STUDIES ON DIVERSE PLANT GROWTH PROMOTING RHIZOBACTERIA ASSOCIATED WITH RHIZOSPHERE OF KIWI VINES

Thesis

by

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(F-2013-19-M)

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CERTIFICATE-I

This is to certify that the thesis entitled, **"Studies on diverse plant growth promoting rhizobacteria associated with rhizosphere of kiwi vines",** submitted in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE MICROBIOLOGY** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) is a bonafide record of research work carried out by **Ms Malvika Sharma (F-13-19-M)** daughter of Sh Parveen Kumar under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation has been fully acknowledged.

Place : Nauni-Solan
Dated: , 2015

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CERTIFICATE-II

This is to certify that the thesis entitled, **"Studies on diverse plant growth promoting rhizobacteria associated with rhizosphere of kiwi vines",** submitted by **Ms Malvika Sharma (F-13-19-M)** daughter of Sh Parveen Kumar to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.), in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE MICROBIOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"Studies on diverse plant growth promoting rhizobacteria associated with rhizosphere of kiwi vines"** submitted to Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) by **Ms Malvika Sharma (F-13-19-M)** daughter of Sh Parveen Kumar in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE MICROBIOLOGY.**

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I solely claim the responsibility for errors and omissions in this work.

Nauni- Solan
Date: **(Malvika Sharma)**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

%	:	Per cent
µg	:	Micro gram
ºC	:	Degree centigrade
CAS	:	Chromo azarol–S
CFU	:	Colony forming units
cm	:	Centimeter
CRD	:	Completely randomized design
EDTA	:	Ethylenediaminetetraaciticacid
ER	:	Root endosphere
g	:	Gram
h	:	Hour
HCl	:	Hydrochloric acid
HCN	:	Hydrogen cyanide
HDTMA	:	Hexadecyltrimethylammoniumbromide
L	:	Litre
M	:	Molar
MEA	:	Malt extract agar
mg	:	Milligram
min	:	Minute
ml	:	Millilitre
mm	:	Millimeter
mM	:	Millimolar
N	:	Nitrogen
NA	:	Nutrient agar
Nif	:	Nitrogen fixing gene
nm	:	Nano meter
O.D.	:	Optical density
P	:	Phosphorous
PCR	:	Polymerase chain reaction
PGPR	:	Plant growth promoting rhizobacteria
PGRs	:	Plant growth regulators
ppm	:	parts per million
PSB	:	Phosphate solubilizing bacteria
PSF	:	Phosphate solubilizing fungus
psi	:	Per square inch
PVK	:	Pikovskaya's medium
rpm	:	Rotation per minute
RS	:	Rhizosphere soil
sp.	:	Species
TCP	:	Tri-calcium phosphate
UV	:	Ultra violet
w/v	:	Weight by volume
v/v	:	volume by volume

Chapter-1

INTRODUCTION

'Kiwi-fruit' (*Actinidia deliciosa*) is a temperate fruit grown all over the world. As a native to Yangste valley of China, it was originally named as "Yang Tao" and commonly named as "Chinese gooseberry" (Ferguson and Huang, 2007).

Kiwi is a highly acclaimed fruit for its nutritive, medicinal and ornamental value. It is a rich source of Vitamin C and E, sugars and several minerals such as phosphorus, potassium and calcium. Since kiwi plays an important role in human health, organic kiwi production has gained importance and there is an increased interest among farmers in nursery materials for its organic production (Basim and Uzun, 2003).

Kiwi-vines when grown in nurseries often lack rooting ability due to insufficient growth promoting factors present in the environment which has become a serious issue in its cultivation. This can be overcome by the use of fertilizers and application of plant growth promoting regulators such as indole-3-acetic acid, gibberlic acid etc. But increase in cost and hazardous nature of chemical fertilizers has led to the resurgence of interest in the use of biofertlilizers for enhanced environmental stability, crop production and good crop yield.

Biofertilizers are products containing living cells of different types of microorganisms which have the ability to convert nutritionally important elements from unavailable form to available form through biological processes. These plant growth promoting rhizobacteria (PGPR) are also involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turnover and sustainable for crop production (Gupta *et al*. 2015). The use of PGPR has gained importance in obtaining nursery material in various horticultural crops.

A considerable number of bacterial species are able to exert a beneficial effect on plant growth. These bacteria (PGPR) significantly effect the plant growth by providing the host plant with fixed atmospheric nitrogen, solubilization of soil phosphorus compounds, producing biologically active substances such as auxins and other plant hormones and thereby suppressing pathogens with the production of certain antibiotics and siderophores (Stefan, 2012). The utilization of plant growth promoting rhizobacteria (PGPR) may help to

develop an environmentally benign biological approach for managing fungal diseases and enhancing the plant health resulting in increase in the yield.

PGPR stimulate plant growth through mobilizing nutrients in the soil, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds like pesticides (Noumavl *et al*. 2013). Indeed, the bacteria lodging around/in the plant roots (rhizobacteria) are more versatile in the transforming, mobilizing, solubilising the nutrients compared from those of bulk soils. This extensive diversity of the soil ecosystem is confined to the rhizosphere. Rhizobacterial diversity is influenced by both plant and soil type. Therefore, the rhizobacteria are the dominant deriving forces in recycling the soil nutrients and consequently they are crucial for soil fertility (Ahemad and Kibret. 2013).

In turn, the plant supply root borne carbon compounds, mainly sugars which can be metabolized for bacterial growth (Deubel *et al*. 2000). The discovery of this mutual beneficiary relationship between plants and rhizobacteria encouraged the development of new technology such as the use of phosphate solubilizers for IAA production in kiwi vines. Although much information is available on diversity of plant growth promoting rhizobacteria for various crops, very limited information is available in respect to IAA producing rhizobacteria with multiple PGP traits associated with kiwi vines.

In particular, the production of IAA seems important to be one of the most prevalent plant growth promoting traits among plant growth promoting bacteria. Higher auxin level impairs plant defence mechanisms making colonization easier. It stimulates both rapid (increases cell elongation) and long term (cell division and differentiation) responses in plants. IAA production is also widespread among soil and plant associated bacteria, and its biosynthesis in an integral core trait of symbiotic species within the genera of *Rhizobium*, *Bradyrhizobium* and *Nostoc* as well as other plant associated saprophytic plant growth promoting rhizobacteria (PGPR) (Shah *et al*. 2013).

However, application of exogeneous plant hormones such as auxins by the method of spraying is not an effective method due to presence of wax layer on the surface of leaves (Kafrawi *et al*. 2014). However, additional input of indole-3-acetic acid (IAA) by

rhizobacteria can modify endogenously synthesized IAA by the plants to an optimal level, resulting in the induction of growth and development.

Although the mechanisms are not completely understood, root induction by PGPR is the accepted result of auxin production. Moreover, PGPR alters several hormonal pathways. This could account for the different morphological changes observed, for example, lateral root elongation and root hair development. Previous studies in our laboratory have described beneficial effect of PGPR on growth and nutrient uptake by apple (Shirkot and Sharma, 2005) and the diversity on phosphate solubilising bacteria (PSB) associated with apple, tomato and capsicum and other vegetable crops (Mehta *et al*. 2013; Walia *et al*. 2013; Gupta *et al*. 2014 and Sharma *et al*. 2015).There are few reports on the application of PGPR for enhanced growth of kiwi-vines by stimulating rooting and shoot growth of kiwi vines under nursery.

Keeping this in view, the purpose of present investigation was to isolate indigenous strains of rhizobacteria associated with different cultivars of kiwi-vines for their plant growth promoting attributes. Moreover, no systematic genetic and functional diversity associated with the rhizosphere had been explored till date. Therefore, the present work was based on the following objectives:

i) Isolation and enumeration of plant growth promoting rhizobacteria from kiwi vine.
ii) Screening, optimization and molecular characterization of plant growth promoting rhizobacteria isolated from kiwi- vine.

Chapter-2

REVIEW OF LITERATURE

Soil microorganisms belonging to different groups are known to play prominent role in plant-soil interactions in the rhizosphere. Among these bacteria that colonize the rhizosphere (rhizobacteria) are very important and beneficial for plant health. They are referred to as plant growth promoting bacteria (PGPB) or plant growth promoting rhizobacteria (PGPR) (Kumar *et al*. 2014). Microorganisms that colonize the rhizosphere can be classified according their effects on plants and the way they interact with roots, some being pathogens, whereas other trigger beneficial effects. Rhizobacteria inhabit plant roots and exert a positive effect ranging from direct mechanisms to an indirect effect. In the last few years, the number of PGPR that have been identified has seen a great increase, mainly because of the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere. Various species of bacteria like *Pseudomonas, Azospirillium, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have been reported to enhance the plant growth (Saharan and Nehra, 2011).

Currently the biological approaches for improving crop production are gaining strong status among agronomists and environmentalists following integrated plant nutrient management system. In this context, there is an ongoing rigorous research worldwide with greater impetus to explore a wide range of rhizobacteria possessing novel traits like heavy metal detoxifying potentials (Ma *et al.* 2011), pesticide degradation/tolerance (Ahemad and Khan, 2012), salinity tolerance (Tank and Saraf, 2010), biological control over phytopathogens and insects along with the normal plant growth promoting properties such as, phytohormone, siderophore (Jahanian *et al.* 2012), 1-amino-cyclopropane-1-carboxylate, hydrogen cyanate (HCN), and ammonia production, nitrogenase activity and phosphate solubilization etc. Hence, diverse symbiotic (*Rhizobium, Bradyrhizobium, Mesorhizobium*) and non-symbiotic (*Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azospirillium, Azomonas*), rhizobacteria are now being used worldwide as bio-inoculants to promote plant growth and development (Ahemad and Kibret, 2013).

In developing countries like India, demand of chemical fertilizers for crop production has increased tremendously due to the release of several high yielding and nutrient

demanding varieties of crop plants. The use of chemical fertilizers has resulted not only in the deterioration of soil health but also has lead to some major environment problems, such as soil and water pollution and other health hazards, besides increasing the input cost for crop production especially on the marginal farmers. So there is an urgent need to recycle available organics and manipulate rhizospheric microflora in a more efficient way and improve and expand their usage. The present review highlights the plant growth promoting rhizobacteria as an alternative to chemical fertilizers for sustainable and environment friendly agricultural practices (Das *et al*. 2013).

Erturk *et al*. (2010) studied the effects of plant growth promoting rhizobacteria (PGPR) on the rooting and root growth of semi-hardwood and hardwood kiwifruit stem cuttings. The PGPR used were *Bacillus* RC23*, Paenibacillus polymyxa* RC05, *Bacillus subtilis* OSUI142, *Bacillus* RC03, *Comamonas acidovorans* RC41, *Bacillus megaterium* RC01 and *Bacillus simplex* RC19. All the bacteria showed indole-3-acetic acid (IAA) producing capacity. Among the PGPR used, highest rooting ratios were obtained at 47.50% for semi-hardwood stem cuttings from *Bacillus* RC03 and *Bacillus simplex* RC19 treatments and 42.50% for hardwood stem cuttings from *Bacillus* RC03. As well, *Comamonas acidovorans* RC41 inoculations indicated higher value than control treatments. The results suggested that these PGPR could be used in organic nursery material production; pointing to the feasibility of synthetic auxin (IBA) replacement by organic management based on PGPR.

2.1 PGPR AND THEIR HOSTS: RHIZOSPHERIC AND ENDOPHYTIC REGION

2.1.1 Rhizospheric Region

The narrow zone of soil directly surrounding the root system is referred to as rhizosphere (Walker *et al*. 2003), while the term 'rhizobacteria' implies a group of rhizosphere bacteria competent in colonizing the root environment (Kloepper *et al*. 1991). Most rhizosphere microorganisms occur within 50 mm of root surface and populations within 10 mm of root surface may reach 1.2×10^8 cells kg^{-1} soil. In addition to providing the mechanical support and facilitating water and nutrient uptake, plant roots also synthesize, accumulate, and secrete a diverse array of compounds. These compounds secreted by plant roots act as chemical attractants for a vast number of heterogeneous, diverse and actively metabolizing soil-microbial communities. The chemicals which are secreted by roots into the soils are generally called as root exudates. The exudation of a wide range of chemical compounds modifies the chemical and physical properties of soil and thus regulates the

structure of microbial community in the immediate vicinity of root surface (Dakora and Phillips, 2002). The composition of these exudates is dependent upon the physiological status and species of plants and microorganisms (Kang *et al*. 2010; Ahemad and Kibret, 2013).

Moreover, these exudates also promote the plant-beneficial symbiotic interactions and inhibit the growth of the competing plant species (Nardi *et al*. 2000). It is reported that approximately 5-21% of photosynthetically fixed carbon is transported to the rhizosphere through root exudation (Marschner, 1995).

2.1.2 Endophytic Region

Rhizobacteria that establish inside plant roots, forming more intimate associations are endophytes. To aid in this conceptualization, simple terms have been adopted: intracellular plant growth promoting rhizobacteria (iPGPR), bacteria residing inside plant cells, producing nodules and being localized in those specified structures. These include a wide range of soil bacteria forming less formal associations than the rhizobia-langume symbiosis; endophytes may stimulate plant growth directly or indirectly include the rhizobia. Soil bacteria in genera *Rhizobium, Sinorhizobium, Azorhizobactria, Mesorhizobium*, *Allorhizobacteria* and *Mesorhizobacterium* belonging to family *Rhizobiaceae* invade plant roots from root nodules (Wang *et al*. 2000). Collectively they are often referred to as rhizobia. These iPGPR are mostly gram negative and rod shaped, with a lower proportion being gram positive rods, cocci and pleomorphic forms. The primary mechanism by which rhizobia increase plant growth is nitrogen fixation.

Different genera of bacteria are present as endophytes in roots and nodules of legumes and roots of non legumes. Predominantly endophytes belong to three major phyla, Actinobacteria, Proteobacteria and Firmicutes and include members of *Strptobyces, Azocareus, Gluconobacter, Pseudomonas, Serratia, Stenophomonas, Bacillus, Paenibacillus* and *Enterobacter*. Majority of the endophytes show plant growth promotion and have other beneficial traits like enhancement of biological nitrogen fixation, phytohormone production, phosphate solubilisation, inhibition of ethylene biosynthesis in response to biotic or abiotic stress and above all have biocontrol activity (Dudeja and Giri, 2014).

Arora *et al*. (2014) isolated endophytic bacteria from leaves of four dominant halophytes and other salt tolerant plant species of coastal Gujarat. The bacterial counts on nutrient agar were found maximum in *Spharanthus indicus* (40%) and minimum in

Salicornia brachiata (10%). Twenty bacterial isolates were selected and were characterized through morphological characters and biochemical tests.

2.2 MODE OF ACTION OF PGPR

PGPR promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or by indirectly decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Glick, 2012).

2.2.1 Direct mechanism

The rhizosphere is a narrow region of soil that is directly influenced by root secretions and associated microbial activity. Plant growth-promoting bacteria (PGPB) occupy the rhizosphere of many plant species and have beneficial effects on the host plant. The main mechanisms by which PGPR directly contribute to the plant growth are phytohormone production like auxins, cytokinins and gibberellins, enhancing plant nutrition by solubilization of minerals like phosphorus and iron, production of siderophores and enzymes, lowering of ethylene levels and induction of systemic resistance (Bhattacharyya and Jha, 2012).

2.2.1.1 PGPR as IAA (Indole-3-acetic acid) producers

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L- tryptophan metabolism produced by several microorganisms including Plant Growth-Promoting Rhizobacteria (PGPR). PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (like auxin, gibberellin, and ethylene), siderophores, HCN and antibiotics (Parmar and Dufrense, 2011). Bacteria synthesize auxins in order to perturb host physiological processes for their own benefit (Shih-Yung, 2010). The microorganisms isolated from rhizosphere region of various crop have an ability to produce indole acetic acid as secondary metabolites due to rich supply of substrates. Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake (Datta and Basu, 2000). IAA stimulates cell elongation by modifying certain conditions like, increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase in cell wall synthesis

and inducing specific protein synthesis. It promotes embial activity, inhibit or delay abscission of leaves, induce flowering and fruiting (Zhao, 2010).

Microorganisms possess several different IAA biosynthetic pathways. The metabolic routes are classified in terms of their intermediates as the indole-3-pyruvic acid (IPyA), indole-3-acetonoitrile, and tryptamine pathways. One major route, the IAM pathway, is employed mostly by many bacteria. First, oxidative decarboxylation of tryptophan leading to indole-3-acetamide is catalyzed by IaaM (indole-2-acetamide monooxygenase). The conversion of indole-3-acetamide to IAA is catalyzed by IaaH (indole-3-acetamide hydrolase). Another common pathway, the IPyA pathway is the major IAA biosynthetic pathway, used by PGPR including *Pseudomonas putida* GR-12-2. In many cases, a single bacterial strain may possess more than one pathway (Yang *et al*. 2007).

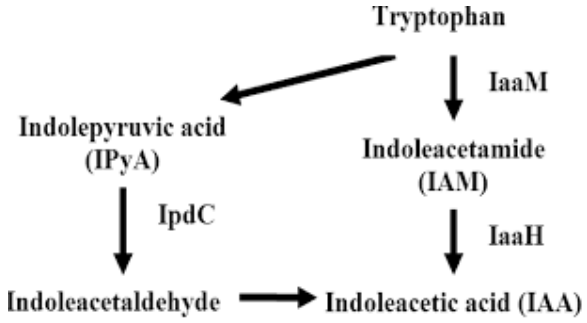


Fig. 1 Mechanism of IAA production

Indole acetic acid production by indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan ranging from 1 to 5 mg/L exhibited toxic effects on plant growth and resulted in decreased growth (Ahmad *et al.* 2005).

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

Sachdev *et al*. (2009) isolated *Klebsiella* strains from rhizosphere of wheat (*T. aestivum*), carried out screening and characterization of these strains for *in vitro* indole acetic acid (IAA) production and studied the effect of these strains on plant growth under xenobiotic

conditions. Nine strains of *Klebsiella* were isolated from rhizosphere of wheat (var. Lokwan) and identified as *K. Pneumonia* by 16S rRNA gene sequencing. Six *K. pnemoniae* showed *in vitro* IAA production. Calorimetric analysis showed that K8 produced maximum IAA (27.5 mg/L) in the presence L-tryptophan (1 mg/ml) at 72 h of incubation with optimum conditions as pH 8.0, 37°C and 0.5% (w/v) NaCl concentration. GC-MS analysis and IR studies confirmed presence of IAA in the cell filtrates of strain K8. Effect of six IAA producing *Klebsiella* strains on plant growth was studied by performing a series of seed germination test using moth bean seeds under anoxic conditions and pot experiments using sterilized soil and wheat seeds (var. Lokwan). Strain K11 and K42 demonstrated increase in root length of inoculated moth beans (~92.71% over the control). Results of pot experiments indicated that almost all six IAA producing *Klebsiella* strains significantly increased the root length and the shoot height of inoculated wheat seedlings over the control.

Indigenous soil samples were tested for auxin production. These bacteria were determined via bioassay and high performance liquid chromatography by the bacteria in liquid culture. Indole acetic acid and indole butyric acid were produced by these bacteria in varying concentration with and without the addition of tryptophan. These bacteria showed stimulatory effects on the growth of root and shoot elongation of mung beans (*Vignaradiata*). Results showed that the growth hormones produced by the bacteria increase growth rates and improve yields of the host plants (Shahab *et al.* 2009).

Ashraf *et al*. (2011) isolated twelve bacteria from root and rhizosphere samples collected from different sugarcane growing areas. Of these strains, ten strains were identified as *Pseudomonas* and two as *Azotobacter* on the basis of colony- and cell-morphology. All isolates showed IAA production in growth medium containing tryptophan as a precursor. Maximum IAA production (4.49 mg/L) was detected in isolate A17 where as IAA production in strains A4 and A11 was also significant. Values for IAA production by nitrogen fixing isolates Azoto1 and Azoto2 were comparatively low (0.2 and 0.1 mg/L respectively). For rapid screening of bacterial isolates from sugarcane, heterologous plant host (sorghum) was used as a test plant. Most of the strains showed beneficial effects on root length, root area and plant dry weight which were comparable to those observed in treatments where confirmed PGPR were used as positive control. Beneficial effects of inoculation on sugarcane grown in pots were also observed.

Kumar *et* al. (2012) analysed six French bean rhizospheric soil samples that were collected from different location of Shimla and Solan in H.P (India). A total of thirty bacteria were isolated and in vitro screening was done for different plant growth promotion activities i.e. phosphate solublization, IAA production, ammonia production, ACC deaminase activity, HCN production and catalase. In the present work twelve bacterial isolates were positive for phosphate solublization. IAA production was shown by almost all the bacterial isolates. Seven isolates were showing maximum plant growth promotion activities and further identified on the basis of colony morphology, Gram staining and biochemical tests. These isolates were identified as *Acinetobacter* sp., *Bacillus* sp., *Enterobacter* sp., *Micrococcus* sp. and *Pseudomonas* sp. As PGPR are environmental friendly and offer sustainable approach to increase production of crops and health. Therefore, these isolates can be utilized for biofertilizer formulation under local agro-climatic conditions of Himachal Pradesh.

Dias *et al.* (2013) screened fluorescent pseudomonads that are able to produce IAA and phosphate solubilization. Seven strains which promoted kale growth, presented low or intermediate solubilization efficiency and IAA production ability and were characterized as *Pseudomonas putida*. This study also shows that growth promoting capability was not consistently related *in vitro* to IAA production and phosphate solubilization.

Mohite, (2013) documented that indole acetic acid (IAA) production is a major property of rhizosphere bacteria that stimulate and facilitate plant growth. Their work dealt with isolation, characterization and identification of indole acetic acid producing bacteria from the rhizospheric soil. Out of ten IAA producing isolates, five were selected as efficient producers. Optimization of indole acetic acid production was carried out at different cultural conditions of pH and temperature with varying media components such as carbon and nitrogen source, tryptophan concentration. Partial purification of IAA was done and purity was confirmed with thin layer chromatography. Subsequently, effect on plant growth was tested by pot assay. In conclusion the study suggested IAA producing bacteria as efficient biofertilizer inoculants to promote plant growth.

Shah *et al*. (2013) studied that interaction between plants and diazotrophs influence the synthesis of phytohormone IAA. Four diazotrophic bacteria were used in the study, *Herabaspirillium seropedicae* (Z78), *Microbacterium* sp. (E7), *Acetobacter* sp. (E9) and *Microbacterium* sp. (E)14 successfully produced IAA under free-living conditions. These diazotrophs exhibited optimum productivity of IAA during the log growth phase. Isolates of

Microbacterium sp.E7 and E14 produced the higher concentration of IAA compared to the other isolates tested. In order to observe plant growth-promoting effects of the phytohormones produced by diazotrophs were inoculated onto the *in vitro* (tissue-cultured) oil palm shoots (*Elaeis guinensis* Jacq.). In associative conditions, inoculation of Z78 showed a significant increment in number of secondary roots, protein content and the increment of shoot fresh weight of oil palm shoots compared to those receiving control treatments. Significant responses of isolates E7 and E14 were also observed in the initiation of secondary roots, protein content and the increment of shoot fresh weight. This study concluded that IAA produced by Z78, E7 and E14 could contribute to enhanced growth and development of *in vitro* palm shoots that could further be developed into a potential biofertilizer.

Pant and Aggarwal, (2014) studied and identified IAA producing rhizobacteria from the rhizosphere. A total ten bacterial isolates were recovered from rhizospheric soil associated with *Withania 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 thin layer chromatography (TLC). The impact of PGPR was evaluated on *Withania somnifera* that showed significant elevation in germination %, root and shoot length as compared to untreated seeds.

Ghosh *et al.* (2015) studied the production of IAA in roots, nodules, and symbionts of an aquatic legume *Neptunia oleracea* and its possible role in nodular symbiosis. The symbiont (N37) was isolated from nodules of this plant and identified as *Rhizobium undicola* based on biochemical characteristics, 16S rDNA sequence homology, and DNA-DNA hybridization results. The strain N37 was found to produce copious amount of IAA in YEM broth medium with tryptophan.

2.2.2.2 PGPR as phosphate solubilizers

The improvement of soil fertility is one of the most common strategies to increase agricultural production. Phosphorus (P) is a major essential macronutrient for biological growth and development. Microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants. P is the least

mobile element in plant and soil contrary to other macronutrients. It precipitates in soil as orthophosphate or is adsorbed by Fe and Al oxides through legend exchange. Phosphorus solubilizing bacteria play role in phosphorus nutrition by enhancing its availability to plants through release from inorganic and organic soil P pools by solubilization and mineralization. The ability of some microorganisms to convert insoluble phosphorus (P) to an accessible form, like orthophosphate, is an important trait in a PGPR for increasing plant yields (Saharan and Nehra, 2011).

Chen *et al*. (2005) carried out a study on isolation, screening and characterization of 36 strains of phosphate solubilizing bacteria (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 of incubation at 30°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 to genus *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 phosphate solubilizing bacteria (PSB) after confirming their capacity to solubilize considerable amount of tricalcium phosphate in the 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 broadens the spectrum of phosphate solubilizers available for field application.

The rhizospheric phosphate utilizing bacteria could be a promising source for plant growth promoting agent in agriculture. The use of phosphate solubilizing bacteria as inoculants increases the P uptake by plants. Among the heterogeneous and naturally abundant microbes inhabiting the rhizosphere, the phosphate solubilizing microorganisms (PSM) including bacteria have provided an alternative biotechnological solution in sustainable agriculture to meet the P demands of plants (Egamberdieva, 2008). Greater efficiency of P solubilizing bacteria has been shown through co-inoculation with other beneficial bacteria and mycorrhiza (Khan and Joergensen, 2009).

Mamta *et* al. (2010) evaluated the effect of four phosphate-solubilizing bacteria (PSB), (*Burkholderia gladioli* 10216, *Burkholderia gladioli* 10217, *Enterobacter aerogenes* 10208 and *Serratia marcescens* 10238) as identified on the basis of 16S rRNA gene

sequencing on plant growth and commercially important glycosides, stevioside (ST) and rebaudioside-A (R-A) of *Stevia rebaudiana* in pots containing tricalcium phosphate (TCP) supplemented soil. The PSB were isolated from the rhizosphere of *S. rebaudiana* plants and tested for P-solubilization ability, biocompatibility, indole acetic acid (IAA) and siderophore production. In greenhouse study, treatment of either individual PSB or a consortium (of PSB) resulted in increased plant growth, ST and R-A contents. The stimulatory effect was observed with consortium treatment in plant growth parameters (shoot length, 22.5%; root length, 14.7%; leaf dry weight, 89.0%; stem dry weight, 76.3% and shoot biomass, 82.5%) and glycoside contents (ST, 150% plant−1 and R-A, 555% plant−1) as compared to the un-inoculated plants. Among individual PSB treatments, *B. gladioli* 10216 showed most promising response in majority of the parameters studied. The root colonization potential of PSB, assayed by RAPD technique, showed the colonization of all PSB isolates, though their extent of colonization varied.

Quereshi *et al*. (2012) conducted a field experiment to assess the ability of P-solubilizing rhizobacteria to enhance the growth and yield of cotton. Isolation and screening of P-solubilizer on Pikovskaya's medium was carried out at the Soil Bacteriology Section Faisalabad. The trial was conducted on clay loam soil with pH 8.3, EC 2.8 dSm^{-1}, N 0.040 %, organic matter 0.75% and available P 10.3 mg/kg with three fertilizer levels viz. 120-30, 120-60, 120-90 kg NP ha^{-1} with and without P-solubilizer (*Bacillus* sp.*)* inoculum. Results revealed that bacterial inoculum produced significantly higher seed cotton yield 1630 as compared to 1511 kg ha^{-1}. The highest seed cotton yield was observed at highest fertilizer level i.e. 1733 kg ha^{-1} with inoculum. The physical parameters like plant height, number of bolls per plant and boll weight and soil available P determined at regular intervals (30, 60, 90, days after sowing) was also found higher in the inoculated treatments. More exploration of this area of research should be carried out in different ecologies to compensate the mineral fertilizers.

Hamdali *et al.* (2012) isolated one hundred and fifty bacteria from a phosphate mine and tested their ability to grow on a synthetic minimum medium (SMM) containing insoluble rock phosphate (RP) as unique phosphate source. Only 29 isolates (19 per cent) were able to weather RP in SMM medium. Five isolates showed the most active growth and were able to solubilize RP in liquid cultures. Four of these strains belonged to the genus *Micromonospora* and one to the genus *Streptomyces.*

Shankarrao, (2012) studied total 28 phosphate solubilising bacteria, isolated from rhizospheric soil of Neem, Mango and Jatropha plant. 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 day incubation .The isolates were identified and characterized for the 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 is the rich source for isolation of phosphate solubilizing bacteria which promote the plant growth by more than one PGPR trait and have wide application in soil ecology.

Dugar *et* al. (2013) enumerated phosphate solubilizers from the rhizosphere of a widely growing weed, *Parthenium hysterophorus*, on Pikovskaya's medium, with an aim to screen for their plant growth promoting abilities for crops. The isolates were further assayed for multi-trait plant growth promoting properties. Two potential isolates, P1 and P2, were employed in seed germination and pot experiments with crop species. While bacterization led to an increase of 70 and 200% in shoot length in both seedling germination and pot experiments with *Cajanus cajan* (red gram), *Vigna radiata* (green gram) displayed an increase in shoot length by about 20% in pot assay using isolates P1 and P2, respectively. The study revealed the presence of phosphate solubilizers in the rhizosphere of *P. hysterophorus* with plant growth promoting effect on other crop species. Due to their potential in exhibiting plant growth promoting properties, these phosphate solubilizing isolates provide a new dimension to the significance of weeds in agricultural ecosystems. The study opened up possibilities of utilization of this property of weeds in plant growth promotion, disease suppression and subsequent enhancement of yield in agriculture**.**

Luna *et al*. (2014) evaluated soybean seeds and the bacterial mechanisms related to plant growth promotion. Isolates was genotypically compared and identified by amplification of partial sequences of 16S rDNA as *Bacillus amyloliquefaciens* strain LL.2012. Isolate was grown until exponential growth phase to evaluate the atmospheric nitrogen fixation, enzymatic activities, phosphate solubilisation, siderophores and phytohormone production. LL.2012 strain was able to produce high levels of auxins, gibberlins and salicyclic acid in

chemically defined medium. Co-inoculation of soybean plants with LL.2012 strain and the natural symbiont (*Bradyrhizobium japaonicum*) altered plant growth parameters and significantly improved nodulation. The results showed that association of LL.2012 with *B. japonicum*, enhanced the capacity of the latter to colonize plant roots and increase the number of nodules.

Kesaulya *et al*. (2015) isolated bacteria of rhizosphere origin of potato cv. Hartapel which has the ability to phosphate solubilization. In this study, the isolate that showed the best capability in solubilizing phosphate was isolate HB3 (14.2376 mg l-1) and isolate HB18 also was able to solubilize phosphate but resulted in lower concentration of soluble phosphate (4.457 mg l -1). Bacteria rhizosphere phosphate solubilization as agents of plant growth promoters, which along its use as inoculants could increase phosphate uptake by plants.

2.2.2.3 Nitrogen fixation in PGPR

Nitrogen is an essential element for all forms of life; a basic requisite for synthesizing nucleic acids, proteins and other organic nitrogenous compounds. Regrettably no plant species is capable for fixing atmospheric dinitrogen into ammonia and expend it directly for its growth. Thus the plants depend on biological nitrogen fixation (BNF). BNF imparts 180×10^6 metric tonnes per year globally, out of which symbiotic nitogen fixation contributes 80% and the remaining comes from free living nitrogen fixation. *Rhizobium*, is an example of symbiotic nitrogen fixing forms, while *Azospirillum, Cyanobacteria, Azoarcus, Azotobacter, Acetobacter diazotrophicus* etc. are the examples of free-living N2-fixing forms (Das *et al.* 2013).

Tripathi *et al.* (2005) isolated a nitrogen-fixing phytohormone-producing bacterial isolate (strain K1) from kallar grass (strain K1) and was identified as *Pseudomonas* sp. by 16S ribosomal RNA gene sequence analysis. Partial *nifH* gene amplified from strainK1 showed 93% and 91% sequence similarities to those of *Azotobacter chroococcum* and *Pseudomonas stutzeri* respectively. The effect of *Pseudomonas* strain K1 on rice varieties Super Basmati and Basmati 385 was compared with those of three non-*Pseudomonas* nitrogen-fixing PGPR (*Azospirillum brasilense* strain Wb3, *Azospirillum lipoferum* strain N4 and *Zoogloea* strain Ky1) used as single-strain inoculants. The results showed that nitrogen-fixing pseudomonads deserve attention as potential PGPR inoculants for rice.

Figueiredo *et al*. (2007) performed a greenhouse experiment to evaluate the effects of PGPR on nodulation, biological nitrogen fixation (BNF) and growth of the common bean (*Phaseolus vulgaris L. cv. Tenderlake*). Bean was single and dual inoculated with *Rhizobium* and other PGPR strains (*Bacillus endophyticus, B. pumilus, B. subtilis, Paenibacillus lautus, P. macerans, P. polymyxa, P. polymyxa (Loutit L.)* and *Bacillus* sp.*)*. Beans co-inoculated with *Rhizobium tropici* and *Paenibacillus polymyxa* had higher leghemoglobin concentrations, nitrogenase activity and N_2 fixation efficiency and thereby formed associations of greater symbiotic efficiency.

The effect of nitrogen fixing *Azotobacter* and phosphate solublising *Bacillus megaterium* on the growth of two trees; Teak *(Tectona grandis)* and Indian redwood (*Chukrasia tubularis*) were tested under nursery condition. The pottery mixture for the Teak and Burmese almond wood seedlings were inoculated with both nirogen fixing bacteria and phosphate solublising bacteria. The co-inoculation effects were also monitored along with the application of either of single super phosphate or rock phosphate alone or in combination of both days of trial. The present study demonstrated the positive effects of co-inoculation of NFB and PSB on *C. tubularis and T. grandis*. This finding could be useful for developing a new formulation for biofertilizer under field conditions (Bhrigu *et al.* 2009*).*

El-Sayed *et al*. (2014) isolated native bacteria from rhizospheric arid soils and evaluated them for both growth-promoting abilities and antagonistic potential against phytopathogenic fungi and nematodes. The phylogenetic affiliation of these representative isolates was also characterized. Rhizobacteria associated with 11 wild plant species were investigated. From a total of 531 isolates, only 66 bacterial isolates were selected based on their ability to inhibit *Fusarium oxysporum*, and *Sclerotinia sclerotiorum.* The selected isolates were screened for *in vitro* for activities related to plant nutrition and plant growth regulations. Isolated bacteria were found to exhibit capabilities in fixing atmospheric nitrogen and also showing other PGP traits. Application of such results in agricultural fields may improve and enhance plant growth in arid soils.

Rehmani *et al*. (2015) studied the effect of co-inoculation with plant growth-promoting rhizobacteria (PGPR) and *Rhizobium*, on nodulation, nitrogen fixation, and yield of common bean (*Phaseolus vulgaris L.*) cultivars was investigated in two consecutive years under field conditions. The PGPR strains Pseudomonas fluorescens P-93 and Azospirillum lipoferum S-21 as well as two highly effective *Rhizobium* strains were used. Common bean

seeds of three cultivars were inoculated with *Rhizobium* singly or in a combination with PGPR to evaluate their effect on nodulation and nitrogen fixation. A significant variation of plant growth in response to inoculation with *Rhizobium* strains was observed. Treatment with PGPR significantly increased nodule number and dry weight, shoot dry weight, amount of nitrogen fixed as well as seed yield and protein content. Co-inoculation with *Rhizobium* and PGPR demonstrated a significant increase in the proportion of nitrogen derived from atmosphere. These results indicate that PGPR strains have potential to enhance the symbiotic potential of rhizobacteria.

2.2.3 Indirect plant growth promotion by PGPR

PGPR provide different mechanisms for suppressing plant pathogens. These include competition for nutrients and space (Ahemad and Kibret, 2014), antibiosis by producing antibiotics viz., pyrrolnitrin, pyocyanine, 2, 4-diacetyl phloroglucinol (Pierson and Thomashow, 1992) and production of siderophores viz., pseudobactin which limits availability of iron necessary for growth of pathogens. Other important mechanisms include production of lytic enzymes such as chitinases and β-1, 3-glucanases which degrade chitin and glucan present in the cell wall of fungi, HCN production and degradation of toxin produced by pathogen (Jha and Saraf, 2015).

2.2.3.1 PGPR as biocontrol agent

The term “biological control” and its abbreviated synonym “biocontrol” have been used in different fields of biology, but in plant pathology, this term is applied for the use of microbial antagonists (the biological control agent or BCA) to suppress diseases. Most narrowly, biological control refers to the suppression of a single pathogen (or pest) by a single antagonist in a single cropping system. Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. Growing cost of pesticide free food has lead to the search for substitutes for these products. Biological control is thus being considered as an alternative to reduce the use of chemical fertilizers in agriculture (Gerhardson, 2002).

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% with a plant-growth promotion and biocontrol efficiency of 180 and 151%, respectively. In general, application of the strain or its extracellular metabolites increased plant growth parameters. The strain produced mycolytic enzymes *viz*. chitinase, chitosanase, ß-1,3glucanase 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.

Bhakthavatchalu *et al*. (2012) carried out a study on the screening of effective PGPR isolate with multiple traits related to biocontrol of phytopathogenic fungi. A total of 51 bacterial isolates from the rhizosphere soil samples were isolated and screened for their antagonistic activity against wide range of phytopathogens. Bacterial antagonist showing highest percent and broad spectrum antagonism against fungal phytopathogens was selected and further identified as *Pseudomonas aeruginosa* FP6 on the basis of 16S rDNA gene sequence analysis.

Devi and Sowndaram, (2014) studied the plant growth promoting (PGP) activity and antagonistic activity of two rhizobacteria *Pseudomonas fluorescence* and *Rhizobium* sp., isolated from rhizosphere area of rice, against two major rice pathogens; where IAA was found maximum in *Pseudomonas fluorescence* (30 mg / ml) and trace amount in *Rhizobium* sp. Maximum amount of siderophore production was noticed in both isolates. *Pseudomonas fluorescence* showed positive result for the HCN production but *Rhizobium* sp., was found to be negative and both the bacterial isolates solubilized phosphate in very effective manner. Both rhizobacteria were found to produce ammonia and catalase. The cross streak studies revealed the complete inhibition of mycelia growth of *Rhizotonia solani* (85%) and partial inhibition of *Sarocladium oryzae* (45%) by two rhizobacteria. Antifungal compound extracted from both rhizobacteria were found to exhibit maximum antagonism against rice pathogens. These results concluded that both the PGPR's can be used as effective bio-control agents as effective bio-control agents against rice pathogens.

Wang *et al*. (2015) screened 1223 isolates for antifungal activity and about 24% inhibited *Rhizoctonia solani* or *Sclerotinia sclerotiorum.* Twenty-four strains inhibitory *to R. solani, Gaeumannomyces graminis* and/or *S. sclerotiorum* were assayed for PGPR activity. Selected isolates belonging to genus *Pseudomonas* were similar to model PGPR strains *Pseudomonas protegens Pf-5, Pseudomonas chlororaphis subsp. aureofaciens* 30–84 and *P.*

brassicacearum Q8r1-96. *Pseudomonas protegens*- and *P. chlororaphis*-like strains had the greatest biocontrol activity against *Rhizoctonia* root rot and take-all of wheat. *Pseudomonas protegens* and *P. brassicacearum*-like strains showed the greatest promotion of canola growth. These results indicated that strains from contaminated soils were similar to well-described PGPR found in agricultural soils worldwide.

Noumavo *et al*. (2015) studied plant growth promoting characteristics of 15 bacteria isolated from maize (*Zea mays L.*) rhizosphere in Benin. Most rhizobacteria strains were found to produce catalase(100%), exopolysaccharides (100%), ammonia (86.66%), HCN (80%) and IAA (60%). *Pseudomonas putida, Pseudomonas flourescens* and *Azospirillium lipoferum* inhibited mycelial growth of *F. verticiloides* and *Aspergillus ochraceus.* These results suggested the possibility to use these rhizobacteria as biological fertilization to increase crop yield and biological control of *F. verticilliodes* and *A. ochraceus.*

2.2.3.2 PGPR as siderophore producers

Iron is one of the bulk mineral present in plenteous amount on earth, yet it is unavailable in the soil for plants. This is because Fe^{3+} (ferric ion) is common form of iron found in nature and meagerly soluble. To overcome this problem, PGPR's secretes siderophores that are iron binding protein of low molecular mass and have a high binding affinity with ferric ion. Siderophores secreted by PGPR's improve plant growth and development by increasing the accessibility of iron in the soil surrounding the roots. Plants such as Oats, Sorghum, Cotton, Peanut Sunflower and Cucumber, demonstrate the ability to use microbial as sole source of iron than their own siderophores (phytosiderophores). Microbial siderophores were also reported to increase the chlorophyll content and plant plant biomass in Cucumber (Das *et al*. 2013).

Rachid and Ahmed, (2005) employed four basal media, supplemented with different concentration of iron to study the effect of iron and different organic carbon sources on the siderophore production in *Pseudomonas fluorescens.* The highest siderophore concentration was obtained in succinate medium. Ferric iron increased the growth yield and completely repressed siderophore production above 200 g/l but had a positive effect below 160 g/l. Penicillin and lead elicited the production of siderophore in the presence of excess iron.

Vassilev *et al*. (2006) observed siderophore production by various fungi on modified CAS agar plate assay. These fungi solubilize rock phosphate, presumably by releasing metal

chelating metabolites. This showed that they can be applied as bio control microorganism with P-solubilizing activity.

Thirty-two bacterial isolates were obtained from wheat rhizosphere in black cotton soils of North Maharashtra region and -subsequently tested for *in-vitro* siderophore production. Seed bacterization with siderophoregenic *A. calcoaceticus* improved plant growth in pot and field studies. Such PGPR activity was attributed to the ability of strain to solubilize phosphates and produce IAA. Siderophore mediated antagonism was observed against common phytopathogens *viz., Aspergillus flavus*, *A. niger, Colletotrichum capsicum* and *Fusarium oxysporum* (Sarode *et al.* 2008).

Solano *et al.* (2010) evaluated PGPR isolated from rhizosphere of wild populations of *Nicotiana glauca* Graham in south-eastern Spain for siderophore and chitinase activity. They observed that ninety six isolates were siderophore producers, and 56 of them were also able to produce chitinases. The ability of these strains to induce systemic resistance against the leaf pathogen *Xanthomonas campestris* in tomato was evaluated. Most of the strains effectively reduced disease symptoms upto50%.

A rhizobacterium isolated from groundnut rhizosphere and identified as *Alcaligens faecalis* BCCM 2374 produced siderophore during 24 h submerged growth in modified succinic acid medium (SM) prepared under tap water without deferration.Inoculation of *Alcaligens faecalis* enhanced seed germination, root length ,shoot length, chlorophyll content in *Arachis hypogeal* over control treatment under pot culture conditions (Sayyed *et al.* 2010).

Srivastava and Kumar, (2011) studied biochemical characterization of siderophore producing plant growth promoting rhizobacteria of rice rhizosphere. Out of 74 isolates, only 29 (39.2%) diazotrophic rhizobacterial isolates showed siderophore production by CAS (chrome azurol S) plate assay. Optimum siderophore production was recorded after 72 h of growth with 2 mM at 30°C temperature in shaking condition in JNFb-liquid medium. The amount of siderophore produced by these isolates ranged between1.45 to 22.22 µg/mg. 15 (51.7%) isolates were catecholate and 14 (48.3%) were hydroxamate type. TLC analysis of catecholate type siderophores revealed that only 13.8% was exactly similar to 2, 3-dihydroxybenzoic acid (DHBA) whereas 86.2 % showed more than one type of siderophores or other catecholate siderophore with varying molecular weights.

Nakouti and Hobbs, (2012) isolated organisms on the basis of their survival in an iron-limited environment. The survivors of this treatment were largely actinomycetes. The most prolific producers as assessed by the Chromoazurol sulphate assay were further characterized and belonged to the genus *Streptomyces*.

Bholay *et al.* (2012) screened *Pseudomonas fluorescens* NCIM 5164 and *Pseudomonas aeruginosa* NCIM 2036 for siderophore production. Quantitative estimation of siderophore was done by the Chrome Azurol S assay (CAS). *P. fluorescens* and *P. aeruginosa* produced 88% and 83% siderophore units respectively. The type of siderophore was detected by chemical as well as spectrophotometric assay. *Pseudomonas fluorescens* was found to produce hydroxamate type of siderophore and *Pseudomonas aeruginosa* produced both the hydroxamate (Pyoverdin) and phenol-catecholate (Pyochelin) siderophore. Evaluation study of siderophore showed that maximum siderophore production was obtained on succinate medium at pH 7.0. Both the *Pseudomonas spp*. were further tested as seed inoculants and found to be very effective in seed germination and plant growth promotion of *Triticum aestivum* and *Apios americana* plants under pot culture conditions.

Parray *et al*. (2013) isolated bacterial strains from saffron rhizoshere soil during the flowering stage of corms. All these isolates were screened for siderophore production. The maximum percentage of the bacterial isolates was of Gram negative rod shaped type The *Bacillus subtilis* showed 62% siderophore production. It was concluded that these rhizobacterial strains isolated could be a promising source for plant growth promoting yield and enhancement of saffron.

2.2.3.3 HCN production by PGPR

Cyanide production is one of the possible ways by which rhizobacteria may suppress plant growth in soil by cyanogenesis. It is dependent on the temperature of incubation within the ranges which allow complete growth. Cyanide is produced after cessation growth. Seventy-four of 110 strains of *Pseudomonas aeruginosa* tested produced detectable amounts of HCN after growth in 2% peptone or nutrient agar. Of the 25 species of 12 bacterial and fungal genera tested, other than *P. aeruginosa*, only *P. fluorescens* and *P. polycolor* mM allows growth but inhibits HCN production. Inorganic phosphate in concentrations between 90 and 300 μm, while copper, zinc, cobalt, and manganese at concentrations of 20μm gave

positive HCN tests. Cyanide is produced after cessation of active growth. Iron was stimulatory to cyanogenesis in concentrations above 1 (Castric, 1975).

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 the major inhibitory compound produced.

Supraja *et al.* (2011) isolated fifteen bacterial isolates from rhizospheric soils of redgram and maize crops in the Rangareddy district. The results indicated that all the 15 isolates inhibited the growth of fungal pathogen except MPF-1 and concluded that fluorescent *Pseudomonas* inhibited the growth of *Fusarium moniliforme* due to production of HCN and siderophores.

Shobha and Kumudini, (2012) screened isolates of *B. megaterium* JUMB1, JUMB2, JUMB3, JUMB4, JUMB5, JUMB6 and JUMB7 *in vitro* for their plant growth promoting traits. All the isolates exhibited all PGP traits including HCN production, which promotes plant growth directly or indirectly.

Sunar *et al.* (2015) isolated 18 bacterial isolates from the rhizosphere of *Sechium edule* growing in the lower foothills of Darjeeling, India. The bacterial isolates were tested for PGPR traits *in vitro* such as phosphate solubilization, HCN, siderophore, IAA, chitinase, protease production as well as inhibition of pthytopathogens. The best isolate identified as s *Bacillus altitudinis* showed a significant improvement in growth measured in terms of increase in root length, shoot length, and increase in root and shoot biomass of *Vigna radiata*, *Cicer arietinum*, and *Glycine max* were bacterized prior to sowing in field condition.

2.2.3.4 Lytic enzymes production by PGPR

Enzymes are the organic catalysts produced generally by microorganisms, differing from other catalysts and constitute the tools which determine the course of the multitude of life process. Various kinds of enzymes are produced by microorganisms. The antagonistic activity against different type of microbes may also be due to the production of lytic enzymes that are produced *in vitro* or *in vivo* by microorganism. Lytic enzymes (chitinase, β-1,3-glucanase, protease) are responsible for the lysis and hyper parasitism of antagonists against deleterious fungal pathogens. In these mechanisms, chitin, β-1,3-glucan and protein

components of the fungal cell wall are digested by these extracellular enzymes. Such bacterial strains have been implicated in the inhibition of plant pathogenic and deleterious rhizobacteria with a significant increase in root colonization and plant growth. These attributes make fluorescent pseudomonads as the effective biocontrol agents (Ramyasmruthi, 2012).

Rani *et al.* (2012) isolated, enumerated and characterized the PGPR from the rhizosphere soil of pigeon pea for the enhancement of growth of pigeon pea. Rhizosphere soils were collected from different areas of Samalkot, Pithapuram, Peddapuram and Kakinada. Sixty five (65) isolates were identified and characterized for their morphological, cultural, stainning and biochemical characteristics, of which 35 was selected for the screening of PGP traits. Sixteen isolates were successfully characterized for the PGPR traits like indole acetic acid (IAA) production, phosphorus solubilization, and production of enzymes like urease, chitinase, amylase, cellulase, protease and β-1, 3 glucanase and were assayed. Three isolates were shown to be promising in IAA production, phosphate solubilization, antagonism towards fungi, and mineralizing capacity. Thus, this study suggested the use of these isolates as inoculant biofertilizers which might be beneficial for pigeon pea cultivation.

Geetha *et al*. (2014) isolated 180 PGPR strains from the rhizosphere soils of green gram and screened for their antifungal activity against *Macrophomina* phaseolina, *Colletotrichum capscici*, *Rhizoctonia solani*, *Fusarium oxysporum.* Twenty antagonistic isolates were tested for their Plant growth promoting (PGP) traits, seed germination ability, extra cellular enzyme production, salt and temperature tolerance. Six isolates were most effective, which may be useful as biofertilizers, they enhanced the growth of green gram due to the production of ammonia, IAA, HCN, phosphate solubilization, and also having antifungal activity against phyto pathogenic fungi.

Reetha *et al*. (2014) conducted a study to determine the Production of cellulase and pectinase enzyme by using plant growth promoting rhizobacteria like *Pseudomonas fluorescence* and *Bacillus subtilis.* These microorganisms were isolated by serial dilution method. These bacteria *Pseudomonas fluorescence* was able to produce high amount of cellulase compared to *Bacillus subtilis. Pseudomonas fluorescence* was also able to produce high amount of Pectinase compare to *Bacillus subtilis*.

Bhatt and Vyas, (2014) isolated rhizobacteria possessing multiple plant growth promoting activities from the rhizospheric soils of plants growing in semi arid region and

screened for their plant growth promoting activities like phosphate solubilization, production of indole-acetic acid, ammonia, hydrogen cyanide (HCN), catalase, amylase, cellulase, and chitinase. All the isolates solubilized phosphate, 33% of the isolates produced ammonia, 27% produced lipase, 53% produced amylase, 50% chitinase, 40% produced indole-acetic acid and a single isolate produced HCN. PGPR isolates in pot trial experiments increased plant dry biomass up to 5-27%, root length 14-154% and shoot length up to 9-70% against control plants.

2.3 PGPR AND PLANT GROWTH PROMOTION

Introduction and exploitation of plant growth promoting rhizobacteria (PGPR) in agro-ecosystems enhance plant–microbes interactions that may affect ecosystems sustainability, agricultural productivity, and environmental quality. Zahid *et al.* (2015) conducted a study to isolate and identify PGPRs associated with maize (*Zea mays* L.) from twenty sites of Himalayan region of Hajira-Rawalakot, Azad Jammu and Kashmir (AJK), Pakistan. A total of 100 isolates were isolated from these sites, out of which eight were selected *in vitro* for their plant growth promoting ability (PGPA) including phosphorus solubilization, indole-3-acetic acid (IAA) production and N_2 fixation. The 16S rRNA gene sequencing technique identified that these isolates belong to *Pseudomonas* and *Bacillus* genera. The isolates promoted plant growth by solubilizing soil P which ranged between 19.2 and 35.6 μg mL^{-1}. The isolates HJR_1, HJR_2, HJR_3, and HJR_5 showed positive activity in acetylene reduction assay showing their N_2-fixation potential.

Lucas *et al.* (2001) studied the effects of inoculation with *Bacillus licheniformis* on the growth of pepper and tomato in three experiments, one under seed bed conditions, and two under green house production conditions. In this study, the bacterium significantly increased the height of plants and the leaf area. Their results suggested that strain had considerable colonization and competitive ability, and it could be used as a biofertilizer or biocontrol agent without altering normal management in green house.

Yadav *et al.* (2010) isolated five isolates of PGPR designated *as Pseudomonas aeruginosa* strain BHUPSB02, *Pseudomonas putida* strain BHUPSB04, *Bacillus subtilis* strain BHUPSB13, *Paenibacillus polymyxa* strain BHUPSB17 and *Bacillus boronophilus* strain BHUPSB19 characterized through 16S rDNA gene sequencing. Subsequently, an experiment was conducted under plant growth chamber where chickpea plants were grown in

plastic cups containing soil and mixed with these isolates to investigate the effect of PGPR on the growth of chickpea plant. Most of isolates resulted in a significant increase in shoot length, root length and dry matter production of shoot and root of chickpea seedlings. Therefore, present study suggests that PGPR isolates viz. BHUPSB02, BHUPSB04 and BHUPSB13 may be used as biofertilizers to enhance the growth and productivity of chickpea.

Kaushal *et al.* (2011) isolated PGPR from cauliflower rhizosphere growing in different 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 on the growth and yield of cauliflower. All the isolates induced the production of indole acetic acid (IAA) and were able to solubilize phosphorus. The conjoint use of PGPR and N and P fertilizers resulted in a significant increase in number of nonwrapper leaves, curd diameter, curd depth and curd weight of cauliflower. Furthermore, PGPR isolates remarkably increased the yield of cauliflower.

Mehta *et al*. (2014) isolated P-solubilizing bacterial isolate CB7 from apple rhizosphere soil of Himachal Pradesh, India. It was identified as *Bacillus circulans* on the basis of phenotypic characteristics, biochemical tests, fatty acid methyl esters analysis, and 16S rRNA gene sequence. The isolate exhibited plant growth-promoting traits of P-solubilization, auxin, 1-aminocyclopropane-1-carboxylate deaminase activity, siderophore, nitrogenase activity, and antagonistic activity against *Dematophora necatrix*. P-solubilization activity was associated with the release of organic acids and a drop in the pH of the Pikovskaya's medium. Remarkable increase was observed in seed germination (22.32%), shoot length (15.91%), root length (25.10%), shoot dry weight (52.92%), root dry weight (31.4%), nitrogen (18.75%), potassium (57.69%), and phosphorus (22.22%) content of shoot biomass over control. These results demonstrate that isolate CB7 has the promising PGPR attributes to be developed as a biofertilizer to enhance soil fertility and promote plant growth.

Dasgupta *et al*. (2015) isolated twelve isolates of bacteria from the rhizospheric soil of Dhaincha (*Sesbania bispinosa*) and were further screened on their efficiency in PGPR characteristics. The most efficient strains were further selected and identified as *Escherichia coli, Pseudomonas fluorescens* and *Burkholderia* sp. A pot culture and a field experiment was conducted using a complete block design with 3 replications and 9 treatments. The maximum

rates of plant height, number of leaves/plant, pod bearing branches, pods/plant, nodules/plant and 100 seed weight were recorded after application of combined inoculation of *Escherichia coli* DACG2+ *Pseudomonas fluorescens* DACG3+ *Burkholderia* sp DACG1 as compared with other inoculation treatments and uninoculated control. Therefore, the study suggested that PGPR isolates viz. *Escherichia coli*, *Pseudomonas fluorescens*, and *Burkholderia* sp. may be used as biofertilizers to enhance the growth and productivity of chickpea.

Sharma *et al*. (2015) assessed plant growth promoting activity of rhizobacteria isolated from mid hill region of Himachal Pradesh (India). Out of one hundred isolates, 26 were further screened for PGP traits as well as antifungal activity. Strain S25 showed significantly higher phosphate solubilization and IAA production. Of the 26 isolates 18 were nitrogen fixers while 23 and 20 showed chitinase and protease activity, respectively. On the basis of *in vitro* screening, 10 most efficient isolates were screened for growth promotion of tomato under net house conditions. Strain S25 exhibited highest seed germination, root length; root dry weight, shoot length and shoot dry weight over all the other treatments. The strain S25 was identified as *Bacillus subtilis* could be used as a PGPR inoculants for growth and yield enhancement of tomato under mid hill regions of Himachal Pradesh.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled "**Studies on diverse plant growth promoting rhizobacteria associated with rhizosphere of kiwi vines**" was conducted in the laboratory of Microbiology section of Department of Basic Science at Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh during the year 2013-15. An account of the material used and methodology adopted is discussed in this chapter.

3.1 MEDIA

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

3.1.1 Nutrient agar (NA)

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

3.1.2 Malt extract agar (MEA)

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

3.1.3 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

3.1.4 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%
$MnSO_4$	:	0.0002%
$FeSO_4$	:	0.0002%
Yeast extract	:	0.05%

3.1.5 Pikovskaya's agar

Pikovskaya's broth + 2.0% agar

3.1.6 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

3.1.7 Luria Bertani (LB) agar

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

3.1.8 Chromeazurol-S (CAS) agar

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

3.1.9 Skim milk agar medium

Pancreatic digest of casein	:	0.50%
Yeast extract	:	0.25%
Glucose	:	0.10%
Skim milk	:	7.00%
Agar	:	2.0%

3.1.10 Tributyrin Agar (TBA)

Peptone	:	0.5%
Yeast extract	:	0.3%
Tributyrene	:	1%
Agar	:	2%

3.1.11 Starch Agar

Starch	:	2%
Peptone	:	5.0%
Beef extract	:	0.3%
Agar	:	2%

3.1.12 Peptone water

Potassium iodide	:	1%
Sodium chloride	:	0.5%

3.1.13 MRVP Broth

Peptone	:	0.7%
Dextrose/Glucose	:	0.5%
Potassium phosphate	:	0.5%

3.1.14 Simmon's citrate Agar

Ammonium dihydrogen phosphate	:	0.1%
Dipotassium phosphate	:	0.1%
Sodium chloride	:	0.5%
Sodium citrate	:	0.2%
Magnesium sulphate	:	0.02%
Agar	:	2%
Bromothymol blue	:	0.08%

3.1.15 Sulfide indole motility Agar (SIM Agar)

Peptone	:	3%
Beef extract	:	0.3%
Ferrous ammonium sulfate	:	0.02%
Sodium thiosulphate	:	0.0025%
Agar	:	2%

3.1.16 Carbohydrate fermentation medium

Peptone	:	1%
Carbohydrate	:	0.5%
Sodium chloride	:	1.5%
Phenol red	:	0.0018

3.2 CHEMICALS AND REAGENTS

Analytical grade chemicals and reagents obtained from Hi-Media, BDH or E. merck were used for most of the investigations.

3.3 MICROBIOLOGICAL METHODS

3.3.1 Microorganisms

3.3.1.1 Microorganism with phosphate solubilizing activity and its maintenance

Microorganisms were isolated from the roots and rhizospheric soil of the kiwi vines collected from Experimental farm at Dr. Y. S. Paramar University of Horticulture and Forestry, Nauni, Solan. The bacterial cultures were maintained in LB broth containing 30% (v/v) glycerol at – 20ºC.

3.3.1.2 Fungal pathogen and their maintenance

Fusarium oxysporum and *Phytophthora capsici* was obtained from the preserved fungal cultures in laboratory of Microbiology, University of Horticulture and Forestry, Nauni, Solan. These cultures were maintained on malt extract agar at 4ºC. Sub-culturing was done periodically on same medium at incubation temperature of 24±1 ºC.

3.3.2 Sterilization

Glassware used was thoroughly washed in detergent water, running tap water followed by rinsing in distilled water. The flasks used were tightly plugged with cotton gauge

and were covered with aluminium foil. Glassware was then kept in hot air oven at 180ºC temperature for one hour. All the media, distilled water solutions etc. were sterilized at 15 lbs per square inch pressure for 20 minutes, unless mentioned otherwise. Laminar air flow chamber was sterilized by ultra violet (UV) irradiation for 15 minutes.

3.4 MEASUREMENT OF GROWTH

3.4.1 Preparation of inoculum

A bacterial cell suspension (O.D. 1.0 at 540 nm) of 24h old culture grown on nutrient agar slants at the rate of 10 per cent was used as inoculum in all experiments, unless mentioned otherwise.

3.4.2 Turbidity

Growth was monitored by measuring the change in absorbance of cells in the medium at 540 nm using uninoculated medium as blank.

3.4.3 Viable count

Appropriate dilutions of bacterial population were used to seed the medium. The number of viable cells in the initial population was obtained by counting the number of colonies that developed after incubating the plates and multiplying this figure by dilution factor.

3.5 SITE CHARACTERIZATION AND SAMPLING

The soil and root samples used for rhizobacterial and endorhizobacterial isolation were collected from the rhizosphere of different cultivars of kiwi vines with varying age groups. Three plants were selected randomly from each cultivar and a total of 21 soil and root samples were used for isolation of total bacterial population. Sampling was done in the month of May of the year 2014. Samples were immediately stored at 4ºC in plastic bags loosely tied to ensure sufficient aeration and to prevent moisture loss until assaying of bacterial community structure.

3.6 ISOLATION, ENUMERATION AND SCREENING FOR PLANT GROWTH PROMOTING RHIZOSPHERIC AND ENDORHIZOBACTERIAL POPULATION ASSOCIATED WITH KIWI VINES

3.6.1 Isolation of rhizospheric soil bacterial population

Bacterial isolates were isolated from the rhizosphere soil and roots of plants during the month of May 2014. Root samples collected from different sites under natural conditions

were shaken vigorously to remove the soil tightly adhered to the roots. One gram of this rhizosphere soil was placed in 9 ml of sterilized distilled water under aseptic conditions. To estimate the number of soil bacteria, counts were calculated on the basis of serial 10 fold dilutions in duplicate, using the pour plate method on nutrient agar (master plate).

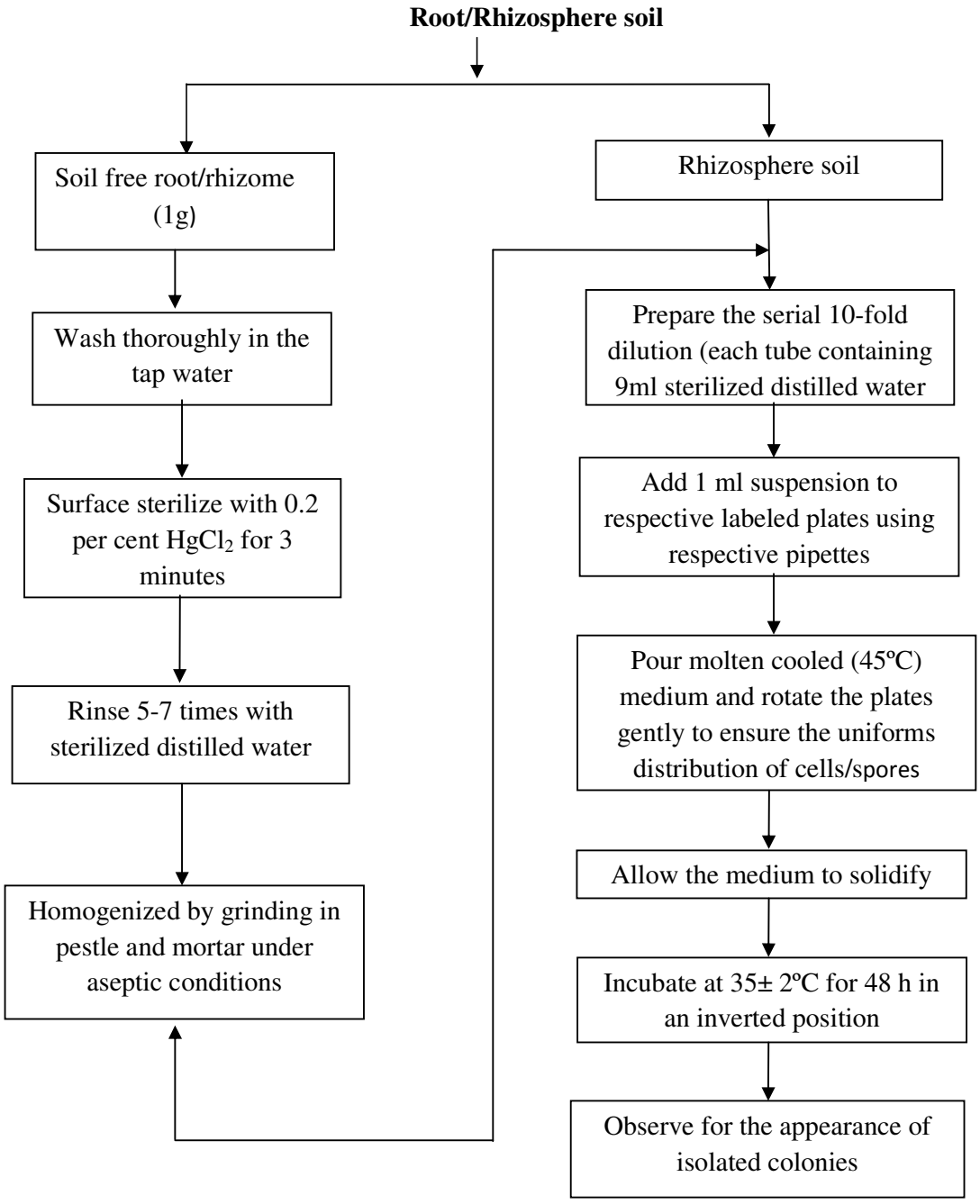


Fig. 2 Generalized flow chart for the isolation of rhizospheric soil and endorhizobacteria

Colony forming units (CFU) were recorded on nutrient agar plates after 48 hour, the average number per gram oven dry weight of soil was calculated as:

$$\text{CFU} = \frac{\text{Bacterial plate count} \times \text{Dilution factor}}{\text{Amount of sample plated}}$$

3.6.2 Isolation of endorhizobacteria

The root sample was surface sterilized by 0.2 per cent mercuric chloride ($HgCl_2$) for three minutes followed by repeated washing in sterilized distilled water.

The surface sterility of roots was cross checked by incubating the surface sterilized roots in sterilized nutrient broth overnight One gram of surface sterilized root sample was placed in 9 ml of sterilized distilled water and was ground to produce slurry using pestle and mortar under aseptic conditions. The root suspension was diluted in 10 fold series and bacterial count was determined by standard pour plate technique (Fig. 2). Populations were expressed as colony forming unit (CFU) per gram of the root weight. The isolates were maintained on specific medium for further studies.

3.6.3 Screening of bacterial isolates for PGPR activities using modified replica technique

Replica plating technique was originally developed to isolate auxotrophic mutants. Here, it was used to screen PGPRs with plant growth promoting activities. Rhizospheric and Endorhizospheric bacterial populations obtained previously on nutrient agar (master plate) were replica plated onto the selective media: CAS medium (Schwyn and Neilands, 1987) for siderophore producing ability, nitrogen free medium for nitrogen fixing ability and P ikovskaya medium (Pikovskaya, 1948) for phosphate solubilizing ability. All the colonies were transferred to the same position as the master plate with the help of wooden block, covered with sterilized velveteen cloth. At the end of the incubation period (72h), the location of the colonies appeared on the replica plates were compared to master plate.

3.7 *IN VITRO* SCREENING OF BACTERIAL ISOLATES FOR QUALITATIVE AND QUANTITATIVE ESTIMATION OF MULTIPLE PLANT GROWTH PROMOTING ACTIVITIES

3.7.1 Quantitative estimation of indole-3-acetic acid (IAA) (Gorden and Paleg, 1957)

For the production of auxins, bacterial cultures were grown in Luria Bertani broth (amended with 5 mM L-tryptophan, 0.065 per cent sodium dodecyl sulphate and 1%

glycerol) for 72h at 37°C under shake conditions. Supernatant was prepared/collected by centrifugation of cultures at 15,000 rpm for 20 minutes and was stored at 4°C.

In measuring the IAA equivalents, 3 ml of supernatant was pipetted into test tube and 2 ml of Salkowski's reagent (2 ml of 0.5 M $FeCl_3$ + 98 ml 35% $HClO_4$) was added to it. The tubes containing the mixture were left for 30 minutes (in dark) for the development of pink colour. Intensity of the colour was measured at 535 nm. Concentration of indole-3-acetic acid was estimated by preparing calibration curve using Indole-3-acetic Acid (IAA, Hi-media) as standard (10-100µg/ml) (Appendix I).

3.7.2 Screening of Phosphorus solubilzing PGPRs (Pikovskaya's 1948)

3.7.2.1 Qualitative estimation of phosphate solubilization

The ability of bacteria to solubilize phosphorus was tested by streaking on the PVK agar plates containing known amount of tri-calcium phosphate ($Ca_3(PO_4)_2$). The plates were incubated at 37ºC for 72h. Each treatment was replicated three times. The bacterial solubilization of phosphorus was exhibited with yellow coloured zones produced around the isolated bacterial colony.

Per cent solubilization efficiency was calculated as:

$$\text{SE } (\%) = \frac{Z+C}{C} \times 100$$

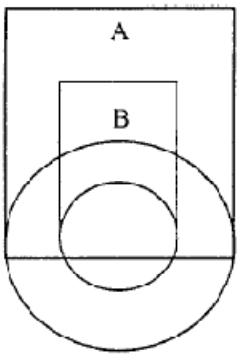
Where,

SE = Solubilization efficiency

Z = Halozone diameter (mm)

C = Colony diameter

The halo diameter around the colony was calculated by subtracting colony size from total size. Phosphate solubilization index (PSI) was measured using the formula (Edi-Premono *et al.* 1996).



Solubilization index = A/B

A = diameter (colony + halozone)

B = diameter colony

3.7.2.2 Quantitative estimation of Tricalcium phosphate solubilization

Pikovskaya's medium with composition given in (3.1.4) was used for the solubilization of phosphate. Fifty ml of medium was dispensed in 250 ml of Erlenmeyer flask containing 0.5 per cent tri-calcium phosphate (TCP) and autoclaved at 15 psi for 20 min. The flasks were inoculated with 1 per cent of the 24h old bacterial suspension (OD 1.0 at 540 nm) and incubated at 37ºC under shaken conditions for 72h. Simultaneously, two controls of PVK broth were run, one with TCP without inoculum and the other with inoculum, but without TCP. Flasks were withdrawn at 72h and contents were centrifuged at 15000 rpm for 20 min at 4ºC. Prior to centrifugation, the samples were withdrawn aseptically for determination of viable number of cells by standard viable plate count technique. The culture supernatant was used for determination of the soluble phosphate as described by Bray and Kurtz, (1945).

3.7.2.3 Assay of soluble phosphate estimation (Bray and Kurtz, 1945)

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 colorimetrically and the results were extrapolated from standard curve (Appendix I) drawn using potassium di-hydrogen phosphate.

An aliquot (0.1-1.0 ml) from the culture supernatant was made to final volume of 25 ml with distilled water and 5 ml ammonium molybdate was added. The mixture was thoroughly shaken. The contents of the flasks were diluted to 20 ml. 1.0 ml of chlorostannous acid was added and its volume was made to 25ml in the volumetric flask. The contents were mixed thoroughly and the blue coloured intensity was measured after 10 minutes at 660 nm. An appropriate blank was kept in which all reagents were added except the culture. P-solubilization was calculated as:

P -solubilizaton = T- C

where,

T = PVK with TCP, inoculated

C = PVK with TCP, un-inoculated

3.7.3 SIDEROPHORE PRODUCTION

3.7.3.1 Qualitative estimation of siderophore by Chrome-azurol-S (CAS) plate assay (Schwyn and Neilands, 1987)

Siderophore production was detected by CAS plate assay method. Sterilized CAS 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 with 25 ml of the medium.

Twenty-four hours old culture of the test bacteria was spotted on pre-poured blue coloured CAS agar plates. Plates were incubated for 72h, at 37ºC. Formation of a bright zone with a yellowish (hydroxamate), pinkish (catecholate) and whitish (carboxylate) colour in the dark blue medium indicated the production of siderophore.

3.7.3.2 Quantitative estimation of siderophore using Chrome-azurol-S (CAS) liquid assay method (Schwyn and Neilands, 1987)

0.1 ml of cell free extract of culture supernatant was mixed with 0.5 ml Chrome-azurol-S (CAS) assay solution (Appendix I) along with 10 µl of shuttle solution (0.2M 5-Sulfosalicyclic acid). It was kept at room temperature for ten minutes and absorbance was recorded at 630 nm. 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:

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

Where,

A_r is defined as absorbance at 630 nm of reference.

A_s is the absorbance at 630 nm of the test.

3.7.4 AGAR STREAK METHOD FOR ANTIFUNGAL ACTIVITY

In order to test the efficacy of the rhizobacterial antagonists, a loopfull of 48h old culture of each isolate was streaked a little below the centre of the prepoured petriplates of malt extract agar (MEA) and incubated at 37ºC. After 24h, a 5mm diameter actively growing test fungal pathogen was placed simultaneously on one side of the streak.

A check inoculated with the test pathogen only was kept as control for comparison. Each treatment was replicated 3 times. The plates were incubated at 24±1ºC and per cent growth inhibition was calculated according to Vincent, (1947).

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

Where,

I = Per cent growth inhibition

C = Growth of fungus in control

T = Growth of fungus in treatment

3.7.5 HCN production (Baker and Schippers, 1987)

The bacterial cultures were streaked on prepoured plates of King's medium B amended with 4.4g/L glycine. Whatman No.1 filter paper strips were soaked in 0.5% picric acid in 0.2% sodium carbonate and was placed in between the petriplates. Petriplates were sealed with parafilm and were incubated at 37ºC for 1-4 days. Uninoculated control was kept for comparison. Plates were observed for change of colour of filter paper from yellow to orange brown to dark brown.

3.7.6 Nitrogen fixing ability

To test the efficacy of the rhizobacteria as nitrogen fixer, loopful of 24h old culture of each isolate were streaked on nitrogen free glucose agar medium described in (3.1.6), incubated for 72h and the colonies showing growth were selected.

3.7.7 Cell wall degrading enzymes production

3.7.7.1 Proteolytic enzymes (Fleming *et al*. 1975)

All bacterial isolates were screened for proteolytic activity by plate assay method on skim milk agar (nutrient agar (100 ml) supplemented with separately sterilized skim milk). Spot inoculation of 24 h old bacterial culture was done on skim milk agar plate. Plates were incubated at 37ºC for 48 h and observed for proteolysis i.e. clear zone (diameter, mm) produced around the spot.

3.7.7.2 Amylolytic enzymes (Shaw *et al*. 1995)

All bacterial isolates were screened for amylolytic activity by plate assay method. Spot inoculation of 24 h old bacterial culture was done on starch hydrolysis agar plate. Plates were incubated at 37ºC for 48 h. Iodine was poured on the agar plates after incubation. Agar plates were observed for starch hydrolysis i.e. clear zone (diameter, mm) produced around the spot.

3.7.7.3 Lipase enzymes (Kumar *et al.* 2012)

All the bacterial isolates were screened for lipase production by plate assay method. Spot inoculation of 24h old bacterial culture was done on TBA medium. Plates were incubated at 37°C for 48h. Agar plates were observed for clear zone (diameter, mm) produced around the spot.

3.8 PHENOTYPIC CHARACTERIZATION OF IAA PRODUCING BACTERIAL ISOLATES

The most effective bacterial isolates exhibiting maximum IAA production and production of siderophore and P-solubilization and also capable of causing maximum inhibition of fungus under *in vitro* conditions were identified on the basis of morphological, cultural and biochemical characteristics by criteria of Bergey's Manual of Systematic Bacteriology (Claus and Berkley, 1986).

3.8.1 Morphological characterization

Morphological characteristics of isolates including colony morphology, Gram's reaction and cell shape were investigated for isolates exhibiting multifarious plant growth promoting traits.

3.8.2 Biochemical characterization

Biochemical characteristics of isolates including citrate utilization, H_2S production, Ammonia production, methyl red, indole production and ability to ferment sugars *viz.* sucrose, lactose, dextrose were investigated.

3.9 TOMATO SEED GERMINATION AND GROWTH PROMOTION ASSAY UNDER NET HOUSE CONDITIONS

3.9.1 Type and preparation of potting mixture

Soil obtained from a depth of 0-20 cm from a fallow area in forest block of the Department of Silviculture and Agro forestry, UHF, Solan was sieved through 2 mm sieve and used for pot culture experiment. The sand, soil and farm yard manure (FYM) was mixed in a ratio of 1:1:1 in order to make the potting mixture. The mixture was then filled in the pots and moistened to one third saturation capacity**.**

3.9.2 Seed surface sterilization

Seeds were surface sterilized by 0.2 per cent mercuric chloride ($HgCl_2$) solution for 3 minutes and rinsed several times with sterilized distilled water. Surface sterilization of

seeds was cross checked by inoculating the surface sterilized nutrient broth. The bacterial growth, if any, around the seeds were recorded after 24h of incubation.

3.9.3 Seed treatment

48h old culture (× 10^8 CFU) of different bacterial isolates was prepared in nutrient broth. About thirty tomato seeds were dipped in these 48h old culture for 6h (Walia, 2008). Uninoculated control was also kept.

3.9.4 Sowing of seeds

Seeds were sown in pots containing potting mixture. Forty seeds treated or untreated with cell suspension of 1.5 O.D were sown at equidistance and covered with moss grass till emergence of plumule. After 3-4 days of seedlings emergence thinning was done and 3 plants per pot were maintained. Three replicate pots per treatment with 35-40 plants in each pot were placed in a randomized block design in net house.

3.10 SEED GERMINATION STUDIES

Germination studies were conducted under net house conditions.

3.10.1 Germination per cent

Germination per cent was calculated as the number of seeds sown and the number of seeds germinated expressed in percentage**.**

3.10.2 Vigour index

The vigour index was calculated using the formula as described by Abdul Baki and Anderson, (1973).

Vigour Index = (Mean root length + Mean Shoot length) X Germination (%)

3.11 ANALYSIS OF SEEDLING TRAITS

Tomato seedlings obtained from net house and in nurseries under field conditions were analyzed for different traits such as: leaf characteristics, shoot characteristics, root characteristics and root – shoot ratio.

3.11.1 Shoot characteristics

3.11.1.1 Shoot length

Shoot length was recorded in centimeters from the ground level to the apical bud of stem.

3. 11.1.2 Shoot weight

Shoot dry weight was taken after drying to constant weight in an oven at 60^{o}C for 72h.

3. 11.2 Root characteristics

3. 11.2.1 Root length

The length of tap root was recorded in centimeters using measuring scale by placing it horizontally on the ground.

3. 11.2.2 Root weight

Root dry weight was taken after drying to constant weight in an oven at 60^{o}C for 72h.

3.12 GROWTH PROMOTION ASSAY OF KIWI SEEDLINGS UNDER NET HOUSE CONDITIONS

3.12.1 Seedling treatment

48h old culture ($\times 10^{8}$ cfu) of different bacterial isolates was prepared in nutrient broth. About thirty kiwi seedlings were dipped in these 48h old culture for 60 mins and were further drenched with the same liquid formulation of bacterial cultures. Uninoculated control was also kept.

3.12.2 Planting of seedlings

Seedlings were planted in pots containing potting mixture. Six treated seedlings with cell suspension of 1.5 O.D were sown at equidistance. After 6-7 days of growth of seedlings thinning was done. Three replicate pots per treatment with three plants in each pot were placed in a randomized block design in net house.

3.13 ANALYSIS OF SEEDLING TRAITS

Kiwi seedlings grown in net house and nurseries under field conditions were analyzed for different traits such as: leaf characteristics, shoot characteristics, root characteristics and root – shoot ratio.

3.13.1 Shoot characteristics

3.13.1.1 Shoot length

Shoot length was recorded in centimeters from the ground level to the apical bud of stem.

3. 13.1.2 Shoot weight

Shoot dry weight was taken after drying to constant weight in an oven at 60^{o}C for 72h.

3.13.1.3 Stem diameter

Stem diameter was measured using vernier calipers from the base of the stem.

3. 13.2 Root characteristics

3. 13.2.1 Root length

The length of tap root was recorded in centimeters using measuring scale by placing it horizontally on the ground.

3. 13.2.2 Root weight

Root dry weight was taken after drying to constant weight in an oven at 60^{o}C for 72h.

3.13.3 Leaf surface area

Leaf surface area was measured with the help of surface area meter.

3.14 MOLECULAR CHARACTERIZATION OF SELECTED ISOLATE BY 16S rDNA SEQUENCE ANALYSIS

3.14.1 Genomic DNA extraction by conventional method (Sambrook *et al.* 1989)

Bacterial isolate B_2S_{10} was grown overnight at 37ºC in nutrient broth at 200 rpm. The cells were harvested and processed for DNA isolation.

Requirements:

96-100% Ethanol

Sterile, DNase- free pipette tips and microcentrifuge tubes

RNase A (50 mg/ml)

10 % SDS

Phenol : Chloroform (1:1)

Extraction buffer (20 mg/ml lysozyme, 100mM Tris- HCl, 50mM EDTA, 500 mM NaCl)

TE buffer (10mM Tris HCl, 1mM EDTA)

Procedure

1. 5 ml of overnight grown culture was transferred to a micro-centrifuge tube and centrifuged at 13,000 rpm for 1 minute and supernatant was discarded.
2. Bacterial pellet was suspended in 500µl of extraction buffer and 50 µl of 10 % SDS. Cell pellet was resuspended by vortexing or pipetting.
3. Incubation at 65ºC water bath was done for 30 min until the sample lysate becomes clear. During incubation, tube was inverted at every 3 min.
4. After 65ºC incubation, 2 µl of RNase A (50mg/ml) was added to sample lysate and mixed by vortexing. Then incubated at room temperature for 5 min.
5. To the lysate equal volume of phenol:chloroform (1:1) was added and mixed well.
6. Centrifuged the above mixture at 10,000 rpm for 5 min at room temperature. Two layers were formed. Upper aqueous layer was collected in new eppendorf tube with the help of pipette.
7. The phenol:chloroform extraction step was repeated.
8. Centrifuged the above mixture at 10,000 rpm for 5 min at room temperature. Two layers were formed. Upper aqueous layer was collected in another new eppendorf tube with the help of pipette.
9. 1/10 volume of 5M NaCl and 2.5 volume of absolute ethanol was added to aqueous phase collected in eppendorf tube.
10. Incubation was done at - 20ºC overnight.
11. Centrifuged the above mixture at 12,000 rpm for 20 min at room temperature and supernatant was discarded.
12. The DNA pellet was washed with 1 ml of 70% ethanol.
13. Centrifuged the above mixture at 12,000 rpm for 5 min at room temperature and supernatant was discarded.
14. DNA pellet was air dried for about 15 min until all the residual ethanol got evaporated.
15. Finally the DNA pellet was suspended in appropriate amount of TE and quantify.

3.14.2 Gel electrophoresis

The isolated DNA was finally suspended in 100 µl of elution buffer and quantified on 1% agarose gel.

3.14.3 Primer

Universal 16S rDNA primers were used for the amplification of P-solubilizing selected bacterial isolates.

3.14.4 PCR amplification of 16S rDNA

PCR reaction was carried out in 20 µl reaction containing ~50ng of template DNA, 20 pmoles of each primers, 0.2 mM dNTPs and 1 U Taq polymerase (Banglore Genei) in 1x PCR buffer. Reaction were cycled 35 times as 94ºC for 30 s, 58ºC for 30 s, 72ºC for 1 min 30 sec. followed by final extension at 72ºC for 10 min.

The PCR products were analyzed on 1% agarose gel in 1x TAE buffer, run at 100V for 1 hr. Gels were stained with ethidium bromide and photographed. Amplified PCR products were eluted from the gel using gel extraction kit (Hi Yield Gel/ PCR DNA Extraction Kit from Real Genomics).

3.14.5 Gel elution was done by Hi Yield Gel/ PCR DNA Extraction Kit

Agarose gel slice containing relevant DNA fragments was excised and extra agarose was removed to minimize the size of the gel slice. 300 mg of the gel slice was transferred into a microcentrifuge tube. 500 µl of DF buffer was added to the sample and mixed by vortexing. Incubation was done at 55^{o}C for 10-15 min until the gel slice gets completely dissolved. During incubation, the tube gets inverted at every 2-3 min and dissolved sample mixture get cool down to room temperature.

DNA binding:

A DF column was placed in 2 ml collection tube. 800 µl of sample mixture (from above step) was applied into the DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube.

Wash:

600 µl of wash buffer (ethanol added) was added into DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube. Centrifuged again for 3 min at 13,000 rpm to dry the column matrix.

DNA elution:

Dried column was transferred into a new microcentrifuge tube. 15-30 µl of elution buffer or distilled water was added into the center of column matrix. Stand for 2 min until

elution buffer or distilled water was absorbed by the matrix. Centrifugation was done for 2 min at 13,000 rpm to elute purified DNA eluted fragment was then sequenced using PCR primers.

Sequence and phylogenetic analysis

Sequencing was done as per manufacturer instructions (Eurofins Genimocs). The sequence was aligned with corresponding sequences of 16S rDNA from the database using BLAST from the website http://www.ncbi.nlm.nih.gov/blast (Altschul *et al.* 1997).

Multiple alignments were generated by the MULTALIN program from the web site: http://prodes.toulouse.inra.fr/multialin/multialin.html (Corpet, 1988). Phylogenetic tree was constructed using alignments drawn using Clustal W from the website http://www2.ebi.ac.uk/clustalw/ (Higgins *et al*.1994) and Mega 6. Tree will be viewed with the help of Tree view from the website http://taxonomy.zoology.gla.ac.uk/rod/treeview.html (Page, 1996).

3.15 OPTIMIZATION Of IAA PRODUCTION OF MOST EFFICIENT BACTERIAL ISOLATE

3.15.1 OPTIMIZATION OF IAA PRODUCTION

3.15.1.1Effect of incubation period on IAA production

Effect of incubation time on IAA production was studied by growing the test organism at different incubation period (24, 48,72, 96 and 120h). The optimum incubation time suited for the IAA production was maintained for further experiments.

3.15.1.2 Effect of temperature on IAA production

Effect of different temperature on IAA production was studied by growing the test at different temperature (30, 35, 40 and 45°C). The optimum temperature studied for IAA production was maintained for further experiments.

3.15.1.3 Effect of pH on IAA production

IAA production by the test bacterial isolate was studied at different pH. The medium was adjusted to various pH (6.0, 7.0, 8.0, 9.0 and 10.0). The optimum pH was maintained for all further experiments.

3.15.1.4 Effect of inoculum size on IAA production

Inoculum size of different population densities (0.5%, 1.0%, 1.5%, 2.0%) will be added to IAA broth and IAA production will be determined after incubating flask at

optimized temperature and incubation period. The inoculums size showing maximum IAA production will be used for all further experiments.

3.15.1.5 Effect of different concentration of tryptophan on IAA production

Different concentration of tryptophan (0.1, 0.25, 0.5, .75, and 1.0%) will be used to determine the concentration at which the bacteria produce IAA effectively. The optimum concentration will be maintained for all further experiments.

3.15.1.6 Effect of various ingredients of Luria Bertani broth on IAA production

Quantitative estimation of IAA production was carried out using bacterial isolate grown on Luria Bertani medium for 3 days. To elucidate the influence of each ingredients of the medium, IAA production was estimated by deleting one component from the medium composition.

3.16 EVALUATION AND ASSESSMENT OF EXTRACTED IAA BY BIOASSAY AND THIN LAYER CHROMATOGRAPHY

Bacterial colony of the best IAA producing bacteria was grown in nutrient broth medium at 30°Cfor 120 h under shake conditions (90rpm). Supernatant was extracted by centrifugation at 10,000 rpm for 30 minutes at 4°C (Chaiharn and Lumyong, 2011).

3.16.1 Extraction of IAA

Mix 100 ml of supernatant with 250 ml of saturated $NaHCO_3$ solution in separatory funnel

↓

Extract with 300 ml ethyl acetate (2X)

↓

Acidify aqueous layer to pH 2.5 with 5 N HCl

↓

Add equal volume of ethyl acetate and shake vigorously for 5 minutes

↓

Separate the ethyl acetate fraction; and re-extract the aqueous layer (2X) with 300 ml of ethyl acetate solvent

↓

Pool all ethyl acetate fractions and dried over Na_2SO_3

↓

Evaporate the ethyl acetate extract on a rotary evaporator at 40°C

↓

Dissolve residue in 2 ml of methanol for thin layer chromatography

3.16.2 Separation of IAA

IAA extracts thus obtained are then separated by thin layer chromatography (TLC) (Chaiharn and Lumyong, 2011).

Spot 100 µl of the methanol extract on thin layer plate pre spread with silica gel G. Develop in solvent system i.e. isopropanol; water (30:20 v/v) for 12-14 hours. Plates were sprayed with Salkowsky's reagent. Observe development of pink colour spots. Calculate the R_f value as:

$$R_f = \frac{\text{Distance covered by solute}}{\text{Distance run by solvent}}$$

3.17 STATISTICAL ANALYSIS

The data obtained was subjected to appropriate statistical analysis (completely randomized design/ variability analysis/ t-test/ correlation studies) as per the requirement of the experiment.

Chapter-4

RESULTS AND DISCUSSION

This chapter deals with the experimental results and discussion and most likely causes and effects, Underlying patterns, resulting in the predictions, evidence or even a line of reasoning, which support interpretation, agree or perhaps contradict previous work, in the light of the available literature. The salient findings obtained during the course of investigation are presented under following main headings:

4.1 Culturable rhizospheric and endorhizospheric bacterial population associated with different cultivars of kiwi vines with varying age group

4.2 Screening of total culturable bacterial populations from rhizospheric soil and root endosphere associated with different cultivars of kiwi vines using modified replica plating technique

4.3 P-solubilizing bacterial population in rhizospheric soil and root endosphere among different cultivars of kiwi vines of kiwi vines

4.4 Siderophore producing bacterial population in rhizospheric soil and root endosphere among different cultivars of kiwi vines

4.5 Nitrogen fixing bacterial population in rhizospheric soil and root endosphere among different cultivars of kiwi vines

4.6 Comparison of rhizospheric soil and root endophytic bacterial isolates possessing plant growth promoting traits (phosphate solubilization, siderophore production and nitrogen fixation)

4.7 Screening of IAA producing population in rhizospheric soil and root endosphere of kiwi vines

4.8 Screening of culturable isolates for multifarious plant growth promoting activities

4.9 Selection of rhizospheric and endophytic iaa producing bacterial isolates on the basis of overall plant growth promoting traits

4.10 Metabolic characterization of selected bacterial isolates

4.11 Tomato seed germination and growth promotion assay under net house conditions

4.12 Kiwi seedlings growth promotion assay under net house conditions

4.13 Relationship (r) studies between growth parameters IAA production, phosphate solubilization and siderophore production

4.14 Molecular identification of most efficient antagonistic bacterial isolate b_2s_{10} based on 16s rDNA gene sequencing

4.15 Optimization of cultural conditions for the production of IAA by selected bacterial isolate B_2S_{10}

4.16 Evaluation and assessment of extracted IAA by bioassay and thin layer chromatography

Rhizosphere is a special micro-ecosystem of plant-microorganism interactions. Rhizosphere bacterial communities play an important role in plant growth promotion and suppressing soil borne diseases (Sun *et al*. 2013). The race for increasing crop yields by adopting intensive agronomic practices and applying more fertilizers is thought to have adverse effects on the diversity of bacteria in agricultural fields. The beneficial microorganisms in bulk soil and rhizosphere in natural agro ecosystems contributing to soil health and plant productivity can be exploited as bioinoculants to increase more crop productivity (Rawat and Mushtaq, 2015). Moreover, PGPR are largely responsible for functioning of ecosystems because of their role in transformation of most of nutrients and their beneficial effects on plant growth and health (Verma *et al*. 2010). Keeping this in view, in the present study an attempt was made to isolate the plant growth promoting rhizobacteria associated with different cultivars of kiwi vines and evaluation of their plant growth promoting effect under net house conditions.

4.1 CULTURABLE RHIZOSPHERIC AND ENDORHIZOSPHERIC BACTERIAL POPULATION ASSOCIATED WITH DIFFERENT CULTIVARS OF KIWI VINES WITH VARYING AGE GROUP

Rhizospheric soil and root samples were collected from four different cultivars of kiwi vines of age group 30 (Hayward, Monty, Bruno and Allison) and 2 years kiwi vines of cultivar Allison with varying genotype (Staminate and Pistillate) from Experimental farm at Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni (Solan). The population densities of bacteria capable of growing on different media were enumerated and reported as $CFUg^{-1}$ of whole root system or ($CFUg^{-1}$) of rhizospheric soil on dry weight basis using surface sterilization, aseptic trituration and dilution plating assay (Shishido *et al*. 1999).

4.1.1 Colony library of culturable bacterial population associated with rhizospheric soil of kiwi vines

The population level of total culturable bacteria, colony morphotypes and per cent IAA-producers colonizing rhizospheric soil of kiwi vines from four different cultivars of kiwi vines of age group 30 (Hayward, Monty, Bruno and Allison) and 2 years kiwi vines of cultivar Allison with varying genotype (Staminate and Pistillate) growing at Nauni, Solan is presented in Fig.3a and Fig 3b respectively. The results revealed that the detectable growth (colony forming unit) of rhizospheric soil bacterial population was observed in all soil samples of each cultivar. Fifty percent of total twenty one soil samples harboured IAA-producers. IAA, a member of the group of phytohormones is considered as one of the most important native auxin (Harikrishnan *et al*. 2014). On the basis of colony morphology obtained on nutrient agar medium two to ten morphotypes were obtained from twenty one rhizospheric soil samples of different cultivars with varying age group. In contrast to our study, only two morphotypes were predominant in rhizospheric soil samples of apple collected from five different locations of Himachal Pradesh, India (Mehta *et al*. 2013).

Two to ten different morphotypes were observed in 30 years old kiwi vines of all four types of cultivars. The total viable bacterial population present in rhizospheric sample of kiwi cultivar Hayward of age group 30 years (Pistillate) on nutrient agar plate ranged from 76 to 112×10^6 CFU g^{-1}. Two colony morphotypes were prevalent in all three soil samples of Hayward and were 100% IAA producers.

Total culturable bacterial population present in three rhizospheric soil samples of kiwi cultivar Bruno of age group 30 years (Pistillate) varied from 90 to 162×10^6 CFU g^{-1}. Based on colony morphology, sample B_1 showed five morphotypes, sample B_2 showed ten morphotypes and sample B_3 showed four morphotypes. The per cent IAA producers in the total culturable bacterial population of the three samples of cultivar Bruno ranged between 0 to 50%.

The total number of viable cultivable bacteria recovered from rhizospheric soil of cultivar Monty (Pistillate, 30 years) ranged from 90 to 104×10^6 CFU g^{-1}. M_1 rhizospheric soil sample showed two morphotypes whereas, three different morphotypes were obtained in rhizospheric soil samples M_2 and M_3. Among these the total the total IAA producers ranged between 70 to 100%.

The total viable bacterial population from kiwi cultivar Allison (Pistillate, 30 years) ranged from 61 to 90×10^6 CFU g^{-1}. Rhizospheric soil sample A_1F and A_3F showed the presence of two morphotypes whereas A_2F showed only one morphotype. IAA producers ranged between 0 to 50% in all the three samples.

The total cultivable bacterial population present in rhizospheric soil of kiwi cultivar Allison (Staminate, 30 years) varied from 42 to 80×10^6 CFU g^{-1}. Rhizospheric soil sample A_1M showed the presence of three morphotypes, sample A_2M was harboured by four morphotypes and sample A_3M harboured only one morphotype. IAA producing bacteria were present in only two of the three samples whereas, per cent IAA producers ranged from 30 to 50 %.

Total culturable bacterial population present in rhizospheric soil samples of kiwi cultivar Allison (Pistillate, 2 years) varied from 34 to 100×10^6 CFU g^{-1}. Rhizsospheric soil sample $A_1F_{(2)}$ showed the presence of two morphotypes while sample $A_2F_{(2)}$ and $A_3F_{(2)}$ showed the presence of only one morphotype. Per cent IAA producers ranged from 0 to 50%.

The total viable bacterial population present in rhizospheric soil samples of kiwi cultivar Allison (Staminate, 2 years) obtained on nutrient agar medium ranged from 60 to 110×10^6 CFU g^{-1}. Rhizospheric soil samples $A_1M_{(2)}$ and $A_3M_{(2)}$ showed the presence five different morphotypes while soil sample $A_2F_{(2)}$ harboured only four morphotypes. Culturable bacterial population harbouring IAA producers ranged from 30 to 50%.

The enumeration of total cultivable bacteria from different cultivars of kiwi vines with different genotype and varying age groups (Table 1) revealed that among these cultivars, Bruno (Pistillate, 30 years) had maximum rhizospheric soil population (105.66×10^6 CFU g^1) while the minimum bacterial population (61.00×10^6 CFU g^{-1}) on nutrient agar medium was observed for cultivar Allison (Staminate, 30 years).

The above results indicate that a large diversity of rhizobacteria depicting variable morphotypes were associated with the kiwi vines of different cultivars of different age group. Variability in IAA production was also observed among rhizospheric isolates with respect to different cultivars, age group and genotypes. These IAA producing bacterial population can exogenously supply plant hormones that may affect plant growth by changing the balance of endogenous levels of hormones. To date, no study has been conducted related to the bacterial diversity of IAA producing bacterial population associated with different cultivars and age

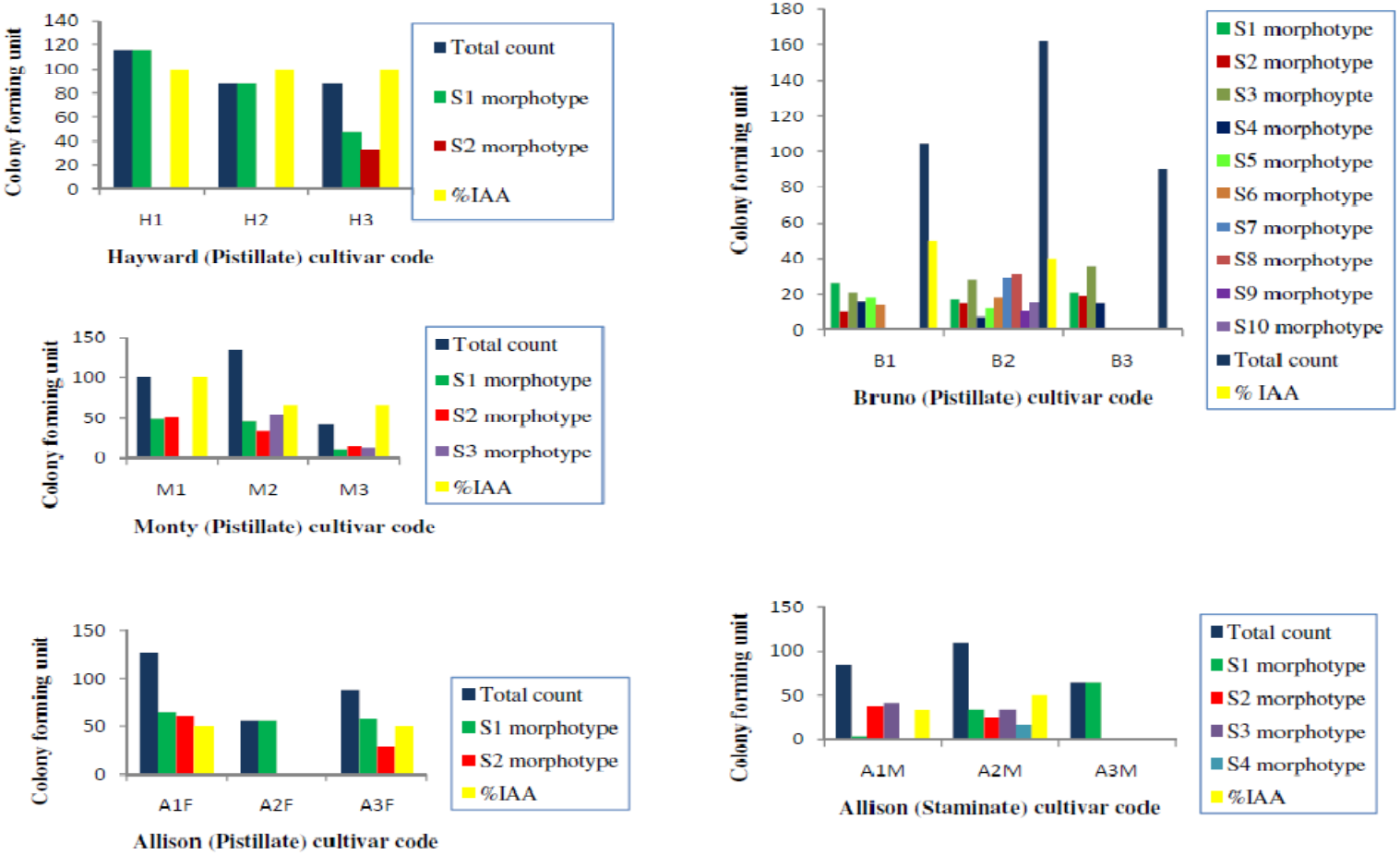


Fig. 3a Enumeration of culturable rhizospheric soil bacterial population, IAA producers and morphotypes in different cultivars of kiwi vines of age group 30

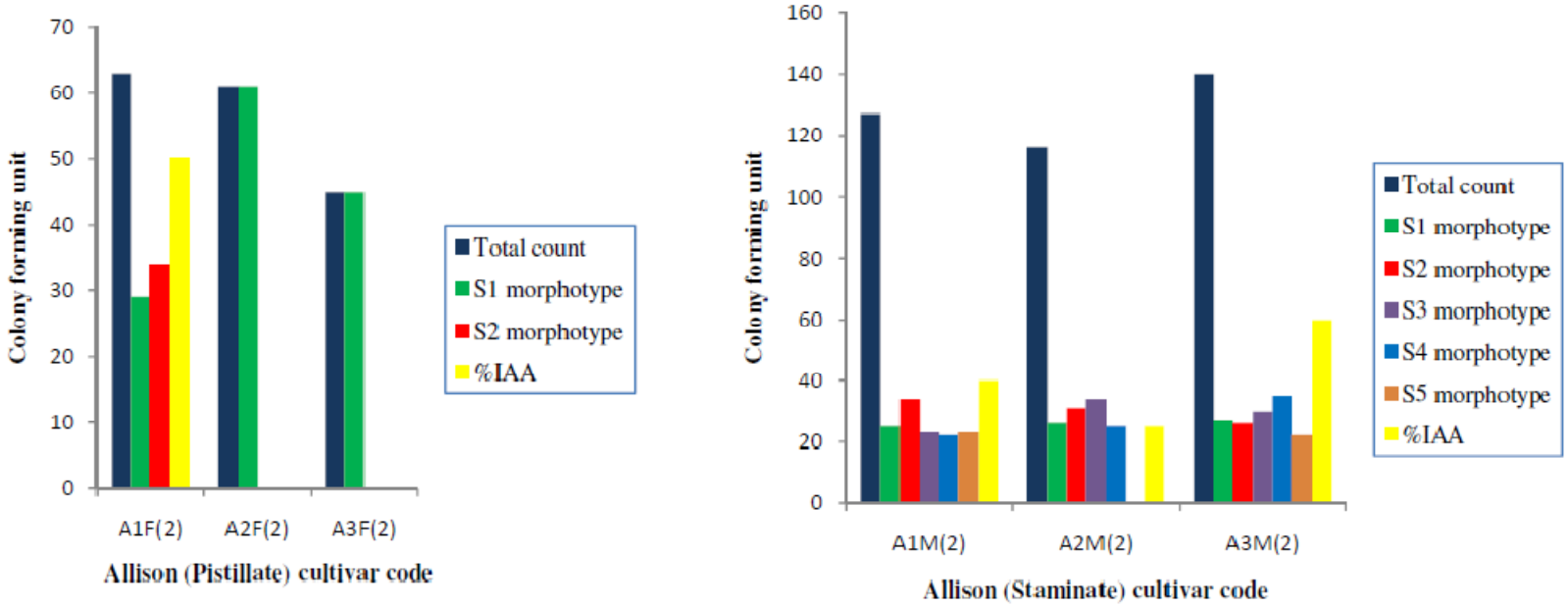


Fig. 3b Enumeration of culturable rhizospheric soil bacterial population, IAA producers and morphotypes of kiwi vines of age group 2

group of kiwi vines. However, Mehta (2012) had studied the diversity of P-solubilizing bacterial population associated with apple trees. Asghar *et al*. (2000) had reported variable IAA producing population from different rapeseed varieties selected from different sites of Punjab (Pakistan).

4.1.2 Colony library of cultivable root endophytic bacterial population associated with kiwi vines of different cultivars with varying genotype and age group

The population level inhabited inside the roots of kiwi vines from four different cultivars with varying genotype and age group is represented in Fig.4a and Fig.4b. It was observed that (76.19%) of total of twenty one root samples showed the presence of IAA producers.

The total culturable root endophytic bacterial population associated with kiwi cultivar Hayward (Pistillate, 30 years) ranged from 48 to135 $\times 10^3$ CFU g^{-1}. Root endophytic samples H_1 and H_2 showed two colony morphotypes and H_3 showed a total of six different morphotypes. All the three root samples harboured IAA producers ranged between 50 to 100%.

The total endophytic bacterial population in root samples of kiwi cultivar Bruno (Pistillate, 30 years) ranged from 78 to 108 $\times 10^3$ CFU g^{-1}. Sample B_1 showed the presence of four morphotypes, B_2 showed six morphotypes whereas sample B_3 showed three different morphotypes. Out of total three samples two harboured IAA producers and the range varied between 33.34 to 50%. Presence of distinct morphotypes from root samples of apple has been reported by Mehta *et al*. (2013).

The total viable root endophytic bacterial population present in root samples of kiwi cultivar Monty (Pistillate, 30 years) ranged from 55 to 124 $\times 10^3$ CFU g^{-1}. Root endophytic samples M_1 and M_2 showed six different morphotypes while sample M_3 showed two morphotypes. All three root samples harboured IAA producing bacteria ranging between 33.34 to 50%. Variation in occurence of morphotypes from root samples of same cultivar has been observed which is for the first time in case of kiwi cultivars.

The total viable root endophytic bacterial population in root samples of kiwi cultivar Allison (Pistillate, 30 years) ranged from 75 to 112 $\times 10^3$ CFU g^{-1}. Samples A_1F and A_3F showed four different morphotypes while A_2F showed only two morphotypes. Two out of three samples harboured IAA producers ranging between 33.34 to 50%.

The total number of cultivable bacteria recovered from root samples of kiwi cultivar Allison (Staminate, 30 years) ranged from 86 to 132 × 10^3 CFU g^{-1}. Sample A_1M showed seven morphotypes, sample A_2M showed six morphotypes while A_3M showed four different morphotypes. Only two of the three root samples showed the presence of IAA producers ranging between 25 to 66.60%. Maximum variation among morphotypes was observed in endophytic samples of Allison (Staminate) of age group 30 years. In this context it is concluded that the mature (30 years) kiwi cultivars might constitute an ideal niche for exploring new IAA producers with diverse mechanisms of PGP traits. This is supported by the fact that, while studying diversity more importance should be given to the richness of niche and habitant (Bell *et al*. 2004).

The total number of cultivable bacteria recovered from root samples of kiwi cultivar Allison (Pistillate, 2 years) ranged from 65 to 110 × 10^3 CFU g^{-1}. Root endophytic sample $A_1F_{(2)}$ showed four morphotypes, $A_2F_{(2)}$ showed six morphotypes and $A_3F_{(2)}$ showed three morphotypes. IAA producers were present in all three root samples ranging 30 to 50 %.

The total viable root endophytic bacterial population in root sample of kiwi cultivar Allison (Staminate and 2 years) varied from 109 to 130 × 10^3 CFU g^{-1}. Sample $A_1M_{(2)}$ showed six morphotypes, $A_2M_{(2)}$ showed four morphotypes while $A_3M_{(2)}$ showed seven morphotypes. Only two of the three samples harboured IAA producers ranging from 33.34 to 42.85%.

Table 1 revealed that analysis of variance for enumeration of total cultivable endophytes and the result showed that among all the kiwi cultivars, Hayward (Pistillate, 30 years) harboured maximum endophytic bacterial population (95.34 × 10^3 CFU g^{-1}). Minimum root endophytic bacterial population (51.34 × 10^3 CFU g^{-1}) was found in Allison (Pistillate and 30 years) cultivar.

The presence of large population of bacterial isolates in all the rhizospheric and endorhizospheric samples may be due to positive influence exerted by root exudates in microbial colonization. Overall our results revealed that more rhizospheric and endophytic bacterial population was obtained from soil and root samples associated with cultivars of age group 30. Similar to our work Recuenco and Vuurde, (2000) carried out studies on root endophytic colonization of 11 old-grown pea cultivars of different age group. They observed that more endophytic bacreria were associated with pea cultivars at the mature stage. This may be because bacterial communities residing in the rhizosphere respond, in particular, with

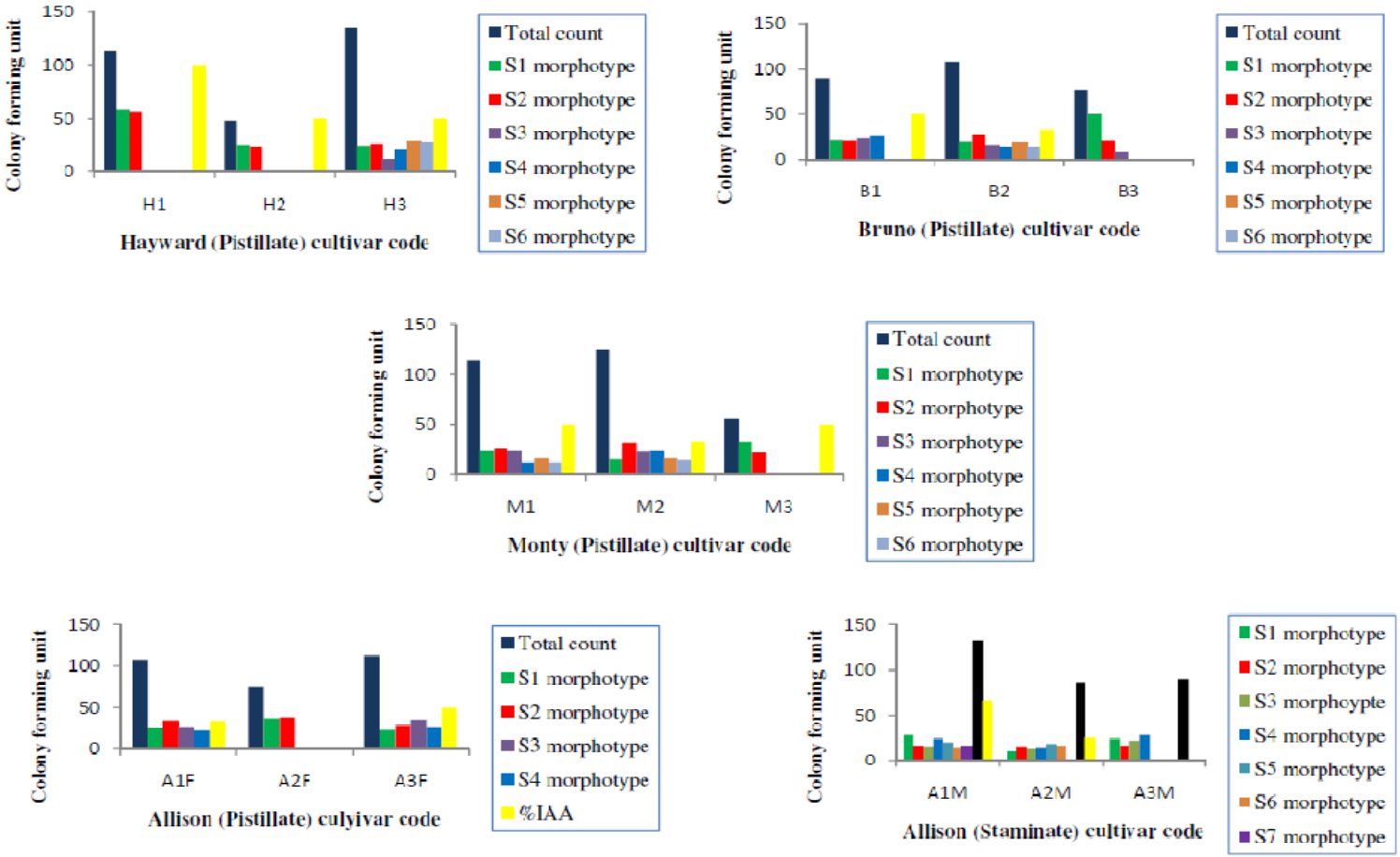


Fig. 4a Enumeration of root endophytic bacterial population, IAA producers and morphotypes of kiwi vines of age group 30

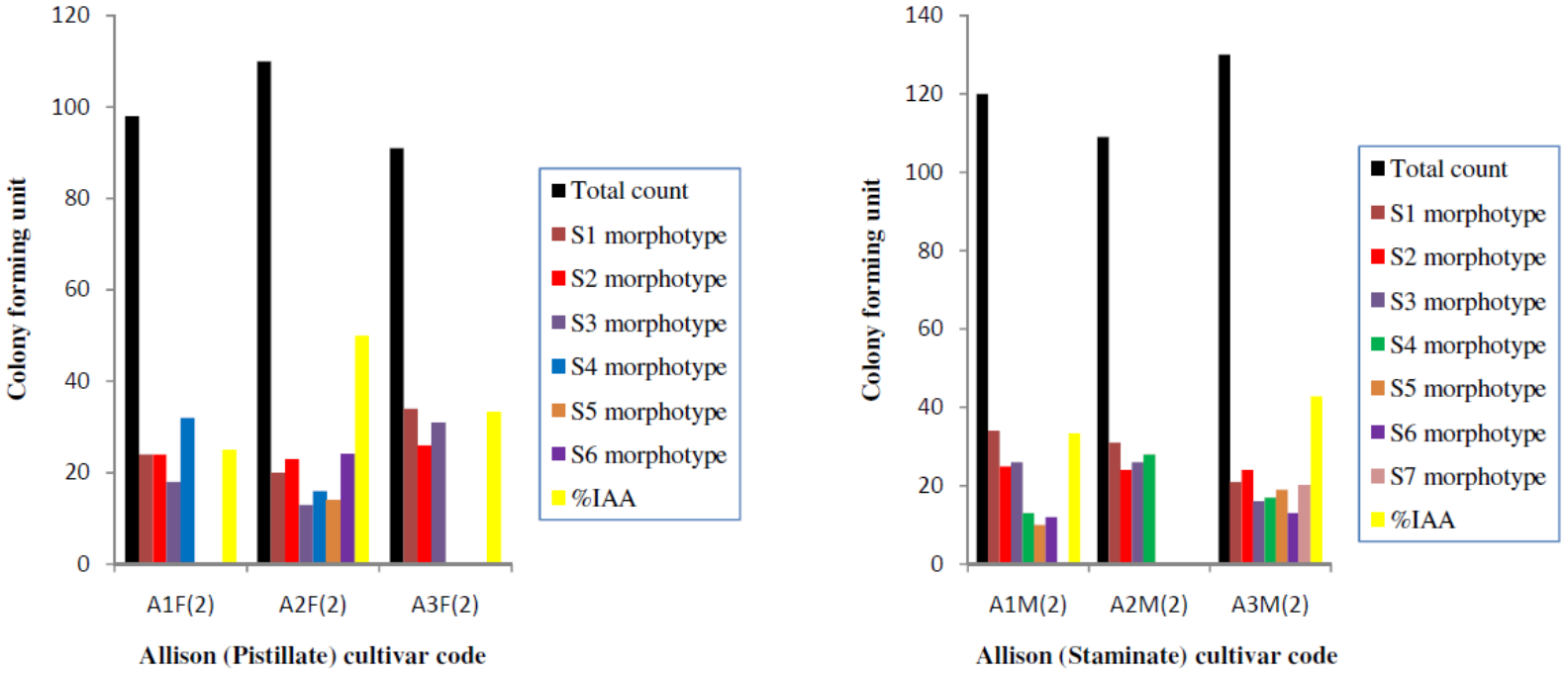


Fig. 4b Enumeration of root endophytic bacterial population, IAA producers and morphotypes of kiwi vines of age group 2

respect to density, composition, and activity, to the plethora and diversity of organic root exudates, resulting in plant species-specific microflora which may eventually vary in stage of plant growth (Wieland *et al*. 2001).

Both rhizospheric soil and the roots of kiwi vines seem to be the preferential sites for IAA producers suggesting that IAA producers proliferate both in rhizosphere and endorhizosphere. To best of our knowledge, this is first report of IAA producing endophytes residing within the kiwi vines root tissues. However P-solubilizing endophytes were reported from apple trees by Mehta *et al.* (2014). The plant-associated habitat is a dynamic environment in which many factors affect the structure and species composition of the microbial communities that colonize roots, stems, branches, and leaves. It has previously been shown that endophytic communities vary spatially in the plant or may be dependent on the interaction with other endophytic or pathogenic bacteria (Araujo *et al*. 2002).

Table 1 Enumeration of total culturable rhizospheric soil and root endophytic bacterial populations of kiwi vines

Age group	**Genotype**	**Cultivar**	***Rhizosphere soil bacterial population($\times 10^6$CFU/g soil) on Nutrient agar medium**	***Root endophytic bacterial population($\times 10^3$CFU/g soil) on Nutrient agar medium**
30 years	Pistillate (Female)	Hayward	88.66	95.34
		Bruno	105.66	55.34
		Monty	98.00	70.00
		Allison	76.00	51.34
	Staminate (Male)	Allison	61.00	79.67
2 years	**Genotype**	**Cultivar**	***Rhizosphere soil bacterial population($\times 10^6$CFU/g soil) on Nutrient agar medium**	***Root endophytic bacterial population($\times 10^3$CFU/g soil) on Nutrient agar medium**
	Pistillate (Female)	Allison	76.00	91.00
	Staminate (Male)	Allison	90.00	80.34

*Average of three samples

4.2 SCREENING OF TOTAL CULTURABLE BACTERIAL POPULATIONS FROM RHIZOSPHERIC SOIL AND ROOT ENDOSPHERE ASSOCIATED WITH DIFFERENT CULTIVARS OF KIWI VINES USING MODIFIED REPLICA PLATING TECHNIQUE

The population densities obtained on NA media were replica plated on three different media (PVK, CAS and nitrogen-free media) in order to screen for P-solubilizers, siderophore producers and nitrogen fixers. The population densities of bacteria capable of growth on different media were enumerated and reported as CFU $g^{-1}$of whole root system or colony CFU g^{-1} of rhizospheric soil on dry weight basis.

4.2.1 Culturable rhizospheric bacterial population associated with rhizospheric soil of kiwi vines

Study on culturable communities investigates a partial collection of rhizsophere is consequently a proper approach to study the whole system and plate count populations could provide quantitative yields and specific information on diversity (Torsvik *et al*. 1990 and Palomino *et al*. 2005).

A summary of population level of bacteria colonizing rhizosphere of kiwi vines from different cultivars with varying age group is presented in Table 2.1, 3.1, 4.1 and 5.1. The results revealed that the detectable growth (colony forming unit) of rhizospheric soil bacterial population was observed in all samples of kiwi. The colony forming units (CFU), determined for rhizospheric soil was rather different for different samples belonging to different cultivars of different age group and varied with medium used for enumeration. The significant variations in population of indegenous rhizosphere soil bacteria may be attributed to plant source, age, variety/cultivars type, time of sampling and environmental conditions, thus suggesting a close association between bacterial population and kiwi vines. The observed rhizobacterial population difference may be related to changes in the soil moisture, soil temperature and rhizodeposition (Yao *et al*. 2006).

The total bacterial population present in rhizospheric soil samples of kiwi cultivar Hayward (Pistillate, 30years) ranged from 76 to 112 × 10^6 CFU g^{-1}. The per cent phosphate solubilizers of the total rhizosphere population in Pikovskaya's medium for different samples ranged between 44.73 to 52.56 per cent while siderophore producers on Chromo azural-S (CAS) medium ranged from 7.69 to 13.15 per cent. Out of total culturable bacterial population 18.75 to 36.84 per cent were able to grow on nitrogen free medium.

Total culturable bacterial population present in rhizospheric soil samples of kiwi cultivar Bruno (Pistillate, 30 years) varied from 90 to 162 $\times 10^6$ CFU g^{-1}. Per cent phosphate solubilizers on Pikovskaya's medium for different samples ranged from 36.88 to 51.11 per cent while siderophore producers on CAS medium ranged from 4.09 to 15.23 per cent. Growth on nitrogen free medium ranged between 29.50 to 51.10 per cent of the total population.

Similarly the total viable bacterial population present in rhizospheric soil samples of kiwi cultivar Monty (Pistillate, 30 years) ranged from 90 to 104 $\times$ 10^6 CFU g^{-1}. Culturable bacterial population harbouring P-solubilizing bacteria ranged between 26.00 to 34.45 per cent on Pikovskaya's medium and on CAS medium ranged between 6.67 to 15.00 per cent. Growth on nitrogen free medium ranged between 13.46 and 55.56 per cent of the total population.

The total viable bacterial population present in rhizospheric soil samples of kiwi cultivar Allison (Pistillate, 30 years) ranged from 61 to 90 $\times$ 10^6 CFU g^{-1}. Culturable bacterial population harbouring P-solubilizing bacteria ranged between 37.66 to 42.23 per cent and siderophorec producers ranged between 0 to 5.56 per cent. Growth on nitrogen free medium ranged between 46.67 to 62.33 per cent of the total population.

The total viable bacterial population present in rhizospheric soil samples of kiwi cultivar Allison (Staminate and 30 years) ranged from 42 to 80 $\times$ 10^6 CFU g^{-1}. Culturable bacterial population harbouring P-solubilizing bacteria ranged between 38.09 to 47.54 per cent on and siderophore producers ranged between 0 to 11.47 per cent. Culturable nitrogen fixers ranged between 30.95 to 57.50 per cent.

The total viable bacterial population present in rhizospheric soil samples of kiwi cultivar Allison (Pistillate, 2 years) ranged from 34 to 100 $\times$ 10^6 CFU g^{-1}. Culturable bacterial population harbouring P-solubilizing bacteria ranged between 30.00 to 61.76 per cent on Pikovskaya's medium and 0 to 11.74 per cent on CAS medium whereas, total culturable nitrogen fixers ranged between 0 to 59 per cent of total bacterial population.

The total viable bacterial population present in rhizospheric soil samples of kiwi cultivar Allison (Staminate, 2 years) were detected using isolation method on nutrient agar plate ranged from 60 to 110 $\times$ 10^6 CFU g^{-1}. Culturable bacterial population harbouring P-solubilizing bacteria ranged between 40.00 to 45.00 per cent on Pikovskaya's medium,

siderophore producers ranged between 0 to 7.27% whereas, growth on nitrogen free medium ranged between 45.00 to 46.36 per cent of the total population.

Table 2.1 Enumeration of total culturable rhizospheric soil bacterial population associated with different cultivars of kiwi vines using modified replica plating technique

Rhizospherc bacterial population ($\times 10^6$ CFU/g soil)							
Age group	**Genotype**	**Cultivar**	**Samples**	***NA medium (Master plate)**	****PVK medium**	*****CAS medium**	**N_2-free medium**
30 Years	Pistillate (Female)	Hayward	H1	112	56	12	21
			H2	78	41	6	24
			H3	76	34	10	28
		Bruno	B1	105	51	16	35
			B2	162	45	5	36
			B3	90	46	4	46
		Monty	M1	100	26	15	53
			M2	90	31	6	50
			M3	104	28	14	59
		Allison	A1 F	61	25	0	32
			A2F	90	38	5	42
			A3F	77	29	0	48
	Staminate (Male)	Allison	A1M	42	16	0	13
			A2M	61	29	7	25
			A3M	80	32	0	46
2 years	Pistillate (Female)	Allison	A1F(2)	100	30	8	59
			A2F(2)	40	13	0	10
			A3F(2)	34	21	0	0
	Staminate (Male)	Allison	A1M(2)	60	24	0	27
			A2M(2)	110	46	8	51
			A3M(2)	100	45	6	45

* NA: Nutrient Agar medium; ** PVK: Pikovaskaya's Agar medium ***CAS: Chromo-azurol-S-medium;

Our results showed that the viable rhizosphere soil bacterial count ranged from 34 to 162 × 10^6 CFU g^{-1} soil of kiwi vines (Table 2.1). Our study supports the statement that rhizosphere effect increases progressively with increase in plant age until the maturity. Rhizospheric samples of kiwi vines of age group 30 harboured higher bacterial density than kiwi vines of age group 2.

All the collected samples of rhizospheric soil were evaluated for P-solubilizing bacteria as well as for the ability to grow on nitrogen free medium and production of siderophore. Rhizobacteria exhibiting all these plant growth promoting traits were found in all samples. Similar to our results, the rhizobacteria isolated from strawberry rhizosphere were subjected to replica plating on different media for one step screening of plant growth promoting traits (Balnatah, 2013). The rhizobacteria showing activities of P-solubilization, nitrogen fixation and siderophore production was found in all the soil samples from strawberry rhizosphere.

4.2.2 Culturable root endophytic bacterial population associated with roots of kiwi vines

The experiment was conducted to isolate and enumerate endophytic bacteria from internal plant tissue of roots using surface sterlization-trituration-plating technique. The results represented in Table 2.2 revealed that roots from different cultivar samples of kiwi vines harboured culturable bacterial population capable of growth on different media.

The population level of total culturable bacteria, per cent phosphate solubilizers, per cent siderophore producers and per cent nitrogen fixers colonizing roots of different cultivars of kiwi vines is presented in Table 2.2, 3.2, 4.2 and 5.2.

The viable culturable total population present in root samples of kiwi cultivar Hayward (Pistillate and 30 years) were detected by using isolation method on nutrient agar plate ranged from 70 to 115 × 10^3 CFU g^{-1}. All the samples harboured P-solubilizing bacteria and per cent phosphate solubilizers of the total endospheric population on Pikovskaya's medium for different samples ranged between 40.00 to 56.52 per cent whereas, siderophore producers on CAS medium ranged between 0 to 6.93 per cent. Out of total culturable bacterial population 38.61 to 51.42 per cent were nitrogen fixers.

Total culturable bacterial population present in root samples of cultivar Bruno (Pistillate, 30 years) varied from 37 to 75 × 10^3 CFU g^{-1}. Per cent phosphate solubilizers

ranged between 0 to 32.96 per cent while no growth was observed on CAS medium for siderophore production. Out of total culturable bacterial population 29.62 to 54.05 per cent were nitrogen fixers.

Total culturable bacterial population present in root samples of cultivar Monty (Pistillate, 30 years) varied from 43 to 113×10^3 CFU g^{-1}. Per cent phosphate solubilizers ranged between 45.13 to 70.37 per cent while siderophore producers ranged from 0 to 3.53 per cent. Out of total culturable bacterial population 18.51 to 51.32 per cent were nitrogen fixers.

The total viable culturable bacterial population present in root samples of kiwi cultivar Allison (Pistillate, 30 years) varied from 40 to 59×10^3 CFU g^{-1}. Per cent phosphate solubilizers on Pikovskaya's medium for different samples ranged between 0 to 85.45 per cent while siderophore producers ranged from 0 to 13.56 per cent. Out of total culturable bacterial population 20.00 to 40.00 per cent were able to grow on nitrogen free medium.

The total viable culturable bacterial population present in root samples of kiwi cultivar Allison (Staminate, 30 years) varied from 37 to 108×10^3 CFU g^{-1}. Per cent phosphate solubilizers on Pikovskaya's medium for different samples ranged between 27.02 to 37.23 per cent while siderophore producers ranged from 0 to 6.38 per cent. Out of total culturable bacterial population 51.06 to 56.75 per cent was able to grow on nitrogen free medium.

The total viable culturable bacterial population present in root samples of kiwi cultivar Allison (Pistillate and 2 years) varied from 65 to 110×10^3 CFU g^{-1}. Per cent phosphate solubilizers on Pikovskaya's medium for different samples ranged between 24.61 to 34.69 per cent while siderophore producers ranged from 5.45 to 21.53 per cent. Out of total culturable bacterial population 40.90 to 44.73 per cent were able to grow on nitrogen free medium.

Total culturable bacterial population present in root samples of kiwi cultivar Allison (Staminate, 2 years) varied from 67 to 98×10^3 CFU g^{-1}. Per cent phosphate solubilizers ranged between 31.57 to 50.74 per cent while siderophore producers ranged from 5.97 to 18.42 per cent. Total culturable nitrogen fixers ranged between 40.90 to 44.73 per cent of the total population.

Table 2.2 Enumeration of culturable plant growth promoting root endophytic bacterial population of different cultivars of kiwi vines using modified replica plating technique

Endophytic bacterial population ($\times 10^6$ CFU/g soil)							
Age group	**Genotype**	**Cultivar**	**Samples**	***NA medium (Master plate)**	****PVK medium**	*****CAS medium**	**N_2-free medium**
30 Years	Pistillate (Female)	Hayward	H1R	115	65	0	48
			H2R	70	28	0	36
			H3R	101	51	7	39
		Bruno	B1R	54	34	0	16
			B2R	75	19	0	40
			B3R	37	0	0	20
		Monty	M1R	113	51	4	58
			M2R	54	38	0	10
			M3R	43	25	0	18
		Allison	A1 FR	59	25	8	22
			A2FR	40	0	0	8
			A3FR	55	47	4	22
	Staminate (Male)	Allison	A1MR	108	40	4	57
			A2MR	94	35	6	48
			A3MR	37	10	0	21
2 years	Pistillate (Female)	Allison	A1FR(2)	65	16	14	31
			A2FR(2)	98	34	18	42
			A3FR(2)	110	47	6	45
	Staminate (Male)	Allison	A1MR(2)	76	24	14	34
			A2MR(2)	67	34	4	25
			A3MR(2)	98	31	15	47

* NA: Nutrient Agar medium; ** PVK: Pikovaskaya's; Agar medium ***CAS: Chromo-azurol-S-medium

Bacterial endophytes have been reported from range of host plants including grape vine (West *et al.* 2010), European deciduous and coniferous trees (Izumi *et al.* 2008), poplar (Ulrich *et al.* 2008) and olives (Prieto and Blanco, 2008). However diversity of root endophytic phosphate solubilizing, nitrogen fixers and siderophore producing bacterial population from different cultivars of kiwi vines has been documented for the first time. In

different cultivars of different age groups, root endophytic bacterial population of kiwi vines ranged from 37 to 115 × 10^3 CFU g^{-1}; which was much higher than the endophytic bacterial population obtained from the endorhizosphere of potato (Gerber *et al*. 2004).

In the present study considering the higher cultivable biomass of root endophytes (10^3 CFU g^{-1} root), it is probable that the root endophytic communities might have become established through a selection or active colonization process, rather than by simple, passive diffusion of most abundant members of soil communities. While studies addressing the mechanisms of such selection have not been reported, it is possible that the niche differentiation may play a role for different distributions of bacteria inside and outside the root (Izumi *et al*. 2008).

4.3 P-SOLUBILIZING BACTERIAL POPULATION IN RHIZOSPHERIC SOIL AND ROOT ENDOSPHERE AMONG DIFFERENT CULTIVARS OF KIWI VINES

All the collected soil and root samples were evaluated for the status of P-solubilizing bacterial population. Since variation in P-solubilizers of each site can be estimated by standard error of mean (SE_m) and to generate the results for larger area for each cultivar, an appropriate statistical measure i.e. 95% fiducial limits for mean was applied. To gauge the variation status of P-solubilizing rhizobacterial population, these measures viz., SE_m and 95% fiducial limits for mean along with range, average and P-solubilizers status have been worked out and presented in Table 3.1 and 3.2 for rhizospheric soil and root endophytic bacterial population, respectively. The results showed that the number of P-solubilizers varied in samples with respect to different cultivars of different age groups.

The results are in agreement with the earlier reports on dominant population of P-solubilizing bacterial isolates inhabiting different altitude of temperate Himalayas (Mehta *et al*. 2015; Kumar *et al*. 2015). There was a large variation in P-solubilizing bacterial population within and between different cultivars of Kiwi vines. Perusal of data in Table 3.1 revealed that average per cent P-solubilizers were highest (49.09%) in rhizospheric soil of kiwi cultivar Hayward (Pistillate) followed by 45.52% in kiwi cultivar Bruno (Pistillate) of age group 30. The status of P-solubilizing bacterial population depending on the average per cent P-solubilizers was rated good for Hayward (Pistillate), Bruno (Pistillate), Allison (Pistillate), Allison (Staminate) and was rated fair for Monty (Pistillate) cultivars of kiwi vines of age group 30.

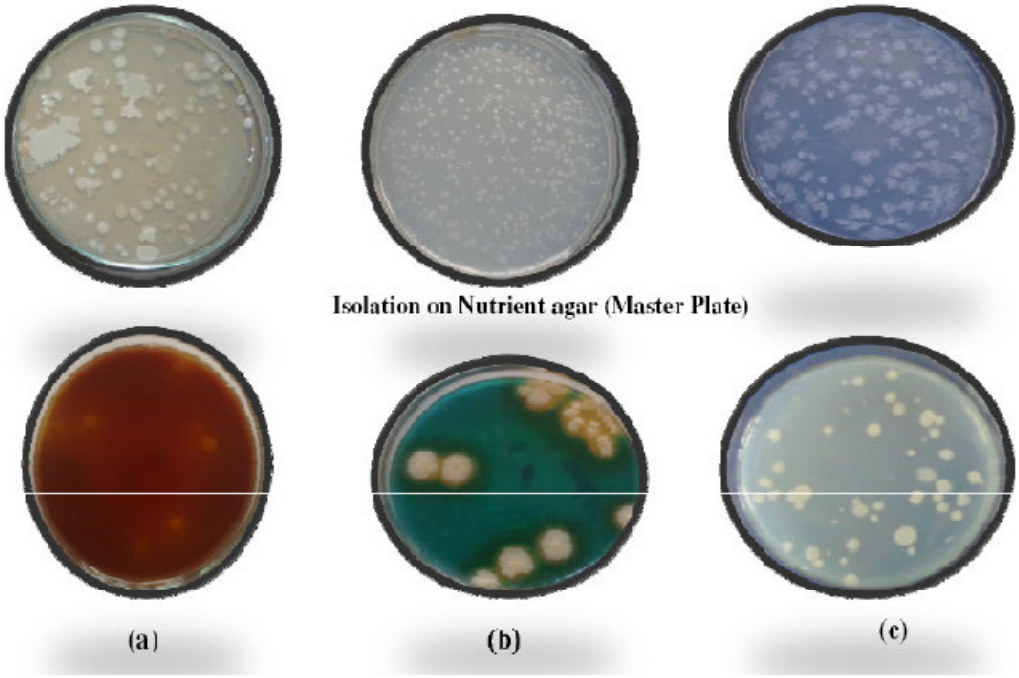


Plate 1. **Isolation and enumeration of culturable plant growth promoting rhizobacterial population associated with different cultivars of kiwi vines of different age group with varying genotype using modified replica plating technique on (a) PVK medium (b) CAS medium (c) nitrogen free medium**

Table 3.1 Cultivar-wise status of rhizospheric soil phosphate solubilizing bacterial population

Age group	Genotype	Cultivars	Range of P-solubilizers (%)	Average P-solubilizers (%)	*Status of P-solubilizing Population	Standard error of mean (SE_m)	95% fiducial limits for mean
30 Years	Pistillate (Female)	Hayward	44.73-52.56	49.09	Good	2.30	49.09±4.60
		Bruno	36.88-51.11	45.52	Good	4.38	45.52±8.76
		Monty	26.00-34.45	37.18	Fair	2.67	37.18±5.34
		Allison	37.66-42.23	40.29	Good	1.36	40.29±2.72
	Staminate (Male)	Allison	38.09-47.54	41.87	Good	2.88	41.87±5.76
2 Years	Pistillate (Female)	Allison	30.00-61.76	41.42	Good	10.19	41.42±20.38
	Staminate (Male)	Allison	40.00-45.00	42.27	Good	1.46	42.27±2.92

*Poor, 0-20 per cent P-solubilizers; Fair, 20-40 per cent P-solubilizers; Good, 40-80 per cent P-solubilizers

Between kiwi cultivars of age group 2, Allison (Staminate) showed highest P-solubilization (42.27%) followed by Allison (pistillate). The status of both the cultivars was rated as good P-solubilizers depending on the average per cent of P-solubilization. Table 3.2 inferred that the average per cent of root endophytic P-solubilizers among kiwi cultivars of age group 30 were highest (57.87%) in Monty (Pistillate) followed by Hayward (Pistillate) (48.90%).

Table 3.2 Cultivar-wise status of root endophytic phosphate solubilizing bacterial population

Age group	Genotype	Cultivars	Range of P-solubilizers (%)	Average P-solubilizers (%)	*Status of P-solubilizing Population	Standard error of mean (SE_m)	95% fiducial limits for mean
30 Years	Pistillate (Female)	Hayward	40.00-56.21	48.90	Good	4.76	48.90±9.52
		Bruno	0.00-32.96	19.43	Poor	9.96	19.43±19.92
		Monty	45.13-70.37	57.87	Good	7.28	57.87±14.56
		Allison	0.00-85.45	42.60	Good	24.66	42.00±49.32
	Staminate (Male)	Allison	27.02-37.23	33.76	Fair	3.37	33.76±6.74
2 Years	Pistillate (Female)	Allison	24.61-34.69	30.31	Fair	2.98	30.31±5.96
	Staminate (Male)	Allison	31.57-50.74	41.67	Good	5.55	41.67±11.1

*Poor, 0-20 per cent P-solubilizers; Fair, 20-40 per cent P-solubilizers; Good, 40-80 per cent P-solubilizers

The status of P-solubilizing bacterial population depending on the average per cent of P-solubilizers was rated good for Hayward (Pistillate), Monty (Pistillate) and Allison (Pistillate), whereas P-solubilizers were rated fair and poor for Allison (Staminate) and Bruno (Pistillate) respectively. The kiwi cultivars of age group 2 showed highest P-solubilizing bacterial population (41.67%) in Allison (Staminate) followed by Allison (Pistillate) (30.31%). The status of P-solubilizing bacterial population depending on the average per cent of P-solubilizers was later good for Allison (Staminate) and fair for Allison (Pistillate).

4.4 SIDEROPHORE PRODUCING BACTERIAL POPULATION IN RHIZOSPHERIC SOIL AND ROOT ENDOSPHERE AMONG DIFFERENT CULTIVARS OF KIWI VINES

All the collected samples of soil and root were evaluated for the status of siderophore producing bacterial population. To gauge the variation status of siderophore producing rhizobacterial population, these measures viz., Standard error of mean (SE_m), average and siderophore producers status have been worked out in Table 4.1 and 4.2 for rhizospheric soil and root endophytic bacterial population, respectively. The results showed that the number of siderophore producers varied in soil and root samples of each cultivar.

Perusal of data in table 4.1 revealed that average per cent siderophore producers among different cultivars of kiwi vines of age group 30, was highest (23.76%) in Bruno (Pistillate) followed by 11.71% in Monty (Pistillate). The status of siderophore producing bacterial population depending on the average per cent of siderophore producers was rated fair for Bruno (23.76%) and poor for rest of the cultivars, viz., Hayward (Pistillate), Bruno (Pistillate), Allison (Pistillate) and Allison (Staminate).

Between the two cultivars of kiwi of age group 2, Allison (Staminate) showed greater siderophore production (4.42%) than Allison (Pistillate) (3.82%).Depending on average per cent Siderophore producers the status of Siderophore producers was rated poor in rhizosphere soil of both the cultivars.

Table 4.2 inferred that average per cent of root endophytic siderophore producers in cultivars of age group 30 was highest (20.83%) in Allison (Pistillate) followed by 3.36% in Allison (Staminate). The status of siderophore producing bacterial population depending on the average per cent of siderophore producers was rated fair for Allison (Pistillate) and poor for all the other cultivars viz., Hayward (Pistillate), Bruno (Pistillate), Monty (Pistillate) and Allison (Pistillate).

Between the two cultivars of age group 2, Allison (Pistillate) showed greater siderophore production (15.11%) than Allison (Staminate) (5.97%). But the status of siderophore producing bacterial population was rated poor in both the cultivars of age group 2.

Table 4.1 Cultivar-wise status of soil siderophore solubilizing bacterial population

Age group	Genotype	Cultivars	Range of Siderophore producers (%)	Average Siderophore producers (%)	*Status of Siderophore producing Population	Standard error of mean(SE_m)	95% fiducial limits for mean
30 Years	Pistillate (Female)	Hayward	7.69-13.15	10.51	Poor	1.57	10.51±3.14
		Bruno	4.09-15.23	23.76	Fair	3.65	23.76±7.30
		Monty	6.67-15.00	11.71	Poor	2.55	11.71±5.10
		Allison	0.00-5.56	0.15	Poor	1.85	0.15±3.70
	Staminate (Male)	Allison	0.00-11.47	3.82	Poor	3.82	3.82±7.64
2 Years	Pistillate (Female)	Allison	0.00-11.47	3.82	Poor	3.82	3.82±7.64
	Staminate (Male)	Allison	0.00-7.27	4.42	Poor	3.82	3.82±7.64

*Poor, 0-20 per cent siderophore producers; Fair, 20-40 per cent siderophore producers; Good, 40-80 per cent siderophore producers

Table 4.2 Cultivar-wise status of root endophytic siderophore producing bacterial population

Age group	Genotype	Cultivars	Range of Siderophore producers (%)	Average Siderophore producers (%)	*Status of Siderophore producing Population	Standard error of mean(SE_m)	95% fiducial limits for mean
30 Years	Pistillate (Female)	Hayward	0.00-6.93	2.31	Poor	2.31	2.31±4.62
		Bruno	0.00	0.00	Poor	0.00	0.00
		Monty	0.00-3.53	1.16	Poor	1.16	1.16±2.32
		Allison	0-13.56	20.83	Fair	3.91	20.83±7.82
	Staminate (Male)	Allison	0-6.38	3.36	Poor	1.84	3.36±3.68
2 Years	Pistillate (Female)	Allison	5.45-21.53	15.11	Poor	4.91	15.11±9.82
	Staminate (Male)	Allison	5.97-18.42	5.97	Poor	3.74	5.97±7.48

*Poor, 0-20 per cent siderophore producers; Fair, 20-40 per cent siderophore producers; Good, 40-80 per cent siderophore producers

4.5 NITROGEN FIXING BACTERIAL POPULATION IN RHIZOSPHERIC SOIL AND ROOT ENDOSPHERE AMONG DIFFERENT CULTIVARS OF KIWI VINES

All the collected samples of soil and root were evaluated for the status of nitrogen fixing bacterial population. To gauge the variation status of nitrogen fixing population, these measures viz., Standard error of mean (SE_m), 95% confidential level for mean along with range, average and nitrogen fixers status have been worked out and are presented in Table 5.1 and 5.2 for rhizospheric soil and root endophytic bacterial population, respectively. There was large variation in nitrogen fixing bacterial population among different cultivars of kiwi vines**.**

Perusal of data in Table 4.5 revealed that average per cent nitrogen fixers among different Kiwi cultivars of age group 30 were highest in Allison (Pistillate) (53.81%) followed by in Monty (Pistillate) (40.67%). The status of nitrogen fixing population depending on the average per cent of nitrogen fixers was rated good for Monty (Pistillate) and Allison (Pistillate) and fair for Hayward (Pistillate), Bruno (Pistillate) and Allison (Staminate).

Between the two cultivars of kiwi of age group 2, Allison (Staminate) showed greater nitrogen fixing bacterial population (45.45%). The status of nitrogen fixing bacterial population was rated good and fair for Allison (Staminate) and Allison (Pistillate) repectively.

Table 5.2 inferred that the average per cent of root endophytic nitrogen fixers among cultivars of kiwi of age group 30 was highest (53.53%) in Allison (Staminate) followed by Hayward (Pistillate) (43.92). The status of nitrogen fixing bacterial population depending on average per cent of nitrogen fixers was rated good for Hayward (Pistillate), Bruno (Pistillate) and Allison (Staminate) and fair for Monty (Pistillate) and Allison (Pistillate).

Between the two cultivars of age group 2, Allison (Pistillate) showed greater nitrogen fixing bacterial population (43.81%). The status of nitrogen fixing bacterial population was rated good and fair for both Allison (Pistillate) and Allison (Staminate).

Table 5.1 Cultivar-wise status of rhizospheric soil nitrogen fixing bacterial population

Age group	Genotype	Cultivars	Range of Nitrogen fixers (%)	Average Nitrogen fixers (%)	*Status of Nitrogen fixing Population	Standard error of mean (SE_m)	95% fiducial limits for mean
30 Years	Pistillate (Female)	Hayward	18.75-36.84	29.64	Fair	5.53	29.64±11.06
		Bruno	29.5-51.11	37.98	Fair	6.65	37.98±13.30
		Monty	13.46-55.56	40.67	Good	13.62	40.67±27.24
		Allison	46.67-62.33	53.81	Good	4.57	53.81±9.14
	Staminate (Male)	Allison	30.95-57.5	29.81	Fair	7.74	29.81±15.48
2 Years	Pistillate (Female)	Allison	0-59	28.00	Fair	17.09	28±34.18
	Staminate (Male)	Allison	45-46.36	45.45	Good	0.45	45.45±0.9

*Poor, 0-20 per cent nitrogen fixers; Fair, 20-40 per cent nitrogen fixers; Good, 40-80 per cent nitrogen fixers

Table 5.2 Cultivar-wise status of root endophytic nitrogen fixing bacterial population

Age group	Genotype	Cultivars	Range of Nitrogen fixers (%)	Average Nitrogen fixers (%)	*Status of Nitrogen fixing Population	Standard error of mean (SE_m)	95% fiducial limits for mean
30 Years	Pistillate (Female)	Hayward	38.61-51.42	43.92	Good	3.85	43.92±7.70
		Bruno	29.62-54.05	41.60	Good	8.02	41.6±16.04
		Monty	18.51-51.32	37.23	Fair	9.75	37.23±19.50
		Allison	20.00-40.00	32.42	Fair	6.26	32.42±12.52
	Staminate (Male)	Allison	51.06-56.75	53.53	Good	1.68	53.53±3.36
2 Years	Pistillate (Female)	Allison	40.90-44.73	43.81	Good	2.01	43.81±4.02
	Staminate (Male)	Allison	40.90-44.73	43.81	Good	1.10	42.82±2.2

*Poor, 0-20 per cent nitrogen fixers; Fair, 20-40 per cent nitrogen fixers; Good, 40-80 per cent nitrogen fixers

4.6 COMPARISON OF RHIZOSPHERIC SOIL AND ROOT ENDOPHYTIC BACTERIAL ISOLATES POSSESSING PLANT GROWTH PROMOTING TRAITS (PHOSPHATE SOLUBILIZATION, SIDEROPHORE PRODUCTION AND NITROGEN FIXATION)

Table 6 show the result of the plant growth promoting traits (PGPT) tests for the isolates from the RS and ER associated with kiwi vines. A total of forty five rhizospheric and sixty four endophytic bacterial isolates were screened for PGPT among the cultivars of age group 30.Whereas, a total of eighteen RS and thirty ER bacterial isolates were screened for PGPT among the cultivars of age group 2.

Overall, among all the cultivars of age group 30, seven of the total forty five rhizobacterial isolates from the RS samples lacked PGPT (15.56%) and eleven of the total sixty four isolates from the ER samples did not display PGPT (17.18). Among the cultivars of age group 2, seven of the total eighteen IAA producing rhizobacterial isolates from RS samples lacked PGPT (9.37%) and three of the total thirty isolates from the ER samples did not display PGPT (10.00%).

Percentages of bacterial isolates among the kiwi cultivars of age group 30 displaying binary activity of phosphate solubilization and siderophore production was highest (21.42%) in ER of Monty (Pistillate) and RS of Bruno (20.00%). PGPR percentages having nitrogen fixing ability and phosphate solubilization activity was highest (50.00%) for RS of Hayward and no result was shown by any of ER samples. None of these cultivar samples showed combined activities of siderophore production and nitrogen fixation.

Among kiwi cultivars of age group 2, binary activity of phosphate solubilization and siderophore production was highest (11.76%) in ER and (7.41%) in Allison (Staminate). PGPR percentages having nitrogen fixing ability and phosphate solubilizing activity was highest (25.00%) in RS of Allison (Pistillate) and in ER of Allison (Staminate). Highest percentages of PGPR having combined activities of siderophore production and nitrogen fixation was found highest (11.76%) in ER of Allison (Staminate) and in (7.69%) RS of Allison (Pistillate).

Ratios of PGPR showing triple activities of phosphate solubilisation siderophore production and nitrogen fixation was highest (37.5%) in RS of Monty (Pistillate) and (17.64%) in ER of Allison (Staminate) among kiwi cultivars of age group 30. Whereas ratios of PGPR showing triple activities of phosphate solubilisation siderophore production and

Table 6. Characterization of rhizospheric soil and root endophytic bacterial isolates for plant growth promoting traits

Age group	Genotype	Cultivar		Number of isolates without PGP activity	Number of isolates with single PGP activity			Number of isolates with binary PGP traits			Number of isolates with triple PGP traits
					P	S	N	P+S	P+N	S+N	P+S+N
30 Years	Pistillate (Female)	Hayward	RS	0/4 (0.00)	0/4 (0.00)	0/4 (0.00)	0/4 (0.00)	2/4 (50.00)	0/4 (0.00)	0/4 (0.00)	2/4 (0.00)
			ER	1/10 (10.00)	0/10 (0.00)	0/10 (0.00)	1/10 (10.00)	0/10 (0.00)	7/10 (70.00)	0/10 (0.00)	1/10 (0.00)
		Bruno	RS	2/20 (10.00)	4/20 (20.00)	0/20 (0.00)	4/20 (20.00)	0/20 (0.00)	8/20 (40.00)	0/20 (0.00)	2/20 (10.00)
			ER	3/13 (23.07)	1/13 (7.69)	0/13 (0.00)	5/13 (38.46)	0/13 (0.00)	4/13 (30.76)	0/13 (0.00)	0/13 (0.00)
		Monty	RS	1/8 (12.5)	1/8 (12.5)	0/8 (0.00)	1/8 (12.5)	0/8 (0.00)	2/8 (25.00)	0/8 (0.00)	3/8 (37.5)
			ER	4/14 (28.57)	3/14 (21.42)	0/14 (0.00)	1/14 (7.14)	0/14 (0.00)	5/14 (35.71)	0/14 (0.00)	1/14 (7.14)
		Allison	RS	0/5 (0.00)	0/5 (0.00)	0/5 (0.00)	1/5 (20.00)	0/5 (0.00)	4/5 (80.00)	0/5 (0.00)	0/5 (0.00)
			ER	1/10 (10.00)	0/10 (0.00)	0/10 (0.00)	0/10 (0.00)	1/10 (10.00)	4/10 (40.00)	1/10 (10.00)	1/10 (10.00)
	Staminate (Male)	Allison	RS	4/8 (50.00)	1/8 (12.5)	0/8 (0.00)	0/8 (0.00)	1/8 (12.5)	0/8 (0.00)	0/8 (0.00)	2/8 (25.00)
			ER	3/17 (17.64)	0/17 (0.00)	0/17 (0.00)	0/17 (0.00)	0/17 (0.00)	11/17 (64.70)	0/17 (0.00)	3/17 (17.64)
Sum%			RS	7/45 (15.56)	6/45 (13.34)	0/45 (0.00)	6/45 (13.34)	3/45 (6.67)	14/45 (31.12)	0/45 (0.00)	9/45 (20.00)
			ER	11/64 (17.18)	4/64 (6.25)	0/64 (0.00)	7/64 (10.93)	1/64 (1.56)	31/64 (48.43)	1/64 (1.56)	6/64 (9.37)
2 Years	Pistillate (Female)	Allison	RS	2/4 (50.00)	0/4 (0.00)	0/4 (0.00)	1/4 (25.00)	0/4 (0.00)	1/4 (25.00)	0/4 (0.00)	0/4 (0.00)
			ER	0/13 (0.00)	2/13 (15.38)	2/13 (15.38)	0/13 (0.00)	1/13 (7.69)	3/13 (23.07)	1/13 (7.69)	4/13 (30.76)
	Staminate (Male)	Allison	RS	5/14 (35.71)	1/14 (7.41)	0/14 (0.00)	2/14 (14.28)	1/14 (7.41)	3/14 (21.42)	1/14 (7.41)	1/14 (7.41)
			ER	3/17 (17.64)	3/17 (17.64)	0/17 (0.00)	0/17 (0.00)	2/17 (11.76)	2/17 (11.76)	2/17 (11.76)	5/17 (29.41)
Sum%			RS	7/18 (9.37)	1/18 (5.56)	0/18 (0.00)	3/18 (16.67)	1/18 (5.56)	4/17 (23.52)	1/18 (5.56)	1/18 (5.56)
			ER	3/30 (10.00)	5/30 (16.67)	2/30 (6.67)	0/30 (0.00)	3/30 (10.00)	5/30 (16.67)	3/30 (10.00)	9/30 (30.00)

P, Phosphate solubilisation; S, Siderophore production; N, Nitrogen fixation, RS, Rhizosphere soil; ER, root endosphere. Figure in parentheses are per cent (%) of isolates with plant growth promoting (PGP) traits

nitrogen fixation was highest (30.76%) in ER of Allison (Pistillate) and (7.41%) in RS of Allison (Staminate) among kiwi cultivars of age group 2.

Percentages of PGPR having phosphate solubilization, siderophore production and nitrogen fixing activity among kiwi cultivars of age group 30 is depicted in Fig5.a. and of age group 2 in Fig.5.b. Percentages of bacteria having phosphate solubilizing activity is arranged in the order Hayward (Pistillate) (97.99%)> Monty (Pistillate) (95.05%)> Allison (Staminate) (83.54%)> Allison (Pistillate) (82.89%)> Bruno (64.95%) among cultivars of kiwi of age group 30. While in cultivars of age group 2, Allison (Staminate) (83.94%) showed higher phosphate solubilizing activity than Allison (Pistillate) (71.73%).

The bacterial isolates ratios showing siderophore synthesis ability were in the order of Bruno (Pistillate) (23.76%)> Allison (Pistillate) (21.34%)> Monty (Pistillate) (12.87%)> Hayward (12.82%)> Allison (Staminate) (7.18%) among cultivars of kiwi of age group 30. While in cultivars of age group 2, Allison (Pistillate) (15.26%) showed higher phosphate solubilizing activity than Allison (Staminate) (10.39%).

Percentages of bacteria having nitrogen fixing activity among cultivars of kiwi of age group 30 is arranged in the order Allison (Staminate) (98.98%)> Allison (Pistillate) (86.23%)> Bruno (Pistillate) (79.58%)> Monty (Pistillate) (77.90%)> Hayward (Pistillate) (73.56%). While in cultivars of age group 2, Allison (Staminate) (88.27%) showed higher phosphate solubilizing activity than Allison (Pistillate) (71.81%).

4.6.1 Correlation analysis

Table 7 shows the results of correlation analysis among the plant growth promoting traits possessing bacterial isolates. A positive correlation was observed between total rhizospheric population of phosphate solubilizing isolates and siderophore producing as well as nitrogen fixing bacterial isolates. Similarly, total population (RS+ER) of phosphate solubilization and nitrogen fixing bacteria. Similar results for correlation between different PGP traits among rhizospheric and endophytic bacterial isolateswere observed by Kumar, 2013.

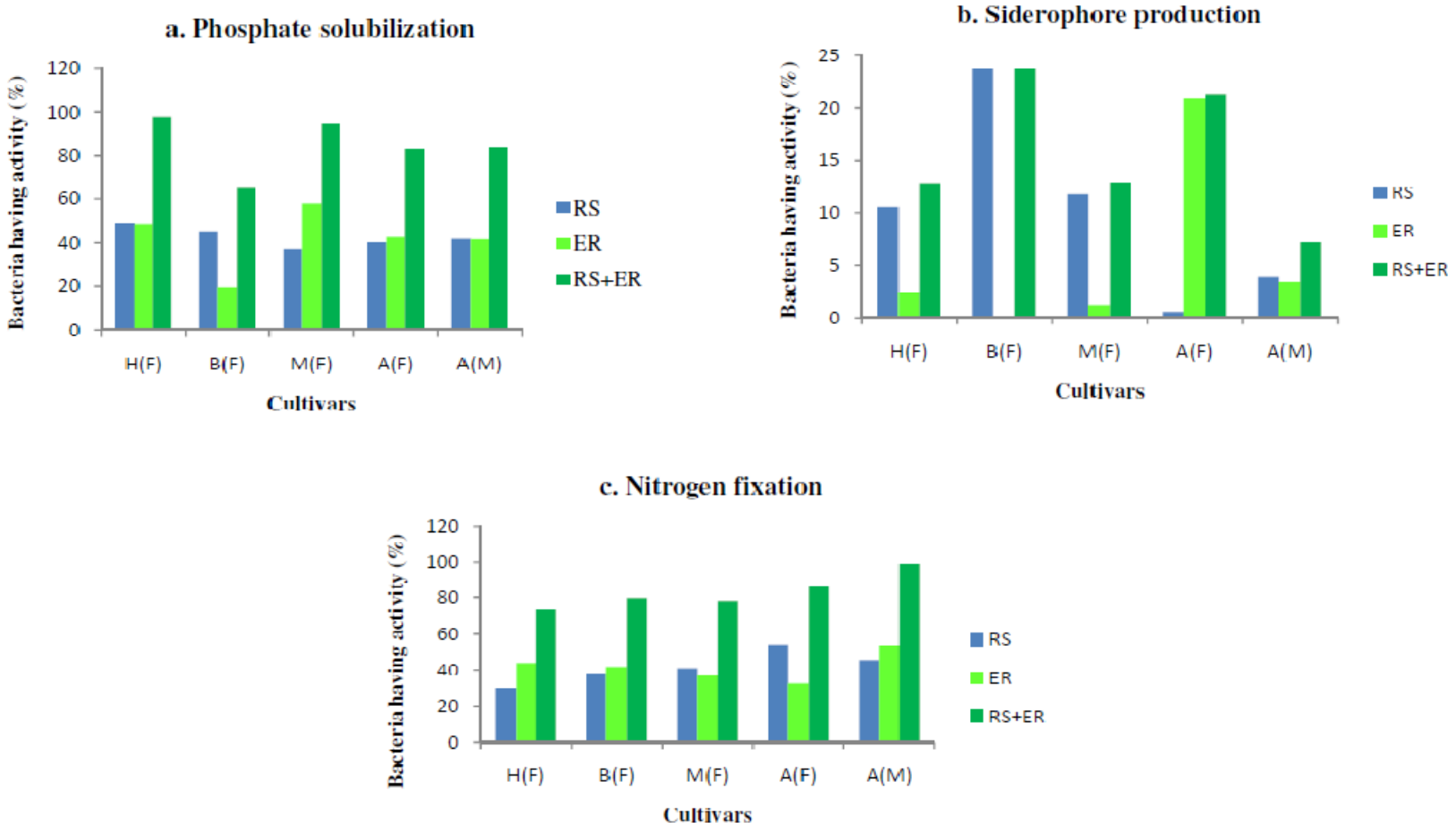


Fig. 5a **Graphical representation of percenteges of rhizosphere soil and root endophytic bacterial isolates of different cultivars of kiwi of age group 30 for plant growth promoting traits: Phosphate solubilization (a); Siderophore production (b); Nitrogen fixation (c)**

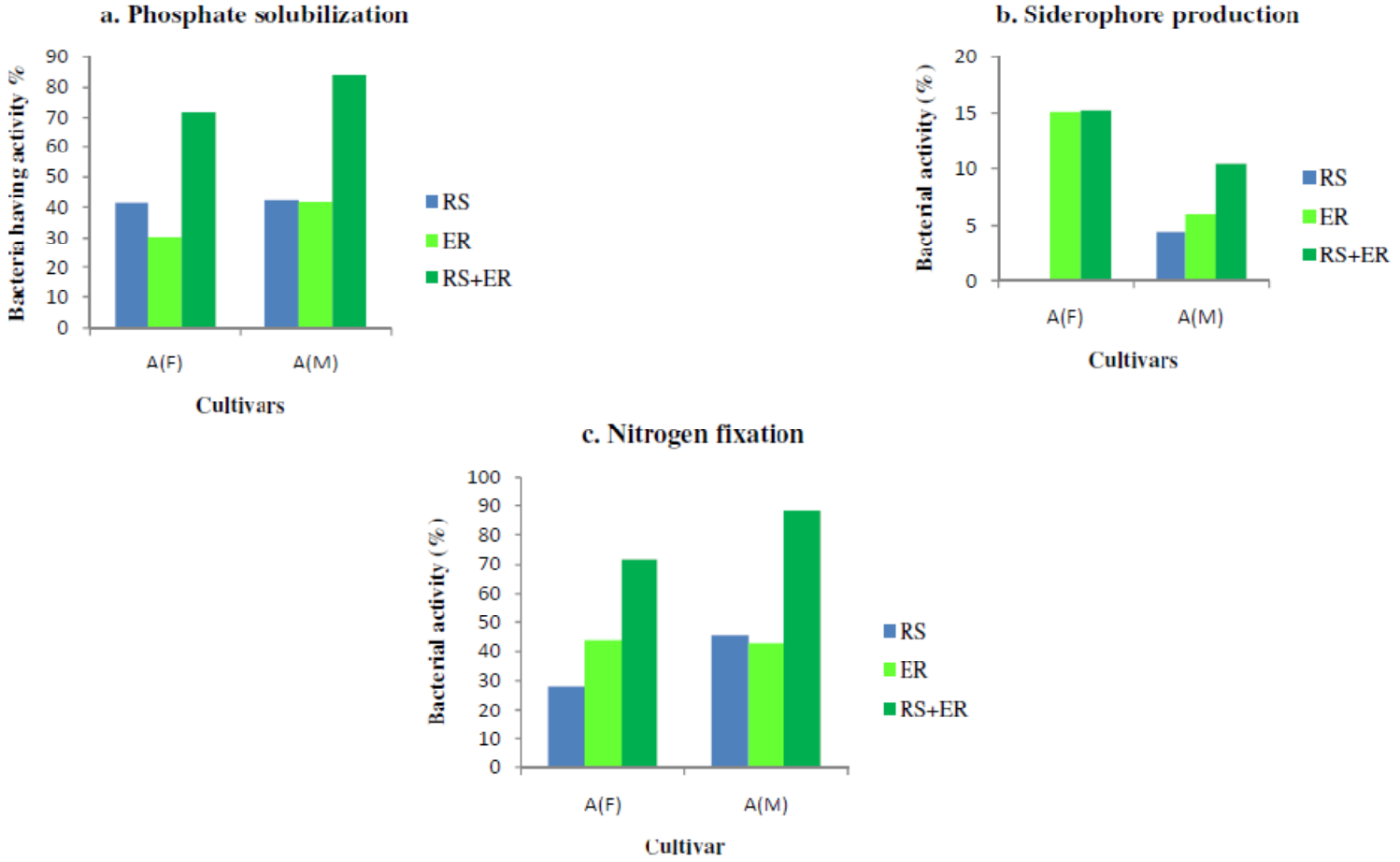


Fig. 5b Graphical representation of percenteges of rhizosphere soil and root endophytic bacterial isolates of different cultivars of kiwi of age group 2 for plant growth promoting traits: Phosphate solubilization(a); Siderophore production (b); Nitrogen fixation (c)

Table 7. Correlation matrices showing relationship among plant growth promoting traits of bacterial isolates

		P			Sid			N		
		RS	**ER**	**RS+ER**	**RS**	**ER**	**RS+ER**	**RS**	**ER**	**RS+ER**
P	**RS**	-								
	ER	-0.49	-							
	RS+ER	0.67	0.31	-						
Sid	**RS**	0.19	0.32	0.49	-					
	ER	-0.46	0.53	-0.05	-0.31	-				
	RS+ER	-0.39	0.71	0.17	0.13	0.89	-			
N	**RS**	0.94	-0.55	0.56	-0.01	-0.30	-0.31	-		
	ER	0.03	0.68	0.62	0.46	0.007	0.20	-0.20	-	
	RS+ER	0.92	-0.14	0.88	0.25	-0.18	-0.18	0.83	0.36	-

*Correlation is significant at 0.05% level of significance

P: Phosphate solubilisation; Sid: Siderophore Production; N: Nitrogen fixation RS: Rhizospheric soil; ER: Root endosphe

4.7 SCREENING OF IAA PRODUCING BACTERIAL POPULATION IN RHIZOSPHERIC SOIL AND ROOT ENDOSPHERE OF KIWI VINES

All the collected samples of soil and root were evaluated for the status of IAA producing bacterial population. There was large variation in IAA producing bacterial population among all the cultivars of kiwi. The highest IAA production by the isolates was observed in rhizosphere soil of Bruno (Pistillate) (42 µg/ml) followed by Allison (Staminate) (40 µg/ml) of age group 30. The root endophytic bacterial isolate producing highest IAA was observed in Hayward (Pistillate) (42 µg/ml) of age group 30 followed by Allison (Pistillate) (40 µg/ml) of age group 2.

The results (Table 8.1 and 8.2) showed that out of sixty three bacterial isolates twenty nine (46.03%) rhizospheric isolates and out of ninety four bacterial isolates 35 (37.23%) endophytic isolates were IAA producers. Data revealed that rhizobacteria isolated from soil and roots from different cultivars of kiwi of different age group 30 and 2 had ability to produce auxins which is supported by Asghar *et al*. (2000) where rhizobacteria from rapeseed varieties produced auxins. Chaiharn and Lumyong,, (2011) also suggested the production of auxins from rhizobacteria.

Many evidences suggested that IAA may be the first PGP trait compared to ACC deaminase activity, siderophore production and phosphate solubilization traits for screening rhizosphere and endophytic bacteria for rice and other crops.

Plant PGP agents such as bacterial IAA contributes to circumvent the host defence by depressing the IAA signalling in the plant; IAA also can have a direct effect on bacterial survival and its resistance to plant defence. The success of invasion and survival within the

Table 8.1 Screening of IAA producing culturable bacterial population from rhizospheric soil of kiwi vines

Age group	Genotype	Cultivar	Isolates	Indole -3-acetic acid (µg/ml)	Viable count (10^6×CFU/ml)	Final pH of supernatant
30 Years	Pistillate (Female)	Hayward	H_1S_1	31.00	112.00	4.9
			H_2S_1	24.00	60.00	5.0
			H_3S_1	27.00	32.00	5.0
			H_3S_2	20.00	110.00	5.0
		Bruno	B_1S_1	30.00	114.00	5.0
			B_1S_2	ND	110.00	5.3
			B_1S_3	ND	57.00	5.4
			B_1S_4	15.00	56.00	5.1
			B_1S_5	ND	36.00	5.5
			B_1S_6	8.00	34.00	5.2
			B_2S_1	14.00	34.00	5.6
			B_2S_2	ND	30.00	5.4
			B_2S_3	ND	36.00	5.5
			B_2S_4	ND	47.00	5.6
			B_2S_5	12.00	100.00	5.6
			B_2S_6	ND	72.00	5.3
			B_2S_7	35.00	116.00	4.4
			B_2S_8	ND	42.00	5.5
			B_2S_9	ND	45.00	5.3
			B_2S_{10}	42.00	111.00	4.5
			B_3S_1	ND	35.00	5.8
			B_3S_2	ND	59.00	5.4
			B_3S_3	ND	64.00	5.5
			B_3S_4	ND	40.00	5.5
		Monty	M_1S_1	21.00	36.00	4.9
			M_1S_2	21.00	32.00	4.8
			M_2S_1	20.00	120.00	5.1
			M_2S_2	ND	87.00	5.6
			M_2S_3	18.00	104.00	5.3
			M_3S_1	ND	36.00	5.4
			M_3S_2	16.00	110.00	5.3
			M_3S_3	11.00	84.00	5.4

Contd. Table 8.1

Age group	Genotype	Cultivar	Isolates	Indole -3-acetic acid (µg/ml)	Viable count (10^6×CFU/ml)	Final pH of supernatant
		Allison	A_1FS_1	11.00	104.00	5.4
			A_1FS_2	ND	38.00	5.4
			A_2FS_1	ND	38.00	5.6
			A_3FS_1	30.00	116.00	4.9
			A_3FS_2	ND	63.00	5.5
		Allison	A_1MS_1	ND	33.00	5.8
			A_1MS_2	3.00	36.00	5.5
			A_1MS_3	ND	32.00	5.6
			A_2MS_1	40.00	104.00	4.8
			A_2MS_2	ND	45.00	5.6
			A_2MS_3	ND	82.00	5.6
			A_2MS_4	20.00	88.00	5.1
			A_3MS_1	ND	46.00	5.4
2 Years	Pistillate (Female)	Allison	$A_1FS_{1(2)}$	ND	48.00	5.4
			$A_1FS_{2(2)}$	6.00	41.00	5.6
			$A_2FS_{1(2)}$	ND	54.00	5.6
			$A_3FS_{1(2)}$	ND	49.00	5.8
	Staminate (Male)	Allison	$A_1MS_{1(2)}$	ND	46.00	5.7
			$A_1MS_{2(2)}$	ND	35.00	5.6
			$A_1MS_{3(2)}$	ND	42.00	5.8
			$A_1MS_{4(2)}$	22.00	96.00	5.5
			$A_1MS_{5(2)}$	2.00	41.00	5.4
			$A_2MS_{1(2)}$	ND	57.00	5.4
			$A_2MS_{2(2)}$	ND	54.00	5.6
			$A_2MS_{3(2)}$	ND	66.00	5.3
			$A_2MS_{4(2)}$	21.00	114.00	5.4
			$A_3MS_{1(2)}$	32.00	84.00	4.7
			$A_3MS_{2(2)}$	28.00	64.00	5.0
			$A_3MS_{3(2)}$	ND	48.00	5.3
			$A_3MS_{4(2)}$	ND	46.00	5.4
			$A_3MS_{5(2)}$	29.00	60.00	5.2
$CD_{0.05}$				0.76	1.16	1.14

ND: Not Detected

Table 8.2 Screening of IAA producing culturable bacterial population from root endosphere of kiwi vines

Age group	Genotype	Cultivars	Isolates	Indole -3-acetic acid (µg/ml)	Viable count (×CFU/ml)	Final pH of supernatant
30 Years	Pistillate (Female)	Hayward	H_1R_1	8.00	60.00	5.6
			H_1R_2	21.00	76.00	5.1
			H_2R_1	ND	34.00	5.5
			H_2R_2	30.00	40.00	4.9
			H_3R_1	20.00	80.00	5.3
			H_3R_2	ND	45.00	5.3
			H_3R_3	42.00	84.00	4.6
			H_3R_4	ND	49.00	5.6
			H_3R_5	8.00	60.00	5.4
			H_3R_6	14.00	114.00	5.3
		Bruno	B_1R_1	ND	54.00	5.3
			B_1R_2	ND	44.00	5.4
			B_1R_3	20.00	40.00	5.3
			B_1R_4	25.00	84.00	4.6
			B_2R_1	5.00	34.00	5.4
			B_2R_2	ND	65.00	4.8
			B_2R_3	ND	41.00	5.6
			B_2R_4	ND	38.00	5.8
			B_2R_5	ND	47.00	5.4
			B_2R_6	25.00	48.00	4.9
			B_3R_1	ND	45.00	5.4
			B_3R_2	ND	45.00	5.3
			B_3R_3	ND	35.00	5.4
		Monty	M_1R_1	29.00	104.00	5.0
			M_1R_2	20.00	104.00	5.0
			M_1R_3	ND	54.00	5.4
			M_1R_4	29.00	88.00	5.3
			M_1R_5	ND	51.00	5.5
			M_1R_6	ND	60.00	5.6
			M_2R_1	23.00	80.00	5.2
			M_2R_2	ND	39.00	5.6
			M_2R_3	22.00	84.00	5.2
			M_2R_4	ND	36.00	5.4
			M_2R_5	ND	47.00	5.5
			M_2R_6	ND	49.00	5.4
			M_3R_1	16.00	60.00	5.4
			M_3R_2	ND	40.00	5.4
		Allison	A_1FR_1	ND	85.00	5.4
			A_1FR_2	ND	48.00	5.5
			A_1FR_3	21.00	88.00	5.3
			A_2FR_1	ND	40.00	5.5
			A_2FR_2	ND	39.00	5.4
			A_2FR_3	ND	46.00	5.4
			A_3FR_1	9.00	60.00	5.6

Contd. Table 8.2

Age group	Genotype	Cultivars	Isolates	Indole -3-acetic acid (µg/ml)	Viable count (×CFU/ml)	Final pH of supernatant
			A_3FR_2	14.00	64.00	5.4
			A_3FR_3	ND	40.00	4.9
			A_3FR_4	ND	56.00	5.4
	Staminate (Male)	Allison	A_1MR_1	7.00	64.00	5.4
			A_1MR_2	ND	36.00	5.4
			A_1MR_3	25.00	80.00	4.9
			A_1MR_4	3.00	68.00	5.4
			A_1MR_5	3.00	76.00	5.3
			A_1MR_6	ND	56.00	5.3
			A_2MR_1	ND	40.00	5.2
			A_2MR_2	ND	60.00	5.4
			A_2MR_3	15.00	76.00	5.3
			A_2MR_4	ND	42.00	5.5
			A_3MR_1	ND	46.00	5.3
			A_3MR_2	ND	42.00	5.3
			A_3MR_3	ND	39.00	5.4
			A_3MR_4	ND	44.00	5.6
2 Years	Pistillate (Female)	Allison	$A_1FR_{1(2)}$	ND	75.00	5.6
			$A_1FR_{2(2)}$	ND	44.00	5.6
			$A_1FR_{3(2)}$	ND	88.00	5.5
			$A_1FR_{4(2)}$	13.00	116.00	5.8
			$A_2FR_{1(2)}$	20.00	64.00	4.9
			$A_2FR_{2(2)}$	25.00	80.00	5.8
			$A_2FR_{3(2)}$	ND	67.00	4.9
			$A_2FR_{4(2)}$	ND	59.00	5.2
			$A_2FR_{5(2)}$	40.00	116.00	4.6
			$A_2FR_{6(2)}$	ND	48.00	4.8
			$A_3FR_{1(2)}$	40.00	120.00	4.6
			$A_3FR_{2(2)}$	ND	40.00	5.4
			$A_3FR_{3(2)}$	ND	48.00	5.3
	Staminate (Male)	Allison	$A_1MR_{1(2)}$	ND	42.00	5.6
			$A_1MR_{2(2)}$	24.00	120.00	5.4
			$A_1MR_{3(2)}$	ND	54.00	5.3
			$A_1MR_{4(2)}$	ND	58.00	5.4
			$A_1MR_{5(2)}$	12.00	110.00	5.4
			$A_1MR_{6(2)}$	ND	36.00	5.4
			$A_2MR_{1(2)}$	ND	41.00	5.4
			$A_2MR_{2(2)}$	ND	38.00	5.4
			$A_2MR_{3(2)}$	ND	42.00	5.3
			$A_2MR_{4(2)}$	ND	48.00	5.4
			$A_3MR_{1(2)}$	ND	78.00	5.6
			$A_3MR_{2(2)}$	12.00	68.00	5.3
			$A_3MR_{3(2)}$	ND	61.00	5.3
			$A_3MR_{4(2)}$	25.00	60.00	4.8
			$A_3MR_{5(2)}$	ND	52.00	5.4
			$A_3MR_{6(2)}$	16.00	116.00	5.3
			$A_3MR_7(2)$	ND	48.00	5.4
$CD_{0.05}$				1.13	1.14	0.73

ND: Not Detected

host also requires that bacteria overcome plant defence responses triggered after microbial recognition, a process in which surface polysaccharides, antioxidant systems, ethylene biosynthesis inhibitors and virulence genes are involved.

It can be speculated that IAA production trait is part of the strategy used by IAA producing bacteria to circumvent the plant defence system. IAA is a plant hormone with no apparent function in bacterial cells however it might improve the fitness of the plant–bacterium interaction (Etesami *et al*. 2015).

4.8 SCREENING OF CULTURABLE ISOLATES FOR MULTIFARIOUS PLANT GROWTH PROMOTING ACTIVITIES

A total of twenty culturable bacterial isolates were selected on the basis of IAA production and further screened for other multifarious plant growth promoting activities (Table 9, 10, 11 and 12). All the twenty isolates exhibited variation in production of different plant growth promoting traits *viz.* P-solubilization, siderophore production, HCN production, and antifungal activity against *Phytophthora capsici* and *Fusarium oxysporum* and enzyme production (protease, amylase and lipase).

4.8.1 Qualitative and quantitative estimation of phosphate solubilization by selected bacterial isolates

P-solubilization is considered as one of the most important attributes of the plant growth promoting rhizobacteria (PGPR) (Patel *et al*. 2008; Yasmin *et al*. 2012). Most of the phosphorus in soil is present as insoluble phosphates and cannot be utilized by plants (Pradhan and Sukla, 2005). PGPR have been known to solubilize precipitated phosphates and enhance phosphate availability to the crops that represented the possible mechanism of plant growth promotion (Verma *et al*. 2014 and Yasmin *et al*. 2012). Therefore in the present study, the selected twenty bacterial isolates were tested for P-solubilization qualitatively and quantitatively on PVK agar medium (Table 9 and Plate 1). Phosphate Solubilization Index (PSI) of bacterial isolates shows a lot of variation. Maximum Phosphate Solubilization Index was observed with H_1S_1 (4.50) which was statistically at par with that of H_2S_1 (2.25) and minimum was recorded with B_1S_4 (0.75).

The phosphate solubilizing activity of selected bacterial isolates was compared on the basis of % P-solubilization efficiency (SE %) in PVK agar medium and P-solubilization (µg/ml) in PVK broth. The results (Table 9) revealed that the individual isolate effectively solubilize the insoluble tri calcium phosphate in liquid medium. Maximum P-solubilization of

380µg/ml was recorded for isolate B_2S_{10} and corresponding viable count (116 10^6 CFU/ml); followed by isolate H_1S_1 (250.00 µg/ml). In PVK agar medium, maximum % P-solubilization efficiency of 233.00% was observed highest by isolate B_1S_4 and it corresponds to P-solubilization of only 65.00 µg/ml in liquid medium.

Table 9 Qualitative and quantitative estimation of tri calcium phosphate solubilization by bacterial isolates

Isolates	Quality estimation Zone size(mm)	Phosphate Solubilization Index **(PSI)	Per cent Solubilization Efficiency ***(%SE)	P-solubilization in liquid medium (µg/ml)	Viable count(10^6× CFU/ml)	pH
H_1S_1	0.9	4.50	122.20	250.00	84.00	5.4
H_2S_1	0.9	2.25	144.40	100.00	112.00	5.5
H_3R_3	0.5	1.25	180.00	75.00	80.00	5.6
H_3R_6	0.5	0.84	80.00	80.00	65.00	5.5
M_2S_1	0.5	1.67	80.00	80.00	51.00	5.6
M_3S_2	0.6	1.50	110.00	100.00	100.00	5.5
M_3S_3	0.6	1.20	183.30	110.00	104.00	5.4
M_1R_1	0.6	1.50	166.60	120.00	60.00	5.6
M_1R_2	0.7	1.40	122.30	80.00	56.00	5.4
B_1S_4	0.6	0.75	233.30	65.00	56.00	5.3
B_2S_1	0.4	0.80	225.00	140.00	34.00	5.4
B_2S_5	0.6	1.50	200.00	40.00	30.00	5.4
B_2S_{10}	0.9	1.80	155.50	380.00	116.00	5.3
A_1FS_1	0.7	1.40	171.42	220.00	110.00	5.4
$A_1FR_{4(2)}$	0.7	0.78	228.57	165.00	44.00	5.6
A_2MS_1	0.6	1.00	200.00	90.00	104.00	5.6
$A_2MS_{4(2)}$	0.6	1.20	183.33	140.00	114.00	5.8
A_2MR_3	0.7	1.00	200.00	225.00	60.00	5.8
$A_1MR_{5(2)}$	0.6	1.50	120.00	120.00	120.00	5.8
$A_3MR_{6(2)}$	0.6	1.00	200.00	180.00	116.00	5.7
$CD_{0.05}$	1.65	4.20	17.47	11.63	4.02	2.02

Correlation coefficient between qualitative and quantitative assay = 0.60
Correlation coefficient between qualitative and quantuitative assay = -0.21

**PSI = Phosphate solubilization Index = $\frac{A}{B}$, A: zone size B: colony size.

***Per cent Solubilization efficiency (%S.E) = $\frac{Z-C}{C} \times 100$
where, C – colony diameter Z – halozone diameter

The isolate B_2S_5 solubilized minimum TCP with the release of 40 µg/ml phosphorus. A large variation in P-solubilization of TCP was recorded amongst different isolates and the difference in P-solubilization was found to be non-significant among all the isolates. The correlation coefficient (r= 0.60) revealed that there was a strong positive significant correlation between qualitative and quantitative assay. Chaiharn and Lumyong, (2011) support our results as they found direct and positive correlation in solid and liquid medium for P-solubilization which is contradictory to earlier studies suggesting no correlation between P-solubilization in solid and liquid medium (Kumar *et al.* 2012).

A correlation cofficient (r= -0.21) revealed that there was a negative correlation between P-solubilization and final pH. Rodriguez and Fraja, (1999) and Sharma, (2012) had shown similar results of negative correlation between phosphate solubilisation and pH of the medium. Chaiharn snd Lumyong, (2011) screened a total of 30 IAA producing strains for P-solubilization that ranged from 0.50 to 334.4 µg/m which in accordance to the present study showed a decline in final pH similar to our study.

Chaiharn and Lumyong S, (2011) screened 216 rhizobacterial strains for various PGP activities *in vitro*. The metabolic capacities of the rhizobacteria isolates assay showed that 100% of isolate-solubilized inorganic phosphate on PVK plate at 30°C. The P-solubilizing zone around the bacterial colony varied from 0.2 to 1.4 cm. Since the direct measurement of P-solubilization in broth assay is likely to give more reliable results than regular plate assay, these 39 IAA-producing strains were tested for their ability to solubilize tri calcium phosphate in PVK broth.

The results showed that P-solubilizing ability of these isolates varied from 0.50 to 334.4 µg/ml using TCP as a source of insoluble *P. acinetobacter* strain CR 1.8 had the highest P-solubilization (334±0.4 µg/ml). The final pH of the medium decreased from 7 to 4.5 after incubation. A direct correlation of P-solubilizing activity in solid and liquid media was found in all 30 strains. However, a further 9 strains did not show this correlation. Plants select plant growth promoting rhizobacteria (PGPR) that are competitively fit to occupy compatible niches without causing pathological stress on them. However, while screening bacteria for plant growth promoting (PGP) agents, it is better to select bacteria for achieving the most promising isolates having suitable colonization and PGP traits. Selected bacterial isolates based on their IAA producing trait have the potential for more PGP traits and colonization of kiwi vines.

4.8.2 Qualitative and quantitative estimation of siderophore production by selected bacterial isolates

The production of siderophore, IAA, HCN and cell wall lytic enzymes are considered important traits for biocontrol activity of PGPR (Hameeda *et al*. 200; Ahemad *et al*. 2008).

Siderophore production was detected among thitrteen isolates (65.00%) out of twenty IAA producing isolates (Table 10). Our findings on siderophore production by 65.00%

isolates are in agreement with Bharucha *et al*. (2013) where the siderophore production was detected in 41% of total isolates.

Maximum zone size was observed by isolate M_2S_1 (5.00 mm) which was significantly higher than all the other isolates. Minimum zone size (0.00 mm) was observed in seven isolates (H_3R_6, M_1R_2, B_2S_1, B_2S_{10}, A_2MS_1, A_2MR_3 and $A_3MR_{6(2)}$).

Table 10 Siderophore estimation of bacterial isolates on CAS medium at 72 hour of incubation

Isolates	Qualitative estimation Zone size (mm)	**Quantitative estimation (Per cent siderophore unit)	Viable count ($10^6 \times$ CFU/ml)	***pH	Siderophore type
H_1S_1	2.00	66.67	45.00	5.4	Carboxylate
H_2S_1	2.00	66.67	42.00	5.5	Carboxylate
H_3R_3	1.00	33.34	31.00	5.6	Carboxylate
H_3R_6	0.00	0.00	0.00	5.5	ND
M_2S_1	5.00	102.67	56.00	5.6	Carboxylate
M_3S_2	2.00	66.67	44.00	5.5	Carboxylate
M_3S_3	3.00	33.33	48.00	5.4	Carboxylate
M_1R_1	4.00	78.43	50.00	5.6	Carboxylate
M_1R_2	0.00	0.00	0.00	5.4	ND
B_1S_4	2.00	33.33	36.00	5.3	Carboxylate
B_2S_1	0.00	0.00	0.00	5.4	ND
B_2S_5	2.00	98.67	47.00	5.4	Carboxylate
B_2S_{10}	0.00	0.00	0.00	5.3	ND
A_1FS_1	1.00	33.33	64.00	5.4	Carboxylate
$A_1FR_{4(2)}$	1.50	100.00	48.00	5.6	Carboxylate
A_2MS_1	0.00	0.00	0.00	5.6	ND
$A_2MS_{4(2)}$	0.50	100.00	31.00	5.8	Carboxylate
A_2MR_3	0.00	0.00	0.00	5.8	Carboxylate
$A_1MR_{5(2)}$	0.50	66.67	36.00	5.8	Carboxylate
$A_3MR_{6(2)}$	0.00	0.00	0.00	5.7	ND
$CD_{0.05}$	1.08	0.52	2.15	4.14	

Correlation coefficient between qualitative and quantitative assay = 0.66

** % Siderophore Unit = $\frac{Ar-As}{Ar} \times 100$ where, Ar= Absorbance of reference at 630 nm; As= Absorbance of reference test (control) at 630 nm.

Quantitative estimation of siderophore using CAS liquid assay revealed that maximum per cent Siderophore Unit (SU) was obtained for M_2S_1 (102.67%) at 72 h of incubation with corresponding viable count of 56 $\times 10^6$ CFU/ml followed by (100.00%) A_1FR_4. Minimum per cent SU (0.00%) was observed for isolates H_3R_6, M_1R_2, B_2S_1, B_2S_{10}, A_2MS_1, and $A_3MR_{6(2)}$.

The correlation coefficient (r= 0.66) between qualitative and quantitative siderophore estimation was found to be positive and statistically significant. The higher study suggests that the occurrence of siderophore producers in kiwi vines of direct significance to plants as it helps in iron sequestering near roots.

4.8.3 Characterization of the bacterial isolates for antifungal activity and HCN production

4.8.3.1 Antifungal activity

The antifungal activity of all twenty isolates against two phytopathogenic fungi *Phytophthora capsici* and *Fusarium oxysporum* was done using dual culture method (Table 11 Plate 2). The selected bacterial isolates exhibited variation in antifungal activity against the fungal pathogens. *In vitro* inhibition of both the fungal pathogens was observed for twelve isolates (60.00 %); four isolates showed inhibition against only one of the two fungal pathogens (20.00 %) however, only one isolate showed contact inhibition with one of the fungal pathogens (5.00 %) while three isolates (15.00 %) showed no inhibition at all. This suggests that the mode of action exerted and/ or the type of antifungal metabolites produced by the isolates may vary not only due to taxonomically different bacterial isolates as well as the type of pathogens to which they are showing antibiosis. It is interesting that IAA producing bacterial isolates associated with kiwi vines antagonize the most common plant pathogens of agricultural crops like tomato, pepper etc.

Among all the twenty isolates fifteen (75.00 %) were able to inhibit the growth of *Phytophthora capsicii* showing clear zone of inhibition whereas five of them showed no zone of inhibition. Maximum per cent growth inhibition was recorded for H_3R_6 (72.72 %) followed by M_1R_2 and B_1S_4 (68.18 %). Larger number of isolates with proper antagonism against *Phytophthora capsici* is supported by Bakthavatchalu *et al.* (2012) where 83.10 % zone of inhibition was obtained against *Phytophthora capsici*

Inhibition against *Fusarium oxysporum* was exhibited by thirteen isolates (65.00 %). One isolate (H_3R_3) showed contact inhibition while the rest six isolates did not show any zone of inhibition. Maximum per cent growth inhibition was recorded for H_3R_6 (74.41 %) followed by $A_1FR_{4(2)}$ (65.11 %).

These isolates controlled *F.oxysporum* growth irrespective of the antagonistic method used. The clear zone of inhibition produced in the *in-vitro* experiment is an indicative of

antibiosis by the biocontrol agent against the fungal pathogens. The present findings on the usage of biocontrol agents for the management of the pathogen revealed that, all the isolates of kiwi vines reduced the growth of *F. oxysporum* and *Phytophthora capsici* as reported earlier by Shobha and Kumudini, 2012) and Ramkumar *et al*. (2015). This suggests the effect of metabolites on the morphology of fungal mycelia of the test pathogen, showing the cell wall degradation and condensation of cytoplasm.

4.8.3.2 HCN Production

All the rhizobacterial isolates along with reference strain were screened for HCN production on King's B medium. None of the isolate was able to change the colour of filter paper from yellow to orange and to dark brown and found negative for HCN production. HCN production, not detected in all twenty four isolates showing *in vitro* suppressiveness, didn't appear to be superficially related to the antifungal activity.

Table 11 Screening of culturable bacterial isolates for antifungal activity against different fungal pathogens

Isolate	*Phytophthora capsici*		*Fusarium oxysporum*	
	Zone of no growth (mm)	***Per cent growth inhibition**	**Zone of no growth (mm)**	***Per cent growth inhibition**
H_1S_1	ND	ND	2.00	62.79
H_2S_1	9.00	63.63	7.00	58.13
H_3R_3	ND	ND	CI	CI
H_3R_6	8.00	72.72	19.00	74.41
M_2S_1	5.00	50.00	7.00	58.13
M_3S_2	7.00	65.90	ND	ND
M_3S_3	1.00	65.90	2.00	46.51
M_1R_1	ND	ND	ND	ND
M_1R_2	8.00	68.18	6.00	62.79
B_1S_4	6.00	68.18	7.00	55.81
B_2S_1	6.00	52.24	ND	ND
B_2S_5	1.00	56.81	5.00	41.86
B_2S_{10}	9.00	63.63	2.00	58.13
A_1FS_1	8.00	56.81	6.00	62.79
$A_1FR_{4(2)}$	1.00	65.90	2.00	65.11
A_2MS_1	6.00	59.09	ND	ND
$A_2MS_{4(2)}$	ND	ND	ND	ND
A_2MR_3	ND	ND	ND	ND
$A_1MR_{5(2)}$	1.00	65.90	8.00	56.21
$A_3MR_{6(2)}$	1.00	65.90	8.00	56.21
$CD_{0.05}$	1.23	7.38	6.65	2.24

* Per cent growth inhibition = $\frac{C-T}{C} \times 100$

4.8.4 Extracellular enzyme activity by culturable bacterial isolates

Table 12 revealed that cell wall degrading enzyme activity of selected bacterial isolates in terms of Enzyme Activity Index (EAI). Out of total twenty isolates, thirteen (65.00%) isolates exhibited protease activity and rest were unable to produce clear halozone on skimmed milk agar plates. Highest protease activity (EAI=1.30) was shown by isolate H_1S_1 followed by 1.00% for isolate $A_2MS_{4(2)}$. Enzyme activity was found to be statistically different among all the isolates. Protease activities were checked on the described media by Kumar *et al*. (2005) and Bakthavatchalu *et al*. (2012).

Among all twenty isolates eighteen isolates (90.00 %) showed amylase activity and two were unable to produce clear halozone on starch agar plates. Highest enzyme activity (EAI=1.34) was shown by isolate H_1S_1 followed by 1.18 for isolate $A_2MR_{6(2)}$. Lytic enzyme production s one of the most important indirect mechanisms involved in biological control of plant pathogens and deleterious control of plant pathogens and microbes (Malleswari and Bagyanarayana, 2013 and Bakthvatchalu *et al*. 2012).

All twenty isolates exhibited lipase enzyme activity (100.00 %) on tributyrin agar plates. Highest enzyme activity (EAI= 1.60) was exhibited by A_2MS_1 and A_1FS_1 followed by 1.50 for isolate B_2S_1. Lipase production was identified on the medium described by Rashid *et al*. (2001). Clear zones around the bacteria growth spot indicated the lipase activity among strains.

Extracellular enzyme production like lipase, protease and amylase had shown contribution to the ability of bacteria to suppress fungal diseases and thus demonstrating the potential of PGPR for biocontrol (Ghodsalavi *et al*. 2013).

All twenty isolates exhibited lipase enzyme activity (100.00 %) on minimal agar media plates. Highest enzyme activity (EAI= 1.30) was exhibited by H_1S_1 followed by 1.20 for isolate H_3R_3. Clear zones around the bacteria growth spot indicated the chitinase activity among strains.

Extracellular enzyme production like lipase, protease, amylase, chitinase had shown contribution to the ability of bacteria to suppress fungal diseases and thus demonstrating the potential of PGPR for biocontrol (Ghodsalavi *et al*. 2013).

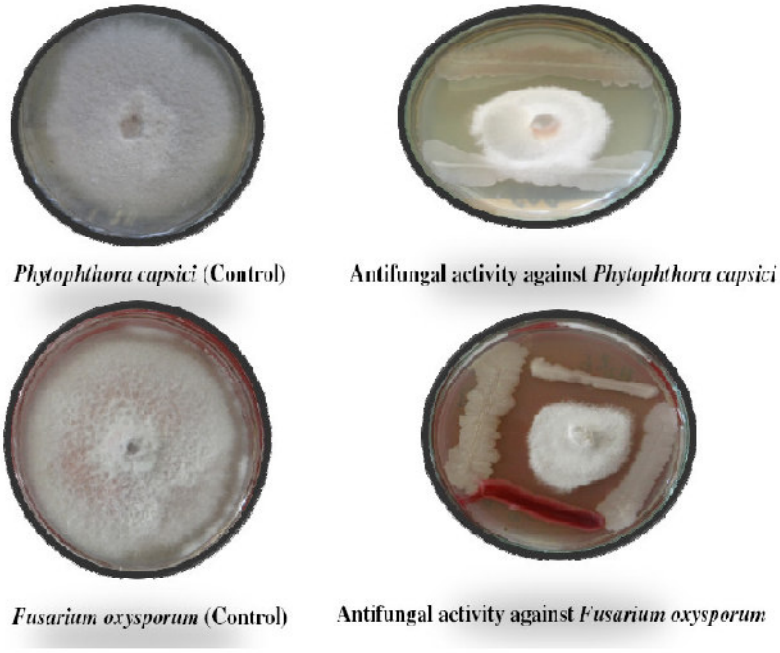


Plate 2. **Antifungal activity of bacterial isolates against *Phytophthora capsici* (a); *Fusarium oxysporum* (b)**

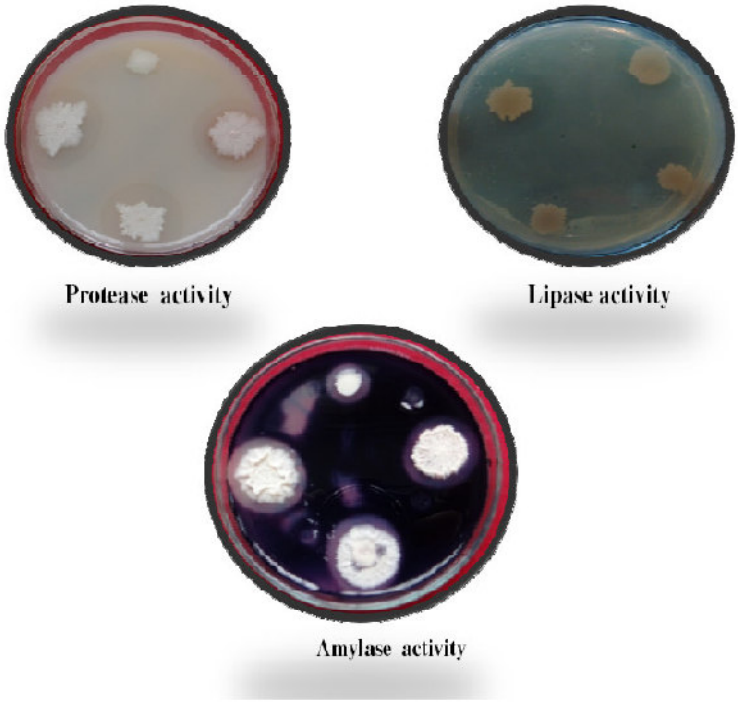


Plate 3. Enzyme production by bacterial isolates

Table 12 Screening of bacterial isolates for extracellular enzyme activity

Isolates	Protease		Amylase		Lipase	
	Zone size (mm)	*EAI	Zone size (mm)	*EAI	Zone size (mm)	*EAI
H_1S_1	13.00	1.30	9.00	1.34	7.00	0.70
H_2S_1	ND	ND	9.00	0.67	12.00	1.09
H_3R_3	2.00	0.08	12.00	0.27	9.00	0.45
H_3R_6	7.00	0.70	8.00	0.89	10.00	0.84
M_2S_1	9.00	0.40	5.00	0.34	9.00	0.81
M_3S_2	ND	ND	4.00	0.40	8.00	0.61
M_3S_3	11.00	0.64	8.00	0.50	11.00	1.10
M_1R_1	11.00	0.73	10.00	0.84	7.00	0.50
M_1R_2	ND	ND	ND	ND	2.00	1.00
B_1S_4	7.00	0.35	4.00	0.27	15.00	1.50
B_2S_1	ND	ND	ND	ND	7.00	0.47
B_2S_5	13.00	1.00	5.00	0.45	9.00	0.50
B_2S_{10}	ND	ND	7.00	1.00	3.00	0.50
A_1FS_1	ND	ND	6.00	0.75	16.00	1.60
$A_1FR_{4(2)}$	11.00	0.55	12.00	0.67	9.00	0.75
A_2MS_1	10.00	0.76	5.00	0.25	16.00	1.60
$A_2MS_{4(2)}$	12.00	1.00	4.00	0.23	12.00	1.09
A_2MR_3	9.00	0.36	8.00	0.45	13.00	1.18
$A_1MR_{5(2)}$	ND	ND	2.00	0.25	1.00	0.04
$A_3MR_{6(2)}$	6.00	0.60	9.00	1.18	19.00	0.22
$CD_{0.05}$	1.33	2.29	1.56	2.01	1.61	1.90

*Enzyme activity (EAI) = $\frac{A}{B}$ Where, A= Halozone diameter ; B= Colony diameter ; ND = Not Detected

4.9 SELECTION OF RHIZOSPHERIC AND ENDOPHYTIC IAA PRODUCING BACTERIAL ISOLATES ON THE BASIS OF OVERALL PLANT GROWTH PROMOTING TRAITS

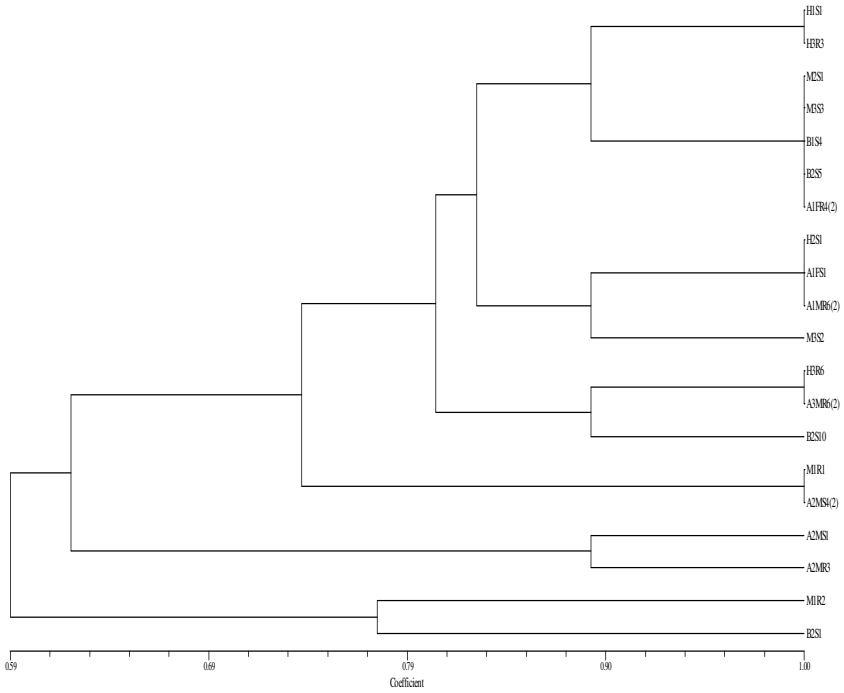
The results after screening of selected twenty isolates for multifarious plant growth promoting traits of selected IAA producing isolates showed a large variation in exhibiting PGP activities. All selected isolates did not possess PGP activities. Six isolates were finally selected on the basis of various plant growth promoting activities for further application as formulation in net house conditions for growth promotion assay which demonstrates the multifunctional properties of the isolates (Mehta *et al*. 2013). Analysis of PGP traits grouped the twenty IAA producing bacteria isolates into two main clusters. These two clusters were 59 % similar with each other (Fig. 6). The upper cluster comprised of eighteen isolates and two isolates were grouped into the lower cluster. In the upper cluster isolates H_1S_1 and H_3R_3 showed 100% similarity as they were positive for IAA production, P-solubilization, siderophore production, growth on nitrogen free media adn lytic enzyme activity. Isolates

M_2S_1, M_3S_3, B_1S_4, B_2S_5 and $A_1FR_{4(2)}$ also showed 100 % similarity as they were found to be positive for all the PGP activities. Isolates H_2S_1, A_1FS_1 and $A_1MR_{6(2)}$ were 100% similar and showed positive results for all the PGP activities except they could not show protease enzyme activity. And isolates M_1R_1 and $A_2MS_{4(2)}$ also showed 100 % similarity showing positive results for all the tested PGP activities except for antifungal activity against any of the two phytopathogens. The upper cluster was divided into six subclusters comprised of eighteen isolates sharing 87 % similarity. The lower cluster comprised of two bacterial isolates with 76.5% similarity where isolate. On the basis of the above PGP activities of IAA producing bacterial isolates, six most efficient isolates (B_2S_{10}, H_1S_1, H_3R_3, M_1R_1, $A_2MS_{4(2)}$ and $A_1FR_{4(2)}$) were further selected.

4.10 METABOLIC CHARACTERIZATION OF SELECTED BACTERIAL ISOLATES

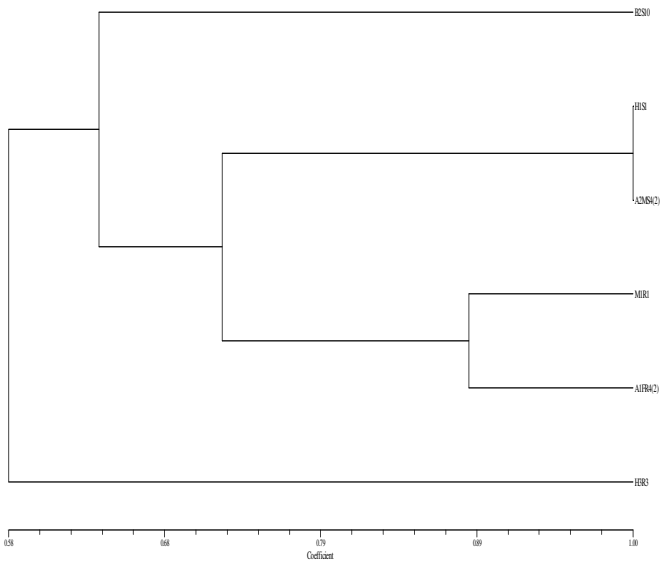
The selected six bacterial isolates were characterized by morphological and biochemical characteristics (Fig.7, Table 13) through Gram's staining method. Most of the isolates stained as Gram positive, coccus or coccobacillus and rods occurring in chains or were single except isolate B_2S_{10} that was Gram negative. Pant and Aggarwal, (2013) obtained similar results for morphological characterization of bacterial isolates in his study on tomato that exhibited wide morphological variation showing dominance of bacilli. Dominance of coccus and coccobacillus in rhizosphere of different crops has been reported by many investigators (Naveed *et al*. 2014 and Mehta *et al*. 2014). The selected six bacterial isolates were further characterized on the basis of biochemical tests. Isolate A_1FR_4 showed utilization of all three sugars i.e. dextrose, sucrose and lactose; isolates B_2S_{10}, H_1S_1 and A_2MS_4 showed utilization of only two sugars i.e. dextrose and sucrose whereas isolate M_1R_1 could utilize sucrose and lactose and all showed only acid production. Isolate M_1R_1 did not utilize any of the sugars. Only isolate B_2S_{10} was indole positive while rest were indole negative. Isolate H_1S_1, H_3R_3 and $A_2MS_{4(2)}$ were Methyl red Positive and isolates B_2S_{10} and H_3R_3 could utilize citrate. All the isolates were Ammonia producers where isolates B_2S_{10} and H_1S_1 were triple positive, H_3R_3 and $A_2\,MS_{4\,(2)}$ were double positive and M_1R_1 and $A_1FR_{4(2)}$ were single positive on the basis of colour production. Only isolate $B_2S_{10}$was catalyse positive while rest of the isolates were catalase negative. Dendrogram based on the biochemical and carbon utilization tests categorised all the isolates into two clusters at 58 % similarity coefficient (Fig. 7). The lower cluster comprised of a single isolate H_3R_3.

PGP traits



Isolates	IAA production (µg/ml)	P-solubilization (µg/ml)	Siderophore production	Growth on Nitrogen free medium	Antifungal activity against *P.capsicii*	Antifungal activity against *F.oxysporum*	Protease enzyme activity	Amylase enzyme activity	Lipase enzyme activity
H_1S_1	11	250.00	66.67	+	ND	62.79	1.30	1.34	0.70
H_2S_1	12	100.00	166.67	+	63.63	58.13	ND	0.67	1.09
H_3R_3	42	75.00	33.34	+++	ND	CI	0.08	0.27	0.45
H_3R_6	14	80.00	0.00	+++	72.72	74.41	0.70	0.89	0.84
M_2S_1	20	80.00	166.67	++	50.00	58.13	0.40	0.34	0.81
M_2S_2	16	100.00	66.67	+	65.90	ND	ND	0.40	0.61
M_2S_3	11	110.00	33.33	++	65.90	46.51	0.64	0.50	1.10
M_1R_1	39	120.00	200.00	++	ND	ND	0.73	0.84	0.50
M_1R_2	21	80.00	0.00	+++	68.18	62.79	ND	ND	1.00
B_1S_4	15	65.00	33.33	++	68.18	55.81	0.35	0.27	1.50
B_2S_1	14	140.00	0.00	+	52.24	NA	ND	ND	0.47
B_2S_5	12	40.00	166.67	++	56.81	41.86	1.00	0.45	0.50
B_2S_{10}	42	380.00	0.00	+	63.63	58.13	ND	1.00	0.50
A_1FS_1	11	220.00	133.33	+	56.81	62.79	ND	0.75	1.60
$A_1FR_{4(2)}$	13	165.00	100.00	++	65.90	65.11	0.55	0.67	0.75
A_2MS_1	40	90.00	0.00	+	59.09	ND	0.76	0.25	1.60
$A_2MS_{4(2)}$	21	140.00	100.00	+	ND	ND	1.00	0.23	1.09
A_2MR_3	23	225.00	0.00	++	ND	ND	0.36	0.45	1.18
$A_1MR_{6(2)}$	12	120.00	66.67	+	65.90	56.21	ND	0.25	0.04
$A_3MR_{6(2)}$	16	180.00	0.00	++	65.90	56.21	0.60	1.18	0.22

Fig. 6 Dendrogram showing relationship amongst selected IAA producing bacterial isolates on the basis of plant growth promoting traits



Isolate	Sugars			Indole	Methyl Red	Vogues Proskauer's	Citrate	H_2S production	Ammonia Production*
	D	S	L						
B_2S_{10}	A	A	-	+	-	-	+	-	+++
H_1S_1	A	A	-	-	+	-	-	-	+++
H_3R_3	-	-	-	-	+	-	+	-	++
M_1R_1	-	A	A	-	-	-	-	-	+
$A_2MS_{4(2)}$	A	A	-	-	+	-	-	-	++
$A_1FR_{4(2)}$	A	A	A	-	-	-	-	-	+

Fig. 7 Dendrogram showing similarity amongst selected six bacterial isolates based on their biochemicals test

D: Dextrose; S: Sucrose, L: Lactorse, A- Acid production only, A+G: Acid and Gas; G: Gas production only; *=+,orange,++, light brown, +++, dark brown.

Table 13. Morpho-Biochemical characteristics of selected six bacterial isolates

Isolate	Sugars			Indole	Methyl Red	Vogues Proskauer's	Citrate	H_2S production	Ammonia Production*	Gram's reaction	Cell Shape	Arrangement
	D	S	L									
B_2S_{10}	A	A	-	+	-	-	+	-	+++	Gram negative	Coccus	Chains
H_1S_1	A	A	-	-	+	-	-	-	+++	Gram Positive	Coccobacillus	Single
H_3R_3	-	-	-	-	+	-	+	-	++	Gram Positive	Coccobacillus	Single
M_1R_1	-	A	A	-	-	-	-	-	+	Gram Positive	Rods	Single
$A_2MS_{4(2)}$	A	A	-	-	+	-	-	-	++	Gram Positive	Rods	Chains
$A_1FR_{4(2)}$	A	A	A	-	-	-	-	-	+	Gram Positive	Rods	Chains

D: Dextrose; S: Sucrose, L: Lactorse, A- Acid production only, A+G: Acid and Gas; G: Gas production only; *=+,orange,++, light brown, +++, dark brown.

The upper cluster was grouped into two subclusters. The upper subcluster comprised of only one isolate B_2S_{10}, while the lower subcluster comprised of two groups at 73 % similarity coefficient. The upper group comprising of H_1S_1 and $A_2MS_{4(2)}$ at 100% similarity while the lower group comprised of two isolates M_1R_1 and $A_2MS_{4(2)}$ showing 88% similarity.

4.11 TOMATO SEED GERMINATION AND GROWTH PROMOTION ASSAY UNDER NET HOUSE CONDITIONS

4.11.1 Effect of liquid base culture of bacterial isolated from kiwi vines on growth promotion of tomato seedlings under net house conditions after 60 days of seed sowing

Perusal of Table 14 and Fig. 8 revealed that seed bacterization resulted in increase in germination overall root shoot parameters over inoculated control. All selected six isolates ($B_2S_{10,}$ H_1S_1, H_3R_3, M_1R_1, $A_2MS_{4(2)}$ and $A_1FR_{4(2)}$) showed increase in seed germination over uninoculated control. Maximum germination per cent recorded was 96.67 per cent in seeds treated with isolate B_2S_{10} followed by H_3R_3, $A_2MS_{4(2)}$ and $A_1FR_{4(2)}$ recorded as 93.34 per cent and minimum was recorded as 90.00 per cent for seeds treated with isolates H_1S_1 and M_1R_1, where as germination per cent was recorded as 86.66 per cent seeds treated with control. In earlier study, bacterization with plant growth promoting bacterial isolate *Pseudomonas* marginally improved seed germination and final standard count of soyabean varieties (Tripathi *et al.* 2005). Maximum root length (4.00 cm) and per cent increase in root length (44.98%) was observed in case of seedlings raised from tomato seeds treated with isolate B_2S_{10} which was significantly higher than roots of seedlings raised from other treated seeds. Minimum root length (3.00 cm) was observed in case of seedlings raised from tomato seeds treated with isolate H_1S_1. PGPR are responsible for growth of seedlings root by reducing the production of hormones such as indole-3-acetic acid (Patten and Glick, 2002). There are many reports suggesting the enhancement of roots and plant height by applying bacterial cultures (Vessey and Buss, 2002). Seed treatment with all the isolates significantly increased the shoot length as compared to untreated control. Isolate B_2S_{10} showed maximum shoot length (89.00 cm) and maximum per cent increase in shoot length (30.54%) which was found to be significantly higher than shoot seedlings raised from other treated seeds. Minimum shoot length (71.00 cm) was found in isolate H_1S_1.Seed treatment with six isolates resulted in increase in vigour index over control. Table 13 illustrated maximum vigour index (8845.30) with seeds treated with isolate B_2S_{10}which was statistically higher than untreated seeds (5892.88). Minimum vigour index was recorded in case of seedlings raised from seeds treated with isolate H_1S_1 (6660.00).

Table 14. Effect of tomato seed treatment with liquid formulation of bacterial isolates on growth parameters in net house (60 days after sowing)

Isolates	Root Length (cm)	% Increase in Root Length ##	Shoot Length (cm)	%Increase in Shoot Length (#)	Root dry weight (mg)	% Increase in Root dry weight (##)	Shoot dry weight (mg)	% Increase in Shoot dry weight (##)	Germination % (##)	Vigour index
Control	2.00	-	66.00	-	35.00	-	1030	-	86.66 (9.30)	5892.88
B_2S_{10}	4.00	44.98 (6.70)	89.00	30.54 (5.52)	49.00 (7.00)	40.18 (6.33)	1720.00	50.85 (7.13)	96.67 (9.83)	8845.30
H_1S_1	3.00	35.25 (5.93)	71.00	15.38 (3.92)	38.00 (6.16)	16.30 (4.03)	1650.00	39.28 (6.26)	90.00 (9.48)	6660.00
H_3R_3	3.50	40.87 (6.39)	88.00	29.98 (5.47)	60.00 (7.74)	32.29 (5.68)	1680.00	38.44 (6.20)	93.34 (9.66)	8680.62
M_1R_1	3.50	40.87 (6.39)	83.00	26.89 (5.47)	44.00 (6.63)	26.87 (5.18)	1420.00	31.59 (5.62)	90.00 (9.48)	7785.00
$A_2MS_{4(2)}$	3.50	40.87 (6.39)	78.00	23.08 (4.80)	35.00 (5.91)	9.71 (3.11)	1041.00	35.87 (5.98)	93.34 (9.66)	7607.21
$A_1FR_{4(2)}$	3.80	43.46 (6.59)	72.00	16.77 (4.09)	48.00 (6.92)	31.34 (5.59)	1450.00	32.54 (5.70)	93.34 (9.66)	7075.17
$CD_{0.05}$	1.64	0.42	2.88	0.48	3.85	0.01	3.30	0.02	2.18	2.96

Per cent increase / decrease = $\frac{T-C}{T} \times 100$ where, T= Treatment; C= Control

Figure in parenthesis ( ) are square root transformed values ## Figure in Parenthesis ( ) are arc sign values

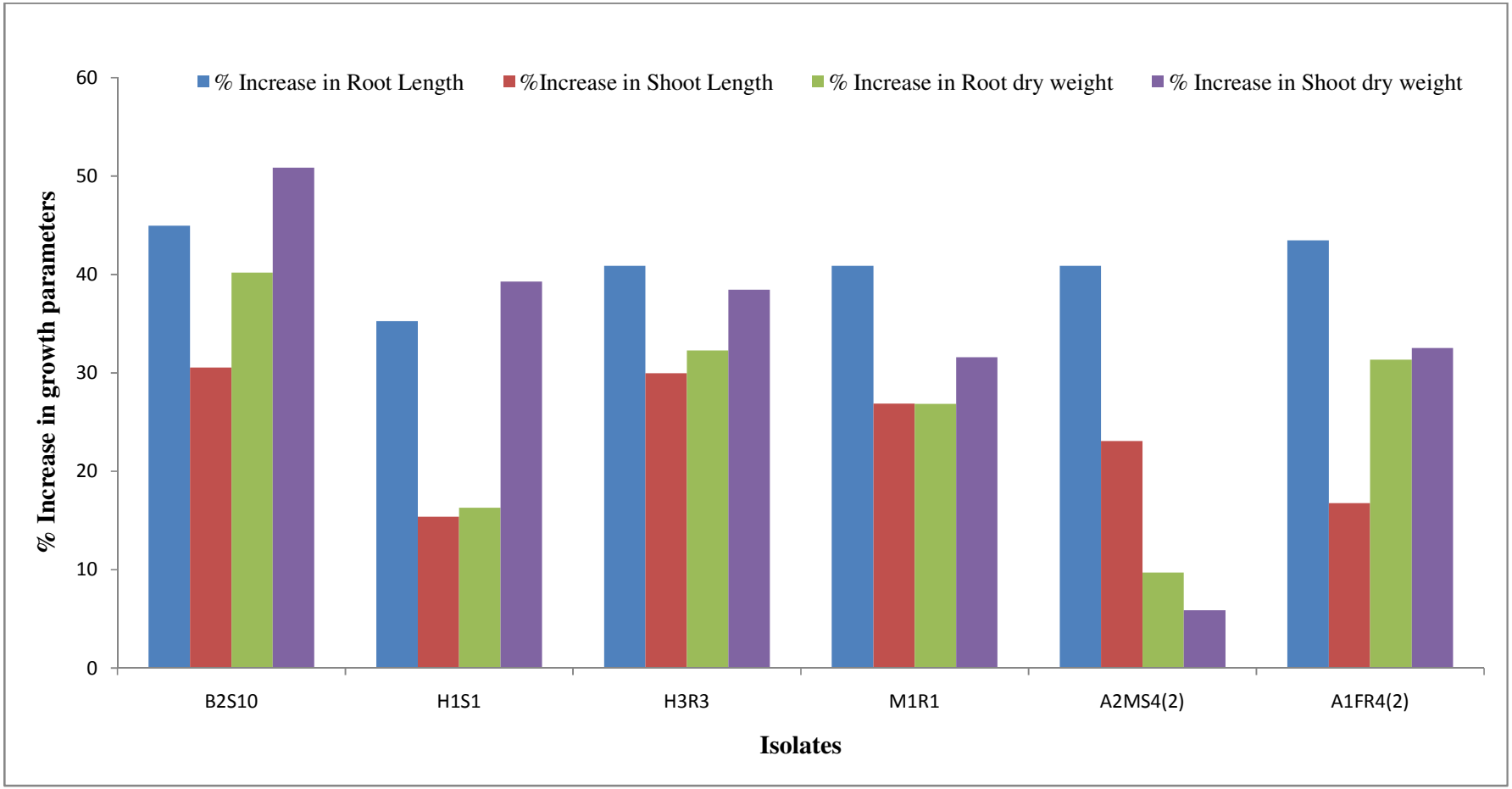


Fig. 8 Graphical presentation of per cent increase in root and shoot growth parameters of tomato seedlings under net house conditions

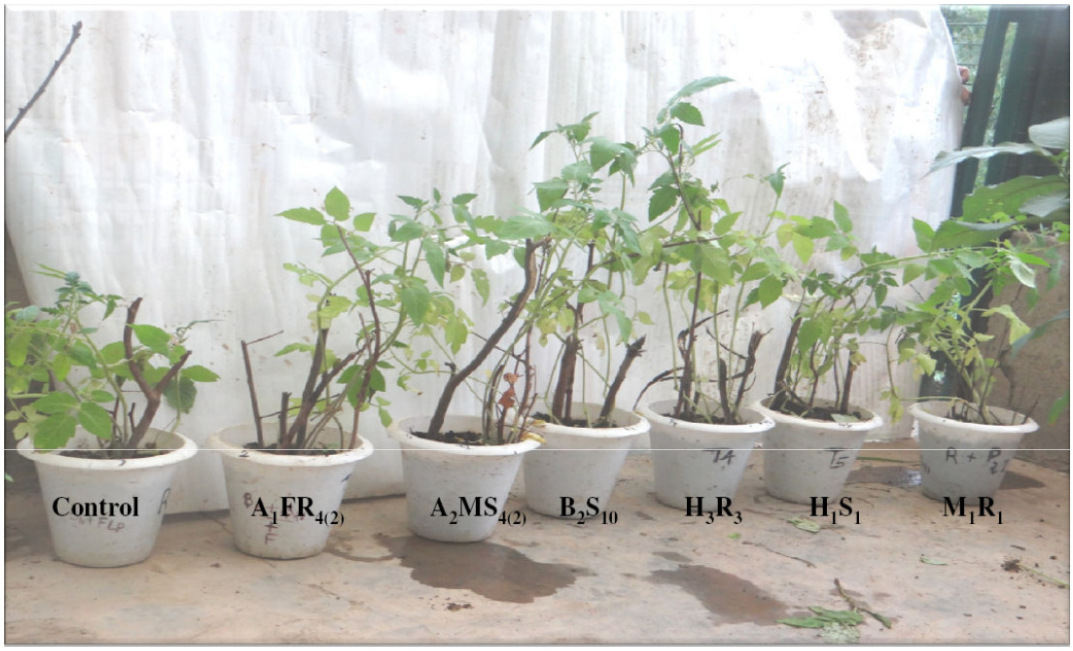


Plate 4. Effect of treatment of selected bacterial isolates on growth of tomato seedlings under net house conditions

4.12 KIWI SEEDLINGS GROWTH PROMOTION ASSAY UNDER NET HOUSE CONDITIONS

4.12.1 Effect of liquid base culture of bacterial isolates from kiwi vines on growth promotion of kiwi seedlings under axenic conditions after 60 days of planting

Several studies have been earlier reported that subjected seedlings of various crops to liquid formulations of bacterial cultures (Sharma *et al*. 2015; Mehta *et al*. 2013). Similarly, in the present study an attempt was made to analyse PGPR isolated from kiwi vines to show growth promoting effect on kiwi seedlings. Thus, suggesting the importance of crop specificity as reported earlier by Bakthavatchalu *et al*. (2002). Perusal of Table 15 (Fig.9) revealed that treatment of kiwi seedlings with liquid base cultures resulted in increase in germination overall root shoot parameters over inoculated control.

All selected six isolates ($B_2S_{10,}$ H_1S_1, H_3R_3, M_1R_1, $A_2MS_{4(2)}$ and $A_1FR_{4(2)}$) showed increase in all plant growth parameters (root length, shoot length, root dry weight, shoot dry weight, laef surface area and stem width) over uninoculated control. Maximum per cent increase root length (30.12%) and shoot length (38.82) was recorded in case of kiwi seedlings treated with isolate $B_2S_{10.}$ Minimum per cent increase in shoot length (12.84) and root length (3.33%) was recorded in seedlings treated with isolate $A_1FR_{4(2)}$.

Maximum per cent increase in root dry weight was recorded as 72.90 in case of kiwi seedlings treated with isolate B_2S_{10} followed by H_1S_1 that showed 65.00 per cent increase in root dry weight as compared to control. Minimum per cent increase in root dry weight was recorded as 53.84 by seedlings treated with isolate H_3R_3. Maximum per cent increase in shoot dry weight was observed in case of kiwi seedlings treated with isolate B_2S_{10} that recorded 58.34 per cent increase followed by seedlings treated with isolate H_3R_3 that recorded a per cent increase of 57.30. Minimum per cent increase in shoot dry weight was recorded as 40.65 in case of seedlings treated with isolate M_1R_1.

Maximum per cent increase in leaf surface area recorded was 7.98 by seedlings treated with isolate B_2S_{10}. Minimum per cent increase in leaf surface area was recorded in case of seedlings treated with isolate M_1R_1. PGPR alters several hormonal pathways, this could account for the different morphological changes observed for example, lateral root elongation and root hair development which supports our finding of using IAA producing PGPR as root inoculants for kiwi (Erturk *et al*. 2010).

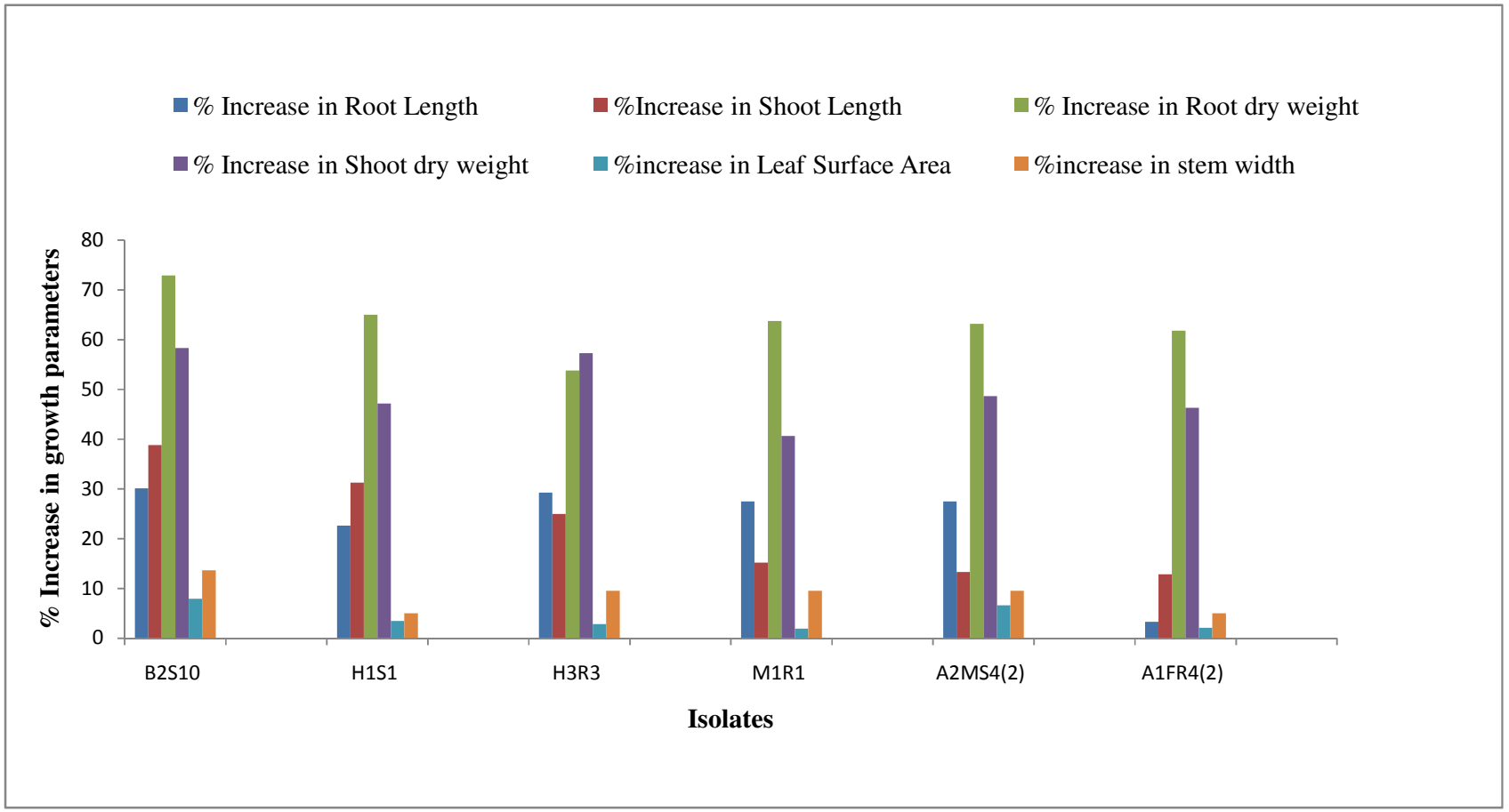


Fig. 9 Graphical presentation of per cent increase in root and shoot growth parameters of kiwi seedlings under net house conditions

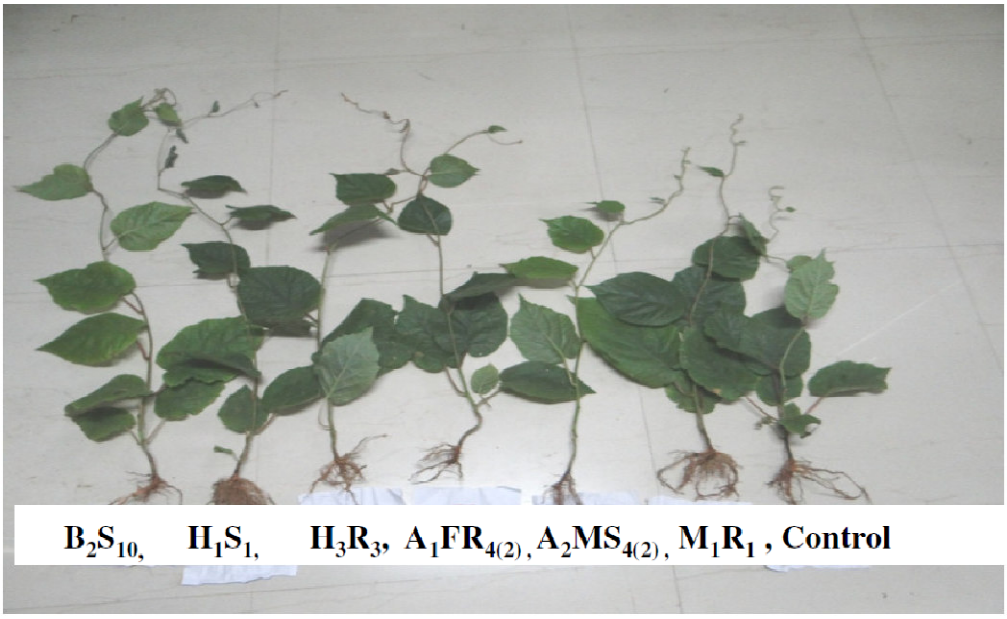


Plate 5. Effect of treatment of selected bacterial isolates on growth promotion of kiwi seedlings

Table 15 Effect of kiwi seedling treatment with liquid formulation of selected bacterial isolates on growth parameters in net house (60 days of planting)

Isolates	Root Length (cm)	% Increase in Root Length #	Shoot Length (cm)	%Increase in Shoot Length#	Root dry weight (g)	% Increase in Root dry weight##	Shoot dry weight (g)	% Increase in Shoot dry weight ##	Leaf Surfcae Area (cm^2)	%increase in Leaf Surface Area #	Stem width (mm)	%increase in stem width (mm) #
Control	5.80	-	78.00	-	0.42	-	2.92	-	140.50	-	19.00	-
B_2S_{10}	8.00	30.12 (5.48)	127.50	38.82 (6.23)	1.55	72.90 (8.53)	7.01	58.34 (7.63)	145.60	7.98 (2.82)	22.00	13.63 (3.69)
H_1S_1	7.50	22.66 (4.76)	113.50	31.27 (5.59)	1.20	65.00 8.06	5.53	47.19 (6.86)	150.45	3.50 (1.87)	20.00	5.00 (2.23)
H_3R_3	8.20	29.26 (5.40)	104.00	25.00 (5.00)	0.91	53.84 (7.33)	6.84	57.30 (7.56)	144.65	2.86 (1.69)	21.00	9.52 (3.08)
M_1R_1	8.00	27.50 (5.24)	92.00	15.21 (3.90)	1.16	63.79 (7.98)	4.92	40.65 (6.37)	143.27	1.93 (1.38)	21.00	9.52 (3.08)
$A_2MS_{4(2)}$	8.30	27.50 (5.24)	90.00	13.34 (3.65)	1.14	63.15 (7.94)	5.69	48.68 (6.97)	152.70	6.61 (2.57)	21.00	9.52 (3.08)
$A_1FR_{4(2)}$	6.00	3.33 (1.82)	89.50	12.84 (3.58)	1.10	61.81 (7.86)	5.44	46.32 (6.80)	143.50	2.09 (1.44)	20.00	5.00 (2.23)
CD (0.05)	0.37	0.08	1.49	0.48	0.09	0.45	0.01	0.04	0.14	0.11	1.76	1.37

Per cent increase /decrease = $\frac{T-C}{C} \times 100$ where, T= Treatment; C= control

Figure in parenthesis ( ) are square root transformed values ## Figure in Parenthesis ( ) are arc sign values

Maximum per cent increase in stem width was observed in case of kiwi seedlings treated with isolate B_2S_{10} with 13.63 per cent increase followed by isolates H_3R_3, $M_1R_1$and $A_2MS_{4(2)}$ that recorded an increase of 9.52 per cent. Minimum per cent increase in stem width was recorded in case of kiwi seedlings treated with isolates H_1S_1 and $A_1FR_{4(2)}$.

The inoculated strains have found to promote and increase the root length and root biomass which augmented growth until increase in mineral uptake in plants (Yan *et al*. 2004 and Pal *et al*. 2003).

4.13 RELATIONSHIP (r) STUDIES BETWEEN GROWTH PARAMETERS IAA PRODUCTION, PHOSPHATE SOLUBILIZATION AND SIDEROPHORE PRODUCTION

The correlation, coefficient between growth parameters viz., shoot length, shoot dry weight, root length, root dry weight and IAA production and phosphate solubilization and siderophore production was determined for 60 days kiwi seedlings under net house conditions.

The results (Table16) revealed that a positive correlation between root length and shoot length (r= 0.26) and shoot dry weight and root dry weight (r= 0.36) at 5% level of significance. A negative correlation was observed between root length and root dry weight (r= -0.10) at 5% level of significance and between shoot length and shoot dry weight (r= 0.62). Shoot dry weight and root length (r= 0.35) and root dry weight and shoot length (r= 0.36) were found at 5% level of significance.

Siderophore production and stem width was found to be negatively correlated (r= -0.24). A positive correlation but non-significant (r= 0.06) was also observed between siderophore production and phosphate solubilization. The present findings are according to the results given by Ghodsalavi *et al*. (2013). A highly positive correlation was found between root length, shoot length, root dry weight and stem width with IAA.

Table 16 Correlation matrix between growth parameters of kiwi vines in net house and IAA production, phosphate solubilization and siderophore production

Growth parameters	Root length	Shoot length	Root dry weight	Shoot dry weight	Leaf surface area	Stem width	IAA	Siderophre production	P-solubilization
Root length	-								
Shoot length	0.26	-							
Root dry weight	-010	0.36	-						
Shoot dry weight	0.35	0.62	-0.04	-					
Leaf surface area	0.34	0.04	0.48	-0.06	-				
Stem width	0.66	0.47	-0.34	0.56	-0.11	-			
IAA	0.63	0.76	0.63	-0.07	-0.15	0.62	-		
Siderophore production	-0.08	0.77	1.00	-0.83	-0.21	-0.24	-0.53	-	
P-solubilization	-0.04	0.36	0.54	0.25	0.12	0.36	0.30	0.55	-

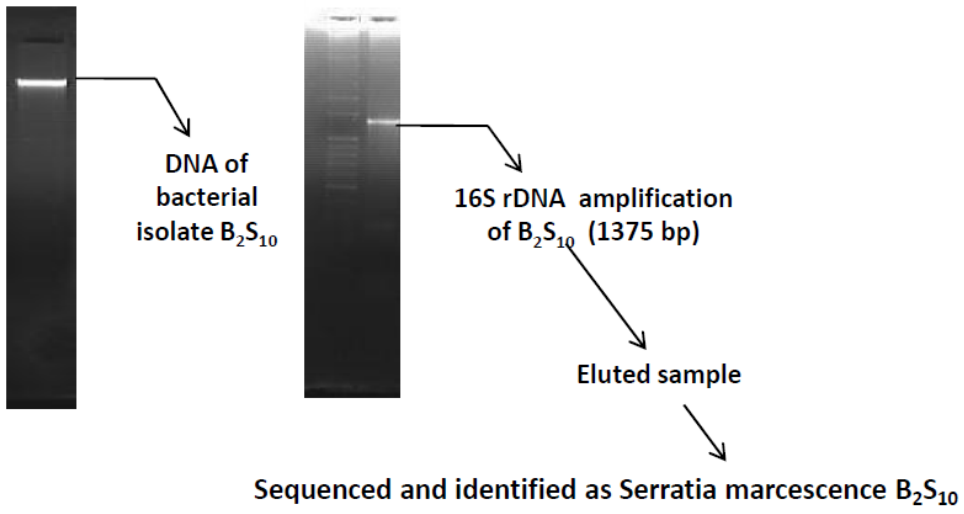
4.14 MOLECULAR IDENTIFICATION OF MOST EFFICIENT ANTAGONISTIC BACTERIAL ISOLATE B_2S_{10} BASED ON 16S rDNA GENE SEQUENCING

Molecular identification of the isolate was done by sequencing part of the 16S rDNA. The amplification of 16S rDNA was confirmed by agarose gel electrophoresis. The PCR product was gel eluted and sequenced (Fig. 10). The sequence data of the 16S rDNA was subjected to BLAST analysis. As 16S rDNA gene sequence provide accurate grouping of organism even at subspecies level it is considered as a powerful tool for the rapid identification of bacterial species (Jill and Clarridge, 2004). The sequence analysis of 16S rDNA sequence of strain B_2S_{10} showed maximum identity of 99.00 per cent to *Serratia marcescenes*. Therefore, the isolate B_2S_{10} can be considered as *Serratia marcescenes*. respectively. The presence of these organisms has not been reported from rhizosphere of kiwi vines. Therefore, the presence of *Serratia marcescenes* in specific habitat of kiwi vines make them much more interesting. This is because these strains can expect to have strain specific plant growth promoting potential.

The phylogenetic analysis of 16S rDNA sequence of the isolates along with the sequences retrieved from the NCBI was carried out with MEGA 6 using the neighbour-joining method with 1,000 bootstrap replicates. The result of phylogenetic analysis showed distinct clustering of the isolate B_2S_{10} with *Serratia marescenes* (KR856282) (Fig.10). *Serratia marcescenes* is a well reported PGPR isolated from many crop systems (Chakraborty *et al*. 2013; Dong *et al*. 2014).

Table 17 Nucleotide base composition in the 16S r RNA gene sequence of B_2S_{10}

Nitrogenous base	Nucleotide count	
	Total	Per cent (%)
Adenine (A)	177	26.11
Thymine (T)	130	19.17
Cytosine (C)	152	22.41
Guanine (G)	219	32.30
G+C	371	54.71
A+T	307	45.28



Corrected Sequence

GTAATGTCTGGGAACTGCCTGATGGAGGGGGATAACTACTGGAA
ACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGA
CCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCAG
TAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCT
GAGAGGATGACCAGCCACAAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAGGATGC
AGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCAC
TTTCAGCGAGGAGGAAGGTAGCTTAATACGCTCATCAATTGACG
TTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAACCGCGTA
TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
CACGCAGGCGTTTGTTAAGTAGATGTGAAATCCCCGGGCTCAAC
CTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGG
GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAG
GAATACCGGTGGCGAAGCGGCCTGGACGAACACTGACGCTCATG
TGCGAAAGCGTGGGGAGCAACAGGATTAAATACCC

Plate 6. Molecular identification of bacterial isolate B_2S_{10} based on 16S rDNA amplification

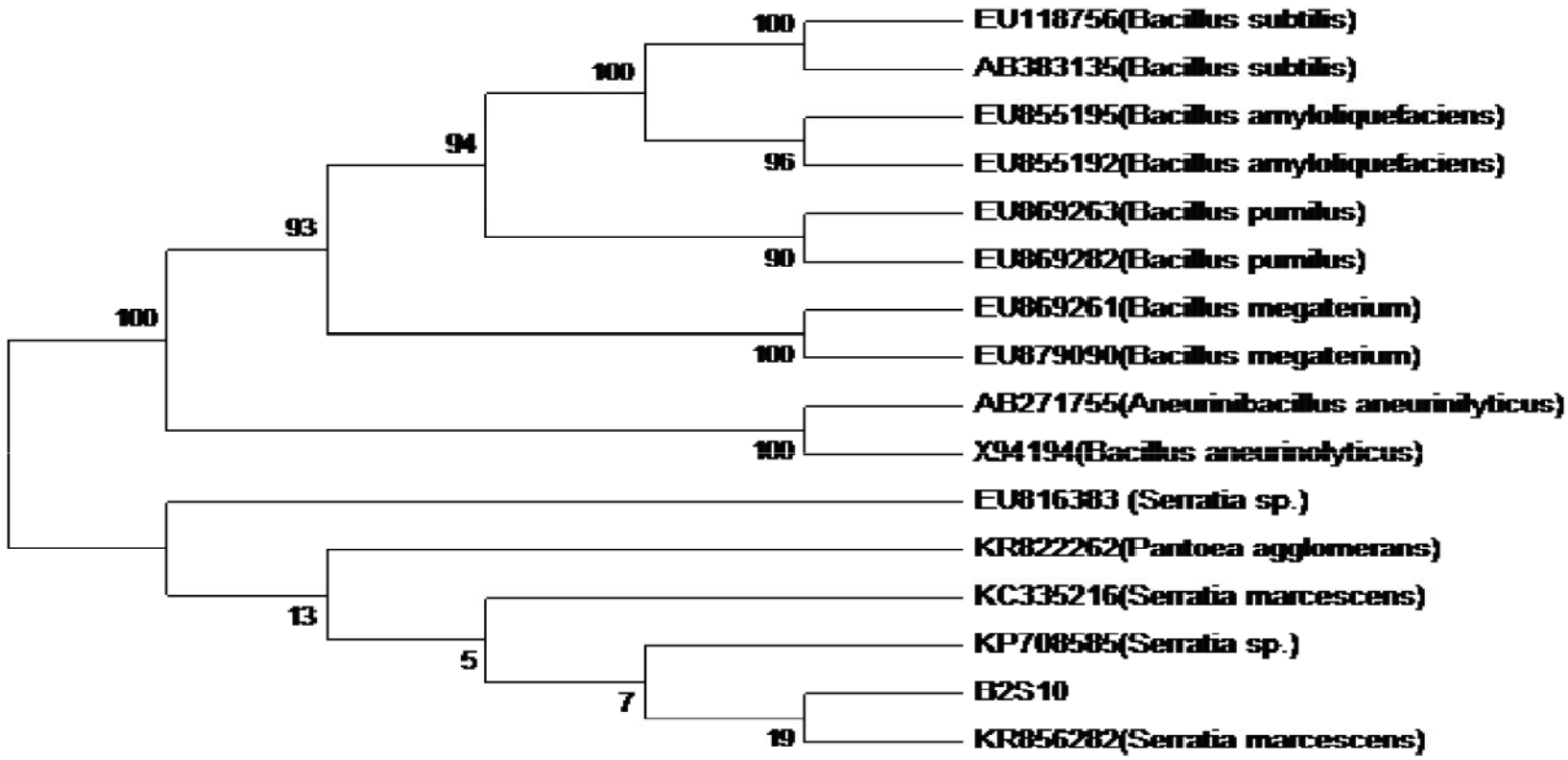


Fig.10. Neighbor joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship of bacterial isolate B_2S_{10} with the analyzed sequences

4.15 OPTIMIZATION OF CULTURAL CONDITIONS FOR THE PRODUCTION OF IAA BY SELECTED BACTERIAL ISOLATE B_2S_{10}

The results of our study suggested that simultaneous screening of IAA producers for *in vitro* phosphate solubilisation is a good tool to select effective PGPR for biofertilizer technology which was reported earlier (Chaiharn and Lumyong, 2011).

4.15.1 Effect of incubation period on IAA production by selected bacterial isolate B_2S_{10} in LB medium

The experiment was conducted in order to determine the optimum incubation period for selected bacterial isolate i.e. B_2S_{10} on IAA production in LB medium. The results revealed that an increase in IAA production was observed upto 96 h of incubation i.e. 42 µg/ml with corresponding viable count (245×10^6 CFU/ml) as well as drop in final pH 7.00 to 4.80 and thereafter a significant decrease was observed upto 120 h. The IAA production was observed highest at 96 h of incubation that further declined in growth medium during late log phase of growth (120 h) (Fig. 11). Earlier reports had also shown that IAA production starts after 48 h of incubation and maximum reached at 10 days of incubation with a decline during late stationary phase of growth (Chaiharn and Lumyong, 2011). Erturk *et al.* (2010) had also reported the IAA production upto 48-168 h of incubation.

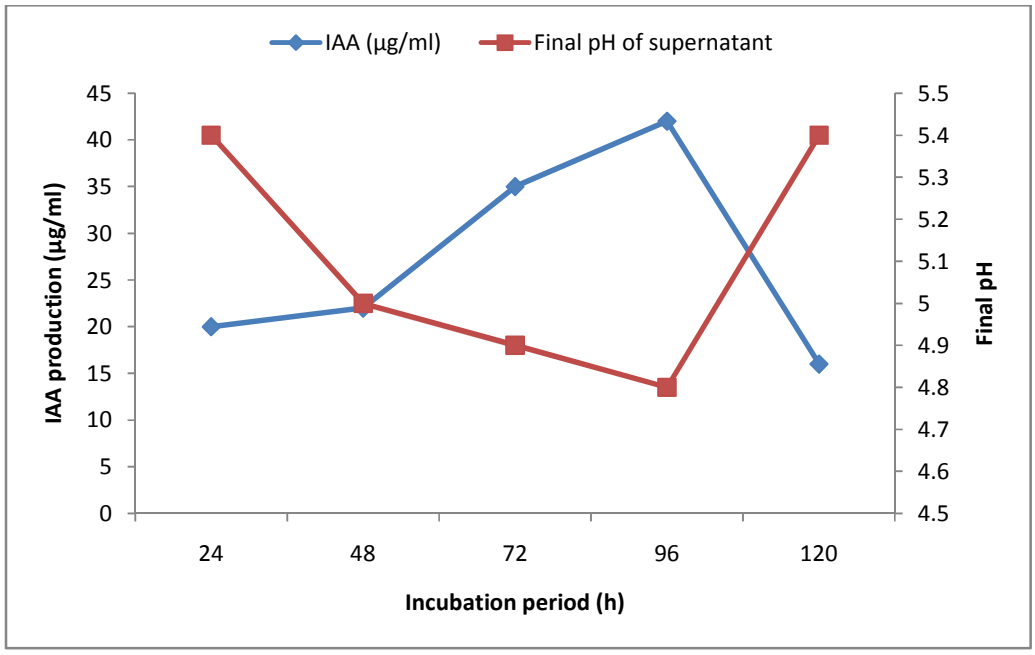


Fig. 11 Effect of incubation period on IAA production by selected bacterial isolate B_2S_{10} in LB medium at 35°C

4.15.2 Effect of temperature on IAA production by selected bacterial isolate B_2S_{10} in LB medium at 96 h of incubation

The effect of different incubation temperatures (25, 30, 35, 40 and 45°C) on IAA production is shown in Fig.12. The results revealed that maximum IAA production was observed at 35°C (51µg/ml) which corresponded to maximum viable count (134.00×10^6 CFU/ml) as well as maximum drop in final pH from 7.0 to 4.7. Increase in temperature beyond 35°C resulted in significant decrease in IAA production and viable count, with the minimum IAA production of 26µg/ml at 45°C and corresponding minimum viable count of 88×10^6 CFU/ml.

Dasgupta *et al.* (2015) also studied the effect of temperature on IAA production and his studies correspond to our results where the minimum increase in IAA is only upto 37°C of temperature. The reason for decrease in IAA production at high temperature may be due to the influence of culture conditions, growth range and availability of substrates that varied greatly among different species (Vijila, 2000).

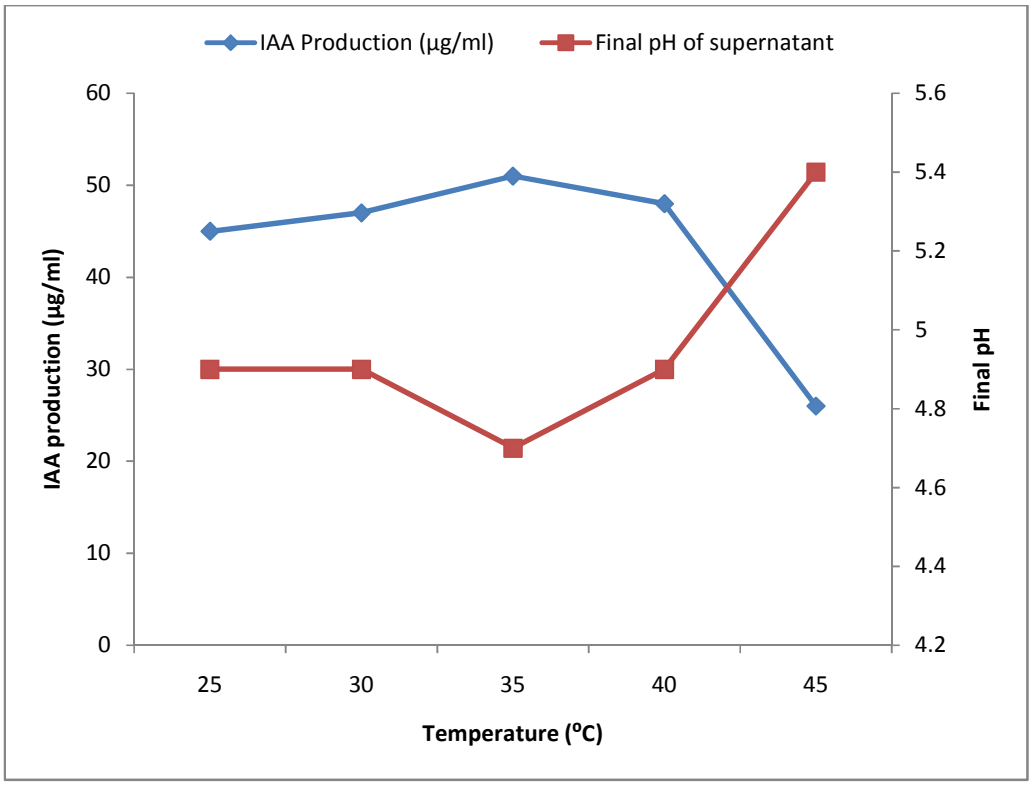


Fig. 12 Effect of temperature on IAA production by selected bacterial isolate B_2S_{10} in LB medium

4.15.3 Effect of pH on IAA production by selected bacterial isolate B_2S_{10} in LB medium at 96 h of incubation

The experiment was conducted in order to determine the effect of pH on selected bacterial isolate i.e. B_2S_{10} for IAA production in LB medium and at 96 h of incubation (Fig.13). The results revealed that maximum IAA was produced at pH 9 (65 µg/ml) with maximum viable count of 164 $\times 10^6$ CFU/ml and final pH of 4.6. However significant decrease in IAA production was observed by either increasing or decreasing the pH from 7.0. In contrast at pH 9 to 10, *Streptomyces* sp. that had shown production of maximum IAA by Harikrishan *et al*. (2014).

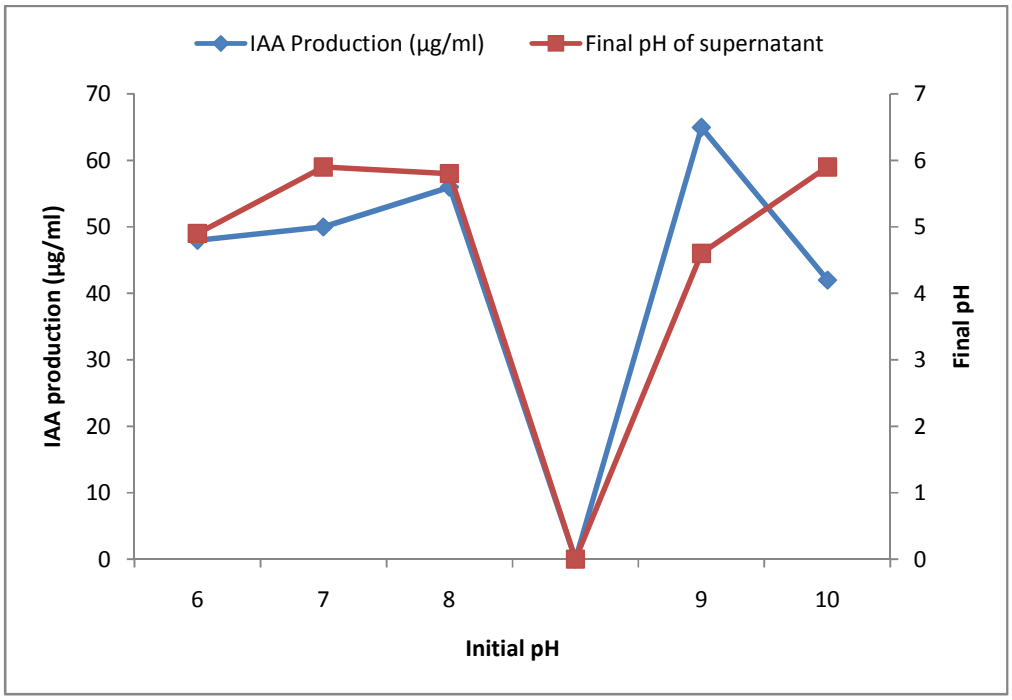


Fig. 13 Effect of pH on IAA production by selected bacterial isolate B_2S_{10} in LB medium at 96 h of incubation

4.15.4 Effect of inoculum size on IAA production by selected bacterial isolate B_2S_{10} in LB medium at 96 h of incubation

The results (Fig.14) revealed that among different concentration of inoculum size, maximum IAA production was found at 1.5% inoculum size i.e.55 µg/ml with corresponding viable count i.e. 110×10^6 CFU/ml at final pH of 4.8. Minimum IAA production was observed at 0.25% inoculum size (29 µg/ml) with viable count 55×10^6 CFU/ml and corresponding decrease in pH from 7.0 to 5.2.

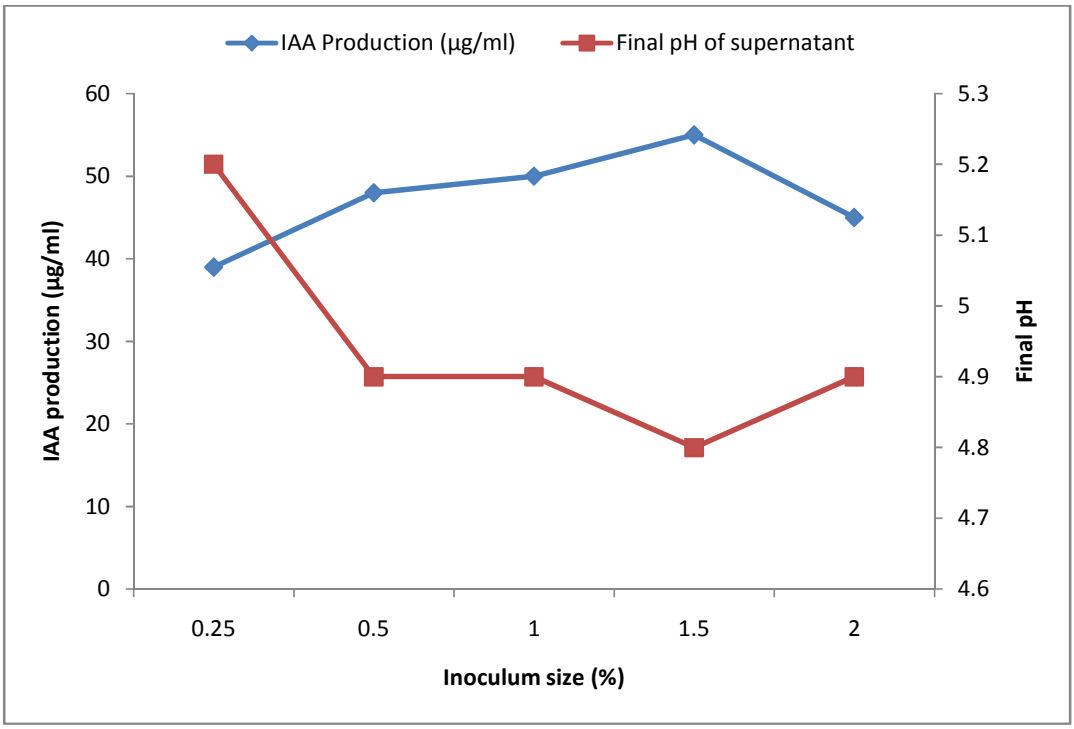


Fig. 14 Effect of inoculums size on IAA production by selected bacterial isolate in LB medium at 96 h of incubation

4.15.5 Effect of different concentration of tryptophan for IAA production and by selected bacterial isolate B_2S_{10} in LB medium at 96 h of incubation

There was a gradual decrease in the final pH with increase in the concentration of tryptophan. Maximum IAA production was observed at 1.00% of tryptophan (65 µg/ml) along with maximum viable count 114×10^6 CFU/ml and final pH was 4.6 (Fig..15). Minimum IAA production was observed at 0.1% of tryptophan (29 µg/ml) with corresponding viable count 32 $\times10^6$ CFU/ml and final pH 5.0. Similar to our results most of the isolates in the literature had shown higher IAA production in the presence of precursor, L-tryptophan (Chaiharn and Lumyong, 2011).

This showed that the strains are dependent on the L-trytophan precursor probably synthesized Tryptophan pathways under natural conditions, plant roots excrete organic compounds including L-tryptophan which could be utilized by rhizobacteria for IAA biosynthesis (Ahemad *et al*. 2005 and Flaishman *et al*. 1996).

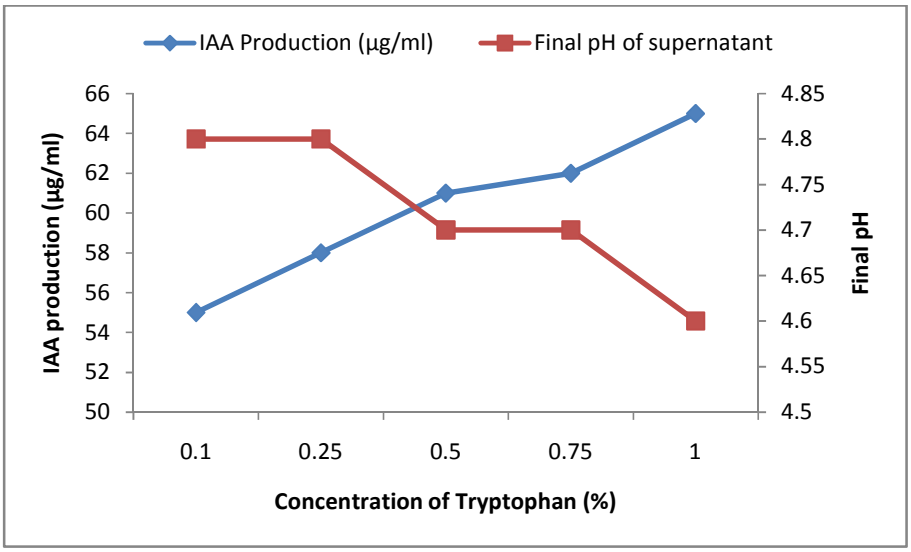


Fig. 15 Effect of different cocentration of tryptophan on IAA production by selected bacterial isolate B_2S_{10} in medium at 96 h of incubation

4.15.6 Effect of different components of LB medium on IAA production by selected bacterial isolate B_2S_{10} at 96 h of incubation

The experiment was conducted in order to determine the effect of different components of LB media on selected bacterial isolate i.e. B_2S_{10} for IAA production at 96h of incubation. The results (Fig.16) revealed that maximum IAA was produced in the absence of NaCl (51µg/ml) with maximum viable count of 57 $\times 10^6$ CFU/ml and final pH of 4.8. Minimum IAA production was observed in the absence of tryptophan (6µg/ml) with a viable count of 38 $\times 10^6$ CFU/ml and final pH of 5.6

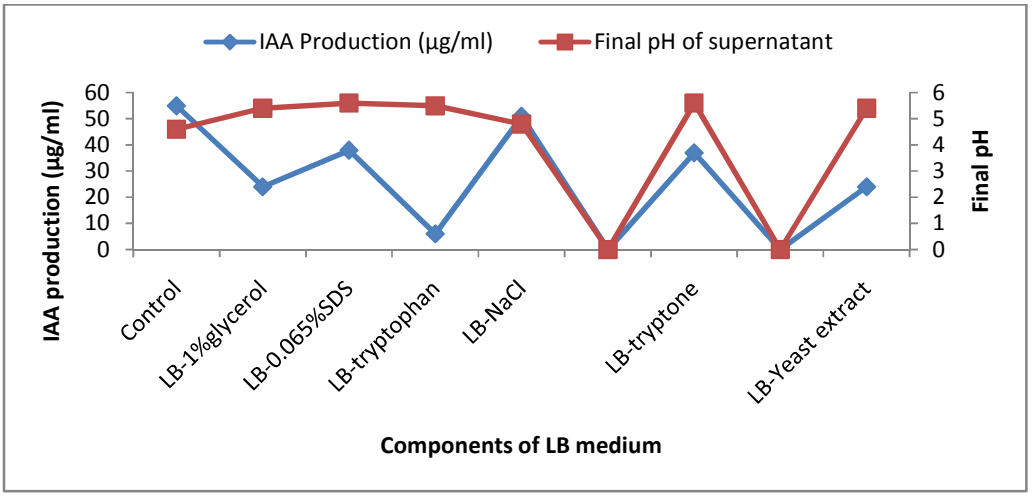
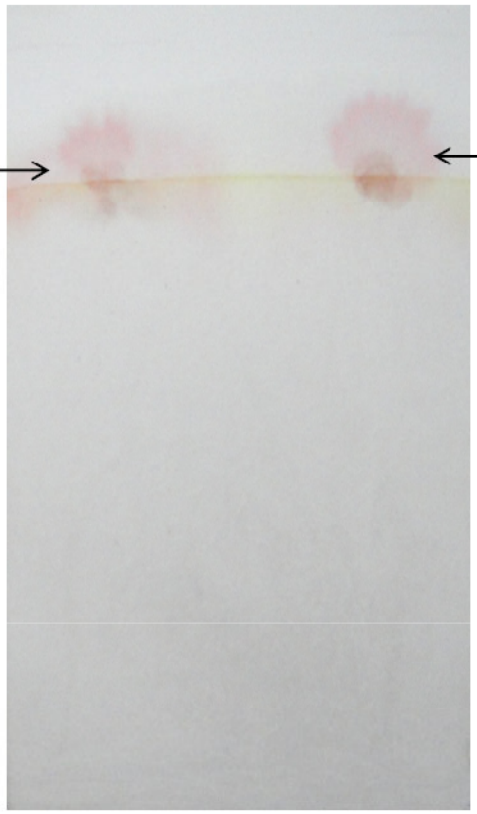


Fig. 16 Effect of different components of LB medium on production of IAA by selected bacterial isolate B_2S_{10} at 96 h of incubation

Similar results were also shown by a study conducted by Gosh *et al*. (2015). The IAA producing ability of the isolated symbiont was tested in tryptophan supplemented by YEM medium. It was observed that both growth and IAA production of the strain N37 started simultaneously and reached the stationary phase after 20 h at 30 ± 2°C. Level of IAA production in the medium was declined during the late stationary phase of growth. Although an increase in the concentration of L-tryptophan was found to enhance growth and IAA production by this N37 strain, it appeared that when concentration of L-tryptophan exceeded 2mg/mL in the medium it inhibited both the growth and IAA production.

4.16 EVALUATION AND ASSESSMENT OF EXTRACTED IAA BY THIN LAYER CHROMATOGRAPHY

Production of IAA by most efficient bacterial isolate B_2S_{10} was further confirmed by extraction of crude IAA and subsequent thin layer chromatography (TLC) analysis. TLC of ethyl acetate extracts from isolate B_2S_{10} culture that showed a clear pink spot at the R_f 0.71 corresponding to standard reference of R_f 0.7 (Plate 7). A specific spot from the extracted IAA production was found to correspond with a standard spot of IAA with similar R_f value. Similar results were documented by Chaiharn and Lumyong, (2011) in which TLC of ethyl acetate extracts from *Klebsiella* SN 1.1 cultures that showed a clear pink spot at the R_f (0.7) corresponding to standard IAA.



- Distance moved by Solvent = 15.30 cm
- Distance moved by reference IAA = 11.10 cm
- Distance moved by B_2S_{10} = 10.90 cm
- R_f value for standard IAA = 0.73
- R_f value for IAA corresponding to B_2S_{10} = 0.71

Plate 7. Separation of crude IAA extract from isolate B_2S_{10} on TLC plate

Chapter-5

SUMMARY AND CONCLUSION

The present study aimed to search and characterize plant growth promoting rhizobacteria inhabiting the rhizospheric soil and endophytic tissues associated with kiwi vines and attempts were also made to observe the effect of liquid formulations of bacterial isolates on tomato and kiwi seedlings. Effect of the inoculants on seed germination and plant growth was investigated under net house conditions.

On the basis of morphological features, one hundred and fifty seven isolates were isolated and screened for IAA production. Twenty most efficient IAA producers selected on the basis of multifarious plant growth promoting traits viz. P-solubilization, siderophore production, nitrogen fixation, antifungal and lytic enzyme activities. Screening of the rhizosphere and endophytic bacteria for their *in vitro* potential of IAA production could provide a reliable base for selection of effective PGPR.

All the twenty bacterial isolates were found to solubilize TCP in PVK agar medium. Maximum P-solubilization was observed with B_1S_4 (233.30 per cent) in solid medium and in liquid medium maximum solubilisation (380.00 µg/ml) was recorded for isolate B_2S_{10}. From total 20 most efficient IAA producers, six rhizobacterial isolates possessed maximum IAA production (P-solubilization) and other plant growth promoting traits. All the twenty bacterial isolates were found to solubilize TCP in PVK agar medium. Maximum P-solubilization was observed with B_1S_4 (233.30 per cent) in solid medium and in liquid medium maximum solubilisation (380.00 µg/ml) was recorded for isolate B_2S_{10}.

The *in vitro* activities of all the twenty isolates against phytopathogenic fungi i.e. *Phytophthora capsici* and *Fusarium oxysporum.* Selected bacterial isolates exhibited variation in antifungal activity against both the fungal pathogens where twelve (60.00%) showed inhibition against both the fungal pathogens; four (20.00%) isolates showed inhibition against only one of the two fungal pathogens; only one (5.00%) isolate showed contact inhibition with one of the fungal pathogens while three isolates (15.00%) showed no inhibition at all.

Finally six plant growth promoting rhizobacteria were evaluated under net house conditions for plant growth promotion of tomato and kiwi seedlings. All the isolates showed

a variation in plant growth parameters for both tomato and kiwi seedlingsin comparison to contol. However isolate B_2S_{10} and H_3R_3 showed maximum increase in plant growth promoting taits under *in vitro* conditions.

Growth promoting potential among six isolates showed maximum germination per cent (96.67) per cent in seeds treated with isolate B_2S_{10} over 86.66 per cent in case of untreated tomato seeds. Plant growth response was variable and dependent on bacterial isolates. Maximum per cent increase in root length (44.98) was observed in case of seedlings raised from tomato seeds treated with isolate B_2S_{10} which was significantly higher as compared to control and other isolates. Isolate B_2S_{10} showed maximum per cent increase in shoot length (30.54) which was found to be significantly higher.

In case of kiwi seedlings maximum per cent increase in root length (30.12) leaf surface area (7.9) shoot length (38.82) stem width (13.63) was observed in case of isolate B_2S_{10} and all these growth parameters were significantly higher as compared to uninoculated control. On the basis of results obtained under *in vitro* conditions and net house conditions, B_2S_{10} exhibited higher level of plant growth promoting traits especially for IAA. Therfore, it was selected for optimization and identification based on 16S rRNA gene sequencing.

Optimization of the cultural conditions for IAA production and phosphate solubilizationby the most efficient bacterial isolate B_2S_{10} was done. Maximum IAA production was observed in LB broth (55µg/ml) after 96 hours of incubation, pH 9 at 35^{o}C temperature amended with 1.5 (%) inoculum size. The most efficient isolate B_2S_{10} was further characterized by 16S rRNA gene sequencing. The isolate had 99% homology with *Serratia marescenes*, therefore identified as *Serratia marcescenes*.

In conclusion, we report for the first time a high diversity of plant growth promoting traits–possessing IAA producing bacteria in rhizospheric soil and root endosphere of kiwi vines, which to a large extent remain hidden and unexplored and represent excellent reservoirs PGPR. The present study elucidates the multifarious role of bacterial isolate *Serratia* sp. with plant growth promoting potential. Thus, the use of plant growth promoting rhizobacterial strain *Serratia marcescenes* with multiple PGP activities as biofertilizers for sustainable tomato as well as growth promotion of kiwi upto nursery stage. Further investigation including efficiency test on kiwi cuttings and seedlings under field conditions should be clarified.

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Title of Thesis	**:**	**"Studies on diverse plant growth promoting rhizobacteria associated with rhizosphere of kiwi vines "**
Name of Student	**:**	**Malvika Sharma**
Admission Number	**:**	F-2013-19-M
Name of Major Advisor	**:**	Dr. C. K. Shirkot
Major field	**:**	Microbiology
Minor field(s)	**:**	i) Biochemistry ii) Biotechnology
Year of award of Degree	**:**	2015
Number of pages in Thesis	**:**	115+V
Number of words in Abstract	**:**	310

ABSTRACT

Plant hormones are chemical messengers that affect a plant's ability to respond to its environment. Hormones are organic compounds that are effective at very low concentration; they are usually synthesized in one part of the plant and are transported to another location. Botanists recognize five major groups of hormones: auxins, gibberellins, ethylene, cytokinins, and abscisic acid. IAA (indole-3-acetic acid) is the member of the group of phytohormones and is generally considered the most important native Auxin. It functions as an important signal molecule in the regulation of plant development including organogenesis, tropic responses, cellular responses such as cell expansion, division, differentiation, and gene regulation. Therefore, the objective of the present study was to isolate and screen IAA producing rhizobacteria from kiwi vines and to optimize cultural conditions for maximum production of IAA. One hundred and fifty seven isolates (sixty three rhizospheric and ninety four root endophytic isolates were isolated from different cultivars of kiwi vines and were screened for IAA production. These IAA producing isolates were further screened for P-solubilization on PVK media, siderophore production on CAS medium and their ability to grow on nirtrogen free medium. Overall maximum IAA producing twenty isolates were selected for screening of other PGP traits. Variation was also observed in their plant growth promoting (PGP) activities such as IAA production, P-solubilization and siderophore production. Out of twenty, majority isolates exhibited antifungal activity and showed enzyme production. Overall six most efficient bacterial isolates were finally selected and evaluated for plant growth promotion of tomato and kiwi seedlings under net house conditions. The most efficient isolate B_2S_{10} was further subjected to optimization of cultural conditions for IAA production. Maximum IAA production of (55µg/ml) was observed after 96 hours of incubation at 35°C temperature, pH 9 in LB broth amended with 1.00 (%) tryptohan. The most efficient isolate was identified as *Serratia marcescens* by 16SrRNAgene sequence analysis

Signature of the Student **Signature of Major Advisor**

Countersigned

Professor and Head
Department of Basic Sciences
Dr. YSP, UHF, Nauni, Solan (H.P.)

APPENDIX-I

1.1 Preparation of standard curve (5-50 µg/ml) for P-estimation

(i) Stock solution (50 ppm) : KH_2PO_4 [Ana(aR)]: 219.3 mg in 1000 ml distilled water in volumetric flask

(ii) Chloromolybdic acid : 15 gm of ammonium molybdate dissolved in 400 ml of warm distilled water. Add 342 ml of concentrated HCl and cool. Make up the volume to 1 lt with distilled water

(iii) Chlorostannous acid (stock) : 25 gm in 40 ml of concentration HCl.

Working solution: (1 ml stock + 65 ml distilled water)

Volume of stock	Final volume (ml)	Final concentration (ppm)	OD at 660
0	25	0	0
0.1	25	5	0.05
0.2	25	10	0.11
0.3	25	15	0.18
0.4	25	20	0.26
0.5	25	25	0.32
0.6	25	30	0.38
0.7	25	35	0.40
0.8	25	40	0.48
0.9	25	45	0.57
1.0	25	50	0.68

1.2 Preparation of standard curve (10-100 µg/ml) for IAA

Stock solution (100 ppm):

10 mg of IAA (99.0% pure) was dissolved in 50 ml distilled water and the final volume was made to 100 ml in a volumetric flask.

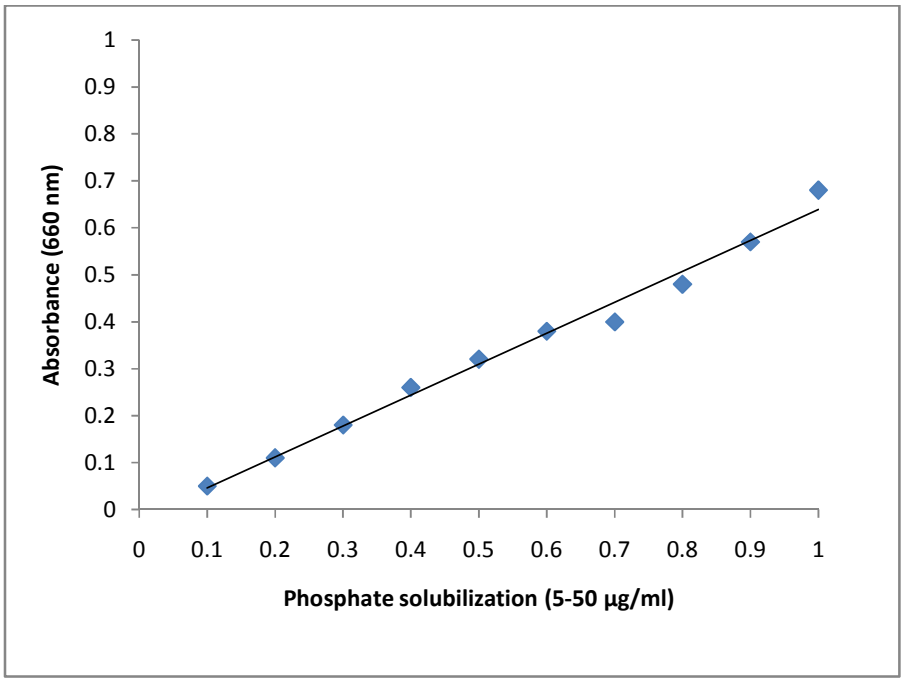
IAA (ml)	Distilled water (ml)	Final volume (ml)	Salkowsky reagent	ppm (ml)	Optical density (O.D.) at 535nm
0.3	2.7	3	2	10	0.09
0.6	2.4	3	2	20	0.19
0.9	2.1	3	2	30	0.28
1.2	1.8	3	2	40	0.36
1.5	1.5	3	2	50	0.47
1.8	1.2	3	2	60	0.53
2.1	0.9	3	2	70	0.64
2.4	0.6	3	2	80	0.68
2.7	0.3	3	2	90	0.78
3.0	0	3	2	100	0.82

1.3 Composition of CAS assay solution

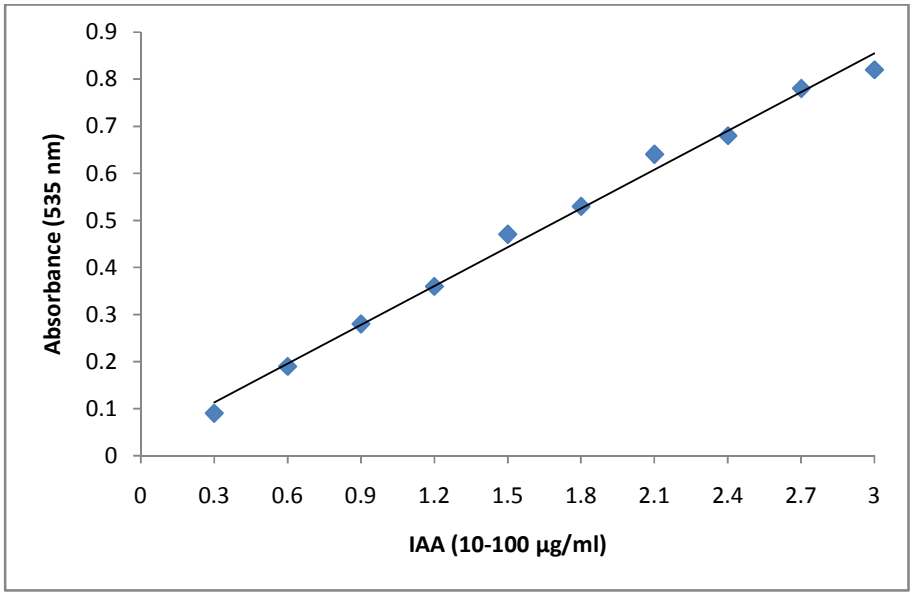
1. **2 mM CAS (stock solution):** 0.121g CAS in 100 ml distilled H_2O
2. **1 mM Fe (stock solution):** 1 mM $FeCl_3.6H_2O$ in 10 mM HCl
3. **Piperazine buffer:** Dissolved 4.307g piperazine in 30 ml distilled water. Added 6.75 ml concentration HCl to bring the pH to 5.6
4. **Hexadecyl trimethyl ammonium bromide (HDTMA):** Dissolved 0.0219 g HDTMA in 50 ml distilled water in a 100 ml mixing cylinder.

Procedure:

Mixed 1.5 ml Fe solution with 7.5 ml CAS solution and added to the HDTMA in the mixing cylinder. Added piperazine solution to the mixing cylinder and brought volume up to 100 ml with water.



Standard curve of phosphate solubilization



Standard curve of Indole-3-acetic acid

APPENDIX-II

Anova for Table 8.1 Screening of IAA producing culturable bacterial population from rhizospheric soil of kiwi vines

Source of variation	DF	MSS	
		pH	IAA production
Treatment (T)	62	0.29	483.08
Error	126	1.00×10^{-2}	0.45
Total	189		

Anova for Table 8.2 Screening of IAA producing culturable bacterial population from root endophytes of kiwi vines

Source of variation	DF	MSS	
		pH	IAA production
Treatment (T)	90	0.20	448.60
Error	182	9.92×10^{-2}	0.99
Total	273		

Anova for Table 9 Qualitative and quantitative estimation of tri calcium phosphate solubilization bacterial isolates

Sources of variation	DF	MSS		
		P-solubilization in solid medium		P-solubilization in liquid medium (µg/ml)
		Zone of P-solubilized (mm)	P-solubilization index	
Treatment (t)	19	5.49×10^{-2}	1.99	1.9×10^{-4}
Error	40	1.00×10^{-2}	2.07×10^{-2}	8.64
Total	59			

Annova for Table 10 Qualtitative and quantitative estimation of siderophore production by selected bacterial isolates

Sources of variation	DF	MSS		
		pH	Qualitative estimation	Quantitative estimation
Treatment (T)	19	7.94×10^{-2}	8.54×10^{-2}	1.40×10^{-4}
Error	40	2.04×10^{-4}	1.03×10^{-1}	6.44×10^{-1}
Total	59			

Anova for Table 11 screening of culturable bacterial isolates for antifungal activity against different fungal pathogens

Sources of variation	DF	MSS			
		Phytophthora capsici		*Fusarium oxysporum*	
		Zone of inhibition (mm)	Per cent growth inhibition	Zone of inhibition (mm)	Per cent growth inhibition
Treatment (T)	19	38.61	2418.5	64.46	2573.0
Error	40	6.12×10^{-1}	3.65×10^{-1}	3.29×10^{-1}	1.11×10^{-2}
Total	59				

Anova for Table 12 Screening of bacterial isolates for extracellular enzymatic activity

Source of variation	DF	MSS					
		Protease		Amylase		Lipase	
		Zone size (mm)	EAI	Zone size (mm)	EAI	Zone size (mm)	EAI
Treatment (T)	19	80.99	5.03×10^{-1}	36.08	4.14×10^{-1}	66.90	5.78×10^{-1}
Error	40	6.58×10^{-4}	1.13×10^{-1}	7.74×10^{-1}	9.99×10^{-2}	8.01×10^{-1}	1.90×10^{-1}
Total	59						

Anova for Table 14 Effect of bacterial isolates from rhizosphere of kiwi vines on growth promotion of tomato seedlings

Source of variation	DF	MSS					
		Germination %	Root length (cm)	Shoot length (cm)	Root dry weight (mg)	Shoot dry weight (mg)	Vigour index
Treatment (T)	6	33.68	1.33	237.43	247.43	2.53×10^{-5}	3.37×10^{-6}
Error	14	1.01	7.67×10^{-1}	1.34	1.79	1.54	1.38
Total	20						

Anova for Table 15 Effect of bacterial isolates from rhizosphere of kiwi vines on growth promotion of tomato seedlings

Source of variation	DF	MSS					
		Root length (cm)	Shoot length (cm)	Root dry weight	Shoot dry weight	Leaf surface area	Stem width
Treatment (T)	**5**	2.6	710.02	0.13	2.08	46.47	1.70
Error	**12**	0.12	0.50	0.00	0.00	0.00	1.00
Total	**17**						

BRIEF RESUME OF THE STUDENT

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(Malvika Sharma)