cucumber against *Colletotrichum orbiculare* (Wei *et al.,* 1991).The HCN production is a common trait of *Pseudomonas* (88.9%) and *Bacillus* (50%) in rhizospheric soil and plant root nodules and also act as biocontrol metabolite in *Pseudomonas* species (Ahmad *et al.,*2008).

Rudrappa *et al.* (2008) elucidated the role of cyanide production in pseudomonad virulence affecting plant root growth and other rhizospheric processes. Growth inhibition of lettuce and barnyard grass by volatile metabolites of the cyanogenic rhizobacteria confirmed that HCN is a major inhibitory compound produced.

Shobha and Kumudini (2012) during *in vitro* screening of 7 isolates of *Bacillus megaterium* from rhizosphere of chilly and beans for plant growth promoting traits found all exhibiting all PGP traits including HCN production.

2.7 Rhizobacteria as producers of lytic enzymes

Lytic enzyme scan degrade several components present in the cell walls of fungi and oomycetes (Chet and Inbar, 1994). A wide variety of bacterial-origin lytic enzymes are known which include cellulases, glucanases, proteases and chitinases. A ß-1,3-glucanase producing PGPR *Pseudomonas cepacia* significantly decreased disease incidence by *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* (Fridlender *et al.,*1993). Chitinase, β-1, 3 glucanase and

cellulase are important fungi controlling enzymes due to their ability to degrade fungal cell wall components such as chitin, β-1,3 glucan and glucosidic (Schroth and Hancock 1982). Chitinase excreting microbes have been reported as efficient biocontrol agents. The role of chitinase in biological control and in plant defense mechanisms has been documented well (Shapira *et al*., 1989).

Nielsen *et al.* (1998) reported that in sugar beet rhizosphere fluorescent pseudomonads inhibited plant pathogenic fungus, *Rhizoctonia solani,* by production of cell wall-degrading endochitinase. Bio-control of *Fusarium solani* mainly via laminarinase and chitinase activities of *P. stutzeri* YPL-1 has been reported. It has also been reported that β-1,3 glucanase producing *P.cepacia* decrease the incidence of root diseases caused by *R. solani*, *Sclerotium rolfsii* and *P. ultimum* (Lim *et al*., 1991).

De Boer *et al.* (1998) demonstrated that besides lytic enzyme production the other mechanisms such as antibiotics production are involved in the suppression of pathogens. Productions of fungal cell wall-degrading enzymes by microbes are frequently involved in the suppression of phytopathogenic fungi (Picard *et al*., 2000).

Garbeva *et al.* (2004) studied the effect of agricultural practices on the composition of *Pseudomonas* spp. and their antagonistic activity towards *R. solani*. They observed that disease suppressiveness against *R. solani* was higher in grassland than in arable land and linked this to increased number of antagonistic *Pseudomonas* spp. possessing chitinolytic activity.

Reetha *et al*. (2014) isolated PGPR like *Pseudomonas fluorescence* and *Bacillus subtilis* by serial dilution method and noticed that *P. fluorescence* produced high amounts of cellulase and pectinase as compared to *B. subtilis*.

2.8 Biocontrol properties of rhizobacteria

Pathogenic microorganisms affect plant health so are a major threat to food production and ecosystem stability. The adverse impact of pesticides and

fertilizers on soil and human health and their escalating cost has forced scientists to search for some safe and ecofriendly alternatives. Biological control is considered a viable option to reduce the use of chemical fertilizers/pesticides in agriculture (Gerhardson, 2002). PGPR are among many microbes identified as potential biological control agents (Kloepper *et al*., 1989). The colonization of plant root system by PGPR reportedly reduces pathogen attack directly through the production of antimicrobial substances *viz.,* siderophores, β-1,3 glucanase, chitinase and antibiotics and through competition for space, nutrients and ecological niches. PGPR suppress pathogens indirectly through induction of systemic resistance (Buchenauer, 1998; Cattelan *et al*., 1999; Viswanathan and Saniyappan, 2002). A variety of antibiotics are produced by PGPR e.g. oligomycin A, kanosamine, zwittermicin A, amphisin, 2,4-diacetylchloroglucinol (DACG), hydrogen cyanide and xanthobaccin produced by *Bacillus* and *Streptomyces* spp. (Milner *et al*., 1996; Kim *et al*., 1999). Oomycin A produced by *Pseudomonas* (Raaijmakers *et al*., 2002).*Bacillus subtilis* RB14 showed antibiotic activities against several phytopathogens *in vitro* by producing the antibiotic inturin A and surfactin and was able to suppress damping off in tomato seedlings caused by *Rhizoctonia solani* in a pot culture assay (Asaka and Shoda, 1996).

Khan and Khan (2002) reported a significant decline in rhizosphere population of *Fusarium oxysporum* f. sp. *lycopersici* after root dip of tomato seedlings with PSM such as *Bacillus subtilis*, *Pseudomonas fluorescens*, *Aspergillus awamori*, *A. niger* and *Penicillium digitatum.* The severity of wilt showed 31.1 and 21.3 per cent decline due to *A. awamori* and *P. digitatum* inoculation, respectively, as compared to the untreated control. Root dip treatment with PSM also increased tomato yield with maximum gain by *A. awamori* and *P. digitatum* in pathogen inoculated (36 and 33% respectively) and uninoculated (19 and 23% respectively) plants. Sultana *et al.* (2004) observed that *Bacillus*

megaterium antagonized the growth of *Dermatophora necatrix*, the causal organism of the white root rot of apple.

Kishore *et al.* (2005) showed that the cell free culture filtrate of *Pseudomonas* spp. GRS 175, *P. aeroginosa* GPS 21, GSE 18, GSE 19 and GSE 30 at 10 per cent (v/v) concentration significantly inhibited spore germination of various species of phytopathogenic fungi.

Nikolay *et al.* (2006) reviewed microbially-mediated solubilization of insoluble phosphates through the release of organic acids and other metabolites such as siderophores, phytohormones and lytic enzymes that take part in biological control against soil borne phytopathogens.

Altindag *et al.* (2006) suggested that *Burkholdria gladii* OSU 7 has the potential to be used as biopesticide for effective management of brown rot disease in apricot. While *Pseudomonas corrugata*, *Bacillus megaterium* and *Flavobacterium* sp. showed consistently good control against *Phytophthora capsici* and Phytophthora blight of pepper (Sang *et al.*, 2008; Akgul and Mirik, 2008).

Cazorla *et al.* (2006) collected 905 bacterial isolates from the rhizosphere of healthy avocado trees and screened them for antagonistic activity against *Dematophora necatrix.* A set of eight strains was selected on the basis of growth inhibition against *D. necatrix* and several other soil-borne phytopathogenic fungi. Isolate PCL1606 exhibited the highest biocontrol activity.

Han *et al.* (2006) identified an antagonistic strain of *Bacillus* sp. against *Streptomyces scabiei* and observed that the culture broth had a suppressive effect on common scab disease in a pot assay, decreasing the infection rate from 75 to 35 per cent. The cell free culture filtrate of *Pseudomonas* sp. GRS 175, *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30 at 10 per cent (v/v) concentration significantly inhibited spore germination of various species of phytopathogenic fungi (Kishore *et al.*, 2005).

Senthilkumar *et al.* (2008) isolated 137 bacterial isolates from surface sterilized root, stem and nodule tissues of soybean and screened them for antifungal activity. They observed that *Paenibacillus* spp. (HKA-15) and *Bacillus* spp. (HKA-121) were potential candidates for biocontrol of charcoal rot as well as for plant growth promotion. The inoculation with PSB not only proved useful in controlling Phytophthora crown blight under field conditions but also increased pepper yield (Akgul and Mirik, 2008).

Ramakrishnan *et al.* (2009) assessed the antagonistic effect of *Streptomyces* sp. SCBT, isolated from rhizosphere of some medicinal plants, against pathogenic bacteria and found that the strain was capable of inhibiting many Gram positive and Gram negative pathogenic bacteria.

Karthikeyan *et al.* (2009) studied individual and combined effects of *Azospirillum bresilense* and *P. fluorescens* on biomass yield and ajmalacine production in *Catharanthus roseus*. Maximum ajmalacine was produced when *A. brasilense* and *P. fluorescens* were co-inoculated in "rosea" variety of *C. roseus* (0.700mg/g dry wt). Thus seed priming and seedling treatments of native PGPR effectively enhanced biomass yield and alkaloid contents in medicinal plants.

Recep *et al.* (2009) reported that PGPR *B. cepacia* strain OSU-7 exhibited inhibition zone ranging from 35.33 to 47.37 mm against *Fusarium* sp. causing dry root of potatoes.

Jha *et al.* (2009) screened 80 fluorescent pseudomonad strains for phosphate solubilization and found 3 strains (BFPB9, FP12 and FP13) able to solubilize tri-calcium phosphate [$Ca_3(PO_4)_2$]. These strains produce IAA and protease as well as exhibited broad spectrum antifungal activity against phytopathogenic fungi. They concluded that owing to the innate potential of these strains for phosphate solubilization and production of siderophore, IAA, protease, cellulase and HCN; these strains can be used as biofertilizers as well as biocontrol agents.

Mafia *et al.* (2009) evaluated 10 PGPR for the control of mini-cutting rot of eucalyptus caused by *Cylindrocladium candelabrum* and *Rhizoctonia solani.* The mycelial growth inhibition of each pathogen differed with PGPR isolate. *Pseudomonas* sp. strain FL2 and *Bacillus subtilis* strain S2 were most effective under *in vitro* conditions. However, under nursery conditions *Pseudomonas fulva* isolate reduced the incidence of mini-cutting rot by 33 and 27 per cent as compared to untreated control and fungicide treatment (epoxiconazole + pyraclostrobin: 0.4 g/L), respectively. Isolate Ca was the most efficient in reducing *C. candelabrum* inoculum in 15 days and in promoting rooting and growth of eucalyptus.

Zhang *et al.* (2010) evaluated the potential of PGPR *Bacillus* sp. in controlling Phytophthora blight, caused by *Phytophthora capsica,* on squash. PGPR strains were applied as a soil drench 1 and 2 weeks after planting (WAP) while *P. capsici* was applied to squash roots at 3 WAP. PGPR strains SE34 and SE49 significantly reduced disease severity as compared to the untreated control. PGPR strains when applied as 2-, 3- and 4-strain mixtures significantly reduced disease severity. Treatment with strains T4 + SE56 demonstrated significantly low level of disease than other individual PGPR strain, thereby revealing either additive or synergistic effect on disease reduction by mixing PGPR strains. They suggested that better disease control can be achieved by multiplexing the PGPR.

Mishra *et al.* (2011) evaluated *Trichoderma harzianum* and a PGPR *Pseudomonas fluorescens* alone and in combinations for their biocontrol potential against many soil-borne plant pathogens *viz.*, *Rhizoctonia solani, Sclerotium rolfsii* and *Macrophomina phaseolina* responsible for root and stem rot disease in soybean. Glass-house and field studies revealed general trend of greater suppression and enhanced consistency against the pathogens by using a mixture of antagonists. The use of more than one antagonists of diverse origin effectively reduced the response variability and increased the reliability of biological control.

Ramyasmruthi *et al.* (2012) isolated 18 bacterial isolates from the

rhizosphere of brinjal, capsicum, chilli and screened them for the production of various PGPR activities. Ten isolates were the most potent chitinolytic bacterial species. These isolates were also found able to produce siderophore, IAA, HCN, phosphate solubilization, NH_3 and catalase. Dual plate assay against few selected soil borne phytopathogens- *Alternaria alternata* OTA36; *Alternaria brassicola* OCA1; *Alternaria brassiceae* OCA3; *Collectotrichum gleosporidose* OGC1 revealed anti-fungal activity by isolate R. The isolate R was identified as *Pseudomonas fluorescens* by biochemical test. Chilli seeds inoculated with *Pseudomonas* showed 100 per cent germination index and almost 50 per cent reduction in disease incidence by *C. gloeosporiodes* OGC1.

A study conducted by Abdel-Aziz *et al.* (2013) revealed that the strain *Bacillus alvei* NRC-14 potentially suppressed fungal growth and prevented root-rot of tomato plants caused by *Fusarium oxysporum* and enhanced plant growth and health. Both *in vitro* and *in vivo* experiments confirmed the efficacy of this strain as an excellent biocontrol agent. When it was applied as soil drench, it significantly reduced wilt incidence by 94 per cent with a plant-growth promotion and biocontrol efficiency of 180 and 151 per cent, respectively. In general, application of the strain or its extracellular metabolites increased plant growth parameters. The strain produced mycolytic enzymes *viz*. chitinase, chitosanase, ß-1,3 glucanase as well as cellulases, proteases and potential bioactive compound(s). These results suggested that the strain may have potential to be considered as a potent biocontrol agent, effective against several plant diseases, pest insects and plant properties.

Koche *et al.* (2013) isolated thirty isolates of *Pseudomonas fluorescens* from citrus rhizosphere and tested them for antifungal activity against *Phytophthora* sp., *P. fluorescens* isolate Pf20 was most efficient in inhibiting the mycelial growth upto 38.9 per cent. The antifungal compounds were extracted with equal volume of ethyl acetate and tentatively identified on TLCat Rf 0.28. The antifungal compounds extracted from *P. fluorescens* at 5 per cent were found

inhibitory to the growth of *Rhizoctonia solani* (42.79%), *Phytophthora parasitica* (28.57%), *P. palmivora* (25.98%) and *Fusarium solani* (20.45%). In HPTLC analysis, the characteristic colour and fluorescent band after derivatization with anisaldehyde reagent showed the presence of secondary metabolites in crude extract.

Wang *et al.* (2015) screened 1223 isolates for antifungal activity and observed that 24 per cent isolates inhibited either *Rhizoctonia solani* or *Sclerotinia sclerotiorum.* Twenty-four strains inhibited *R. solani, Gaeumannomyces graminis* and/or *S. sclerotiorum.* The selected isolates belonged to genus *Pseudomonas* and were similar to model PGPR strains *Pseudomonas protegens Pf-5, P. chlororaphis* sub sp. *aureofaciens* 30–84 and *P.brassicacearum* Q8r1-96. *P. protegens* and *P. brassicacearum*-like strains showed higher promotion of canola growth.

2.9 Plant growth promotion by rhizobacteria

Several phosphate solubilizing bacteria occur in soil and usually their number is not enough to compete with other commonly established bacteria in rhizosphere. Hence, the amount of phosphorus liberated by them is insufficient for substantial increase in *in situ* growth of plant. The inoculation of plants by a target microorganism at a much higher concentration than that normally found in soil is necessary to enhance plant growth and yield. Phosphate solubilizing micro-organisms (PSM) are known to bring about mobilization of insoluble phosphates and stimulate plant growth even under the condition of P-deficiency.

Gull *et al.* (2004) reported that plant growth, phosphorus and nitrogen concentration, nodulation efficiency and nitrogenase activity were significantly enhanced as a result of co-inoculation with phosphate solubilizing bacteria.

Domenech *et al.* (2006) demonstrated synergistic effect of co-inoculation of three PGPR, *Bacillus licheniformis* CECT 5106, *Pseudomonas fluorescens* CECT 5398 and *Chryseobacterium balustinum* CECT 5399 with LS 213 on the

growth promotion and biocontrol on tomato and pepper against *Fusarium* wilt and *Rhizoctonia* damping off. LS213 is a product formed by a combination of two PGPR, *Bacillus subtilis* strain GB03 (a growth-promoting agent), *B. amyloliquefaciens* strain IN937a (an inducer of systemic resistance) and chitosan. They concluded that when individual *Rhizobacterium licheniformis* and LS213 were put together, the biometric parameters showed significant improvement over individual *Rhizobacterium* in both tomato and pepper.

Tamilarasi *et al.* (2006) studied the diversity of root associated microbes of selective medicinal plants and influence of rhizo-microorganisms on anti-microbial property of *Coriandrum sativum* and found that about 71 per cent bacterial isolates were nitrate reducers and 90.6 per cent solubilized phosphate. The rhizosphere bacterial isolates were also capable of hydrolyzing starch, cellulose, casein, urea and gelatin and showed resistance to 14 commercially used antibiotics.

Vikram *et al.* (2007) found a significant association between PSB isolates and organic acid production. Further, they reported that PSB isolates have potential to promote plant growth by producing plant growth promoting substances and organic acids.

Jaleel *et al.* (2009) studied *Pseudomonas fluorescens* as a physiological modulator in the enhancement of medicinally important alkalods of *Catharanthus roseus* and found that *P. fluorescens* has a profound influence on plant growth and caused a significant enhancement in the production of individual alkaloids like ajmalacine, catharanthine, serpentine and vindoline in comparison to untreated control plants.

Gupta *et al.* (2014) isolated and characterized rhizospheric and endorhizospheric bacteria from capsicum (*Capsicum annuum*). Only six isolates (RS2, RS3, RS4, RS7, RS8 and RS10) possessing maximum of PGP traits were evaluated for seed germination and seedling growth studies under controlled

conditions. Maximum seed germination (84.40%), increase in shoot length (41.54%), shoot biomass (41.46%), root length (69.64%), root biomass (42.85%) over uninoculated control were noted for RS7 isolate inoculated seeds.

Verma *et al.* (2014) isolated and characterized ten fluorescent *Pseudomonas* sp. from apple rhizosphere from Shimla District (HP) India. Two isolates were selected on the basis of their high production of plant growth promoting activities. The strains were successfully used to study their effect on apple plants planted in replant area of Deola (Distt. Shimla). The results showed a significant increase in various plant and soil parameters. Further, the disease incidence was reduced after the cyclic treatment of these strains.

2.10 Partial sequencing of 16S rRNA gene for rhizobacterial diversity assessment and identification

Molecular techniques are major tools for the analysis of microorganisms from biological substances. These techniques provide ways to screen for a broad range of agents in a single test (Field and Wills, 1998). It is useful for rapid differentiation of species, strain identification and definition of strain relatedness from different samples. Molecular methods are complementary to the traditional methods and have revolutionized the studies on microbial diversity,their taxonomic positioning and field application. Liu *et al.* (2000) isolated a rhizobacterial strain, designated GP72, from green pepper rhizosphere and identified it as *Pseudomonas chlororaphis* on the basis of morphology; conventional biochemical and 16S rDNA sequence analysis.

Chen *et al.* (2005) carried out a study on the isolation, screening and characterization of36 strains of PSB from Central Taiwan. Mineral phosphate solubilizing (MPS) activities of all isolates were tested on tricalcium phosphate medium by analyzing the soluble-P content after 72 h incubation at 30^{o}C. Identification and phylogenetic analysis of 36 isolates were carried out by 16S rDNA sequencing. Ten isolates belonged to genus *Bacillus*, nine to genus *Rhodococcus*, seven to genus *Arthrobacter*, six togenus *Serratia* and one each to

genera *Chryseobacterium*, *Delftia*, *Gordonia* and *Phyllobacterium*. In addition, four strains namely, *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. were reported for the first time as PSB after confirming their ability to solubilize considerable amount of tricalcium phosphate in medium by secreting organic acids. An inverse relationship between pH and P-solubilized was apparent from this study. Identification and characterization of soil PSB for the effective plant growth-promotion broadened the spectrum of phosphate solubilizers available for field application.

Siddikee *et al.* (2010) isolated 140 halotolerant bacterial strains from the rhizosphere of six naturally growing halophytic plants in the vicinity of Yellow Sea, near the city of Incheon in the Republic of Korea. These strains were characterized for multiple plant growth promoting traits *viz.,* nitrogen fixation, phosphorus and zinc solubilization, thiosulfate (S_2O_3) oxidation and production of IAA, ammonia and hydrolytic enzymes such as protease, chitinase, pectinase, cellulase and lipase under *in vitro* conditions. The 36 halotolerant bacterial strains selected for 16S rRNA gene sequencing analysis revealed that they belonged to 10 different bacterial genera: *Arthrobacter Bacillus*, *Brevibacterium*, *Corynebacterium*, *Exiguobacterium*, *Halomonas*, *Micrococcus, Oceanimonas*, *Planococcus* and *Zhihengliuella.* Three bacteria *viz*., *Brevibacterium epidermidis* RS15, *Micrococcus yunnanensis* RS222 and *Bacillus aryabhattai* RS341 showed more than 40 per cent increase in root elongation and dry weight when compared with uninoculated salt stressed canola seedlings.

Walia *et al.* (2014) isolated and characterized various PGPR from the rhizosphere of tomato with multiple plant growth promoting activities. Pot culture experiment on tomato seedlings conducted under net house revealed that most of the isolates significantly increased shoot length, root length and dry matter of shoot and root. Among the eleven isolates, isolate N11, identified on the basis of 16S RNA as *Bacillus subtilis,* exhibited multiple plant growth promoting activities *viz*., phosphate solubilization, siderophore production, indole-acetic acid

production and hydrogen cyanide production. Single strain inoculum *B. subtilis* strain CKT1 remarkably increased seed germination (36.1%), shoot length (5.2%), root length (21.1%), shoot dry weight (63.5%) and root dry weight(54.1%), nitrogen (18.7%), potassium (57.7%) and phosphorus (22.2%).

Mehta *et al.* (2014) isolated a P-solubilizing bacterial strain (CB7) from apple rhizosphere of Himachal Pradesh (India) and identified it as *Bacillus circulans* on the basis of phenotypic characteristics, biochemical tests, fatty acid methyl esters analysis and 16S rRNA gene sequencing. The isolate exhibited plant growth-promoting traits like P-solubilization, auxin production, 1-aminocyclopropane-1-carboxylate deaminase activity, siderophore production, nitrogenase activity and antagonistic activity against *Dematophora necatrix*. P-solubilization activity was associated with the release of organic acids and drop in pH of Pikovskaya's medium. Remarkable increase in seed germination (22.3%), shoot length (15.9%), root length (25.1%), shoot dry weight (52.9%), root dry weight (31.4%), nitrogen (18.7%), potassium (57.7%) and phosphorus (22.2%) contents in shoot biomass over control was observed. Isolate CB7 appeared promising PGPR to be developed as a biofertilizer for enhancing soil fertility and plant growth.

Chapter-3

MATERIALS AND METHODS

The present study was conducted in the Division of Plant Pathology, SKUAST-Kashmir, Shalimar, Srinagar during the years 2014-2016.

3.1 Materials

3.1.1 Soil samples from rhizosphere and surface

Surface as well as rhizosphere soil samples were collected from four walnut growing districts of Jammu and Kashmir state *viz*., Kupwara, Baramulla, Budgam and Shopian. The sampling was done in the month of June during the year 2014-2015.

3.1.2 Chemicals

Analytical grade chemicals and reagents, obtained from Hi-Media, BDH and E. Merck, were used in the present study. The chemicals used in molecular study were obtained from Banglore Genei Pvt. Ltd. and Bioron. The primers were obtained from Sigma Aldrich Pvt. Ltd., USA.

3.2 Methods

3.2.1 Collection of samples

A field survey was conducted in four walnut growing districts of Jammu and Kashmir *viz*., Kupwara, Baramulla, Budgam and Shopian to collect the rhizosphere soil samples. In each district three commercially walnut growing Blocks were chosen and in each Block three sites were randomly selected. The rhizosphere soil along with root samples were collected from the canopy of young actively growing walnut trees from the selected sites. The rhizopshere soil samples collected from all sides at root depth. The composite samples were used for the isolation of plant growth promoting rhizobacteria (PGPR). The soil samples were also taken from these sites to assess various soil physicochemical

properties. The rhizospheric samples were used for the isolation of rhizosphere bacteria.

3.2.2 Estimation of soil physicochemical characteristics

3.2.2.1 pH and electrical conductivity

The soil pH and electrical conductivity was determined in 1:2.5 soil: water suspension using pH meter (Micro pH System-361; Systronics) and conductivity meter-306 (Systronics) as per Jackson (1973).

3.2.2.2 Organic carbon

The organic carbon content in soil was determined by chromic acid titration method (Walkley and Black, 1934). One gram of soil sample was taken in 500 ml conical flask and 10 ml of 1N $K_2Cr_2O_7$ and 20 ml concentrated H_2SO_4 was added to it. All the three components were mixed thoroughly and the reaction was allowed to proceed for 30 minutes. The reaction mixture was diluted with 200 ml water and 10 ml H_3PO_4 and then 10 ml sodium fluoride solution and 2 ml diphenylamine were added to this mixture. The solution was titrated with standard ferrous ammonium sulphate to a brilliant green colour. A blank without soil was run simultaneously. Organic carbon content was calculated as:

$$\text{OC (\%)} = \frac{10}{\text{Blank}} \text{ (Blank – reading)} \times \frac{0.003 \times 100}{\text{Weight of soil}}$$

3.2.2.3 Available nitrogen

The available nitrogen was determined by alkaline permanganate method (Subbiah and Asija, 1956). Ten grams of soil sample was taken in Kjeldahl distillation flask and 100 ml of $KMnO_4$ (0.32%) was added to it followed by the addition of 100 ml 2.5 per cent NaOH after proper assemblage. The assembly was sealed with paraffin wax to avoid excess frothing. After that 25 ml 0.02N H_2SO_4 was taken in a 100 ml conical flask and 2-3 drops of methyl red indicator was added to it. The end of delivery tube was dipped in to the conical flask. Hot plate was switched onto distil ammonia gas. Approximately 30 ml distillate was

collected in H_2SO_4 (0.02N). The excessive H_2SO_4 in conical flask was titrated against 0.02N NaOH and end point noted by observing the change in colour from pink to yellow. Final volume of NaOH used was noted. The available N was calculated using formulae:

$$\text{Percentage of available N} = \frac{(25\text{-}X) \times 0.00028 \times 100}{10} = e$$

Wherein 10 is weight of soil taken in g, 25 is the volume of 0.02N H_2SO_4 taken in ml; X is the volume of 0.02N NaOH used (titrate value) in ml and 25-X is the volume of 0.02N acid used for ammonia absorption. 1 ml of 0.02N H_2SO_4 = 0.00028 g of N or 0.28 mg N

$$\text{Available N in soil (ppm)} = \text{Percentage of available N x } 10{,}000 = f$$

$$\text{Available N in soil (kg/ha)} = f \times 2.24$$

3.2.2.4 Available phosphorus

The available phosphorus was extracted from soil samples using 0.5 N $NaHCO_3$ at pH 8.5 (Olsen's *et al.*, 1954) and phosphorus content determined spectrophotometrically by using UV-VIS spectrophotometer SL 164 (Systronics). One gram of soil was transferred to 100 ml conical flask, followed by the addition of a pinch of Darco-G 60 and 20 ml 0.5 N sodium bicarbonate. The contents were shaken for 30 minutes on an electric shaker. Then the suspension was filtered through Whatman No. 1 filter paper and 5 ml filtrate was pipetted out in 25 ml volumetric flask and 5 ml ammonium molybdate was added to it. The mixture was thoroughly shaken so as to avoid direct contact to $SnCl_2.2H_2O$ with concentrated ammonium molybdate. Finally, 1.0 ml $SnCl_2.2H_2O$ working solution was added and final volume was made up to 25 ml with distilled water. The solution was kept as such for 10 minutes at room temperature. Intensity of blue colour intensity was measured on Spectronic-20 at a wave length of 660 nm using red filter. An appropriate blank was kept using all the reagents, except soil.

Observations and calculations:

a) Weight of soil taken = 1 g

b) Volume of 0.5 M $NaHCO_3$ solution added = 20 ml

c) First dilution = 20 times

d) Volume of filtrate taken for colour development = 5 ml

e) Final volume made = 25 ml

f) Second dilution = 5 times

g) Total dilution = 20 × 5 = 100 times

h) Absorbance as read from colorimeter = X

i) ppm of P as read from standard curve = A

j) ppm of available P in the given soil = A × 100 = B

k) kg/ha of available P in the given soil = B × 100 x 2.24

l) kg/ha of available P_2O_5 in soil = B × 100 × 2.24 × 2.29

3.2.2.5 Available potassium

The available potassium was extracted from soil samples by normal neutral ammonium acetate (Merwin and Peech, 1951) and K content determined by flame photometer (Flame photometer-128; Systronics). One gram of soil was taken in 150 ml conical flask and 5 ml neutral ammonium acetate solution was added to it. The contents were vigorously shaken on an electric shaker for 5 minutes. The contents were then filtered through Whatman No. 1 filter paper and filtrate was fed into the atomizer of flame photometer and the readings were noted. Flame photometer was set with standards of 5 and 10 ppm of K solution.

Observations and calculations:

a) Weight of the soil = 1 g

b) Volume of the extract added = 5 ml

c) Dilution = 5 times

d) Readings indicated by the flame photometer = X

e) ppm of K as read from the standard curve against X = Y

f) Available K (ppm) in given soil = Y × 5 = Z

g) kg/ha of available K in the given soil = Z × 2.24

3.2.2.6 Soil texture

The soil texture was determined by feel method (Thien, 1979).

3.2.3 Isolation and enumeration of rhizobacteria from walnut rhizosphere

The rhizospheric soil samples were collected from within the walnut tree canopy along with root hairs from all the sites and upto one gram of rhizosphere soil samples were used for enumeration studies. The serially diluted suspension was spread on pre-poured nutrient agar medium. After incubation for 24-48 h, the isolated colonies that developed on nutrient agar medium (master plate) were replica plated (Roberts, 1959) onto the selective media *viz*., nitrogen-free medium for determining the nitrogen fixing ability, CAS medium (Schwyn and Neilands,1987) for assessing siderophore producing ability and Pikovskaya medium (Pikovskaya, 1948) for estimating phosphate solubilizing ability. All the colonies were transferred to the same position as the master plate with the help of a wooden block, covered with sterilized velvet cloth. At the end of the incubation period, the location of the colonies appeared on the replica plates which were compared to the master plate. Populations were expressed as colony forming unit (cfu) per gram of dry soil weight.

3.3 Morphological characterization of bacterial isolates

The morphological characterization was done by observing the isolated colonies under a compound microscope (Gaynor). The colonies were observed for their colour, form, elevation and margin on visual basis and for cell shape, size, endospore presence and Gram's reaction under a microscope at 100X.

3.4 Biochemical characterization

The biochemical characterization *viz*., indole production, methyl red test, Voges-Proskauer reaction, citrate utilization test, oxidase test, catalase production, acid production, H_2S production and starch hydrolysis was carried out as per Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

3.4.1 Indole production

Test culture was inoculated in 5 ml peptone water broth (Annexure-I) and incubated at 28±2^{o}C for 24 hours. After incubation, 0.2 ml Kovac's reagent was added. The appearance of cherry red colour indicated a positive reaction.

3.4.2 Methyl red test

Test culture was inoculated in 5 ml MR-VP broth (Annexure-I) and incubated at 28±2^{o}C for 48 hours. After incubation, 5-6 drops **of** methyl red solution (Annexure-I) were added to it. A bright red colour indicting a pH of 4.2 or less was considered as positive test. Yellow or orange colour indicated a negative reaction. A weakly positive test showed red-orange colour. The uninoculated medium contained glucose and had a neutral pH value (pH 7.0).

3.4.3 Voges-Proskauer (acetoin production) test

Test culture was inoculated in 5 ml MR-VP broth (Annexure-I) and incubated at 28±2^{o}C for 48 hours. After incubation, 1 ml of 40 per cent potassium hydroxide (plus 1 per cent creatine) and 3 ml of 5 per cent solution of α-naphthol in absolute ethanol were added to it. A positive reaction was indicated by the development of a pink colour within 2-15 minutes which became crimson coloured in 30 minutes.

3.4.4 Citrate utilization test

Simmon's citrate medium (Annexure-I) was prepared, dispensed in test tubes and sterilized at 121^{o}C for 15 minutes. Then the slants were allowed to set and inoculated with test cultures. To minimize nutrient carryover, the cells were

rinsed in distilled water before their transfer to Simmon's citrate agar slants. The slants were incubated for 96 hours at 28±2°C. A positive test showed a blue colour on the streak of growth. Retention of original green colour and no growth on the line of streak indicated a negative reaction.

3.4.5 Catalase test

On a clean glass slide, a loopful of culture growth was taken and emulsified with 3-4 drops of hydrogen peroxide. Immediate effervescence or evolution of air bubbles indicated positive results.

3.4.6 Oxidase test

A small piece of filter paper was soaked in 1per cent aqueous solution of tetra-methyl phenylenediamine hydrochloride. Then a loopful of fresh young growth from culture plate was scraped with edge of a clean glass slide and rubbed on impregnated filter paper. Development of blue colour within 10-15 seconds was considered positive oxidase test and delayed reaction showed such colour after 15 seconds.

3.4.7 Starch hydrolysis

The cultures were spotted on starch minimal medium (Annexure-I) plates and incubated at 28±2°C for 48-72 hours. After flooding the surface of starch agar plates with Gram's iodine, a clear zone around the area in which the microorganism was grown indicated hydrolysis of starch.

3.4.8 H_2S production

The test tubes containing 9.0 ml tryptone broth (Annexure-I) were prepared. Each culture was inoculated in these test tubes separately. Tubes without culture served as control. Lead acetate was put into broth tubes and incubated at 28±2°C for 48-72 hours. The tubes were examined after 72 hours for change in colour. Black colouration of the broth indicated positive reaction.

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3.5 Screening of bacterial isolates for various plant growth promoting traits

All rhizobacterial isolates were screened for phosphate solubilization, siderophore production, indole-3-acetic acid production, gibberellic acid production, ammonia production, hydrogen cyanide production, chitinase production and antifungal activity. All the experiments were conducted in a completely randomized design and each treatment replicated four times. Further, all these experiments were repeated twice.

3.5.1 Phosphorus solubilization

3.5.1.1 Qualitative estimation of phosphate solubilization

The ability of bacterial isolates to solubilize phosphorus was estimated by plate assay method (Pikovskaya, 1948). The isolates were streaked on PVK agar (Annexure-I) plates containing a known amount of tricalcium phosphate [$Ca_3(PO_4)_2$] and incubated at 37^oC for 48 hours. Each treatment was replicated three times. Solubilization of phosphorus was observed by formation of yellow coloured zones around the isolated bacterial colonies. Phosphate solubilization index was calculated asunder:

$$PSI = \frac{\text{Colony diameter + Holozoned diameter}}{\text{Colony diameter}}$$

3.5.1.2 Tricalcium phosphate solubilization in liquid medium

Pikovskaya's medium (Annexure-I) was used for the solubilization of phosphate. Fifty millilitre medium was dispensed into 250 ml Erlenmeyer flask containing 0.5 per cent tri-calcium phosphate (TCP) and autoclaved at 103.42 Kpa for 20 min. The flasks were inoculated with 10 per cent bacterial suspension (OD 1.0 at 540 nm) and incubated at 35±2^oC under shake conditions for 72 hours. Simultaneously, a control PVK broth containing TCP without inoculum was also run. Flasks were withdrawn at 72 hours and the contents centrifuged at 15000 rpm for 20 minutes at 4^oC. The culture supernatant was used for the determination of soluble phosphorus as per the method described by Bray and Kartz (1945).

3.5.1.3 Assay of phosphate estimation

The procedure essentially consisted of estimating soluble phosphorus formed by the action of phosphate solubilizing bacteria on tri-calcium phosphate. The soluble phosphorus formed was estimated calorimetrically as per the method of Bray and Kartz (1945) and the data was extrapolated by standard curve drawn using potassium di-hydrogen phosphate.

An aliquot (0.1-1.0 ml) from culture supernatant was made upto the final volume of 25 ml with distilled water and 5 ml ammonium molybdate added to it. The mixture was thoroughly shaken. The contents of the flasks were diluted upto 20 ml followed by the addition of 1 ml chloro-stannous acid and its volume made upto 25 ml. The contents were mixed thoroughly and blue colour intensity measured after 10 minutes at 660 nmon a UV-VIS spectrophotometer SL 164 (Systronics). An appropriate blank was also maintained wherein all the reagents, except culture, were added. Phosphorus solubilization was calculated as under:

P-solubilization = T- C

Wherein, T = PVK with inoculated TCP and C =PVK with uninoculated TCP

3.5.2 Siderophore production

3.5.2.1 Qualitative estimation of siderophore by chrome-azurol-S (CAS)

The siderophore production was detected by CAS plate assay method (Schwyn and Neilands, 1987). Sterilized blue agar was prepared by mixing CAS (60.5 mg/50ml distilled water) with 5ml iron solution (1mM $FeCl_3.6H_2O$) and 5ml 10mM HCl). This solution was slowly added to hexadecyltrimethyl ammonium bromide (HDTMA) (72.9 mg/40ml distilled water). Thus, 50 ml CAS dye was prepared and poured into 500 ml nutrient agar and the plates were prepared.

Twenty four hours old culture of test bacteria was spotted on pre-poured blue coloured CAS agar (Annexure-I) plates. Plates were incubated for 72 hours at 37^oC. The formation of a bright zone with yellowish (hydroxamate), pinkish

(catecholate) and whitish (carboxylate) colour in dark blue medium indicated the production of siderophore.

3.5.2.2 Quantitative estimation of siderophore using chrome-azurol-S

The quantitative estimation of siderophore was done by liquid assay method (Schwyn and Neilands, 1987). Cell free extract of supernatant (0.1 ml) was mixed with 0.5 ml chrome-azurol-S assay solution along with 10 µl of shuttle solution (0.2M 5-sulfosalicyclic acid). It was kept at room temperature for 10 minutes and absorbance recorded at 630 nm using UV-VIS spectrophotometer SL 164 (Systronics). The minimal medium was used as a blank and the reference (r) was prepared using exactly the same components, except the cell free extract of culture supernatant. The siderophore units were calculated using formula:

$$\text{Per cent siderophore unit} = \frac{Ar - As}{As} \times 100$$

Wherein, A_r is the absorbance of reference at 630 nm and A_s is the absorbance of test solution at 630nm.

3.5.4 Quantitative estimation of indole-3-acetic acid (IAA)

IAA was estimated by the method of Gorden and Paleg (1957). For the production of auxins, bacterial cultures were grown in Luria Bertani broth (Annexure-I), amended with 5 mM L-tryptophan, 0.065 per cent sodium dodecyl sulphate and 1 per cent glycerol, for 72 hours at 37^{o}C under shake conditions. Supernatant was collected by the centrifugation of cultures at 15,000 rpm for 20 minutes and stored at 4^{o}C.

For measuring IAA equivalents, 3 ml supernatant was pipetted into test tube and 2 ml Salkowski's reagent (2 ml 0.5 M $FeCl_3$ + 98 ml 35% $HClO_4$) added to it. The tubes containing the mixture was left as such for 30 minutes (in dark) for the development of pink colour. The colour intensity was measured at 535 nm by UV-VIS spectrophotometer SL 164 (Systronics). The concentration of IAA

was estimated by preparing calibration curve using 10-100µgIAA (Hi-media) per ml.

3.5.5 Estimation of gibberellins

The gibberellins were estimated as per the method of Holbrook *et al.* (1961). For this, bacterial cultures were grown in nutrient broth (Annexure-I) for 72 hours at 37°C under incubater shaker. The supernatant was collected by centrifugation of cultures at 15,000 rpm for 20 minutes and stored at 4°C till use. The supernatant (15 ml) was taken and 2 ml zinc acetate reagent (21.9 g zinc acetate + 1 ml glacial acetic acid) and the volume made upto 100 ml with distilled water) added to it. After 2 minutes, 2 ml potassium ferrocyanide (10.6% in distilled water) was added and the contents centrifuged at low speed (2000 rpm) for 15 minutes. Then, to 5 ml supernatant 5 ml of 30 per cent HCI was added and the mixture incubated at 20°C for 75 minutes. For blank, 5 ml of 5 per cent HCI was used. The absorbance was read at 254 nm using UV-VIS spectrophotometer SL 164 (Systronics). The concentration of gibberellins was calculated by preparing standard curve using gibberellic acid (GA_3) as standard (100-1000 µg/ml).

3.5.6 Ammonia production

For the detection of ammonia production, the method of Lata and Saxena (2003) was followed. Bacterial isolates were grown in 5ml peptone water in tubes. The tubes were then incubated at 28±2°C for 4 days. After 4 days, 1ml of Nessler's reagent was added to each tube. The presence of very light brown colour (+) indicated small amount of ammonia production while light brown (++) to orange brown colour (++++) indicated large amount of ammonia production.

3.5.7 HCN production

The method of Baker and Schippers (1987) was adopted for the estimation of HCN production by rhizobacteria. The test cultures were streaked on pre-poured plates of King's medium B (Annexure-I) amended with 4.4 g/L glycine.

The Whatman No.1 filter paper strips were soaked in 0.5 per cent picric acid in 0.2 per cent sodium carbonate and was placed in between the petriplates. The petriplates were sealed with parafilm and then incubated at 37^{o}C for 1-4 days. Uninoculated control was also maintained for comparison. The plates were observed for colour change in filter paper from yellow to orange brown to dark brown.

3.5.8.1 Chitinase assay

Chitinase was assayed as per the method of Robert and Selitrennikoff (1988). For this, colloidal chitin was prepared by digesting powdered chitin overnight with conc. HCl at 4^{o}C. After digestion, distilled water was added carefully and thoroughly mixed. The mixture was centrifuged and supernatant removed carefully. This supernatant was highly acidic. Washing of chitin was continued with distilled water till the pH of solution reached around 4.0. Thereafter, the pH of colloidal chitin solution was adjusted to 6.0-6.5 by using 2N NaOH. Then, 10 ml liquid chitin was added to 100 ml minimal agar medium directly. Bacterial cultures were spotted onto this amended minimal agar medium after incubation for 7 days at 30^{o}C. The iodine was added to the developed colonies. The formation of halo zone around the colonies after iodine addition was considered as positive for chitinase enzyme production.

3.5.8.2 Liquid assay for chitinase production

For production of chitinase, the bacterial isolates were grown in 100 ml fresh medium (3% w/v chitin; 0.1% KH_2PO_4; 0.05% $MgSO_4.7H_2O$; 50 mM sodium phosphate buffer, pH 6.0) in 250 ml Erlenmeyer flasks at 30^{o}C for three days. After incubation, the supernatant (enzyme solution) was collected by centrifuging the mixture at 12,000 rpm for 20 minutes. For the estimation of chitinase activity the method of Berger and Reynolds (1958) was followed. Colloidal chitin was selected as substrate. The reaction mixture containing 0.5 ml 1 per cent w/v colloidal chitin and 0.5 ml enzyme solution was incubated at 45^{o}C

for one hour. Then, 3 ml of 3, 5-dinitrosalicylic acid reagent was added to stop the reaction, followed by heating at 100^{o}C for 5 minutes. The material was centrifuged at 10,000 rpm for 15 minutes and supernatant collected.The amount of reducing sugars released was measured at 530 nm using UV-VIS spectro-photometer SL 164 (Systronics) alongwith substrate and blanks. For the determination of enzyme unit, serial dilutions of N-acetylglosamine (from 0 to 50 mM) were prepared. One unit (U) of chitinase activity was defined as amount of enzyme required to release 1 mmol of N-acetyl D glucosamine (as a standard) from chitin/minute

3.5.9 Antifungal activity of bacterial isolates

Five fungal pathogens i.e. *Dematophora necatrix, Alternaria solani, Pythium aphanidermatum, Fusarium oxysporum* and *Phytophthora capsici* owing to the huge damage caused by them were selected to check the antifungal activity shown by the rhizobacterial isolates. *D. necatrix* is the causal agent of white root rot of many fruit trees like apple, pear, plum and almond, *A. solani* is the pathogen that produces disease in tomato and potato plants called early blight. *P. aphanidermatum* causes damping off, root and stem rots and has a wide host range *viz.,* soybeans, beets, peppers, cucurbits, chrysanthemum and cotton, *F. oxysporum* causes Fusarium wilt and hosts of this pathogen are tomato, tobacco, legumes, cucurbits, sweet potatoes and banana and *P. capsici* causes blight and fruit rot of peppers and too has a wide host range including members of Solanaceae and Cucurbitaceae. To test the efficacy of rhizobacterial antagonists, a loopful of 48-hour old culture of each isolate was streaked a little below the centre of pre-poured petriplates containing malt extract agar (MEA) medium and then incubated overnight at 37^{o}C. The mycelial discs (5 mm Diameter) of 4 days old culture of test fungal pathogens i.e. *D. necatrix*, *A. solani*, *P. aphanidermatum*, *F. oxysporum* and *P. capsici* obtained from the Division of Plant Pathology, SKUAST-Kashmir, Shalimar, Srinagar (J&K) were placed simultaneously on one side of the streak. A check inoculated with test pathogens alone was also

maintained for comparison. The experiment was conducted in a completely randomized design and each treatment replicated three times. The plates were incubated at 24±1^{o}C for seven days and per cent growth inhibition was calculated according to Vincent (1947).

$$I = \frac{C - T}{C} \times 100$$

Wherein, I = Per cent growth inhibition; C = Growth of fungus in control; T = Growth of fungus in treatment

3.6 Performance score of isolates

Each plant growth promoting attribute i.e. phosphate solubilization, siderophore production, IAA production, gibberellic acid production, chitinase activity, hydrogen cyanide production, ammonia production and antifungal activity was evaluated 0-10 scale. The isolates showing maximum production of any of these traits were given ten points and for rest of the isolates were calculated by dividing that isolate's production by maximum production multiplied by 10 (Shahid, 2016). The best performing 12 isolates were chosen for molecular characterization.

3.7 16S rRNA gene amplification and sequencing

The molecular characterization of best rhizobacterial isolates from walnut was carried out on the basis of 16S rRNA sequencing. For this, the isolates were sent to Triyat Scientific 39A, Kannava Nager, Wardha, Nagpur – 440015, Maharashtra (India).

3.7.1 DNA extraction and gene amplification

As per the details shared by Triyat Scientific, the total genomic DNA of isolates was extracted by N-cetyl-N-N-trimethyl-ammonium bromide (CTAB) method (Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992). The forward and reverse primers used for 16S rDNA amplification were: fD1 (5′AGAGTTTGATCCTGGCTCAG3′) and rD1 (5′

AAGGAGGTGATCCAGCCGCA3′) (Luckow *et al*., 2000) used to amplify 1542, 1584, 1500, 1542, 1529, 1512, 1540, 1484, 1557, 1555, 1571 and 1466 bp region of 16S rRNA genes of these isolates using a thermal cycler (BioRad, USA). Amplification products were resolved by agarose-gel electrophoresis (1.5%) and visualized using a gel documentation system (Alfa Imager, Alfa Innotech Corporation, USA). The amplicons were purified using Genei Pure^{TM} quick PCR purification kit (GeNei^{TM}, Bengaluru, India) and quantified at 260 nm using a spectrophotometer taking calf thymus DNA as control. The purified partial 16S rDNA amplicons were sequenced in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, CA, USA).

3.7.2 Analysis of 16S rDNA sequences

The partial sequences of nucleotides were compared with the available sequences from National Center for Biotechnology Information (NCBI) database and the sequences showing >99 per cent similarity were retrieved by Nucleotide Basic Local Alignment Search Tool (BLAST-N) programme available at NCBI server (*www.ncbi.nlm.nih.gov/BLAST*). The retrieved sequences were aligned with our sequences at *http://www.ebi.ac.uk/Tools/msa/muscle/*. The primer impurity was identified and unwanted sequences trimmed. The pure sequences were submitted to NCBI at *https://www.ncbi.nlm.nih.gov/* and accession numbers for each isolate was obtained.

3.8 Statistical analysis

All the data was analyzed statistically using analysis of variance technique (Narayanan and Adorisio, 1983). The significance of treatments was tested at 5 per cent level of probability as per the procedure followed by Gomez and Gomez (1984).

Chapter -4

EXPERIMENTAL FINDINGS

The results of the present study entitled "Studies on plant growth promoting rhizobacteria associated with walnut (*Juglans regia* L.)" are presented as under:

4.1 Physico-chemical characteristics of walnut soils

Surface soil samples were collected from 36 sites belonging to 12 blocks from 4 major walnut growing districts of Kashmir *viz*., Shopian, Budgam, Kupwara and Baramulla. The surface soil samples were evaluated for pH, electrical conductivity, texture, organic carbon content, available nitrogen, available phosphorus and available potassium. The soils from the rhizosphere were evaluated for bacterial population. The results revealed that the soil texture of sites studied showed a great variation with thirteen sites each having silty clay loam and clay loam soil, eight sites having loam soil and two sites having silty loam (Table 1). The soil pH of these sites ranged from 5.9 to 7.7 with highest pH observed in the soil samples from Krewa-manul (Shopian) and lowest in samples from Palhalan (Baramulla). Further, the EC values of soils from these sites ranged from 0.1 to 0.6 dS/m. The organic carbon content of these sites was found to be in the range of 0.62 to 1.65 per cent with maximum content noticed in Krewa-manul site and minimum in Palhalan site. The available nitrogen and phosphorus contents was highest in soils of Krewa-manul site (540.0 and 28.5 kg/ha, respectively). However, highest available potassium content of 205.7 kg/ha was observed in soils of Warsun site (Kupwara) and minimum (90.5 kg/ha) in soil from Gonipora site (Baramulla). The lowest available N and P contents of 252 and 17.8 kg/ha, respectively, was recorded from soils of Gonipora (Baramulla) and Kababmarg (Kupwara).

Table 1: Physico-chemical characteristics of soil samples collected from various walnut growing locations of Kashmir

District	Block	Location	Texture	Chemical characteristics					
				pH	EC (dS/m)	OC (%)	Av.-N (kg/ha)	Av.-P (kg/ha)	Av.-K (kg/ha)
Shopian	Shopian	Krewa-manul	Silty clay loam	**7.7**	0.3	1.65	540.0	28.5	198.4
		Hal	Silty clay loam	**6.6**	0.3	1.03	405.0	23.0	98.0
		Pahelpora	Silty clay loam	**7.5**	0.4	1.42	470.0	26.4	179.0
	Keller	Tangwain	Silty loam	**7.7**	0.5	1.50	485.0	27.2	200.4
		Ganaupur	Silty clay loam	**6.6**	0.4	1.34	425.0	24.0	97.5
		Mujmarg	Silty clay loam	**7.6**	0.6	1.38	428.0	25.5	201.4
	Zainapora	Narawain	Silty clay loam	**6.9**	0.3	1.31	420.0	20.6	117.6
		Safanager	Silty clay loam	**7.5**	0.6	1.52	465.0	27.6	201.2
		Tularan	Silty clay loam	**7.7**	0.7	1.27	435.0	19.6	203.0
Budgam	Budgam	Soibugh	Clay loam	**7.2**	0.5	1.32	420.8	23.7	201.6
		Naidgam	Clay loam	**6.7**	0.3	1.49	404.3	26.8	97.0
		Wadwan	Clay loam	**6.9**	0.4	1.31	365.8	21.5	114.5
	Khansahib	Palpur	Loam	**6.0**	0.3	1.12	294.0	19.9	96.3
		Bugur	Loam	**6.4**	0.2	1.24	474.1	20.7	98.7
		Dur	Loam	**6.6**	0.4	1.35	375.0	23.4	102.5
	Chadoora	Gopalpora	Silty loam	**7.8**	0.4	1.45	470.0	26.3	202.4
		Malikgund	Loam	**6.5**	0.3	1.26	412.5	21.6	115.4
		Malapur	Loam	**6.2**	0.2	1.18	387.8	20.2	95.6

District	Block	Location	Texture	Chemical characteristics					
				pH	EC (dS/m)	OC (%)	Av.-N (kg/ha)	Av.-P (kg/ha)	Av.-K (kg/ha)
Kupwara	Kupwara	Warsun	Clay loam	**7.5**	0.6	1.65	493.0	28.3	205.7
		Hondi	Clay loam	**6.2**	0.3	0.93	374.5	19.2	179.9
		Kababmarg	Clay loam	**6.8**	0.4	0.87	313.5	17.8	198.5
	Trehgam	Hyan	Clay loam	**6.6**	0.3	1.25	414.3	20.8	117.6
		Drug Mulla	Clay loam	**6.5**	0.2	1.05	417.8	21.3	92.5
		Heri	Clay loam	**6.1**	0.2	1.05	412.6	22.0	94.5
	Karnah	Kandi	Loam	**7.5**	0.4	1.23	425.5	21.5	191.2
		Haran	Loam	**6.6**	0.3	1.26	424.3	22.3	110.5
		Tangdar	Loam	**6.5**	0.3	1.38	431.8	25.7	98.8
Baramulla	Wagoora	Kreeri-a	Clay loam	**6.6**	0.3	0.82	305.3	18.4	112.7
		KalantaraBala	Clay loam	**7.0**	0.4	1.16	437.0	21.5	205.5
		Waripora	Clay loam	**6.8**	0.3	0.91	396.5	19.8	162.4
	Tangmarg	Dhobiwan	Clay loam	**6.2**	0.2	0.96	371.3	20.2	94.0
		Gonipora	Silty clay loam	**6.0**	0.1	0.69	252.0	20.5	90.5
		Narpur	Silty clay loam	**6.1**	0.2	0.75	292.5	22.0	93.0
	Pattan	Mirgund	Silty clay loam	**6.3**	0.3	0.68	279.5	18.5	93.5
		Palhalan	Silty clay loam	**5.9**	0.2	0.62	272.0	18.2	92.0
		Hamray	Silty clay loam	**6.8**	0.3	0.75	378.5	21.6	96.7

EC : Electrical conductivity; OC: Organic carbon; Av.-N :Available nitrogen; Av.-P: Available phosphorus; Av.-K: Available potassium

cfu: Colony forming units

4.1.1 Bacterial population in rhizosphere soil of walnut

The rhizosphere soils, collected from all the sites surveyed in 4 districts of Kashmir valley, were grown on 4 different media and the bacterial population observed is presented in Table 2 (Plate 1).Amongst the media tested, nutrient agar medium showed highest rhizobacterial population. The bacterial populations in walnut rhizosphere from different sites on nutrient agar was found in the range of 25.0×10^5 to 95.0×10^5 cfu/g soil, with maximum bacterial count observed at Krewa-manul (95.0×10^5 cfu/g soil),followed by Warsun with bacterial count of (83.0×10^5 cfu/g soil)and minimum at Palhalan site. Further, a significant positive correlation (r=0.90) was observed between rhizosphere bacterial population (on nutrient agar medium) and pH. Similarly, significant positive correlation (r values) of 0.49, 0.70, 0.73, 0.65 and 0.87 were found between rhizosphere bacterial population and electric conductivity, organic carbon and available N, P and K, respectively.

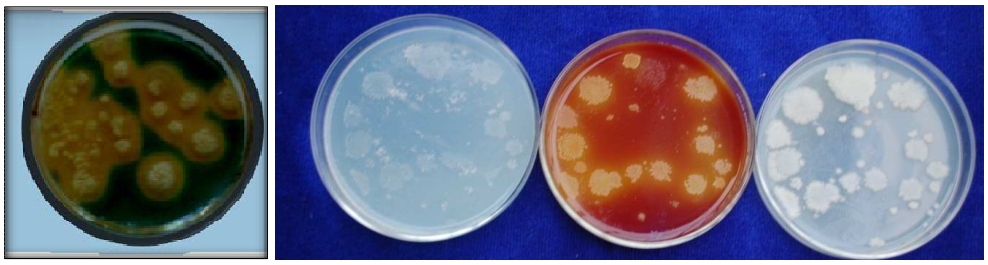
On PVK medium, Mujmarg (Shopian) accounted for highest rhizobacterial population (65.0×10^5 cfu/g soil) while soils from the sites of Gopalpora, Drug Mulla, Heri, Kalantara Bala, Waripora, Gonipora and Palhalan showed no growth on this medium. On CAS medium, the highest bacterial population in walnut rhizosphere was observed at sites Tangwain and Waripora (42.0×10^5cfu/g soil) while no growth was noticed in the rhizosphere soil samples from 17 sites. On nitrogen-free glucose medium (Jensen's medium) the site Gopalpora showed highest population (55.0×10^5 cfu/g soil) while no growth was found at sites Hal, Soibugh and Hondi. During replica plating a total of 98 bacterial isolates apparently morphologically dissimilar were selectedfrom walnut rhizosphere and were designated as WI 1- WI 98. District-wise, 23, 24, 20 and 30 bacterial isolates were selected from Shopian, Budgam, Kupwara and Baramulla.

Table 2: Bacterial population in rhizosphere soil of walnut on different media

District	Block	Location	Rhizosphere bacterial population (×10^5 cfu/g soil)				Isolates collected
			Nutrient agar medium	PVK medium	CAS medium	Jensen's medium	
Shopian	Shopian	Krewa manul	95	50	35	15	WI 1, WI 2, WI 3
		Hal	43	25	20	0	WI 4, WI 5
		Pahelpora	77	45	0	25	WI 6, WI 7
	Keller	Tangwain	80	40	42	42	WI 8, WI 9
		Ganaupur	46	24	0	10	WI 10
		Mujmarg	80	65	30	14	WI 11, WI 12, WI 13, WI 14
	Zainapora	Narawain	54	34	30	26	WI 16, WI 18, WI 19, WI 22
		Safanager	72	37	0	22	WI 15, WI 17, WI 20
		Tularan	82	58	0	33	WI 21, WI 23
Budgam	Budgam	Soibugh	63	43	33	0	WI 24, WI 27, WI 30, WI 33
		Naidgam	47	32	0	23	WI 25, WI 26, WI 28
		Wadwan	49	25	0	22	WI 29, WI 31, WI 32
	Khansahib	Palpur	30	20	0	20	WI 34, WI 35
		Bugur	37	12	13	15	WI 36, WI 39, WI 41
		Dur	39	30	0	12	WI 37, WI 38
	Chadoora	Gopalpora	80	0	0	55	WI 40, WI 45
		Malikgund	50	43	0	5	WI 42, WI 43
		Malapur	37	25	23	19	WI 44, WI 46, WI 48

PVK medium: Pikovskaya's medium; CAS medium : Chrome-azural-s medium; WI : walnut isolate

Master plate for isolation of PGPR (Nutrient Agar)



CAS Media PVK Media Jensen's Media

Replica plating on different media

Plate 1: Bacterial population of rhizosphere soil samples, collected from various locations in Kashmir, on different media

4.2 Morphological characterization of bacterial isolates

The rhizosphere bacterial isolates showed great variations in their morphological characters. The shape of colonies varied from circular, filamentous, punctiform to irregular, with margins varying from, entire, undulate, lobate, erose to curled. The colony elevation varied from flat, raised, convex to umbonate. Overall, 98 isolates were selected from master plates (nutrient agar). Some morphological features of bacterial isolates associated with walnut rhizosphere is presented in Table 3 (Plate 2). About 83.7 per cent isolates were Gram positive ad rest were Gram negative. Rod shaped isolates were most dominant and comprised 82.6 per cent of total rhizosphere bacterial population. Rest isolates (17.4%) were cocci. Among rod shaped bacteria80.3 per cent were Gram positive while 19.7 per cent were Gram negative rods. Among Gram positive rods, 60.2 per cent were long rods, followed by 21.5 per cent medium rods and 12.3 per cent small rods. With respect to colony form 43.8 per cent isolates were circular while 36.7 per cent showed entire margins and 27.5 per cent had convex elevation. The bacterial isolates exhibited great variation with respect to their cell arrangement. A maximum of 47.7 per cent occurred singly, 25.9 per cent arranged in chains, 17.30 per cent showed pair-arrangement and 5.10 per cent were in pairs and chains. Only 4.0 per cent exhibited bunch arrangement. Amongst the isolates, 66.3 per cent were endospore formers with 76.9 per cent having centrally positioned endospore, 20.0 per cent with terminally positioned endospore and only 3.1 per cent having endospore in their sub-terminal position.

4.3 Biochemical characterization of bacterial isolates

All the rhizobacterial isolates from walnut rhizosphere were identified up to genus level on the basis of their morpho-biochemical characteristics using standard procedures. The isolates were grown on Luria Bertani medium at 28±2^{o}C for 24 hours. The biochemical characters of isolates were studied as per the procedures described in Bergey's Manual of Determinative Bacteriology. The results revealed that 65 isolates probably belonged to the genus Bacillus as they

Table 3: Colony character, bacterial cell morphology and Gram reaction of rhizobacterial isolates from walnut

Isolate	Colony morphology	Gram reaction	Cell shape	Cell arrangement	Endospore position
WI 1	Irregular, flat, lobate, creamish	+	Long rods	Pairs and chains	Central
WI 2	Irregular, erose, raised, creamish	+	Medium rods	Singly	Central
WI 3	Irregular, convex, lobate, creamy faint white	+	Small rods	Pairs	Central
WI 4	Irregular, flat, lobate, whitish	+	Minute cocci	Bunches	-
WI 5	Irregular, raised, lobate, creamish	+	Medium rods	Singly	Central
WI 6	Circular, raised, creamish,	+	Long rods	Singly	Terminal
WI 7	Irregular, umbonate, curled, creamish	+	Long rods	Pairs and chains	Terminal
WI 8	Circular, flat, entire, orangish	-	Long rods	Chains	-
WI 9	Circular, convex, entire, yellowish	+	Medium rods	Singly	Central
WI 10	Irregular, erose, umbonate, yellowish	+	Medium rods	Pairs and chains	Central
WI 11	Irregular, flat, undulate whitish	+	Minute cocci	Pairs	-
WI 12	Circular, convex, entire, wax colour	+	Minute cocci	Pairs	-
WI 13	Circular, convex, entire, orange	+	Long rods	chains	Central
WI 14	Irregular, raised, lobate, yellowish	+	Long rods	Singly	Terminal
WI 15	Circular, convex, entire, brownish	+	Medium rods	Pairs	Central
WI 16	Circular, raised, curled, dark yellowish	-	Long rods	Single	-
WI 17	Irregular, flat, lobate, creamish	+	Long rods	Single	Central
WI 18	Wrinkled, lobate, raised, creamish	+	Small rods	Pairs	Central
WI 19	Irregular, undulate, flat with pinpoint center Creamish white	+	Small rods	Single	Central
WI 20	Irregular, raised, erose, pale	+	Long rods	Single	Terminal
WI 21	Circular, convex, entire, dark brown	+	Minute cocci	Bunches	Central
WI 22	irregular, undulate,umbonate, deep orange	-	Long rods	Singly	-

Contd…

Table 3: Contd…

WI 23	irregular, lobate, flat filamentous,white	+	Small rods	Singly	Central
WI 24	irregular, raised wrinkled, undulate, light yellow	+	Long rods	Singly	Central
WI 25	Circular, raised, entire, creamish	+	Minute cocci	Pairs	-
WI 26	Circular, convex, entire, whitish	+	Medium rods	Pairs	Terminal
WI 27	Circular, convex, entire, yellowish	+	Medium rods	Pairs	Central
WI 28	irregular, lobate, flat filamentous,white	+	Small rods	Singly	Central
WI 29	Irregular, umbonate, curled, creamish	+	Small rods	Singly	Central
WI 30	Irregular, umbonate, curled, white	+	Minute cocci	in pairs	-
WI 31	Irregular, flat, lobate, creamish	+	Long rods	Single	Terminal
WI 32	irregular, undulate, umbilicate,creamish white	+	Long rods	Pairs	Terminal
WI 33	small circular, raised entire, white	+	Medium rods	Singly	Central
WI 34	Circular, erose, umbonate, creamish	+	Medium rods	Singly	Central
WI 35	Irregular, raised, erose, creamish	+	Long rods	Pairs	Central
WI 36	Punctiform, raised, entire,yellowish	+	Long rods	Pairs	Central
WI 37	Circular, convex, entire, reddish	+	Minute cocci	Pairs	-
WI 38	Punctiform, raised, entire, yellowish	+	Long rods	Pairs	Sub-terminal
WI 39	Irregular, raised, erose, yellowish	+	Small rods	Pairs	Central
WI 40	Circular, convex, erose, yellowish	-	Long rods	Singly	-
WI 41	Circular, convex, entire, creamish	+	Minute cocci	Chains	-
WI 42	Circular, raised, entire, light orange	+	Medium rods	Singly	Central
WI 43	Irregular, flat, lobate, gummy white	+	Long rods	Singly	Central
WI 44	Irregular, raised, umbonate, white	+	Long rods	Chains	Central
WI 45	Irregular, raised, lobate, creamish	-	Long rods	Singly	-
WI 46	Circular, convex, entire, deep red	+	Long rods	Chains	Central
WI 47	Irregular, raised, serrate, yellowish	+	Long rods	Pairs	Central

Contd…

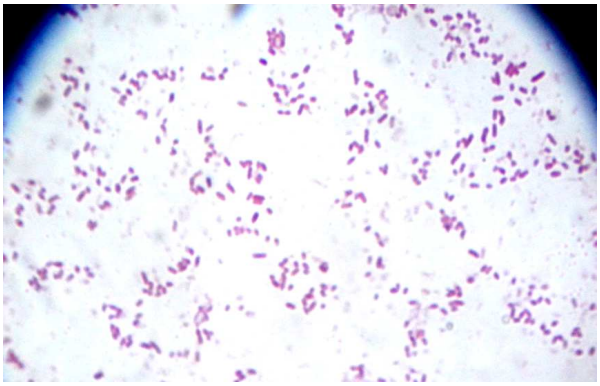
Table 3: Contd…

WI 48	Circular, flat, entire, creamish	+	Medium rods	Pairs	Terminal
WI 49	Circular, convex, entire, deep yellow	+	Long rods	Singly	Central
WI 50	Circular, convex, lobate, yellowish	+	Minute cocci	Bunches	-
WI 51	Circular, flat, undulate whitish	+	Long rods	Singly	Central
WI 52	Irregular, umbonate, curled, creamish	+	Minute cocci	Bunches	-
WI 53	Circular, convex, entire, pale yellow	+	Long rods	Single	Central
WI 54	Irregular, raised, lobate, creamish	+	Small rods	Chains	Central
WI 55	Circular, convex, entire, brownish	+	Small rods	Chains	Terminal
WI 56	Irregular, flat, lobate, creamish	+	Minute cocci	Single	-
WI 57	Irregular, raised, lobate, creamish	-	Long rods	Chains	-
WI 58	Circular, convex, entire, bright orange	+	Long rods	Single	Central
WI 59	Circular, convex, entire, light brown	+	Long rods	Single	Terminal
WI 60	Circular, much convex, entire, yellowish	+	Minute cocci	Chains	-
WI 61	Circular, convex, entire, gummy white	-	Long rods	Singly	-
WI 62	Circular, convex, entire, watery look	+	Long rods	Pairs and chains	Central
WI 63	Irregular, umbonate, curled, creamish	+	Long rods	Singly	Central
WI 64	Irregular, raised, lobate, whitish	+	Long rods	Single	Central
WI 65	Circular, raised, entire, creamish	+	Medium rods	Pairs	Central
WI 66	Circular, convex, entire, whitish	-	Long rods	Singly	-
WI 67	Circular, convex, entire,straw brown	+	Small rods	Chains	Central
WI 68	Circular, raised, entire, gummy white	+	Long rods	Singly	Terminal
WI 69	Irregular, umbonate, curled, yellowish	+	Long rods	Singly	Central
WI 70	Irregular, raised,undulate, creamish	+	Long rods	Singly	Central
WI 71	Irregular, flat, lobate, creamish	+	Long rods	Singly	Central
WI 72	Circular,raised, undulate, whitish	+	Small rods	Singly	Central

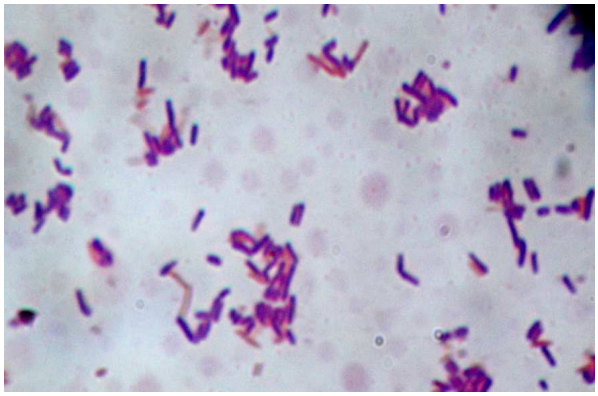
Contd…

Table 3: Contd…

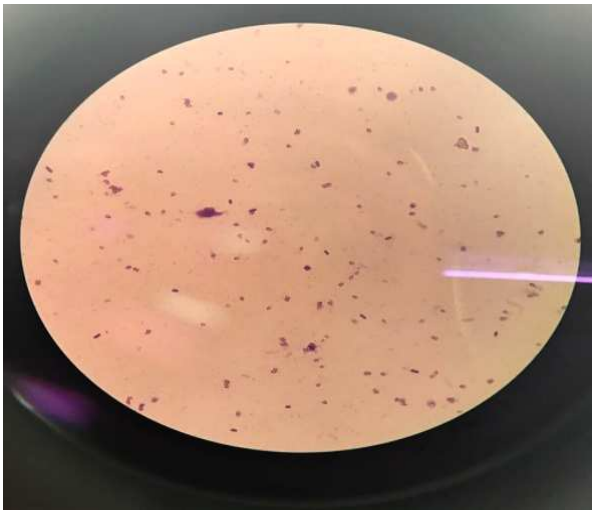
WI 73	circular, flat, undulate, whitish	+	Medium rods	Chains	Terminal
WI 74	Irregular, flat, undulate, yellowish	+	Medium rods	Singly	Central
WI 75	Irregular, flat, undulate, off-white	-	Long rods	Singly	-
WI 76	Irregular, raised, erose, whitish	+	Long rods	Singly	Central
WI 77	Irregular, flat, erose, yellowish	-	Long rods	Singly	-
WI 78	Irregular, flat, undulate, yellowish	-	Long rods	Chains	-
WI 79	Irregular, raised, erose, yellowish	+	Minute cocci	Singly	-
WI 80	Irregular, raised, umbonate, yellowish	+	Minute cocci	Chains	-
WI 81	Circular, raised, lobate, gummy white	-	Long rods	Chains	-
WI 82	Irregular, convex, lobate, creamish	+	Long rods	Pairs	Central
WI 83	Irregular, flat, lobate, creamish	+	Long rods	Singly	Central
WI 84	Irregular, flat, undulate,bright whitish	+	Long rods	Chains	Central
WI 85	Irregular, raised, lobate, creamish	+	Small rods	Singly	Sub terminal
WI 86	Circular, convex, entire, creamish	+	Minute cocci	Chains	-
WI 87	Irregular, flat, undulate, creamish	+	Long rods	Singly	Central
WI 88	Circular, raised, entire, creamish	+	Long rods	Singly	Central
WI 89	circular, convex, entire, yellowish	+	Minute cocci	Chains	-
WI 90	Circular, convex, entire, whitish	+	Long rods	Chains	Central
WI 91	Irregular, flat, undulate whitish	+	Minute cocci	Chains	-
WI 92	Irregular, umbonate, curled, creamish	-	Long rods	Chains	-
WI 93	Circular, concave, entire, creamish	-	Long rods	Singly	-
WI 94	Irregular, raised, lobate, creamish	+	Long rods	Chains	Terminal
WI 95	Circular, concave, entire, light yellow	+	Minute cocci	Singly	-
WI 96	Irregular, raised, serrate, creamish	-	Long rods	Chains	-
WI 97	Irregular, flat, lobate, creamish	-	Long rods	Singly	-
WI 98	Circular, concave, entire, creamish	+	Long rods	Chains	Central



Medium sized rods



Long rods



Minute cocci

Plate 2: Colony morphology and Gram's reaction of rhizobacterial isolates from walnut rhizosphere

were Gram positive rods, endospore formers, positive for catalase test, citrate utilization, starch hydrolysis and Voges-Proskauer test and negative for indole test, acid production, oxidase, gas production, pigmentation and H_2S production (Tables 3 and 4; Plate 3). Twelve isolates as per morpho-biochemical characteristics belonged to the genus *Azotobacter*. They were Gram negative rods and positive for catalase test, oxidase test, citrate utilization, starch hydrolysis, pigment production and H_2S production and negative for indole test methyl red, Voges-Proskauer and gas production. Seventeen isolates tentatively belonged to the genus *Micrococcus* as they were Gram positive cocci and positive for oxidase test, catalase test and starch hydrolysis but negative for citrate utilization, indole test, methyl red, Voges-Proskauer, gas production, pigment production and H_2S production. Four isolates probably belonged to the genus *Pseudomonas* as they were Gram negative rods and positive for oxidase test, catalase test, pigment production, citrate utilization and gas production and negative for starch hydrolysis, Voges-Proskauer test, methyl red test and indole test.

4.4 Screening of rhizobacterial isolates for plant growth promoting activities

A total of 98 rhizobacterial isolates were screened for their multifarious plant growth promoting activities like phosphate solubilization, siderophore production, indole-3-acetic acid (IAA) production, gibberellic acid production, chitinase activity, hydrogen cyanide (HCN) and ammonia production and antifungal activity.

4.4.1 Phosphate solubilization

The phosphate solubilizing activity of rhizobacterial isolates were compared on the basis of their phosphate solubilizing index (PSI) in PVK agar medium and P-solubilization in PVK broth medium. Of the 98 isolates, 79 isolates showed P-solubilization both on PVK agar and liquid media (Table 5; Plate 4a). The results revealed that in PVK agar medium the isolates varied in their PSI values. Amongst the PSI depicting isolates, the isolate WI 65 showed maximum PSI (3.4) followed by isolate WI 91 (3.0) while minimum of 0.7 was noticed in isolates WI 17, WI 35 and WI 46.

Table 4: Biochemical characteristics of rhizobacterial isolates collected from walnut rhizosphere

Isolate	Indole test	Methyl red test	Voges Proskauer test	Starch hydrolysis	Citrate utilization test	Gas production	H_2S production	Oxidase test	Catalase test	Pigment production	Probable genus
WI 1	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 2	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 3	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 4	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 5	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 6	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 7	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 8	-	-	-	-	+	-	-	+	+	+	*Pseudomonas*
WI 9	-	-	-	+	+	-	-	-	+	-	*Bacillus*
WI 10	-	-	-	-	+	-	-	-	+	-	*Bacillus*
WI 11	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 12	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 13	-	-	+	-	+	+	-	-	+	-	*Bacillus*
WI 14	-	-	-	-	+	+	-	-	+	-	*Bacillus*
WI 15	-	-	-	-	+	-	-	-	+	-	*Bacillus*
WI 16	-	-	-	-	+	-	-	+	+	-	*Pseudomonas*
WI 17	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 18	-	-	+	-	+	-	-	+	+	-	*Bacillus*
WI 19	-	-	+	-	+	-	-	+	+	-	*Bacillus*
WI 20	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 21	-	-	-	-	+	-	-	+	+	-	*Bacillus*

Contd…

Table 4: Contd…

WI 22	-	-	-	-	+	-	-	+	+	-	*Pseudomonas*
WI 23	-	-	+	-	-	-	-	-	+	-	*Bacillus*
WI 24	-	-	+	-	-	-	-	-	+	-	*Bacillus*
WI 25	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 26	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 27	-	-	+	+	+	-	-	+	+	-	*Bacillus*
WI 28	-	-	+	-	+	-	-	+	+	-	*Bacillus*
WI 29	-	-	+	+	+	-	-	+	+	-	*Bacillus*
WI 30	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 31	-	-	+	-	+	-	-	+	+	-	*Bacillus*
WI 32	-	-	+	+	-	-	-	+	+	-	*Bacillus*
WI 33	-	-	+	+	-	-	-	-	+	-	*Bacillus*
WI 34	-	-	+	+	+	+	-	-	+	-	*Bacillus*
WI 35	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 36	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 37	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 38	-	-	+	+	+	+	-	+	-	-	*Bacillus*
WI 39	-	-	+	+	+	-	-	+	+	-	*Bacillus*
WI 40	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 41	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 42	-	-	+	+	-	-	-	-	+	-	*Bacillus*
WI 43	-	-	+	-	-	-	-	-	+	-	*Bacillus*
WI 44	-	-	+	-	-	-	-	-	+	-	*Bacillus*
WI 45	-	-	-	+	-	-	-	-	+	-	*Azotobacter*

Contd…

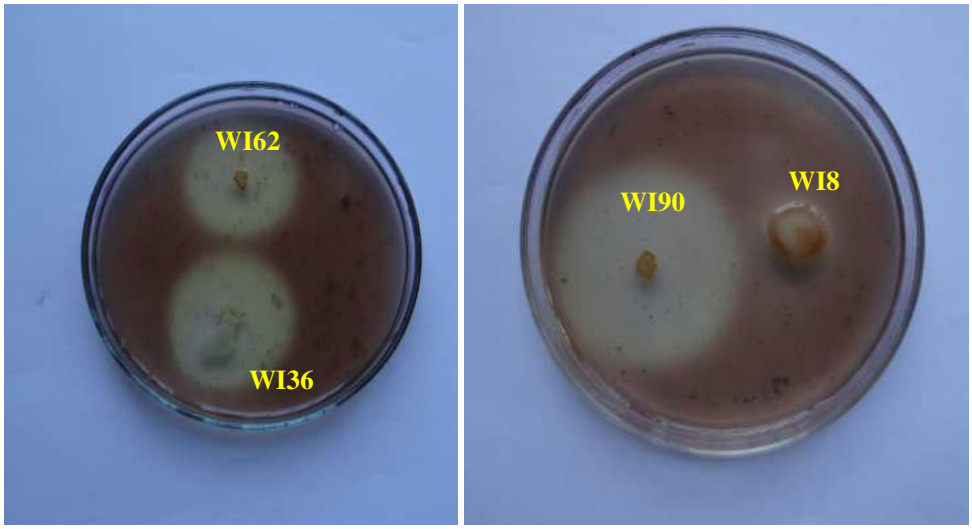
Table 4: Contd…

WI 46	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 47	-	-	-	+	+	-	-	-	+	-	*Bacillus*
WI 48	-	-	+	+	-	-	-	+	+	-	*Bacillus*
WI 49	-	-	+	+	-	-	-	+	+	-	*Bacillus*
WI 50	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 51	-	-	+	-	+	-	-	+	+	-	*Bacillus*
WI 52	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 53	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 54	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 55	-	-	-	-	+	-	-	-	+	-	*Bacillus*
WI 56	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 57	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 58	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 59	-	-	+	-	-	-	-	-	+	-	*Bacillus*
WI 60	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 61	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 62	-	-	+	+	+	+	-	+	+	-	*Bacillus*
WI 63	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 64	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 65	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 66	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 67	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 68	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 69	-	-	+	-	+	-	-	-	+	-	*Bacillus*

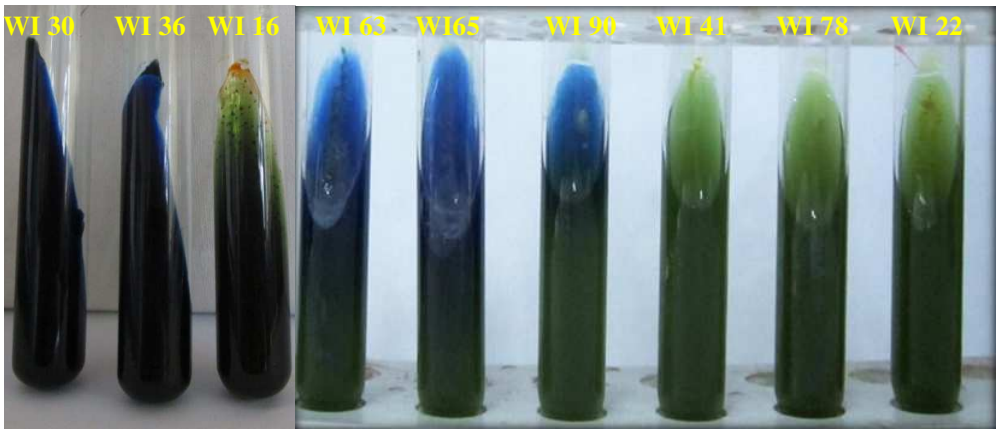
Contd…

Table 4: Contd…

WI 70	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 71	-	-	+	+	+	-	-	+	+	-	*Bacillus*
WI 72	-	-	-	+	-	-	-	+	+	-	*Bacillus*
WI 73	-	-	+	+	-	-	-	+	+	-	*Bacillus*
WI 74	-	-	-	-	-	-	-	-	+	-	*Bacillus*
WI 75	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 76	-	-	+	+	-	+	-	-	+	-	*Bacillus*
WI 77	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 78	-	-	-	-	+	-	-	+	+	-	*Pseudomonas*
WI 79	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 80	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 81	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 82	-	-	+	+	+	-	-	-	+	-	*Bacillus*
WI 83	-	-	+	+	-	-	-	-	+	-	*Bacillus*
WI 84	-	-	-	+	-	-	-	-	+	-	*Bacillus*
WI 85	-	-	+	+	-	-	-	-	+	-	*Bacillus*
WI 86	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 87	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 88	-	-	+	-	-	-	-	-	+	-	*Bacillus*
WI 89	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 90	-	-	-	+	-	-	-	+	+	-	*Bacillus*
WI 91	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 92	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 93	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 94	-	-	+	-	+	-	-	-	+	-	*Bacillus*
WI 95	-	-	-	+	-	-	-	+	+	-	*Micrococcus*
WI 96	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 97	-	-	-	+	-	-	-	-	+	-	*Azotobacter*
WI 98	-	-	+	+	+	-	-	-	+	-	*Bacillus*



Starch hydrolysis



Citrate utilization

Plate 3: Biochemical characterization of rhizobacterial isolates from walnut

a. Starch hydrolysis
b. Citrate utilization
c. Methyl Red test
d. H_2S production

WI

Cont…

Plate 3: Contd…

Methyl Red test (All the isolates were negative)

H_2S production

Table 5: Screening of bacterial isolates from walnut rhizosphere for quantitative and qualitative phosphate solubilization

Isolate	Phosphate solubilization		Isolate	Phosphate solubilization	
	Phosphate solubilization index (PSI)	Quantitative P-estimation (mg/l)		Phosphate solubilization index (PSI)	Quantitative P-estimation (mg/l)
WI 1	2.60	130	WI 42	1.90	72
WI 2	2.60	115	WI 43	2.30	77
WI 3	2.27	135	WI 44	2.20	82
WI 4	2.45	108	WI 46	0.70	37
WI 5	2.88	118	WI 47	0.60	47
WI 6	1.30	100	WI 48	1.40	83
WI 7	0.70	77	WI 49	2.10	77
WI 8	1.70	93	WI 50	2.20	81
WI 9	1.70	127	WI 51	1.20	62
WI 10	2.20	85	WI 52	1.40	68
WI 11	2.40	164	WI 53	1.70	86
WI 12	2.50	205	WI 54	2.10	132
WI 13	2.38	112	WI 55	2.70	116
WI 14	1.60	122	WI 58	1.60	71
WI 15	1.40	42	WI 59	2.20	98
WI 16	2.20	80	WI 60	2.10	172
WI 17	2.50	77	WI 62	2.70	222
WI 18	2.30	62	WI 63	2.35	152
WI 19	2.00	102	WI 64	1.60	78
WI 20	2.20	55	WI 65	3.40	242
WI 21	2.30	82	WI 67	1.27	53

Contd…

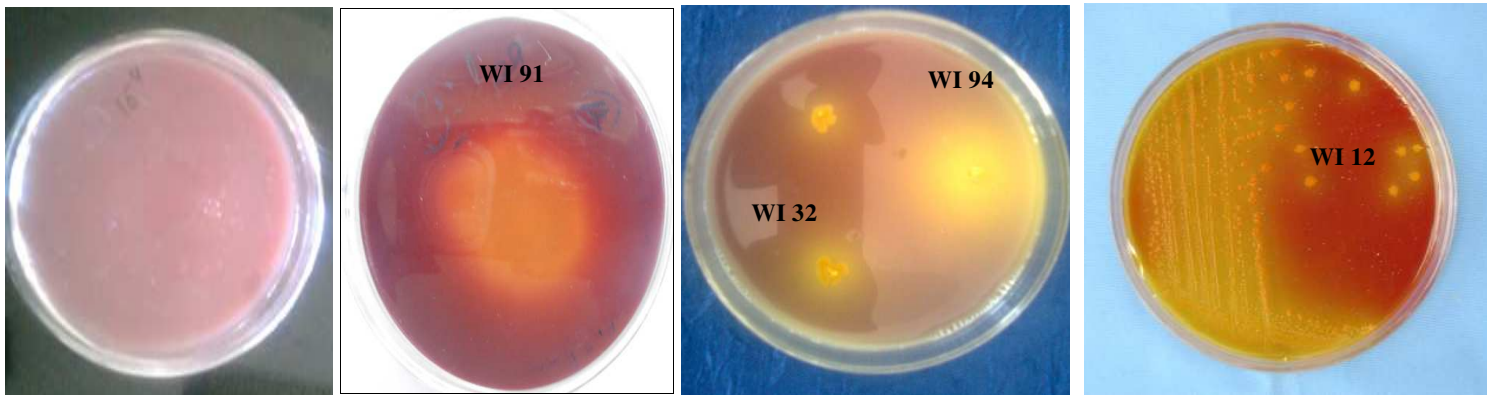
Table 5: Contd…

WI 22	2.35	70	WI 68	2.40	106
WI 23	2.78	90	WI 69	2.40	93
WI 24	2.62	90	WI 71	2.60	60
WI 25	2.40	60	WI 73	1.10	103
WI 26	2.35	72	WI 74	2.20	112
WI 27	1.30	55	WI 76	1.10	42
WI 28	1.70	65	WI 78	0.80	48
WI 29	1.80	90	WI 80	2.60	162
WI 30	1.80	185	WI 83	1.60	78
WI 31	1.60	116	WI 84	2.20	81
WI 32	1.50	129	WI 86	2.40	86
WI 33	2.50	86	WI 87	1.10	42
WI 34	2.20	94	WI 88	1.20	54
WI 35	0.70	42	WI 90	2.70	312
WI 36	1.60	180	WI 91	3.00	267
WI 37	1.30	48	WI 94	2.70	88
WI 38	1.20	58	WI 95	2.20	192
WI 39	2.20	92	WI 98	2.60	60
WI 41	2.20	180			
Mean	**1.98**			**102.30**	
C.D($p \leq 0.05$)	0.501			3.257	
SE(m)	0.179			1.165	
CV(%)	5.629			1.972	

Rest of the isolates showed no phosphate solubilization,

Medium used Pikovskaya's medium, reading taken after 72 hrs

PSI : Clear zone diameter ÷ growth diameter



Control PVK

a. Solid assay

b. Estimation of phosphorus in liquid PVK medium

Plate 4: P-solubilization both on PVK agar and liquid media

In liquid PVK medium the isolates showed great variation in P-solubilization activity (Table 5; Plate 4b). Of the isolates showing P-solubilization, maximum activity was observed in isolate WI 90 (312 mg/l), followed by isolate WI 91 (267 mg/l) and isolate WI 65 (242 mg/l); however, these isolates differed significantly from isolate WI 90. The isolate WI 46 solubilized minimum phosphate (37 mg/l). The maximum PSI was not related to the maximum P solubilization in liquid medium. The correlation coefficient (r=0.09) between PSI and P solubilization by bacterial isolates was positive but non-significant.

4.4.2 Siderophore production

The siderophore production by selected bacterial isolates on chome-azurol-S (CAS) solid medium was compared on the basis of their zone size and per cent siderophore unit. Amongst the 98 rhizobacterial isolates only 53 isolates (54.1%) produced siderophore (Table 6; Plate 5). The results revealed that the isolates WI 63 and WI 90 produced significantly highest siderophore (zone size 20 mm). This was followed by isolates WI 30 (18.0mm),WI 36(17.3mm), WI 65 (16.0mm) and WI 60 (15.6mm) which were statistically at par with one another while least zone size of 3.0 mm observed in isolates WI 72, WI 83 and WI 86.

Quantitative estimation of siderophore using CAS liquid assay revealed that isolate WI 12 produced significantly highest siderophore units (27.2%), followed 25.0 per cent siderophore units by isolate WI 90 and 22.45 units by isolate WI 12. Minimum per cent siderophore units (4.75%) was observed in isolate WI 1. The correlation coefficient (r= 0.10) between qualitative and quantitative siderophore production was positive and non-significant.

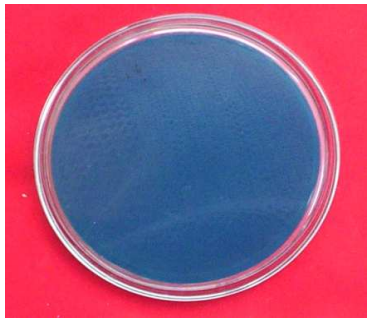
Table 6: Screening of bacterial isolates from walnut rhizosphere for quantitative and qualitative siderophore production

Isolate	Siderophore production		Isolate	Siderophore production	
	Qualitative siderophore estimation (zone size, mm)	Per cent siderophore unit		Qualitative siderophore estimation (zone size, mm)	Per cent siderophore unit
WI 1	9.5	4.75	WI 52	4.00	12.80
WI 2	4.0	8.20	WI 54	6.00	10.80
WI 3	5.0	10.42	WI 56	7.30	13.90
WI 4	14.3	12.38	WI 58	10.00	13.00
WI 5	8.3	8.60	WI 60	15.60	16.50
WI 8	12.0	12.50	WI 62	10.00	22.30
WI 9	12.1	14.20	WI 63	20.00	16.60
WI 11	11.0	18.66	WI 65	16.00	18.83
WI 12	9.0	27.21	WI 67	6.00	12.00
WI 13	12.0	14.80	WI 68	7.70	14.26
WI 14	8.0	12.30	WI 70	7.30	7.70
WI 16	8.0	14.50	WI 72	3.00	7.60
WI 18	5.0	14.20	WI 74	6.00	9.80
WI 19	6.0	10.70	WI 76	5.70	9.60
WI 22	6.0	10.20	WI 78	5.50	11.20
WI 24	8.0	13.65	WI 79	7.00	11.50
WI 27	4.0	9.20	WI 80	11.30	16.00
WI 30	18.0	17.70	WI 82	9.00	10.00
WI 33	7.3	14.64	WI 83	3.00	8.50
WI 36	17.3	17.50	WI 85	10.00	12.33
WI 39	8.0	14.80	WI 86	3.00	8.00
WI 41	14.4	22.45	WI 88	8.00	10.50
WI 44	8.0	22.50	WI 90	20.00	25.00
WI 46	6.0	9.00	WI 91	7.60	15.00
WI 48	6.0	11.00	WI 95	5.50	11.20
WI 50	8.2	14.00	WI 98	9.00	10.00
WI 51	9.0	14.00			
Mean	**9.01**			**13.18**	
C.D.($p \leq 0.05$)	2.724			2.157	
SE(m)	0.970			0.768	
C.V. (%)	6.99			5.089	

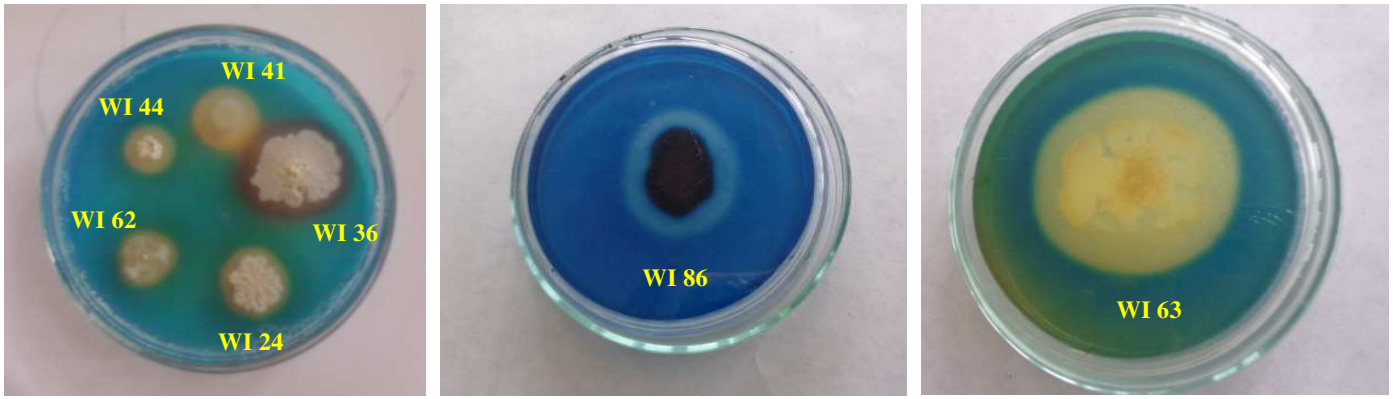
Rest of the isolates did not show any siderophore production

Per cent siderophore unit= $(A_r - A_s) \div A_s \times 100$

Medium used CAS medium, readings taken after 72hrs



CAS control



Siderophore production by various isolates

Plate 5: Rhizobacterial isolates from walnut orchard for quantitative and qualitative siderophore production

4.4.3 Indole-3-acetic acid (IAA) production

Out of 98 bacterial isolates only 52 isolates (53.1%) showed the ability to produce phytohormone IAA (Table 7). The data revealed that bacterial isolates varied in their ability to produce IAA (Plate 6). The isolates WI 36 and WI 41 yielded maximum IAA (30 µg/ml), followed by statistically at par isolate WI 60 (28 µg IAA/ml). The least IAA production (3.3 µg/ml) was observed in isolate WI 87 which was at par with isolates WI 59, WI 4, WI 32, WI 29, WI 69, WI 74 and WI 98.

4.4.4 Gibberellic acid production

Out of 98 rhizobacterial isolates 48 isolates (49%) were positive for gibberellic acid production (Table 8). Among these GA positive isolates, maximum GA production was observed in isolate WI 90 (65.3 µg/ml), followed by isolates WI 91 (65.0 µg/ml) and WI 80 (64.7 µg/ml) which were statistically at par with isolate WI 90. Minimum gibberellic acid was produced by isolate WI 8 (10.0 µg/ml) which was, however, at par with isolates WI 15, WI 70 and WI 44.

4.4.5 Chitinase activity

The screening of rhizobacterial isolates for chitinase activity indicated that only 35 isolates (35.7%) were capable of producing chitinase enzyme (Table 9; Plate 7). The chitinase producing isolates showed average production of 15.71 units chitinase activity/ml. A significantly high chitinase enzyme was produced by isolate WI 63 (30.47 units/ml), followed by isolates WI 62 (25.33units/ml) and WI 65(25.14units/ml), WI 11 (24.05 units/ml) and WI 90 (23.18units/ml). Least enzyme activity was shown by isolate WI 18 (8.90 units/ml). The mean of the ratio of zone of clearance to the colony size in these isolates was 2.70. Amongst the chitinase producing isolates, the isolate WI 63 showed significantly highest CZ:CS ratio (4.05), followed by WI 90 (3.67), WI 15 (3.55) and WI 89 (3.35). The isolate WI 50 depicted lowest CZ:CS ratio of 1.60.

Table 7: Screening of bacterial isolates collected from walnut rhizosphere, for indole acetic acid production (µg/ml)

Isolate	Indole-3-acetic acid	Isolate	Indole-3-acetic acid
WI 1	14.05	WI 51	12.00
WI 2	11.50	WI 54	14.00
WI 4	4.15	WI 57	6.00
WI 6	10.00	WI 59	3.94
WI 8	13.00	WI 60	28.00
W1 11	24.00	WI 61	15.70
WI 12	21.00	WI 62	21.00
WI 15	7.45	WI 63	22.00
WI 18	11.50	WI 65	22.50
WI 20	14.25	WI 66	15.50
WI 22	10.00	WI 69	5.50
WI 25	10.00	WI 71	8.25
W1 27	13.60	WI 74	4.20
WI 29	5.00	WI 75	10.50
WI 30	21.00	WI 76	9.50
WI 32	5.00	WI 78	7.70
WI 34	9.00	WI 80	21.00
WI 36	30.00	WI 84	11.79
WI 39	15.00	WI 86	11.50
WI 40	16.00	WI 87	3.30
WI 41	30.00	WI 88	14.00
WI 42	13.68	WI 90	19.00
WI 45	11.64	WI 91	16.00
WI 47	13.00	WI 93	12.00
WI 49	6.00	WI 95	14.00
WI 50	12.00	WI 98	5.00
Mean		**13.18**	
C.D($p \leq 0.05$)		2.342	
SE(m)		0.834	
C.V (%)		5.950	

Medium used:Luria Bertani broth

Rest of the isolates did not show any IAA production

Table 8: Screening of bacterial isolates isolated from walnut rhizosphere for gibberellic acid production (µg/ml)

Isolate	Gibberellic acid	Isolate	Gibberellic acid
WI 1	32.00	WI 55	43.68
WI 2	22.50	WI 60	49.00
WI 3	43.00	WI 61	45.00
WI 5	12.00	WI 62	60.00
WI 8	10.00	WI 63	62.00
W1 10	34.66	WI 65	48.00
WI 11	60.00	WI 66	35.45
WI 12	51.00	WI 67	19.43
WI 15	32.25	WI 70	12.60
WI 18	27.50	WI 74	44.50
WI 21	23.34	WI 75	30.75
WI 25	34.80	WI 76	21.00
W1 27	27.70	WI 77	23.68
WI 29	24.00	WI 80	64.66
WI 30	46.00	WI 81	15.25
WI 33	31.60	WI 86	35.50
WI 36	59.00	WI 88	37.00
WI 37	14.50	WI 89	25.45
WI 39	15.50	WI 90	65.33
WI 41	45.00	WI 91	65.00
WI 44	12.76	WI 92	45.50
WI 46	39.90	WI 93	35.75
WI 49	19.50	WI 96	24.50
WI 52	35.25	WI 97	40.00
Mean	**35.50**		
C.D($p \leq 0.05$)	4.367		
SE(m)	1.162		
C.V (%)	5.652		

Medium used: Nutrient broth

Rest of the isolates did not show any gibberellic acid production

Table 9: Screening of bacterial isolates isolated from walnut rhizosphere for chitinase activity (units/ml)

Isolate	Chitinase activity	CS:CZ	Isolate	Chitinase activity	CS:CZ
WI 2	11.21	2.75	WI 51	9.08	2.34
WI 3	9.80	2.24	WI 52	13.17	1.87
WI 4	12.43	2.94	WI 53	14.55	3.35
WI 11	24.05	3.06	WI 54	15.76	2.85
WI 12	18.2	2.50	WI 60	19.75	2.75
WI 13	10.88	1.85	WI 62	25.33	3.06
WI 14	9.65	2.26	WI 63	30.47	4.05
WI 15	20.13	3.55	WI 65	25.14	3.11
WI 16	17.88	3.25	WI 73	10.92	1.85
WI 17	12.00	2.44	WI 80	17.67	2.50
WI 18	8.90	1.93	WI 81	13.45	1.93
WI 30	16.45	3.25	WI 82	10.23	1.85
WI 36	18.30	3.30	WI 83	12.75	3.05
WI 37	11.36	2.20	WI 84	14.98	3.25
WI 38	9.46	2.05	WI 89	16.15	3.35
WI 41	21.03	3.00	WI 90	23.18	3.67
WI 44	19.00	2.85	WI 91	17.50	3.00
WI 50	11.05	1.60			
Mean	**15.71**			**2.70**	
C.D($p \leq 0.05$)	0.035			0.045	
SE(m)	0.013			0.016	
C.V (%)	1.156			1.256	

CS:CZ Colony size : Colony zone

Rest of the isolates did not show any chitinase activity

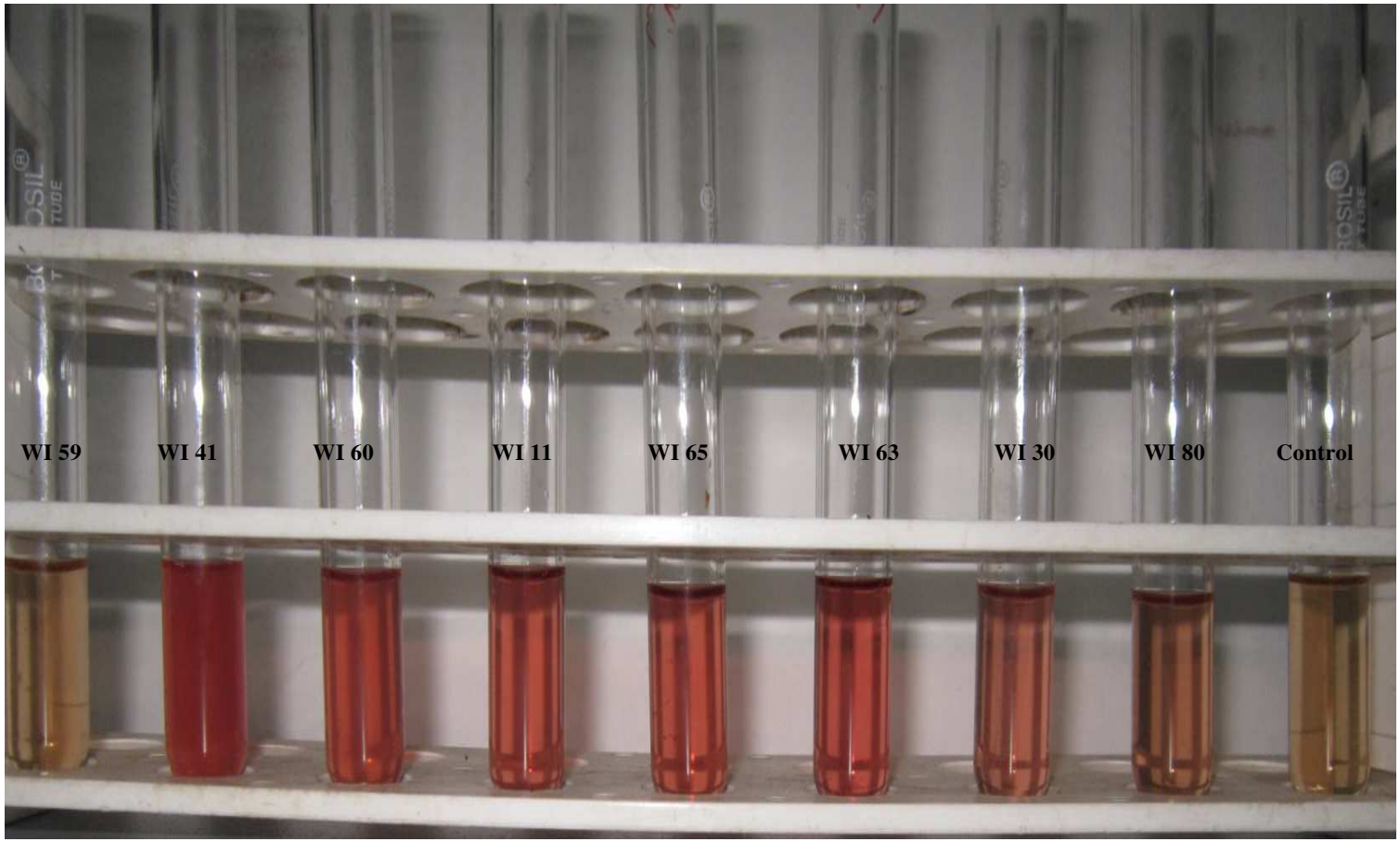


Plate 6: IAA production by rhizobacterial isolates

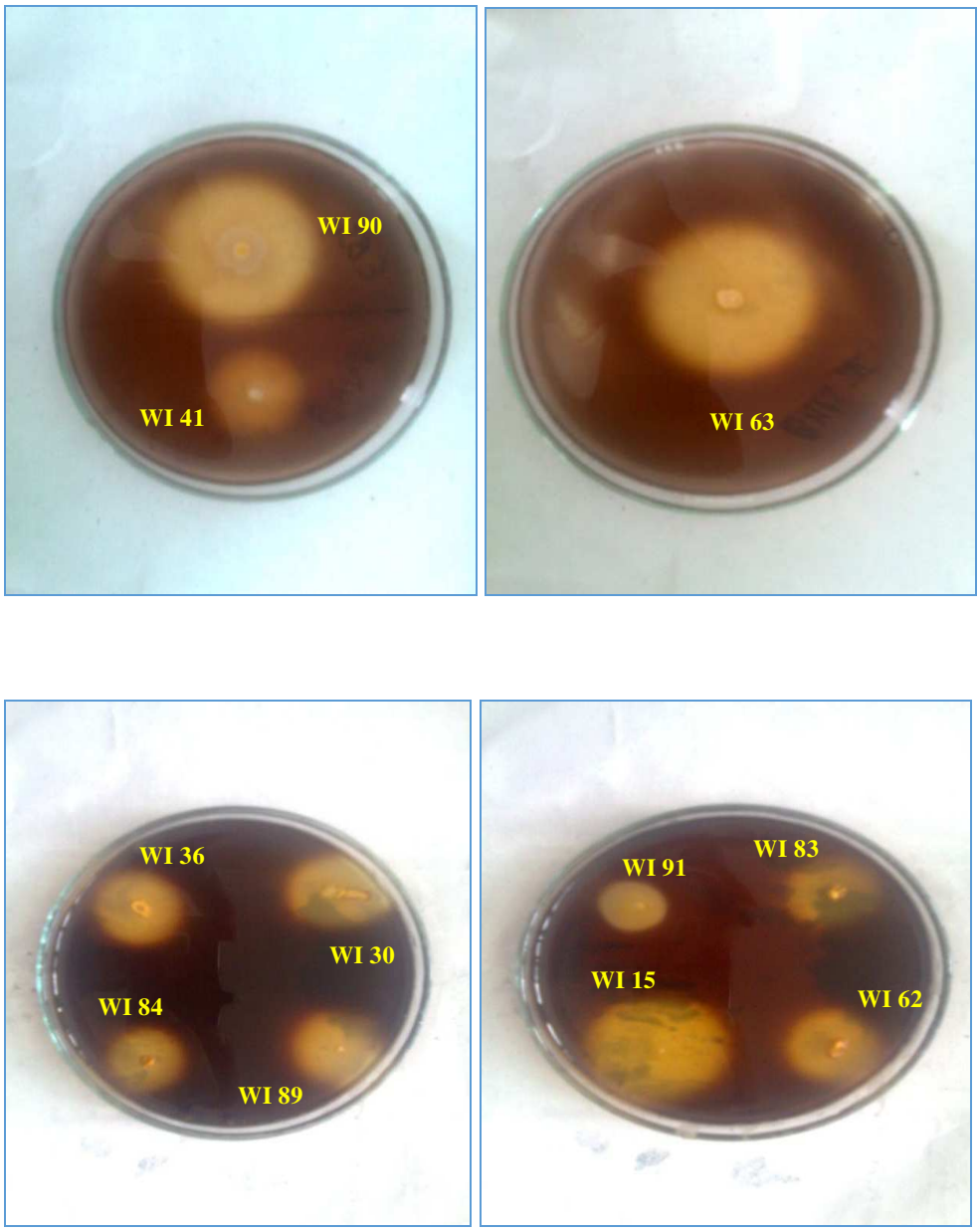


Plate 7: Chitinase activity shown by various isolates

4.4.6 Hydrogen cyanide (HCN) and ammonia production by rhizobacterial isolates

All the 98 rhizobacterial isolates were screened for HCN and ammonia production. These isolates showed great variation in HCN and ammonia production (Table 10; Plate 8 and 9). Seven isolates (7.14%) *viz*., WI 12, WI 21, WI 36, WI 60, WI 62, WI 90 and WI 91 showed very high HCN production and in these cases the colour of filter paper changed from yellow to brown. Thirteen isolates (13.26%) i.e. WI 4, WI 7, WI 14, WI 19, WI 22, WI 41, WI 44, WI 61, WI 63, WI 65, WI 73, WI 80 and WI 85 showed change in the colour of half of filter paper from yellow to brown. The colour of filter paper turned yellow to orange in 30 isolates (30.61%) i.e. WI 2, WI 3, WI 5, WI 9, WI 11, WI 13, WI 15, WI 16, WI 17, WI 18, WI 20, WI 30, WI 32, WI 33, WI 35, WI 37, WI 38, WI 42, WI 50, WI 51, WI 59, WI 74, WI 75, WI 77, WI 78,WI 86, WI 87, WI 89, WI 92 and WI 98. However, 16 (16.32%) isolates i.e. WI 8, WI 10, WI 23, WI 24, WI 27, WI 34, WI 55, WI 56, WI 67, WI 68, WI 70, WI 39, WI 40, WI 43, WI 93 and WI 97did not show HCN production.

Six isolates (6.12%) *viz*., WI 30, WI 36, WI 61, WI 62, WI 63 and WI 65 showed very high ammonia production wherein the colour of peptone water changed from faint yellow to dark brown on the addition of Nessler's reagent. Nineteen isolates i.e. WI 9, WI 14, WI 16, WI 26, WI 27, WI 28, WI 29, WI 33, WI 34, WI 37, WI 38, WI 47, WI 48, WI 64, WI 79, WI 81, WI 82, WI 86 and WI 87 did not produce ammonia.

4.4.7 Antifungal activities ofrhizobacterial isolates against various fungal pathogens

All the 98 rhizobacterial isolates were screened for their antifungal activities against five fungal pathogens *viz*., *Dematophora necatrix, Alternaria solani, Fusarium oxysporum*, *Pythium aphanidermatum* and *Phytophthora capsica.* Only 23 isolates (23.46%) were antagonistic to *D. necatrix* (Table 11). Amongst these, isolate WI 90 exhibited maximum fungal inhibition of 66 per cent (Plate 10a), followed by isolate WI 60 (43.0% inhibition), WI 11 (41.4%

Table 10: Screening of bacterial isolates isolated from walnut rhizosphere for hydrogen cyanide (HCN) and ammonia production

Isolate	HCN	Ammonia	Isolate	HCN	Ammonia
WI 1	+	++	WI 51	++	+
WI 2	++	+	WI 52	+	+
WI 3	++	+	WI 53	+	+
WI 4	+++	+++	WI 54	+	++
WI 5	++	+++	WI 55	-	++
WI 6	+	++	WI 56	-	+
WI 7	+++	++	WI 57	+	+
WI 8	-	+	WI 58	+	+
WI 9	++	-	WI 59	++	+
WI 10	-	+	WI 60	++++	++
WI 11	++	+++	WI 61	+++	++++
WI 12	++++	+++	WI 62	++++	++++
WI 13	++	+	WI 63	+++	++++
WI 14	+++	-	WI 64	+	-
WI 15	++	++	WI 65	+++	++++
WI 16	++	-	WI 66	+	+
WI 17	++	++	WI 67	-	+
WI 18	++	++	WI 68	-	+
WI 19	+++	+	WI 69	+	+
WI 20	++	++	WI 70	-	+
WI 21	++++	++	WI 71	+	++
WI 22	+++	+	WI 72	+	++
WI 23	-	+	WI 73	+++	++
WI 24	-	+	WI 74	++	++
WI 25	+	+	WI 75	++	+
WI 26	+	-	WI 76	+	+

Contd…

Table 10: Contd…

WI 27	-	-	WI 77	++	+
WI 28	+	-	WI 78	++	+
WI 29	+	-	WI 79	+	-
WI 30	++	++++	WI 80	+++	++
WI 31	+	+	WI 81	+	-
WI 32	++	+	WI 82	+	-
WI 33	++	-	WI 83	+	+
WI 34	-	-	WI 84	+	+
WI 35	++	+	WI 85	+++	+
WI 36	++++	++++	WI 86	++	-
WI 37	++	-	WI 87	++	-
WI 38	++	-	WI 88	+	+
WI 39	-	++	WI 89	++	+
WI 40	-	+++	WI 90	++++	+++
WI 41	+++	+++	WI 91	++++	++
WI 42	++	+	WI 92	++	++
WI 43	-	++	WI 93	-	+
WI 44	+++	++	WI 94	+	+
WI 45	+	+	WI 95	+	+
WI 46	+	+	WI 96	+	+
WI 47	+	-	WI 97	-	++
WI 48	+	-	WI 98	++	++
WI 49	+	++			
WI 50	++	++			

Medium used :kings B

- : nocolour change

+ :colour change of filter paper only on edges from yellow to orange

++: colour change of filter paper from deep yellow to orange

+++: colour change of filter paper from deep yellow to orange brown

++++: colour change of filter paper from deep yellow to dark brown

and for Ammonia change of colour occurs in media (peptone water)

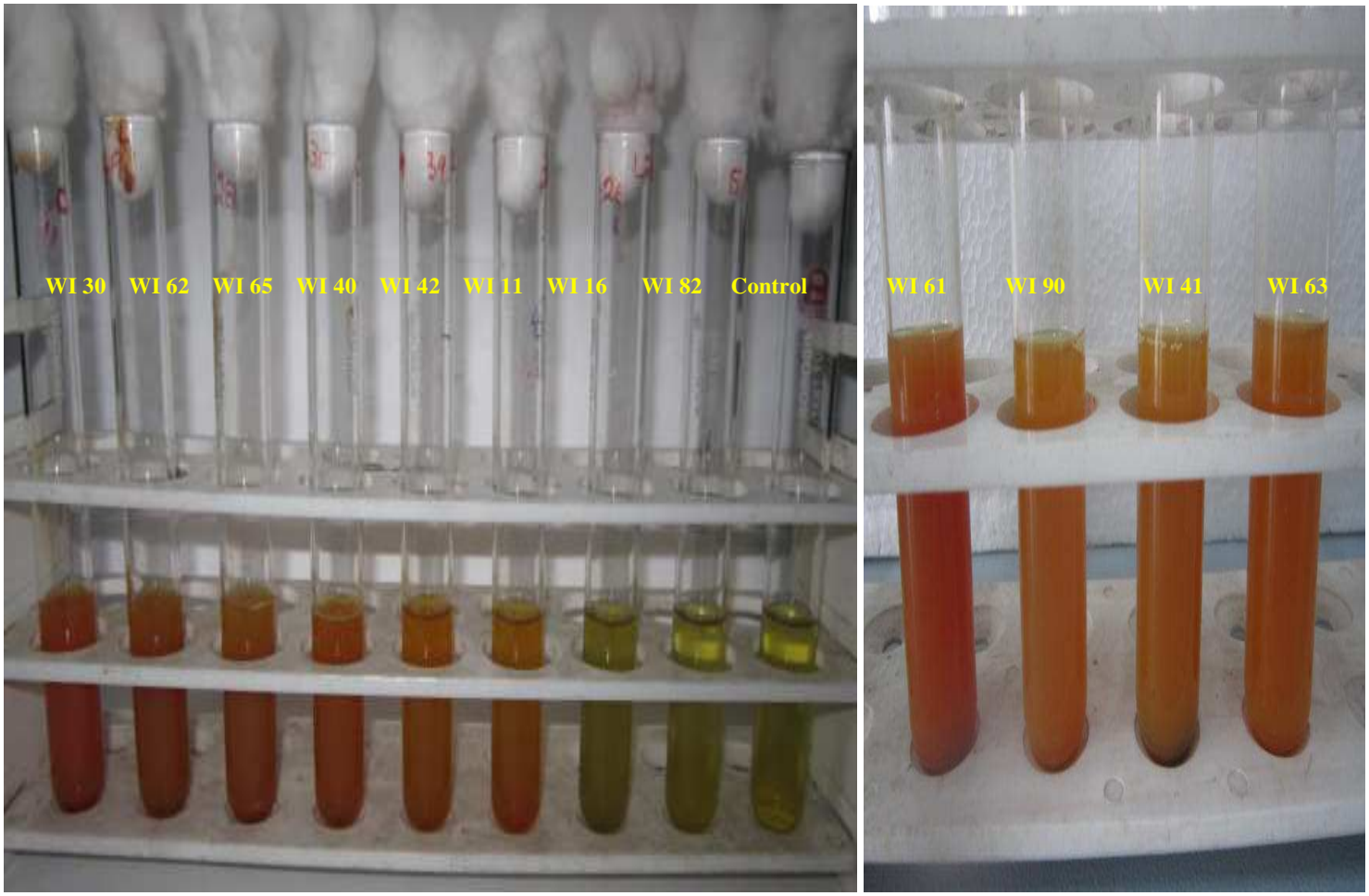


Plate 8: Ammonia production by various isolates in peptone water

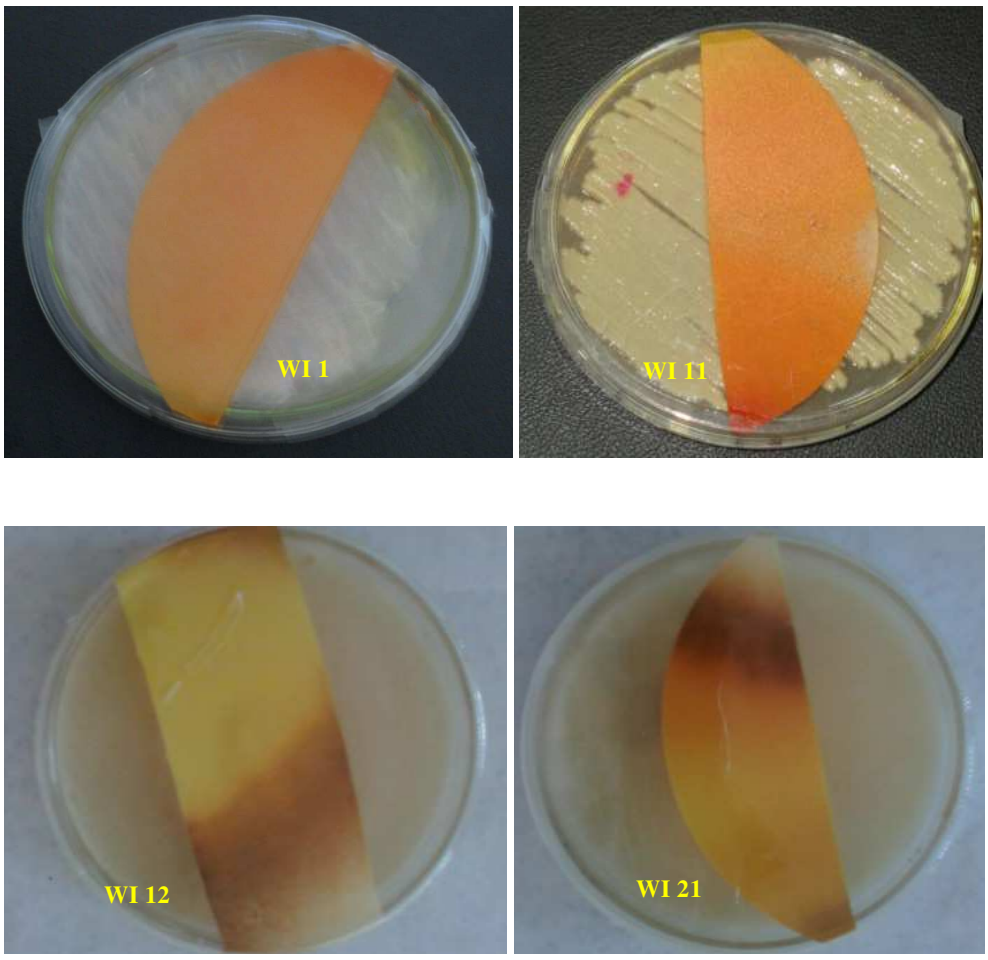


Plate 9: HCN production by rhizobacterial isolates

Table 11: Antifungal activity of various rhizobacterial isolates, collected from walnut rhizospehere, against different fungal pathogens

Dematophora necatrix		*Alternaria solani*		*Fusarium oxysporum*		*Pythium aphanidermatum*		*Phytophthora capsici*	
Isolate	**Inhibition %**	**Isolates**	**Inhibition %**	**Isolate**	**Inhibition %**	**Isolate**	**Inhibition %**	**Isolate**	**Inhibition %**
WI 2	15.5	WI 4	17.3	WI 2	10.7	WI 2	16.5	WI 2	18.8
WI 3	11.7	WI 7	8.9	WI 3	18.8	WI 3	22.5	WI 3	17.5
WI 4	7.6	WI 11	45.5	WI 11	39.7	WI 7	21.2	WI 4	12.7
WI 7	16.0	WI 12	30.8	WI 12	22.2	WI 11	30.4	WI 11	28.7
WI 11	41.4	WI 14	16.6	WI 19	13.2	WI 12	35.5	WI 12	35.3
WI 12	34.7	WI 18	31.5	WI 30	37.0	WI 13	19.2	WI 13	21.5
WI 13	12.01	WI 30	21.4	WI 36	32.3	WI 21	26.0	WI 22	10.0
WI 14	17.3	WI 36	67.0	WI 41	33.3	WI 30	35.3	WI 30	38.7
WI 30	29.6	WI 37	22.45	WI 44	12.3	WI 36	38.2	WI 36	25.4
WI 36	38.4	WI 41	26.6	WI 50	19.2	WI 38	18.5	WI 38	8.8
WI 37	16.0	WI 42	12.9	WI 51	12.4	WI 41	26.0	WI 41	23.2
WI 38	29.22	WI 51	17.7	WI 60	20.3	WI 49	15.4	WI 50	18.8
WI 41	23.3	WI 59	17.5	WI 62	43.8	WI 51	24.7	WI 51	24.3
WI 44	12.0	WI 60	29.8	WI 63	35.5	WI 60	43.2	WI 60	27.1

Dematophora necatrix		*Alternaria solani*		*Fusarium oxysporum*		*Pythium aphanidermatum*		*Phytophthora capsici*	
Isolate	**Inhibition %**	**Isolates**	**Inhibition %**	**Isolate**	**Inhibition %**	**Isolate**	**Inhibition %**	**Isolate**	**Inhibition %**
WI 50	10.0	WI 62	39.6	WI 65	36.6	WI 62	38.4	WI 62	29.8
WI 51	17.3	WI 63	55.6	WI 85	16.5	WI 63	45.5	WI 63	21.4
WI 60	43.0	WI 65	44.0	WI 90	33.9	WI 65	30.0	WI 65	49.0
WI 62	38.2	WI 87	19.5	WI 91	25.0	WI 85	17.5	WI 86	16.0
WI 63	36.5	WI 90	21.8	WI 98	13.3	WI 89	13.67	WI 90	27.3
WI 65	35.5	WI 91	23.3			WI 90	31.5	WI 91	24.5
WI 73	8.7					WI 91	19.5		
WI 89	16.5								
WI 90	66.0								
Mean	**25.08**		**24.95**		**24.47**		**25.58**		**23.19**
CD($p \leq 0.05$)	0.62		3.66		3.54		4.66		6.4
SE(m)	0.33		1.64		1.60		2.60		3.12
CV(%)	3.54		2.45		2.45		2.18		3.40

Medium used : Malt extract agar, readings taken after 7-14 days

Rest of the isolates showed no fungal inhibition or contact inhibition

inhibition), WI 36 (38.4% inhibition) and WI 62 (38.2% inhibition). Minimum inhibition of *D. necatrix* was observed in isolate WI 4 (7.6%).

Only 20 isolates (20.4%) showed antagonistic activity against *A. solani* (Table 11). The isolate WI 36 showed maximum inhibition of 67.0 per cent against this fungus (Plate 10b), followed by isolate WI 63 (55.6% inhibition), WI 11 (45.5% inhibition) and WI 65 (44.0% inhibition). Minimum inhibition against *A. solani* was depicted by isolate WI I7 (8.9%).

Of the 98 isolates evaluated against *F. oxysporum*, only 19 isolates inhibited its growth with maximum inhibition shown by isolate WI 62 (43.8%) [Table 11; Plate 10c] and least by isolate WI 2 (10.7%). Similarly, only 21 isolates were found antagonistic to *P. aphanidermatum* with maximum inhibition depicted by isolate WI 63 (45.5%) [Table 11; Plate 10d], followed by statistically at par isolate WI 60 (43.2%) and least inhibition was shown by isolate WI 49 (15.4%). Only 20 isolates exhibited growth inhibition of *P. capsici* with maximum inhibition by isolate WI 65(49.0%) [Table 11; Plate 10e] and minimum by isolate WI 38 (8.8%).

4.4.8 Performance score of rhizobacterial isolates

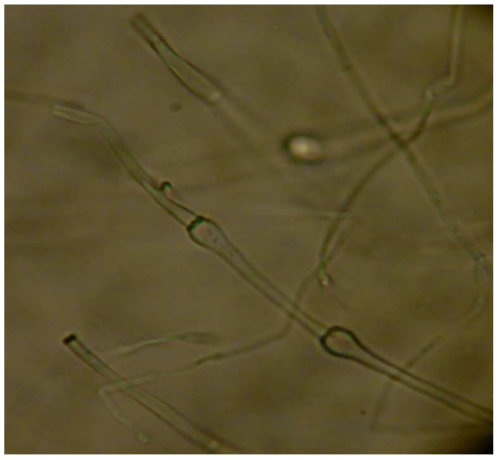
The overall performance of all the isolates was quantified by giving weightage to each attribute of plant growth promotion. Twelve isolates namely WI 90, WI 62, WI 36, WI 63, WI 65, WI 12, WI 41, WI 11, WI 60, WI 91, WI 30 and WI 80 proved superior with overall performance score of 67.29, 66.86, 64.40, 62.41, 62.31, 60.67, 57.79, 56.39, 55.80, 53.69, 53.05 and 46.25, respectively (Table 12). The best performing isolates were selected for molecular characterization.

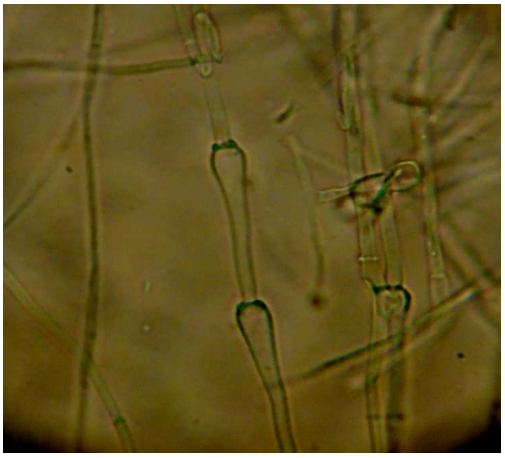
4.5 16S rRNA gene amplification and sequencing

Twelve best isolates in terms of their plant growth promoting attributes were identified by amplifying their 16S rRNA gene sequences of different lengths (Table 13). The partial sequences of nucleotides were compared with the available

Dematophora necatrix (Control)

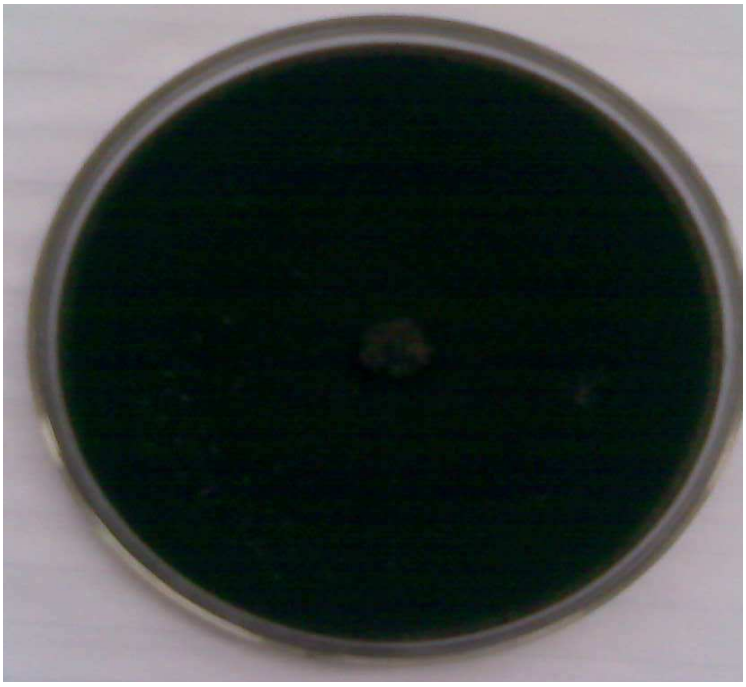
Dematophora necatrix + WI90, (*Bacillus licheniformis)*





Pear shaped bulging at septa formation, a unique characteristic of *Dematophora necatrix*

Plate 10(a): Fungal inhibition

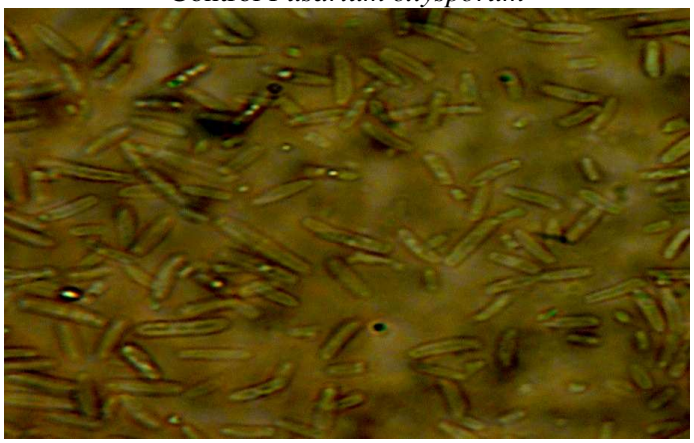


Alternaria solani (Control)

Alternaria solani + WI63 (*Bacillus subtilis*)

Plate 10(b): Inhibition shown against *Alternaria solani*

Control *Fusarium oxysporum*



Conidia of *Fusarium oxysporum*

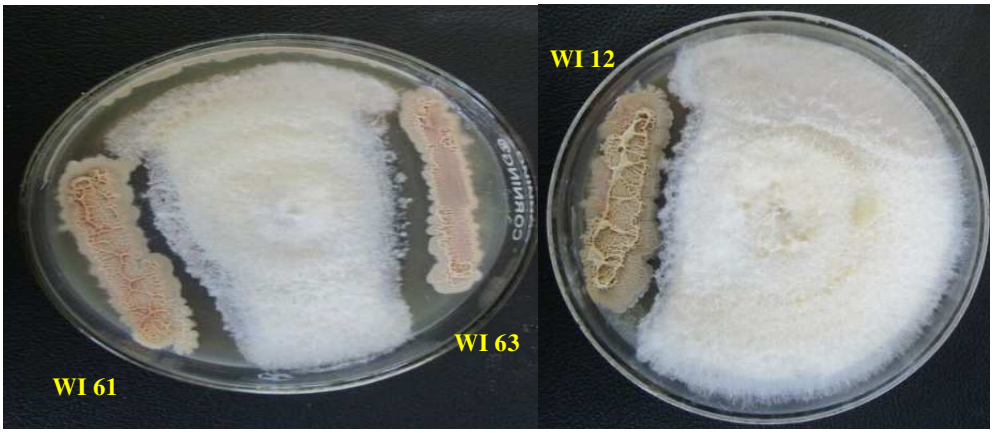
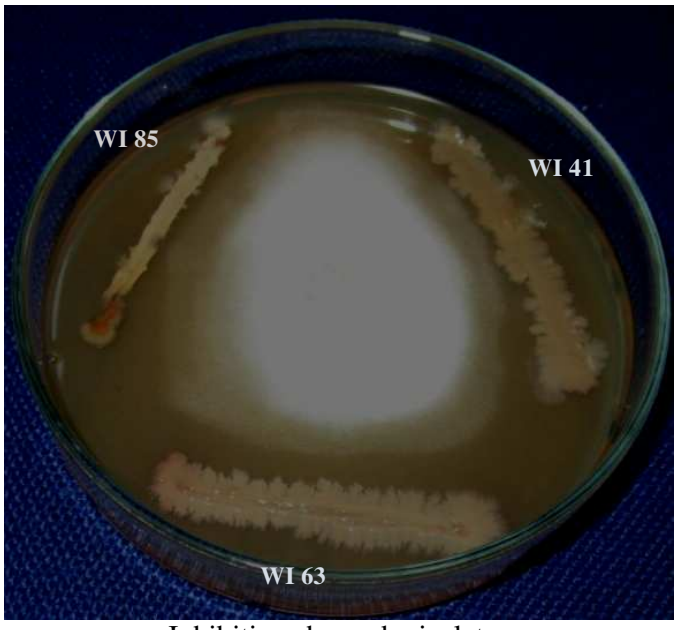


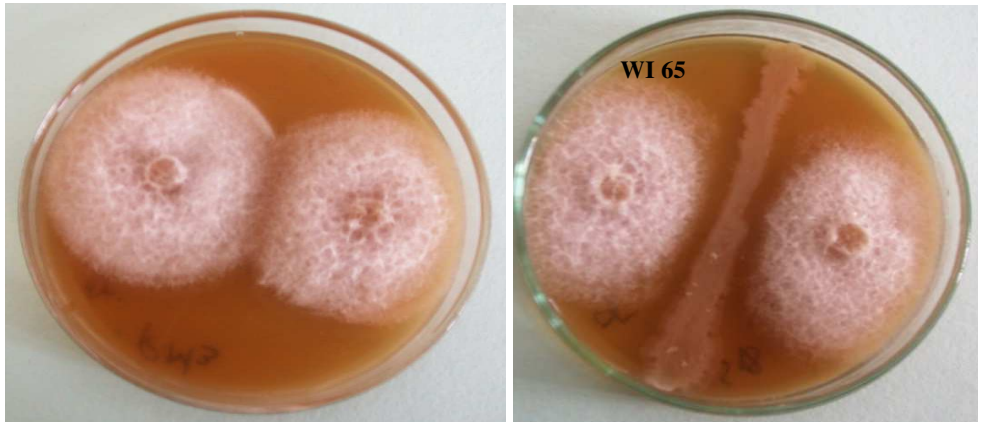
Plate 10(c): Inhibition shown by bacterial isolates

Control : *Pythium aphanidermatum*



Inhibition shown by isolates

Plate 10d): Inhibition shown against *Pythium aphanidermatum*



Control: *Phytophthora capsici*

Plate 10(e): Inhibition of fungal growth by various rhizobacterial isolate

sequences from NCBI database and the sequences showing>99 per cent similarity were retrieved by Nucleotide Basic Local Alignment Search Tool (BLAST-N) program available at National Center for Biotechnology Information (NCBI) (*www.ncbi.nlm.nih.gov/BLAST*). The sequences were submitted to NCBI and accession numbers and number of base pairs amplified are indicated in Table 13. The result of phylogenetic analysis revealed that the bacterial isolates resembled with many reference sequences existing in the global bacterial gene pool and accordingly they were identified on the basis of maximum sequence similarity. The rhizobacterial isolate WI 90 was identified as *Bacillus licheniformis* strain WI 90. Similarly, isolate WI 62 was identified as*Bacillus tequilensis* strain WI 62, isolate WI 36 as *Bacillus cereus* strain WI 36, isolate WI 63 as *Bacillus subtilis* strain WI 63and isolate WI 65 as *Bacillus subtilis* strain WI 65. The isolate WI 12 was identified as *Micrococcus luteus* strain WI 12, isolate WI 41 as *Micrococcus luteus* strain WI 41 and isolate WI 80 as *Micrococcus luteus* strain WI 80.The isolates WI 11 was identified as *Micrococcus* sp. strain WI 11 and isolate WI 91 as *Micrococcus* sp. strain WI 91. The isolate WI 60 was identified as *Micrococcus yunnanensis* strain WI 60 and isolates WI 30 as *Micrococcus yunnanensis* strain WI 30.All the above rhizobacterial species are reported for the first time from walnut rhizosphere; though there is one report from Jammu & Kashmir wherein walnut rhizobacteria have been described up to genera level only.

Table 12: Performance score of rhizobacterial isolates based on various plant growth promoting attributes

Isolate	PSOL	S pdn	IAA pdn	GA pdn	Chitinase activity	HCN	Ammonia	Antifungal activity					Total
	10	10	10	10	10	10	10	2	2	2	2	2	80
WI 1	4.16	1.74	4.60	4.89	0	2.5	5.0	0	0	0	0	0	22.89
WI 2	3.68	3.01	3.83	3.44	3.67	5.0	2.5	0.40	0	0.48	0.72	0.76	27.49
WI 3	4.32	3.82	0	6.58	3.21	5.0	2.5	0.30	0	0.85	0.98	0.71	28.27
WI 4	3.46	4.54	1.38	0	4.07	7.5	7.5	0.20	0.51	0	0	0.51	29.67
WI 5	3.78	3.16	0	1.83	0	5.0	7.5	0	0	0	0	0	21.27
WI 6	3.20	0	3.33	0	0	2.5	5.0	0	0	0	0	0	14.03
WI 7	2.46	0	0	0	0	7.5	5.0	0.42	0.26	0	0.93	0	16.57
WI 8	2.98	4.59	4.33	1.53	0	0	2.5	0	0	0	0	0	15.93
WI 9	4.07	5.21	0	0	0	5.0	0	0	0	0	0	0	14.28
WI 10	2.72	0	0	5.3	0	0	2.5	0	0	0	0	0	10.52
WI 11	5.25	6.83	8.0	9.18	7.89	5	7.5	1.08	1.35	1.81	1.33	1.17	56.39[8]
WI 12	6.57	10	7	7.80	5.97	10	7.5	0.91	0.91	1.01	1.56	1.44	60.67[6]
WI 13	3.58	5.43	0	0	3.57	5.0	2.5	0.31	0	0	0.84	0.87	22.1
WI 14	3.91	4.52	0	0	3.16	7.5	0	0.45	0.49	0	0	0	20.03
WI 15	1.34	0	2.48	4.93	6.60	5.0	5.0	0	0	0	0	0	25.35
WI 16	2.56	5.32	0	0	5.86	5.0	0	0	0	0	0	0	18.74
WI 17	2.46	0	0	0	3.93	5.0	5.0	0	0	0	0	0	16.39
WI 18	1.98	5.21	3.83	4.20	2.92	5.0	5.0	0	0.94	0	0	0	29.08
WI 19	3.26	3.93	0	0	0	7.5	2.5	0	0	0.60	0	0	17.79
WI 20	1.76	0	4.75	0	0	5.0	5.0	0	0	0	0	0	16.51
WI 21	2.62	0	0	3.57	0	10.0	5.0	0	0	0	1.14	0	22.33
WI 22	2.24	3.74	3.33	0	0	7.5	2.5	0	0	0	0	0.40	19.71
WI 23	2.88	0	0	0	0	0	2.5	0	0	0	0	0	5.38

Contdd…

Table 12: Contdd..

WI 24	2.88	5.01	0	0	0	0	2.5	0	0	0	0	0	10.39
WI 25	1.92	0	3.33	5.32	0	2.5	2.5	0	0	0	0	0	15.57
WI 26	2.30	0	0	0	0	2.5	0	0	0	0	0	0	4.80
WI 27	1.76	3.38	4.53	4.24	0	0	0	0	0	0	0	0	13.91
WI 28	2.08	0	0	0	0	2.5	0	0	0	0	0	0	4.58
WI 29	2.88	0	1.66	3.67	0	2.5	0	0	0	0	0	0	10.71
WI 30	5.92	6.50	7.0	7.04	5.39	5.0	10	0.77	0.63	1.68	1.55	1.57	53.05[11]
WI 31	3.71	0	0	0	0	2.5	2.5	0	0	0	0	0	8.71
WI 32	4.13	0	1.66	0	0	5.0	2.5	0	0	0	0	0	13.29
WI 33	2.75	5.38	0	4.83	0	5.0	2.5	0	0	0	0	0	17.96
WI 34	3.01	0	3.0	0	0	0	0	0	0	0	0	0	6.01
WI 35	1.34	0	0	0	0	5.0	2.5	0	0	0	0	0	8.84
WI 36	5.76	6.43	10	9.03	6	10	10	1.01	2	1.47	1.67	1.03	64.40[3]
WI 37	1.53	0	0	2.21	3.72	5.0	0	0.42	0.67	0	0	0	13.55
WI 38	1.85	0	0	0	3.10	5.0	0	0.76	0	0	0.81	0.35	11.87
WI 39	2.94	5.43	5.0	2.37	0	0	5.0	0	0	0	0	0	20.74
WI 40	0	0	5.33	0	0	0	7.5	0	0	0	0	0	12.83
WI 41	5.76	8.25	10	6.88	6.90	7.5	7.5	0.61	0.79	1.52	1.14	0.94	57.79[7]
WI 42	2.30	0	4.56	0	0	5.0	2.5	0	0.38	0	0	0	14.74
WI 43	2.46	0	0	0	0	0	5.0	0	0	0	0	0	7.46
WI 44	2.62	8.26	0	1.95	6.23	7.5	5.0	0.31	0	0.56	0	0	32.43
WI 45	0	0	3.88	0	0	2.5	2.5	0	0	0	0	0	8.88
WI 46	1.18	3.30	0	6.10	0	2.5	2.5	0	0	0	0	0	15.58
WI 47	1.50	0	4.33	0	0	2.5	0	0	0	0	0	0	8.33
WI 48	2.66	4.04	0	0	0	2.5	0	0	0	0	0	0	9.2
WI 49	2.46	0	2.0	2.98	0	2.5	5.0	0	0	0	0.67	0	15.61

Contdd…

Table 12: Contdd..

WI 50	2.59	5.14	4.0	0	3.62	5	5	0.26	0	0.87	0	0.76	27.24
WI 51	1.98	5.14	4.0	0	2.97	5.0	2.5	0.45	0.52	0.56	1.08	0.99	25.19
WI 52	2.17	4.70	0	5.39	4.32	2.5	2.5	0	0	0	0	0	21.58
WI 53	2.75	0	0	0	4.77	2.5	2.5	0	0	0	0	0	12.52
WI 54	4.23	3.96	4.66	0	5.17	2.5	5.0	0	0	0	0	0	25.52
WI 55	3.71	0	0	6.68	0	0	5.0	0	0	0	0	0	15.39
WI 56	0	5.10	0	0	0	0	2.5	0	0	0	0	0	7.6
WI 57	0	0	2.0	0	0	2.5	2.5	0	0	0	0	0	7.0
WI 58	2.27	4.77	0	0	0	2.5	2.5	0	0	0	0	0	12.04
WI 59	3.14	0	1.31	0	0	5.0	2.5	0	0.52	0	0	0	12.47
WI 60	5.51	6.06	9.33	7.50	6.48	10	5	1.13	0.88	0.92	1.89	1.10	55.80[9]
WI 61	0	0	5.23	6.88	0	7.5	10.0	0	0	0	0	0	29.61
WI 62	7.11	8.19	7	9.18	8.31	10	10	1.0	1.18	2.0	1.68	1.21	66.86[2]
WI 63	4.87	6.12	7.33	9.49	10	7.5	10	.96	1.65	1.62	2	.87	62.41[4]
WI 64	2.5	0	4.0	0	0	2.5	0	0	0	0	0	0	9.0
WI 65	7.75	6.92	7.33	7.34	8.25	7.5	10	0.93	1.31	1.67	1.31	2.0	62.31[5]
WI 66	0	0	5.16	5.42	0	2.5	2.5	0	0	0	0	0	15.58
WI 67	1.69	4.41	0	2.97	0	0	2.5	0	0	0	0	0	11.57
WI 68	3.39	5.24	0	0	0	0	2.5	0	0	0	0	0	11.13
WI 69	2.98	0	1.83	0	0	2.5	2.5	0	0	0	0	0	9.81
WI 70	0	2.82	0	1.92	0	0	2.5	0	0	0	0	0	7.24
WI 71	1.92	0	2.75	0	0	2.5	5.0	0	0	0	0	0	12.17
WI 72	0	2.79	0	0	0	2.5	5.0	0	0	0	0	0	10.29
WI 73	3.30	0	0	0	3.58	7.5	5.0	0.22	0	0	0	0	19.6
WI 74	3.58	3.60	1.40	6.81	0	5.0	5.0	0	0	0	0	0	25.39
WI 75	0	0	1.5	4.70	0	5.0	2.5	0	0	0	0	0	12.2

Contdd…

Table 12: Contdd..

WI 76	1.34	3.52	3.16	3.21	0	2.5	2.5	0	0	0	0	0	16.23
WI 77	0	0	0	3.62	0	5.0	2.5	0	0	0	0	0	11.12
WI 78	1.53	4.11	2.56	0	0	5.0	2.5	0	0	0	0	0	15.7
WI 79	0	4.22	0	0	0	2.5	0	0	0	0	0	0	6.72
WI 80	5.19	5.88	7	9.89	5.79	7.5	5	0	0	0	0	0	46.25[12]
WI 81	0	0	0	2.33	4.41	2.5	0	0	0	0	0	0	9.24
WI 82	0	3.67	0	0	3.35	2.5	0	0	0	0	0	0	9.52
WI 83	2.5	3.12	0	0	4.18	2.5	2.5	0	0	0	0	0	14.8
WI 84	2.59	0	3.93	0	4.91	2.5	2.5	0	0	0	0	0	16.43
WI 85	0	4.52	0	0	0	7.5	2.5	0	0	0.75	0.76	0	16.03
WI 86	2.75	2.94	3.83	5.43	0	5.0	0	0	0	0	0	0.65	20.6
WI 87	1.34	0	1.1	0	0	5.0	0	0	0.58	0	0	0	8.02
WI 88	1.73	3.85	4.66	5.66	0	2.5	2.5	0	0	0	0	0	20.9
WI 89	0	0	1.78	3.89	0	5.0	2.5	0.43	0	0	0.60	0	13.42
WI 90	10	9.18	6.33	10	7.6	10	7.5	2	0.65	1.54	1.38	1.11	67.29[1]
WI 91	8.55	5.51	5.33	9.94	5.74	10	5	0	0.69	1.14	0.79	1	53.69[10]
WI 92	0	0	2.0	6.96	0	5.0	5.0	0	0	0	0	0	18.96
WI 93	0	0	4.0	5.47	0	0	2.5	0	0	0	0	0	11.97
WI 94	2.82	0	0	0	0	2.5	2.5	0	0	0	0	0	7.82
WI 95	6.15	4.11	4.66	0	0	2.5	2.5	0	0	0	0	0	19.92
WI 96	0	0	0	3.75	0	2.5	2.5	0	0	0	0	0	8.75
WI 97	0	0	0	6.12	0	0	5.0	0	0	0	0	0	11.12
WI 98	1.92	3.67	1.66	0	0	5.0	5.0	0	0	0.60	0	0	17.85

Psol: phosphate solubilization, S pdn: siderophore production, IAA pdn: Indole-3-acetic acid production, GA pdn: gibberellic acid production

Numbers as superscripts denote the rank of that isolate

Table 13: Molecular characterization of selected rhizobaterial isolates based on16s rRNA sequencing (submitted to NCBI, USA)

Rhizobacterial isolates	Accession number	Isolate identified	No. of base pairs amplified
WI 91	KY777463	*Micrococcus* sp. strain WI91	1542
WI 60	KY777460	*Micrococcus yunnanensis* strain WI60	1584
WI 11	KY777456	*Micrococcus* sp. strain WI11	1500
WI 80	KY777455	*Micrococcus luteus* strain WI80	1542
WI 12	KY777454	*Micrococcus luteus* strain WI12	1529
WI 30	KY777453	*Micrococcus yunnanensis* strain WI30	1512
WI 41	KY777452	*Micrococcus luteus* strain WI41	1540
WI 36	KY777451	*Bacillus cereus* strain WI36	1484
WI 90	KY777445	*Bacillus licheniformis* strain WI90	1557
WI 65	KY777444	*Bacillus subtilis* strain WI65	1555
WI 62	KY777443	*Bacillus tequilensis* strain WI62	1571
WI 63	KY777442	*Bacillus subtilis* strain WI63	1466

Chapter-5

DISCUSSION

Exploring microbial and plant diversity from unusual and unexplored habitats offers new prospects for bio-prospecting which might be exploited for future biotechnological interventions (Tamilarasi *et al*., 2006). Soil microorganisms are integral component of diverse habitat, fulfilling critical roles in nutrient cycling, maintenance of soil health and regulation of plant growth. A very special ecological niche is present around the roots of a plant that supports a group of metabolically versatile and active microorganisms (Raaijamakers, 2001; Upadhyay and Srivastava, 2010; Chauhan *et al*., 2014). Keeping this in view, the importance of walnut in temperate areas of North Western Himalaya it was considered worthwhile to assess the diversity of plant growth promoting bacteria in walnut rhizosphere. The soil attached to root system is a hotspot for microbial abundance and their intense activity is owing to the presence of root exudates and rhizo-depositions around the plants (Smalla *et al.,* 2006).

Significant positive correlation of rhizosphere bacterial population with soil pH (r=0.90), EC (r=0.49), organic carbon (r=0.70), available N (r=0.73), available P (r=0.65) and available K (r=0.87) was observed. These results are in conformity with Chatli *et al.* (2008). Fierer and Jackson (2006) considered pH as the best predictor of soil bacterial diversity and richness. The results revealed that rhizosphere bacterial population in the sites studied in four districts of Kashmir valley showed great variation. Chauhan *et al.* (2014) had also reported diverse PGPR population from *Valeriana jatamansi* collected from five different sites of Chamba district in Himachal Pradesh (India).

The bacterial population from walnut rhizosphere soil taken from different sites exhibited variable growth on different test media *viz.,* nutrient agar, chromo-azurol S medium (CAS), Pikovskaya's (PVK) medium and nitrogen-free medium with better response in nutrient agar medium. Root exudates play a key role in

plant-microbe interaction by influencing the structure and function of soil microbial communities (Shi *et al.*, 2011). Significant variations observed in the rhizosphere population of phosphate solubilizers on PVK medium, nitrogen fixers on nitrogen-free medium and siderophore producers on CAS agar medium may be attributed to the plant source, root exudates produced and soil and environmental conditions, which influence the association of bacterial population in rhizosphere of medicinal plants (Rathaur *et al.*, 2012). The extent of response of root exudation to microorganisms or a response by microorganisms to root exudation suggests a certain degree of co-evolution between the plant and the microorganisms inhabiting rhizosphere (Nannipieri *et al.*, 2007). The rhizosphere is a very complex environment in which the effects of plant on soil microorganisms and vice versa are interactive and interdependent (Mukerji *et al.*, 2006). Isolation of different bacterial groups from rhizosphere samples from different plants on specific media have been reported earlier by Ahmed *et al.* (2014).

The rhizobacterial population in soil samples, collected from the selected sites in four districts of Kashmir valley, on nutrient agar medium ranged from 25.0x 10^5 to 95.0×10^5cfu/g. This is in agreement with Lamado *et al.* (2013) who have suggested that rhizosphere bacterial population can vary between 7.3 × 10^4 to 1.3 × 10^5 cfu/g. The rhizobacterial isolates showed wide morphological variation. Among the 98 isolates, 65 bacterial isolates were Gram positive rods and had variable colony morphology. The dominance of genus *Bacillus* in rhizosphere of several crops has been reported earlier also (Illmer and Schinner, 1992; Motsara *et al.*, 1995; Tilak and Reddy, 2006). The majority of rhizobacterial associated with *Salix alba* from Lahaul and Spiti valleys of Himachal Pradesh were reported to be Gram positive rods (Chatli *et al.*, 2008). Agarwal and Agrawal (2013) reported similar wide morphological variation in the bacterial isolates from tomato rhizosphere. They also observed variation in colony shape *viz.*, irregular, circular, etc., although Gram positive bacilli with entire edge

and creamy white appearance were dominant; which is contrary to our finding where rods and cocci type cells were observed instead of rods only. Our findings are also supported by Naveed *et al.* (2014) who found rods, cocci and coco-bacillus dominant in rhizosphere. All the isolates were identified upto genus level through their biochemical characters determined as per the procedures described in *Bergey's Manual of Determinative Bacteriology.* Sixty-five isolates were Gram positive rods, endospore formers, positive for catalase test, citrate utilization, starch hydrolysis and Voges Proskauer test and negative for indole test, acid production, oxidase, gas production, pigmentation and H_2S production and based on these observations were placed under genus *Bacillus*. Twelve isolates were placed in genus *Azotobacter* as they were Gram negative rods and positive for catalase test, oxidase test, citrate utilization, starch hydrolysis, pigment production and H_2S production and negative for indole test methyl red, Voges Proskauer and gas production. Seventeen isolates tentatively belonged to genus *Micrococcus* as they were Gram positive cocci and positive for oxidase test, catalase test and starch hydrolysis but negative for citrate utilization, indole test, methyl red, Voges Proskauer, gas production, pigment production and H_2S production. Four isolates probably belonged to genus *Pseudomonas* as they were Gram negative rods and positive for oxidase test, catalase test, pigment production, citrate utilization and gas production and negative for starch hydrolysis, Voges Proskauer test, methyl red test and indole test.

The findings are supported by Vedan *et al.* (2010) who reported predominance of *Bacillus* sp. and *Pseudomonas* sp. in plant rhizosphere. Vega *et al*. (2005) observed that high number of bacteria isolated from coffee rhizosphere belonged to genera *Bacillus*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Escherichia*, *Micrococcus*, *Pantoea*, *Pseudomonas*, *Serratia* and *Stenotrophomonas*. In conformity to our observations, Panchal and Ingle (2011) isolated five bacterial species from safed musli (*Chlorophytum borivilianum*) and

identified them as *Bacillus pumilus*, *B. subtilis*, *B. megaterium*, *Pseudomonas mendocina* and *Staphylococcus pasteuri*.

P-solubilization is considered as one of the most important attributes of plant growth promoting rhizobacteria (Patel *et al*., 2008; Yasmin *et al*., 2012). Isolate WI 90 showed a maximum P-solubilization of 312 mg/l; while WI 46 depicted minimum (37 mg/l) activity. A significant variation in P-solubilization was noticed in different rhizobacterial isolates. The correlation coefficient of r=0.09 was found between plate assay and liquid assay. Similar to our observation, Kumar *et al.* (2012) found no correlation between P-solubilization in solid and liquid media.

Siderophore production was detected in 54 isolates i.e.55.1 per cent isolates which is in agreement with Baharucha *et al.* (2013) who detected siderophore production in 41 per cent isolates. Also, amongst the siderophore yielding isolates, isolate WI 12 produced highest per cent siderophore units of 27.21 and isolate WI 1 produced minimum of 4.75 per cent siderophore unit. Significantly highest and lowest siderophore zones were produced by isolates WI 36 (16.3 mm) and WI 86 (3.0 mm). Shobha and Kumudhini (2012) reported various bacterial isolates as efficient siderophore producers and reported that *Bacillus* isolate JUMB7 produced 10 per cent siderophores. Pal and Gokarn (2010) reported that *Klebsiella* sp. were able to produce 3.22 and 11.99 per cent siderophore units which falls within our observed range. Kaushal and Kaushal (2011) reported that isolate MK7 produced a zone of 13.33mm. Our findings are also supported by Prasad and Dagar (2014).

The production of IAA is an important plant growth promoting trait in PGPR. Higher auxin level impairs plant defense mechanisms making colonization easier. It stimulates both rapid (increases cell elongation) and long term (cell division and differentiation) responses in plants. IAA production is widespread among soil and plant associated bacteria and its biosynthesis in an integral core trait of symbiotic species within genera of *Rhizobium*, *Bradyrhizobium* and

Nostoc and other plant-associated PGPR (Shah *et al*., 2013). IAA is considered an important signal molecule in the regulation of plant development. Among 98 isolates, 52 isolates showed IAA production on Luria Bertani broth after 72 hours of incubation, with a maximum of 30.0 µg/ml IAA produced by isolate WI 36 and a minimum of 4.15 µg IAA/ml by isolate WI 42. Our results are in agreement with Beneduzi *et al.* (2008) who reported that many *Bacillus* sp. and *Paenibacillus* sp. produces IAA in Luria Bertani broth. Our results are in agreement with Kaur and Sharma (2013) who reported IAA production in the range of 61.58 to 70.05 µg/ml. Husain (2003) also reported a similar range of IAA production (2.09 to 33.28 µg/ml). Shobha and Kumudini (2012) reported that *Bacillus* isolates produced IAA in varying quantities from 35 to 217 µg/ml. Ashrafuzzaman *et al.* (2009) reported that IAA production by PGPR varies among different species and strains and is influenced by cultural condition, growth stage and substrate availability. Khin *et al.* (2012) reported that *Bacillus* sp. produced IAA in the range of 53.1 to 71.1 ppm under optimum conditions whereas *Serratia* sp. were found poor IAA producers.

Of the isolated rhizobacteria, 48 isolates were gibberellic acid (GA) producers with maximum production of 65.33 µg/ml by isolate WI 90 and a minimum of 10.0 µg/ml by isolate WI 8. These observations are in agreement with Karakoc and Aksoz, (2006) who optimized cultural parameters for GA production by *Pseudomonas* sp., isolated from wastes of processed olive, with highest GA production (250.06 mg/l) obtained in nutrient broth (pH 7.0) incubated at 30ºC for 72 hours on a rotatory shaker and in dark conditions.

Among different lytic enzymes chitinases are particularly useful in agriculture as biocontrol agents against various fungal pathogens owing to their ability to hydrolyze chitinous fungal cell wall (Maisuria *et al*., 2008; Chaiharn and Lumyong, 2009; Suresh *et al*., 2010; Wahyudi *et al*., 2011). In present study, 35 isolates (35.7%) exhibited chitinase activity and isolate WI 63 showed maximum production of 30.47 units/ml and isolate WI 8 showed minimum production of

8.90 units/ml. Our results are in agreement with Bhatt and Vyas (2014) who isolated and screened bacterial isolates for various PGPR activities *viz.*,P-solubilization and production of IAA, HCN, chitinase, amylase, etc. They found that 27 per cent strains produced lipase, 53.0 per cent amylase and 50.0 per cent chitinase. Our results are in agreement with Wang *et al*. (2015) who reported the chitinase activity in 22 out of 72 bacterial isolates. Chitinase production is induced in a colloidal chitin containing environment (Gupta *et al.*, 1995; Mahadevan and Crawford, 1997). Our findings are supported by Dhar and Kaur (2010) who observed highest chitinase activity of (2.64-35.08 U/ml) in 17 bacterial isolates after 120 hours of incubation.

Hydrogen cyanide is a secondary metabolite produced commonly by rhizobacteria. The HCN production reportedly is a common trait of *Bacillus* (88.9%) and *Pseudomonas* (50%) in rhizospheric soil (Heydari *et al.*, 2008). HCN is postulated to play a role in biological control of pathogens (Defago *et al.*, 1990). In present study, 82 isolates i.e.83.67 per cent showed the ability to produce HCN though in varying amounts. Noumavo *et al.* (2015) reported that of the 15 rhizo-bacterial isolates from maize 86.7, 80.0 and 60.0 per cent produced ammonia, HCN and IAA, respectively. Antibiosis has widely been accepted as a PGP property (Cazorla *et al.,* 2006; Pliego *et al.,* 2011) and siderophores, HCN and antibiotic production plays vital role in disease suppression (Voisard *et al.,* 1989).

In present study 80.6 per cent isolates produced ammonia which is in line with Chaiharn and Lumyong (2009) who reported the production of ammonia by P-solubilizing microorganisms and found more than 64 per cent isolates able to produce ammonia. Ahmad *et al.* (2006) too reported PGPR able to produce ammonia. Our findings are in conformity with Malleswari and Bagyanarayana (2013) who isolated 219 rhizobacterial strains from different locations of Andhra Pradesh and reported that 201 bacterial isolates (91.7%) showed ammonia production, 186 (84.9%) IAA production, 43 (19.6%) phosphate solubilizing

activity, 58 (37.8%) HCN production and 43 (19.6%) antifungal activity against *Macrophomina phaseolina*. Four of these isolates were identified as *Pantoea* sp., *Bacillus* sp. and *Pseudomonas* sp.

Rhizobacteria can antagonize phytopathogens through competition, antibiotics production or lytic enzymes secretion (Van Loon and Bakker, 2003) that make them a potent tool for reducing damages through prevention of deleterious effects of phytopathogens. The main bacteria were the representatives of genera *Pseudomonas*, *Bacillus* and *Streptomyces*. In present study 23, 20, 19, 21 and 20 isolates were found to be antagonistic to *Demotophora necatrix, Alternaria solani, Fusarium oxysporum, Pythium aphanidermatum* and *Phytophthora capsici,* respectively, with maximum inhibition in each respective case by isolate WI 90 (66%), WI 63(55.6%), WI 62 (43.8%),WI 63 (45.5%) and WI 65 (49%). These rhizobacteria on morpho-biochemical and molecular characters were later identified as *Bacillus licheniformis* strain WI 90, *Bacillus subtilis* strain WI 63, *Bacillus tequilensis* strain WI 62, *Bacillus subtilis* strain WI 63 and *Bacillus subtilis* strain WI 65. In the present study, most of the rhizobacterial isolates exhibiting maximum inhibition of various soil borne pathogens belonged to genus *Bacillus*. A relatively wide range of antagonistic performances among the isolates was observed in present study and similar observation have been previously been reported in other studies involving the same or different fungi (Idris *et al*., 2007; Calvo *et al*., 2010). Our findings are in agreement with many studies showing the production of maximum antifungal compounds at the end of exponential phase because the production of peptide antibiotic usually begins at late log phase of growth and continues with stationary phase as found in case of *Bacillus megaterium* (Sultana *et al*., 2004) and *Bacillus* spp. (Sarkar and Paules, 1972; Flio *et al.*, 2004). Almost all the selected bacterial isolates showed antifungal activity against *F. oxysporum*, *P. aphanidermatum, P. capsici, A. solani* and *D. necatrix*. This is in agreement with those of Virdi *et al.* (1994), Quesado *et al.* (1996); Khan and Khan (2002); Ramirej *et al.* (2004) and

Cazorla *et al.* (2007) who reported that *Bacillus* strain UCR 236 and *Bacillus* spp. produced antifungal substances against a number of mycelial fungi. Other workers have reported that growth inhibition of phytopathogens was due to the production of some specific siderophores, antibiotics, secondary metabolites or hydrolytic enzymes (Buysens *et al*., 1996; Kirner *et al*., 1998; Srivastav *et al*., 2004). Similar to our observations, Geetha *et al.* (2014) isolated and screened 180 PGPR strains from green gram rhizosphere for their antifungal activity against *Macrophomina phaseolina*, *Colletotrichum capscici*, *Rhizoctonia solani* and *F. oxysporum.* They evaluated 20 antagonistic isolates for PGP-traits, seed germination ability, extracellular enzyme production and salt and temperature tolerance and found 6 isolates most effective in enhancing the growth of green gram due to the production of ammonia, IAA and HCN, phosphate solubilization and antifungal activity against pathogenic fungi. These isolates inhibited the growth of *F.oxysporum*, irrespective of the antagonistic method used. The clear zone of inhibition produced in *in-vitro* experiment is an indicative of antibiosis by biocontrol agent against the fungal pathogens. In conformity to our study, Dalal and Kulkarni (2013) isolated 31 bacteria belonging to *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Acetobacter*, *Burkholderia*, *Rhizobium* and *Xanthomonas* from soybean and screened them *in vitro* for antagonistic activity against soil-borne fungal pathogens *viz*., *R. solani*, *F. oxysporum*, *Sclerotium rolfsii*, *Collectotrichum truncatum*, *Macrophomina phseolina* and *Alternaria alternata*. Five isolates, JDB 3 (*Pseudomonas* spp.), JDB 5 (*Pseudomonas* spp.), JDB 9 (*Bacillus* spp.), JDB 11 (*Bacillus* spp.) and JDB 14 (*Bacillus* spp.) were found to exhibit maximum number of PGP traits *viz*., production of plant growth regulators (auxins, gibberellins and cytokinins), siderophores and HCN, so were considered as efficient isolates possessing dual abilities *i.e.* antagonistic and plant growth promotion with the view of plant health and productivity. Kim *et al.* (1997) reported that a potential biocontrol strain *Bacillus* spp. L324-92 has a broad spectrum inhibitory activity against take all, root rot caused by *R. solani*, *Pythium irregulare* and *Pythium ultimum.* In agreement to our observations,

Sharma *et al.* (2015) reported biocontrol potential of *Bacillus* species against *Phytophthora capsica* and *Fusarium oxysporum* and found that under *in vitro* conditions 9 isolates (45%) inhibited *P.capsici* while ten isolates (50%) inhibited *F. oxysporum.* Jamali *et al.* (2004) and Sharma *et al.* (2015) reported that under greenhouse conditions the rhizobacterial isolates belonging to genera *Bacillus* and *Pseudomonas* were effective against Fusarium wilt of chickpea and collar rot disease caused by *Phytophthora capsici.*

The best twelve isolates in terms of their PGP traits were identified by amplifying their 16S rRNA genes. The rhizobacterial isolate WI 90, identified by its maximum homology and phylogeny with the global reference sequences, was identified as *Bacillus licheniformis* strain WI 90. The strain name was given to represent the walnut rhizosphere isolate (WI) and isolate number. The isolates identified were: one strain each of *Bacillus tequilensis* (strain WI 62), *Bacillus cereus* (strain WI 36) and *Bacillus licheniformis* (strain WI 90); two strains each of *Bacillus subtilis* (strain WI 63 and strain WI 65), *Micrococcus* sp. (strain WI 11 and strain WI 91) and *Micrococcus yunnanensis* (strain WI 60 and strain WI 30); and three strains of *Micrococcus luteus* (strain WI 12, strain WI 41and strain WI 80). These all species are reported for the first time from walnut rhizosphere, although previously Dar *et al.* (2009) have reported the presence of genera *Azotobacter, Azospirillum, Bacillus, Pseudomonas, Aspergillus* and *Penicillium* in walnut rhizosphere but they have not identified them upto species level. Perusal of the literature has revealed that no work has been conducted on the rhizobacteria of walnut based on molecular characterization, especially in India and North-Western Himalayan region and the present work appears first of its kind conducted in Jammu & Kashmir state (Jammaludin *et al*., 2004). *Bacillus subtilis*, *B. cereus* and *B. licheniformis* have earlier been reported as culturable bacterial endophytes of saffron in Kashmir (Sharma *et al*., 2015) while *Bacillus tequilensis* has been isolated from the water samples of Manasbal lake of Kashmir by Shafi *et al*. (2017). There is no report of *Micrococcus luteus, Micrococcus* sp. and

Micrococcus yunnanensis from the state of Jammu and Kashmir. *Micrococcus luteus* though has been reported as being associated with the rhizosphere of black pepper by Dinesh *et al.* (2014) from Kerala (India) and *Micrococcus* sp. found as novel plant growth promoting rhizobacteria associated with cowpea named as *Micrococcus* sp. NII-0909 by Dastager *et al.* (2010) from Trivandrum (India). There is no report of *Micrococcus yunnanensis* as rhizobacteria from India, although it has been reported as an endophyte of *Catharanthus roseus* wherein it has been evaluated for production of antibiotics against antibiotic resistant pathogens (Rajan and Jadeja, 2017) and not for plant growth promoting traits. Hence, *Micrococcus yunnanensis* as PGPR microbe is reported for the first time from India. Worldwide, there are two reports of *M. yunnanensis* being PGPR, one is from Iran (Ghavami *et al.*, 2017) where 45 isolates were obtained from the rhizosphere of *Brassica napus* and evaluated for siderophore production. The highest siderophore producing isolates were molecularly characterized based on 16S rRNA sequence analysis and were identified as *M. yunnanensis* YIM 65004 (T) and *Stenotrophomonas chelatiphaga* LPM-5 (T). These two isolates were also studied for plant growth promoting effect on canola and maize plants and showed increased grain weight and iron content of roots and shoots in comparison to control under greenhouse conditions and another study is from Korea by Siddikee *et al.* (2010) in which 140 halotolerant bacterial strains were isolated from the rhizosphere of six naturally growing halophytic plants in the vicinity of Yellow Sea, near the city of Incheon in the Republic of Korea. Based on multiple plant growth promoting traits only 36 halotolerant bacterial strains were analyzed by 16S rRNA gene sequencing which revealed that they belonged to 10 different bacterial genera: *Bacillus*, *Brevibacterium*, *Planococcus*, *Zhihengliuella*, *Halomonas*, *Exiguobacterium*, *Oceanimonas*, *Corynebacterium*, *Arthrobacter* and *Micrococcus.* Three of the bacteria *viz*., *Brevibacterium epidermidis* RS15 *Micrococcus yunnanensis* RS222 and *Bacillus aryabhattai* RS341 showed more than 40 per cent increase in root elongation and dry weight when compared with uninoculated salt stressed canola seedlings. The results suggested that these

bacteria have a real potential to enhance plant growth under saline stress. Aravind *et al.* (2009) isolated a total of 74 bacteria belonging to six different genera *viz*., *Bacillus* sp. (22 strains), *Pseudomonas* sp. (20 strains), *Serratia* (1 strain), *Arthrobacter* sp. (15 strains), *Micrococcus* sp. (7 strains) and *Curtobacterium* sp. (1 strain). *Bacillus* species were also isolated by Figueiredo *et al.* (2009) from Brazilian sweet corn on the basis of their sequencing of 16S ribosomal gene and amongst the 42 isolates they identified *Bacillus subtilis* and *B. pumilus* as the most frequently encountered species (15 and 12 isolates, respectively) along with *B. licheniformis* (7 isolates), *B. cereus* (5 isolates) and *B. amyloliquefaciens* (3 isolates).

Chapter-6

SUMMARY AND CONCLUSION

The present study was aimed to explore the diversity of bacterial population colonizing the rhizosphere of walnut (*Juglans regia*) and assess its plant growth promoting potential for development of microbial inoculants for use in stressed soils. During survey, surface and rhizosphere soil samples were collected from 36 sites in 12 blocks of four walnut growing districts of Kashmir valley *viz*., Shopian, Budgam, Baramulla and Kupwara. The soils were assessed for physicochemical characteristics and rhizosphere soils for rhizobacterial population. The organic carbon content of all the sites was found in the range of 0.62 to 1.65 per cent with maximum content noticed in Krewa-manul site (1.65%) and minimum in Palhalan (0.62%). The available nitrogen ranged between (52.0 to 540.0 kg/ha with maximum value observed at Krewa-manul (Shopian) and minimum at Gonipora (Baramulla) and phosphorus contents ranged between 17.8 to 28.5 kg/ha with highest value observed at Krewa-manul (Shopian) and minimum at Kababmarg (Kupwara). The highest available potassium (205.7 kg/ha) was found at Warsun (Kupwara) and minimum (90.5 kg K/ha) at Gonipora. The minimum value of pH (5.9) was found in soil samples collected from Palhalan (Baramulla). The EC values ranged between 0.1 to 0.6 dS/m. Among 36 sites 13 sites had sity clay loam texture, 13 sites had clay loam texture, 8 sites had loam texture and 2 sites had silty loam texture. The rhizo-bacterial population was maximum at Krewa-manulsite (95.0 $\times 10^5$ cfu/g soil) on NA and minimum at Palhalan (25.0$\times 10^5$ cfu/g soil).

Modified replica plating technique was adopted to differentiate the population of P-solubilizers, siderophore producers and nitrogen fixing bacteria from the total plate population of all the soil samples. Ninety eight morphologically dissimilar bacterial isolates were selected and characterized on the basis of Gram staining, colony and cell morphology. Gram positive bacteria were most dominant group amongst the isolates. Most of the colonies were

circular in form having entire margins and convex elevation. The isolates were biochemically characterized as per the procedures described in Bergey's Manual of Determinative Bacteriology, Morpho-biochemical characterization revealed that 65 isolates belonged to genus *Bacillus*, 12 to genus *Azotobacter,* 17 to genus *Micrococcus* and 4 isolates to *Pseudomonas*.

All the isolates were screened for P-solubilization, antifungal activities and production of siderophore, IAA, gibberellic acid, chitinase enzyme, HCN and ammonia. Amongst the 98 rhizobacterial isolates, isolate WI 65 had maximum phosphate solubilizing index (3.40) while isolate WI 17 had minimum PSI (0.70). In liquid PVK medium, maximum P-solubilization was recorded for rhizobacterial isolate WI 90 (312 mg/l) while isolate WI 46 solubilized minimum phosphate (37 mg/l). Fifty four isolates produced siderophore with maximum zone size of 16.3 mm by isolate WI 36 and a minimum of 3.0 mm zone size by isolates WI 72, WI 83 and WI 86. Quantitatively, isolate WI 12 yielded maximum per cent siderophore units (27.21) while isolate WI 1 produced minimum per cent siderophore unit (4.75). Only 58 out of 98 isolates exhibited indole-3-acetic acid producing ability with maximum IAA production of 30 µg/ml by isolates WI 36 and WI 41 and minimum (3.94 µg/ml) by isolate WI 59. Forty eight isolates were positive for gibberellic acid production with maximum GA was production by isolate WI 90 (65.33 µg/ml) and minimum by isolate WI 8 (10.0 µg/ml). The screening of bacterial isolates for chitinase activity revealed that only 35.71 per cent isolates were able to produce chitinase enzyme. The chitinase producing isolates showed average production of 15.71 units chitinase activity/ml with higher chitinase activity depicted by isolate WI 63 (30.47 units/ml) and least by isolate WI 18 (8.90 units/ml). CZ:CS was significantly higher for isolate WI 63 (4.05) and least for isolate WI 50 (1.60). Of the 98 isolates, 82 isolates showed HCN production with seven isolates having very high HCN. Similarly, six isolates showed very high ammonia production while 19 isolates exhibited no ammonia production. All isolates were screened for their antifungal activities against five

fungal pathogens *viz*., *Dematophora necatrix, Alternaria solani, Fusarium oxysporum*, *Pythium aphanidermatum* and *Phytophthora capsici.* Twenty three isolates showed antagonistic activity against *D. necatrix* with isolate WI 90 inflicting highest growth inhibition of (66%) and isolate WI 4 showing least inhibition (7.6%). Twenty isolates were antagonistic to *A. solani* with maximum inhibition (55.6%) by isolate WI 63 and minimum by isolate WI 7 (8.9%). Only 19 isolates inhibited the growth of *F. oxysporum.* The isolate WI62 depicted highest antifungal activity of (43.8% inhibition) and least by isolate WI 2 (10.7% inhibition). Only 21 and 20 isolates were antagonistic to *P. aphanidermatum* and *P. capsici.* Isolate WI 63 showed maximum of 45.5 per cent growth inhibition against *P. aphanidermatum* while isolate WI 49 showed least inhibition (15.4%). Against *P. capsica,* isolate WI 65 exhibited highest growth inhibition (49%) while isolate WI 38 showed minimum growth inhibition (8.8%).

On the basis of maximum plant growth promoting traits, twelve bacterial isolates were further selected for molecular characterization. The phylogenetic analysis revealed that rhizo-bacterial isolates resembled with many reference sequences in global bacterial gene pool and were identified on the basis of maximum sequence similarity. The rhizobacterial isolate WI 90 was identified as *Bacillus licheniformis* strain WI 90. Similarly, isolate WI 62 was identified as *Bacillus tequilensis* strain WI 62, isolates WI 36 as *Bacillus cereus* strain WI 36 and strains WI 63 and WI 65 as *Bacillus subtilis* strain WI63 and strain WI65, respectively. Isolates WI 12,WI 41 and WI 80 were identified as *Micrococcus luteus* strain WI 12, strain WI 41 and strain WI 80, respectively. The isolates WI 60 and WI 30 were identified as *Micrococcus yunnanensis* strain WI 60 and strain WI 30, respectively; and isolates WI 11and WI 91 as *Micrococcus* sp. strain WI 11 and strain WI 91, respectively.

CONCLUSION

From the present study, it is concluded that the rhizosphere of walnut possess diverse and potential microbial association which can be explored as

microbial inoculants for improving the growth of a wide variety of horticultural and other plant species growing under nutrient-stress conditions. These microbes have good adaptability to grow in walnut rhizosphere so are suitable bioagents to control soil-/root-borne diseases as well. Present work for the first time reports the presence of a highly diverse PGPR from walnut rhizosphere which possess ability to solubilize phosphates and produce siderophore, IAA, gibberellic acid, chitinase enzyme, ammonia and HCN. The study elucidates the multifarious role of rhizobacterial isolates, especially *Bacillus licheniformis*, *B.tequilensis*, *B. cereus*, *B. subtilis*, *Micrococcus luteus*, *Micrococcus* sp. and *M. yunnanensis.* Use of these PGPR can be successfully exploited as biofertilizers for sustainable crop production. There is need to develop suitable microbial consortium to offset the microclimatic unfavourable effects on individual species. Also, suitable delivery system needs to be evaluated so that these consortia reach target site without any intense competition for niche and nutrients as well as they remain viable and effective for longer periods.

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Appendix-I

Media

Composition of the media (Atlas, 1995) used for the study:

1. **Nutrient agar (NA)**

Beef extract	:	0.3%
Peptone	:	0.5%
NaCl	:	0.5%
Agar	:	2.0%
pH	:	6.5

2. **Malt extract agar (MEA)**

Malt extract	:	2.0%
Agar	:	2.0%
pH	:	5.6

3. **King's medium B**

Proteose peptone	:	2.0%
K_2HPO_4 anhydrous	:	0.15%
$MgSO_4.7H_2O$	:	0.15%
Glycerol	:	1.5%
Ph	:	7.2

4. **Pikovskaya's (PVK) broth**

Glucose	:	1.0%
$Ca_3 (PO_4)_2$	:	0.5%
$(NH_4)_2 SO_4$	:	0.05%

KCl	:	0.02%
$MgSO_4.7H_2O$	:	0.01%
$MnSO_4$	:	0.0004%
$FeSO_4$	:	0.0002%
Yeast extract	:	0.05%

5. Pikovskaya's agar

Pikovskaya's broth + 2.0% agar

6. Nitrogen free glucose medium (Azotobacter agar)

K_2HPO_4 anhydrous	:	0.1%
$MgSO_4.7H_2O$	:	0.02%
NaCl	:	0.02%
$FeSO_4$	:	0.0005%
Soil extract	:	0.5%
Glucose	:	1.0%
Agar	:	2.0%
pH	:	7.6

7. Luria Bertani (LB) agar

Tryptophan	:	1.0%
Yeast extract	:	0.5%
NaCl	:	0.5%
Agar	:	2.0%
pH	:	7.5

8. **Chromeazurol-S (CAS) agar**

CAS	:	0.006%
HDTMA	:	0.007%
HCl	:	0.002%
$FeCl_3$	:	0.002%
Agar	:	2.0%

9. **Minimal agar**

KH_2PO_4	:	0.3%
Na_2HPO_4	:	0.6%
NaCl	:	0.5%
NH_4Cl	:	0.2%
$Mg(SO_4)_2$	:	0.01%
Glucose	:	0.8%
Agar	:	1.5%
pH	:	7.0
Agar	:	2 %
Ph	:	8

10. Peptone water broth

Peptone	:	10.0
NaCl	:	5.0
Distilled water	:	1000 ml

11. Methyl Red-Voges Proskauer (MRVP) broth

Peptone	:	5.0

KH_2PO_4	:	5.0
Distilled water	:	1000 ml
Glucose, 10% solution	:	50 ml

12. Methyl Red indicator solution

Methyl red	:	0.1
Ethanol	:	300 ml
Distilled water	:	200 ml

13. Simmon's citrate medium composition

NaCl	:	5.0
$(NH_4)H_2PO_4$	:	1.0
K_2HPO_4	:	1.0
$MgSO_4.7H_2O$	:	0.2
Sodium citrate	:	5.0
Distilled water	:	1000 ml
Bromothymol blue (0.2%)	:	40 ml
Agar-agar	:	20.0
Ph	:	6.8

14. Starch minimal agar medium

Starch (soluble)	:	20.0
$(NH_4)_2SO_4$	:	1.0
Yeast extrac	:	5.0
K_2HPO_4	:	1.0
$FeSO_4.7H_2O$	:	0.05

$MgSO_4.7H_2O$	:	0.5
Distilled water	:	1000 ml
Agar-agar	:	20.0

15. Tryptone agar medium

Tryptone	:	5.0
Yeast extract	:	2.5
Dextrose	:	1.0
Bromo Cresol purples	:	0.02
Agar	:	20.0
pH	:	7.0
Distilled water	:	1000 ml

16. Tryptone broth

Tryptone	:	5.0
Yeast extract	:	2.5
Glucose	:	1.0
Distilled water	:	1000 ml
pH	:	6.0

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Agriculture, Division of Plant Pathology

CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Dr. A. K. Bhat, Prof. & Head, Division of Microbiology, SKUAST-J during thesis Viva-Voce examination held on 12-10-2017 have been incorporated in the final manuscript entitled ***"Studies on Plant Growth Promoting Rhizobacteria Associated with Walnut (Juglans regia L.)"*** submitted by **Ms. Shakeela Sofi (Regd. No. 2013-463-D)**

(Dr. G. H. Dar)
Chairman
Advisory Committee