CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA AND EVALUATION OF THEIR BIOCONTROL POTENTIAL AGAINST TOMATO BACTERIAL CANKER

Thesis

by

SWATI GAUTAM

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

MICROBIOLOGY

COLLEGE OF FORESTRY
Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni
Solan - 173230 (H.P.), INDIA
2014

Dr C.K. Shirkot
Professor & Head

Department of Basic Sciences
College of Forestry
Dr Y S Parmar University of Horticulture and Forestry, Nauni-173230, Solan (HP)

CERTIFICATE-I

This is to certify that the thesis entitled, **“Characterization of plant growth promoting rhizobacteria and evaluation of their biocontrol potential against tomato bacterial canker”** submitted in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE** in **MICROBIOLOGY (BASIC SCIENCES)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) is a record of bonafide research work carried out by **Ms SWATI GAUTAM (F-2012-24-M)** 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 investigations has been fully acknowledged.

Place: Nauni-Solan
Dated:

Dr C.K. Shirkot
Major Advisor

CERTIFICATE-II

This is to certify that the thesis entitled, **"Characterization of plant growth promoting rhizobacteria and evaluation of their biocontrol potential against tomato bacterial canker"** submitted by **Ms SWATI GAUTAM (F-2012-24-M)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE MICROBIOLOGY (BASIC SCIENCES)** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

Major Advisor
Dr C.K. Shirkot
Professor and Head

External Examiner

Members of Advisory Committee

Dr P.K. Mahajan
Professor

Dr I.M. Sharma
Professor

Dr Rajesh Kaushal
Associate Professor

Dr (Mrs) Neerja Rana
Assistant Professor

Dean's Nominee

Professor and Head
Dept of Basic Sciences, COF, Nauni

Dean
College of Forestry
YSP UHF, Nauni-Solan (HP)

CERTIFICATE-III

This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"Characterization of plant growth promoting rhizobacteria and evaluation of their biocontrol potential against tomato bacterial canker"** submitted to Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP) by **Ms SWATI GAUTAM (F-2012-24-M)** in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE MICROBIOLOGY (BASIC SCIENCES).**

Major Advisor

____

Dr C.K. Shirkot
Professor and Head

____

Dr C. K. Shirkot
Professor and Head
Department of Basic Sciences
Dr Y S Parmar, UHF, Nauni-173230, Solan (HP)

ACKNOWLEDGEMENTS

Every effort is motivated by an ambition an all ambitions are backed up by the strength given by ***God****, a constant source of positive energy. With deep sense of pleasure, I embellish my sojourn by expressing my gratitude to the almighty for bestowing upon me his choices blessings, who blessed me with all favourable circumstances to go through this work.*

With great reverence, I humbly express my gratitude to my guide ***Dr C.K. Shirkot****, Professor and Head, Department of Basic Science. I have no words to express my heartfelt thanks to him for his illuminating guidance, unfailing encouragement, scholarly suggestions, constructive criticism and keen interest during the course of this investigation and preparation of this manuscript.*

I emphatically owe my heartiest thanks to ***Dr P.K. Mahajan****,* ***Dr. I.M. Sharma, Dr. Rajesh Kaushal, Dr (Mrs.) Neerja Rana*** *worthy members of my advisory committee for their valuable suggestions and full cooperation throughout the course of investigation. Profound thanks are due to my faculty members* ***Dr (Mrs.) Nivedita Sharma & Dr (Mrs.) Mohinder Kaur.***

Thanks will be a small word for all support, guidance, kind concern and timely help render to me by ***Mrs. Anjali Chauhan*** *(Assistant Professor). Without her expertise and proficiency, this work won't have been shaped well.*

No words can express my deep regards and eternal gratitude to ***my parents*** *for their prudent persuasion, selfless sacrifices and heartfelt blessings. This piece of work is the culmination of the wishes and prayers for me by my* ***siblings****.*

*I have been fortunate in getting the kind cooperation and valuable help from my seniors (****Abhishek sir, Shiwani mam, Rashmi mam, Shalini mam****) and* ***friends Rutika, Ranjana, Parul, Meenakshi and Girish*** *and last but not the least from my all juniors.*

With personal touch of fervently, I place on record my special thanks to ***Usha di, Richa di, Rani di, Raveena di and Gullu*** *for their continuous encouragement and moral support.*

The cooperation and assistance extended by Daya Ram ji, Satish ji, Joshi ji and office staff members of Department of Basic Sciences is thankfully acknowledged.

I am pleased to pay my ponderous thanks to Edge computers Nauni for their cooperation in helping me to complete this manuscript.

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

Nauni- Solan
Date: **(Swati Gautam)**

CONTENTS

Chapter	Title	Page(s)
1.	INTRODUCTION	1-4
2.	REVIEW OF LITERATURE	5-37
3.	MATERIALS AND METHODS	38-57
4.	EXPERIMENTAL RESULTS	58-88
5.	DISCUSSION	89-102
6.	SUMMARY AND CONCLUSION	103-105
7.	REFERENCES	106-124
	ABSTRACT	125
	APPENDICES	I-II

LIST OF TABLES

Table	*Title*	*Page(s)*
1.	Site characterization and screening of rhizobacterial isolates for antagonism against *Clavibacter michiganensis*	60
2.	Characterization of antagonistic bacterial isolates from different horticultural crops possessing plant growth promoting traits (P solubilization, siderophore production and growth on N free medium)	61
3.	Screening of bacterial antagonists from strawberry rhizosphere for multifarious PGP Traits	65
4.	Screening of bacterial antagonists from apple rhizosphere for multifarious PGP traits	68
5.	Screening of bacterial antagonists from apricot against for multifarious PGP traits	71
6.	Morphological characterization of ten selected antagonistic isolates	75
7.	Metabolic fingerprinting of ten selected antagonistic PGPRs based on biochemical tests	76
8.	Summary of the RAPD amplified products obtained from selected ten antagonistic bacterial isolates using four primers	78
9.	Screening of antagonistic bacterial isolates for quantitative estimation of tricalcium phosphate solubilization at 72 h of incubation	80
10.	Indole-3-acetic acid production by bacterial antagonists in Luria Bertani broth with tryptophan at 72h of incubation	81
11.	Siderophore production by bacterial antagonists on CAS medium after 72h of incubation	82
12.	Biocontrol efficacy of PGPR strains in controlling bacterial canker caused by *Clavibacter michiganensis*	83
13.	Effect of liquid formulation of selected bacterial isolates on tomato seedlings and their growth promotion under net house conditions	85
14.	Intrinsic antibiotic resistance of efficient antagonist S1 towards different antibiotics	87
15.	Nucleotide base composition in the 16S rRNA gene sequence of S1	88

LIST OF PLATES

Plates	*Title*	**Between pages**
1.	Screening of rhizobacterial isolates for antagonism against *Clavibacter michiganensis* from different horticultural crop rhizosphere	59-60
2.	Screening of antagonistic bacterial antagonists from different horticultural crops for multifarious plant growth promoting traits and lytic enzymes production	67-68
3.	Evaluation of antibacterial metabolite from cell free supernatant by selected antagonistic bacterial isolates against *Clavibacter michiganensis*	71-72
4.	Genomic DNA of ten selected antagonistic bacterial isolates	77-78
5a.	RAPD profile of antagonist bacterial isolates (1-10) using primers RBa-1, RBa-2	77-78
5b.	RAPD profile of antagonist bacterial isolates (1-10) using primers RBa-3 and RBa-5	
6.	Biocontrol activity of antagonistic bacterial isolates against *Clavibacter michiganensis* inoculated to tomato plants under net house conditions	85-86
7.	Intrinsic antibiotic resistance (IAR) Pattern of most efficient antagonist S1	87-88
8.	Molecular identification of selected antagonistic bacterial isolate S1 based on 16S rRNA amplification	88-89

LIST OF FIGURES

Figure	*Title*	*Between page(s)*
1.	Percentages of rhizobacteria from rhizospheres of strawberry, apple and apricot having PGP traits of P-solubilization, siderophore production and IAA production	69-70
2.	Percentages of bacterial antagonists from different fruit crops viz. Strawberry, apple, apricot for lytic enzymes production	69-70
3.	Antibacterial activity of selected bacterial antagonists in cell free supernatant against *Clavibacter michiganensis*	71-72
4.	Dendrogram showing relationship among bacterial antagonists based on similarity coefficient derived from their multifarious plant growth promoting traits	74
5.	Cluster analysis of antagonistic bacterial isolates against *C. michiganensis* based on similarity coefficient derived from their metabolic fingerprinting	77
6.	Dendrogram showing the genetic relationship amongst selected 10 antagonistic isolates based on RAPD analysis	79
7.	Per cent increase in plant growth parameters under net house conditions	85-86
8.	Effect of incubation time (h) on antagonistic activity of bacterial antagonist S1 against *Clavibacter michiganensis*	87-88
9.	Effect of temperature (^{o}C) on antagonistic activity of bacterial antagonist S1 against *Clavibacter michiganensis*	
10.	Effect of inoculum size (%) on antagonistic activity of bacterial antagonist S1 against *Clavibacter michiganensis*	
11.	Effect of pH on antagonistic activity of bacterial antagonist S1 against *Clavibacter michiganensis*	
12.	Neighbour-joining tree based on 16S rDNA gene sequence showing the phylogenetic relationship of isolate S1 with the analysed sequence (Gen Bank accession no. KM658175) isolate	88-89

ABBREVIATIONS

%	:	Per cent
µg	:	Micro gram
ºC	:	Degree centigrade
CAS	:	Chromazarol S
cfu	:	Colony forming units
cm	:	Centimeter
CRD	:	Completely randomized design
Cmm	:	*Clavibacter michiganensis* ssp. *michiganensis*
Fig	:	Figure
g	:	Gram
h	:	Hour
HCl	:	Hydrochloric acid
HCN	:	Hydrogen cyanide
HDTMA	:	Hexadecyltrimethylammoniumbromide
l	:	Litre
M	:	Molar
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
ppm	:	parts per million
PSB	:	Phosphate solubilizing bacteria
psi	:	Per square inch
PVK	:	Pikovskaya's medium
rpm	:	Rotation per minute
RS	:	Rhizosphere soil
sp.	:	Species
ssp.	:	Sub species
TCP	:	Tri-calcium phosphate
UV	:	Ultra violet
V	:	Volt
w/v	:	Weight by volume
v/v	:	volume by volume

Chapter-1

INTRODUCTION

The rhizosphere is the narrow zone of soil specifically influenced by the root system and is hot spot of microbial abundance and the activity is due to presence of root exudates and rhizodeposits (Samalla *et al.*, 2006). This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (Gray and Smith, 2005) and the bacteria colonizing this habitat are called rhizobacteria. This situation is reflected by the number of bacteria that are found around the roots of plants, generally 10 to 100 times higher than that in the bulk soil. Infact, the root system, which was traditionally thought to provide anchorage and uptake of nutrients and water, is in fact a chemical factory that mediates numerous underground interactions (Badri *et al.,* 2009; Walker *et al.,* 2003). These plant-associated bacteria can be classified into beneficial, deleterious and neutral groups (Dobbelaere *et al.*, 2003). Beneficial free-living, soil bacteria are usually referred to as plant growth-promoting rhizobacteria, however, deleterious group include pathogenic microorganisms, while neutral group have neither good nor adverse effect on plants.

Beneficial bacteria are reffered to as PGPR, constitutes only 1-2% of the total population (Antoun and Kloepper, 2001) and affect plant growth in two different ways, direct and indirect. The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium, for example phytohormones, or facilitating the uptake of certain nutrients from the environment (Bhattacharya and Jha, 2012). The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. This can happen by producing antagonistic substances or by inducing resistance against pathogens (Sivasakthi *et al.*, 2014). A particular PGPR may affect plant growth and development by using any one, or more, of these mechanisms. PGPR, as biocontrol agents, can act

through various mechanisms, regardless of their role in direct growth promotion, such as by known production of auxin phytohormone, decrease of plant ethylene levels (Glick *et al.*, 2007) or nitrogen fixing associations with roots.

PGPR and their interactions with plants are exploited commercially (Podile and Kishore, 2006) and hold great promise for sustainable agriculture. Applications of these associations have been investigated in maize, wheat, oat, barley, peas, canola, soy, potatoes, tomatoes, lentils, radicchio and cucumber. A variety of soil microorganisms have demonstrated activity in the control of various soilborne and seedborne plant pathogens. Bacteria that reduce the incidence or severity of plant diseases are often reffered to as biocontrol agents whereas those that exhibit antagonistic activity towards a pathogen are defined as antagonists (Beattie, 2006). The antagonistic bacteria might be useful in formulating new inoculants, offering an attractive alternative of environmentally friendly biological control of plant diseases and improving the cropping systems into which it can be most profitably applied (Beneduzi *et al.*, 2012).

Tomato (*Lycopersicon esculentum Mill*) is one of the most widely grown vegetables in the world. Cultivated tomato is a diploid, self-pollinating perennial herb and its popularity among consumers has made it an important source of vitamins A and C in diets. The tomato production throughout the world in an area of 4,803,680 hectares was 161,793,834 tones. During 2012, Indian production was 17,500,000 tones over an area of 87,000 hactares, making India second largest producer of tomato in the world after China (FAO STAT, 2012). There are nearly 200 known tomato diseases of diverse causes and etiologies throughout the world. Among them, bacterial canker of tomato is an economically significant disease.

Bacterial canker is the most contagious and destructive disease of tomato caused by *Clavibacter michiganensis* ssp. *michiganensis* (Cmm) can drastically reduce tomato yield and quality, thus causing substantial economic losses both in greenhouses and in open-field production. The pathogen is seed borne, persists in plant debris in soil and on contaminated greenhouse structures and infected seeds (Agrawal *et al.,* 2012). It infects host plants via roots or wounds and invade the

xylem vessels, followed by a systemic infection of the host which results in leaf lesions, wilting, fruit lesions and ultimately yield loss of marketable fruits (Girish & Umesha, 2005).

Disease control is often difficult due to the unavailability of commercial cultivars with complete resistance to the disease, therefore, progress towards breeding genetic resistant tomato lines has only been modest (Lanteigne *et al.,* 2012). Antibiotics and copper compounds, which prevent bacterial multiplication and further infection, are used as chemical control but their use is banned or severely limited because they leads to the selection of resistant bacterial population and with limited efficacy when conditions favour canker development, respectively (Soylu *et al*., 2003). Further, inorganic chemical fertilizers are immobilized rapidly and become unavailable to plants. Their use not only causes soil erosion but also lowers the crop yield and in turn disturbs the environment, subvert ecology, degrade soil productivity, mismanage water resources. The decreasing efficacy of the chemical pesticides as well as risks associated with their residues on the leaves and fruit, have highlighted the need for a more effective and safer alternative control measures (Haggag, 2010; Deshwal and Kumar, 2013).

Moreover, 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 which has resulted not only in the deterioration of soil health but also has led to some major environmental problems, besides increasing the input cost for crop production. Therefore, there is an urgent need to recycle available organics and manipulation of rhizospheric microflora in a more efficient way to improve and expand their usage. Different combinations of chemical treatments, plant activators and biological control are being used for the control of canker in tomato, but none of them approaches have so far proven to be successful for reliable and constant control (Slusarski, 2008). Search for ecologically adaptable Plant Growth Promoting Rhizobacteria with enhanced plant growth promotory properties and their use to enhance crop productivity and potential biocontrol agents could be

beneficial which in-turn can improve the socio-economic status of poor farmers (Das *et al.,* 2013).

Effective functioning of introduced PGPR as bioinoculants is possible only by exploring the large pool of indigenous soil microbes (Hill, 2000). Biological control through the use of beneficial microorganisms or by the combination of multiple antagonists colonizing on the rhizosphere, surface and inner tissues of healthy plants has emerged as a promising alternative to chemical pesticides as a more rational and safer crop management over disease control. PGPR exhibit several mechanisms of biological disease control, most of which involve competition and production of metabolites like antibiotics, cell wall degrading enzymes, siderophores, and HCN and inducing the systemic resistance (Induced Systemic Resistance).

Therefore, in the present study, our objective was to evaluate the potential of beneficial microbes as a bioinoculant to control bacterial canker of tomato *in-vitro* and on young tomato seedlings inoculated with the pathogen under net house conditions.

Chapter-2

REVIEW OF LITERATURE

Plant diseases have become a permanent threat since human societies started to rely on agriculture as on a major food provider. Back in history, outbreaks of plant diseases resulted in human catastrophes. For example, the Great Potato Famine killed hundreds of thousands of Irish people and forced the emigration to the USA in 1845-1846. Similarly, an epidemic of brown spot rice was the cause of a devastating famine in India in 1943 (Bent, 2002). Even nowadays the crop loss due to phytopathogens is still a serious economical problem in agriculture. It is estimated to cause a 15-20 % reduction of the crop yield worldwide.

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations. However, increasing use of chemical inputs causes several negative effects, i.e., development of pathogen resistance to the applied agents and their non target environmental impacts. Furthermore, the growing cost of pesticides, particularly in less-affluent regions of the world, and consumer demand for pesticide-free food has led to a search for substitutes for these products. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent. Biological control is, thus, being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Compant *et al.,* 2005).

There are nearly 200 known tomato diseases of diverse causes and etiologies and among them, bacterial diseases pose a very big challenge in the production of tomatoes worldwide. Bacterial pathogens are important not only because they reduce crop yield, but also due to certain compounds they produce

during proliferation on/in plants. These compounds, called bacterial toxins, are highly poisonous and can adversely affect human and animal health (Pitt, 2000). Bacterial canker caused by *Clavibacter michiganensis* ssp. *michiganensis,* occurs in all parts of the country and usually limits production of tomatoes. Bacterial canker, the most contagious and destructive disease of tomato can drastically reduce tomato yield and quality, thus causing substantial economic losses both in greenhouses and in open-field production (Menzies and Jarvis, 1994). The pathogen is seed borne and disease is spread from plant to plant by cultural practices such as transplanting and pruning. Pathogen persists in plant debris in soil and on contaminated greenhouse structures and infected seeds. It infects host plants via roots or wounds and invade the xylem vessels, followed by a systemic infection of the host.

For instance, it causes severe yield loss under serious attack. Also it reduces the market value of crops because of the leaf lesions and wilting, lesions, spots and cankers it forms on the fruit (Girish & Umesha, 2005). This reduction in market value leads to monitory losses. Also the cost of managing the disease is quite expensive leading to more monitory losses. The disease has no cure and is very persistent in the soil for about three years. This calls for crop rotation for about three years which may not be economically feasible due to land constraint problem.

The disease was first described by Erwin F. Smith on tomato growing in greenhouses at Grand Rapids, Michigan and called it Grand Rapids disease but later named it bacterial canker. After 1926 it was reported from New York, New Jersey to California and in several southern states. It caused losses in Canada, most European countries, Israel, Morocco, Kenya, South Africa, Australia and New Zealand. It is a serious disease throughout the world in favourable conditions. At 31-83% systemic infection of seedlings, up to 46 per cent loss in yield may occur (Agrawal *et al.,* 2012).

The gram positive actinomycete bacteria enter the plant through natural openings and wounds colonies on xylem vessels and lead to typical disease symptoms: light brown discoloration of the vascular tissues, wilting of leaves,

sometimes one-sided asymmetric and necrotic lesions on leaves, canker lesions on stems and petioles. If plant infection occurs from seed or seedlings a systemic disease infection will develop and the plant will die, otherwise, if infection occurs at late stage of plant development plants will be able to survive and generate fruits that can have bird's eye spots. (Calis *et al.,* 2012).

The bacterial canker account among the most important phytosanitary tomato problems. It is a very contagious and destructive disease in tomato crop both under controlled and field conditions. The bacterial canker can cause significant damage which may go up to the destruction of 100% crop (Boudyach *et al.,* 2001). This disease is very difficult to control. This is because it's hard to detect because it has varying symptoms. No chemicals can effectively control the disease. Some methods of managing the disease include; the use of certified seeds and clean planting materials. This prevents the introduction of the disease to places where it was not in existence.

Soil sterilization on green houses and fields is also another method of controlling the disease. Sterilization ensures that the soil or planting media is free of innoculum either from previous season or other sources and hence helps ensure healthy plants.

So far, many investigations have been carried out to find adequate methods for the control of *Clavibacter michiganensis*, but none has been found to be completely effective. Preventive cultural management recommendations include the use of certified seeds and healthy transplants, greenhouse disinfection, plant debris removal or plowdown and rotation with non-solanaceous plants for at least two years (Marcic *et al.,* 2012)

Applications of fixed copper plus either maneb or mancozeb and copper chelating compounds such as 8-hydroxy-quinoline may reduce populations of *Clavibacter michiganensis* before symptoms appear, but they generally have little impact on disease control. Spraying copper based fungicides to lower disease levels, suppresses the bacteria activity and keeps it below economic injury level (Seebold *et al.,* 2003). The chemical treatments recommended for this disease

only reduced the population of the pathogen in the surface of the infected plants. Although the control of this bacterial disease continues to be difficult, prevention was the first defense line. Given the inefficiency of chemical treatments and their impact on health and the environment, research and development of alternative methods are recommended and biocontrol is the most promising way to control the disease (Talibi *et al.,* 2011).

Some bacteria are associated with the roots of crop plants, have beneficial effects on their host, and are referred to as plant growth-promoting rhizobacteria (PGPR). PGPR are free living bacteria that may have beneficial effects on plants, viz. seedling emergence, colonizing roots, stimulating overall plant growth, mineral nutrition, and water utilization, as well as disease suppression. The manipulation of the crop rhizosphere with PGPR for the biocontrol of plant pathogens has shown considerable promise. Similarly, the presence of rhizobia in the rhizosphere may also protect host roots from damage caused by pathogens (Akhtar *et al.,* 2012).

PGPR are highly diverse and in this review we focus on rhizobacteria as biocontrol agents. Their effects can occur via local antagonism to soil-borne pathogens or by induction of systemic resistance against pathogens throughout the entire plant. Several substances produced by antagonistic rhizobacteria have been related to pathogen control and indirect promotion of growth in many plants, such as siderophores and antibiotics. Induced systemic resistance (ISR) in plants resembles pathogen-induced systemic acquired resistance (SAR) under conditions where the inducing bacteria and the challenging pathogen remain spatially separated. Both types of induced resistance render uninfected plant parts more resistant to pathogens in several plant species. Rhizobacteria induce resistance through the salicylic acid-dependent SAR pathway, or require jasmonic acid and ethylene perception from the plant for ISR. Rhizobacteria belonging to the genera *Pseudomonas* and *Bacillus* are well known for their antagonistic effects and their ability to trigger ISR. Resistance-inducing and antagonistic rhizobacteria might be useful in formulating new inoculants with combinations of different mechanisms of action, leading to a more efficient use for biocontrol strategies to improve cropping systems (Beneduzi *et al.,* 2012).

2.1 ROLE OF PGPR:

2.1.1 PGPR as biocontrol agents

2.1.2 PGPR as growth promoters

2.1.1 PGPR as biocontrol agents

Biological control refers to the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens (Ouda, 2014). This may involve the use of microbial inoculants to suppress a single type or class of plant diseases. Or, this may involve managing soils to promote the combined activities of native soil- and plant-associated organisms that contribute to general suppression. Most narrowly, biological control refers to the suppression of a single pathogen (or pest), by a single antagonist, in a single cropping system (Pal and Gardner, 2006).

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).

Kerr in 1972 discovered and developed the first biocontrol system by isolating non-pathogenic strains of *Agrobacterium radiobacter*, from disease sites, and testing their ability to compete with pathogenic strains in mixed inoculations. He found several non-pathogenic strains helped to reduced infection, but one strain in particular, *A. radiobacter* strain designated as K84 completely prevented disease when added to wound sites at a 1:1 ratio with cells of *Agrobacterium tumefaciens*. This strain is the one that is successfully used against pathogenic strains of *Agrobacterium* on different hosts. It is used until now and marketed globally by several companies under a range of trade names (Tolba and Soliman, 2012).

2.1.1.1 Mechanisms of biocontrol

PGPR provide different mechanisms for suppressing plant pathogens. These include competition for nutrients and space (Bakthavatchalu *et al.,* 2012), antibiosis by producing antibiotics viz., pyrrolnitrin, pyocyanine, 2, 4-diacetyl phloroglucinol (Haas and Keel, 2003) and production of siderophores (fluorescent yellow pigment) viz., pseudobactin which limits availability of iron necessary for growth of pathogens (Krewulak and Vogel, 2008; Osorio *et al.,* 2008). 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 (Zahir *et al.,* 2004; Glick *et al.,* 2007), HCN production and degradation of toxin produced by pathogen (Noori and Saud, 2013).

2.1.1.1.1 PGPR as siderophore producers

Iron is the most important micronutrient used by microorganisms and is essential for their metabolism, being required as a cofactor for a large number of enzymes and iron-containing proteins (Dave *et al.,* 2006). Under iron-limiting conditions, microorganisms produce a range of iron chelating compounds or siderophores which have a very high affinity for ferric ions. These bacterial iron chelators are thought to sequester the limited supply of iron available in the rhizosphere making it unavailable to pathogenic fungi, thereby restricting their growth (Bholay *et al.,* 2012). Some PGPR strains go one step further and draw iron from heterologous siderophores produced by cohabiting micro-organisms.

Neilands and Leong (1986) concluded that there are at least three ways by which siderophores could affect plant life. Firstly, the chelaters act in the soil to solubilize and transport Fe (III), a very important mineral in plant nutrition. A second possible effect of siderophores may be via the facilitation of plant disease and the third mechanism is a type of biocontrol in which certain microbial species, such as fluorescent Pseudomonads discourage the growth or metabolic activities of competing microorganisms.

Rachid and Ahmed (2005) studied the biosynthesis of siderophores in four basal media supplemented with different concentrations of iron. They

observed that ferric ions increased the growth yield and completely repressed siderophores production above 200 g/l, but had a positive effect below 160 g/l and also found that penicillin and lead elicited the production of siderophores in the presence of excess iron.

Sarode *et al.* (2009) obtained thirty-two bacterial isolates from wheat rhizosphere and subsequently tested for *in-vitro* siderophore production. Wheat isolate SCW1, being a strong siderophore producer was selected, identified and confirmed as *Acinetobacter calcoaceticus*. The strain produced catechol type of siderophores during exponential phase which was influenced by iron content of medium. Seed bacterization with siderophoregenic *A. calcoaceticus* improved plant growth in pot and field studies.

Ramos Solano *et al.* (2010) evaluated plant growth promoting rhizobacteria (PGPR) isolated from the rhizosphere of wild populations of *Nicotiana glauca* Graham in south-eastern Spain for siderophore and chitinase activity. They observed that ninty 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 up to 50%.

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 Chromo azurol sulphate assay were further characterized and found to belong to the genus *Streptomyces*.

Tan *et al.* (2013) isolated two tomato root colonizing strains, *Bacillus amyloliquefaciens* CM-2 and T-5, after the enrichment procedure on the roots of tomato seedlings and evaluated for their antagonistic activities against pathogenic *Ralstonia solanacearum* (RS) *in vitro*. Both strains were recovered from the interior of the stems and roots of plants. They showed positive reactions for ammonia, indole acetic acid and siderophores production and phosphate

solubilizing activity. The data proved the potential of isolated strains for biocontrol of tomato bacterial wilt.

Ahemad and Kibret (2014) assessed the role of the siderophore-producing Pseudomonas strain GRP3 on iron nutrition of *Vigna radiate*. After 45 days, the plants showed a decline in chlorotic symptoms and iron, chlorophyll a and chlorophyll b content increased in strain GRP3 inoculated plants compared to control.

2.1.1.1.2 PGPR as HCN producers

A secondary metabolite produced commonly by rhizosphere microorganisms is hydrogen cyanide (HCN), a gas known to negatively affect root metabolism and root growth (Martinez-Viveros *et al.,* 2010). Cyanide production is one of the possible ways by which rhizobacteria may suppress plant growth in soil. Although cyanide acts as a general metabolic inhibitor, it is synthesized, excreted and metabolized by hundreds of organisms, including bacteria, algae, fungi, plants, and insects, as a mean to avoid predation or competition. It affects sensitive organisms by inhibiting the synthesis of ATP mediated cytochrome oxidase and is a potential and environmentally compatible mechanism for biological control of weeds.

Nielson *et al.* (2002) reported HCN production by *Pseudomonas fluorescens* strains inhibit the fungal growth of *Pythium ultimum* and *Rhizoctonia solani* in sugar beet rhizosphere.

Ramette *et al.* (2003) isolated, purified indigenous *Pseudomonas* sp. and evaluated their ability in HCN synthesis. The effects of these strains on stem length, root length and stem length/root length rate in rye, wild barley and wheat were evaluated in 3 different *in vitro* tests examining the effects of gas and liquid metabolites produced by the bacteria. The results showed that the gas metabolites reduced more than 90% of root and shoot growth in weeds and draw conclusion that cyanogenic *Pseudomonas fluorescens* had the potential of biological weed control.

Wani *et al.* (2007) tested the rhizospheric isolates for HCN production *in vitro* and found that most of the isolates produced HCN and helped in the plant growth. The isolates from the rhizospheric soil of chickpea also exhibited more than two or three PGPR traits including HCN production, which promotes plant growth directly or indirectly or synergistically (Joseph *et al.,* 2007).

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.

Mazhar and Hasnain (2011) tested cyanobacterial strains isolated from rice fields for different plant growth promoting traits such as phosphate solubilization, nitrogen fixation and hydrogen cyanide and auxin production. The two selected cyanobacterial strains were identified as *phormidium* SM-14 and SM-15. Both strains were able to solubilize phosphate, fix atmospheric nitrogen and produce hydrogen cyanide.

Lanteigne *et al.* (2012) investigated the antagonistic capacity of DAPG and HCN, both produced by *Pseudomonas* sp. LBUM300, on *C. michiganensis* subsp. *michiganensis* under in vitro and in planta conditions. In planta, *Pseudomonas* sp. LBUM300 was capable of significantly reducing disease development and *C. michiganensis* subsp. *michiganensis* rhizospheric population, suggesting that the production of both DAPG and HCN was involved. Therefore, simultaneous DAPG/HCN production by *Pseudomonas* sp. LBUM300 shows great potential for controlling bacterial canker of tomato.

Pseudomonas sp. is a widespread bacteria in agricultural soils and the most effective strains of *Pseudomonas* are gram negative, motile, rod shaped bacteria and have various phytobeneficial traits. Their plant growth promoting activities include production of HCN, siderophores, protease, antimicrobials, phosphate solubilizing enzymes. Noori and Saud (2013) studied, the production of HCN, siderophores, antimicrobials and phosphate solubilisation by 20 *Pseudomonas* isolated from rhizosphere soils of paddy.

Bhagat *et al.* (2014) studied the effect of sixteen native *Mesorhizbum* sp. on *Fusarium* wilt in chick pea under *in-vitro* conditions. Out of 16 Mesorhizbum sp. along with refrence Mesorhizbum sp. LGR 3 tested, 64% isolate were found positive for HCN production. Three native isolates of Mesorhizbum sp. LGR 14, LGR 15 and LGR 16 were able to produce maximum HCN and essentially a mechanism involved in biocontrol.

2.1.1.1.3 Lytic enzymes produced 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. An enzyme chitinase and chitobiase produced by some bacteria and fungi like *Mucor, Trichoderma* and *Pseudomonas* species possessed lytic effect which was related to antagonistic behavior (Benitez *et al.,* 2004).

Chitinases are particularly useful in agriculture as biocontrol agents against fungal phytopathogens because of their ability to hydrolyse the chitinous fungal cell wall (Suresh *et al.,* 2010; Wahyudi *et al.,* 2011). Different *Paenibacillus* strains are inhibitory to bacteria and or fungi, attributed to the production of antimicrobial substances and cell wall-degrading enzymes (β-1,3-glucanases, cellulases, chitinases and proteases) (Budi *et al.,* 2000).

Radjacommare *et al.* (2004) studied the association of the hydrolytic enzyme chitinase against *Rhizoctonia solani* in rhizobacteria treated rice plants.

Increased induction of the pathogenesis related chitinase isoform in *Pseudomonas* treated rice in response to *R. solani* infection indicated that the induced chitinase has a definite role in suppressing disease development.

Ramos Solano *et al.* (2010) screened 960 strains in the rhizosphere of *N. glauca* to isolate PGPRs associated to this genus. A subset of 442 isolates constituted by the most abundant parataxonomic groups were characterized based on metabolic activities regarded as putative PGPR traits (siderophores and chitinase production).

Kumar *et al.* (2012) studied the hydrolytic enzymes viz., chitinase, protease, β-1, 3 glucanase and cellulase from twenty eight *Bacillus* sp. isolated from tomato rhizospheric soil in IIVR farm (DPNSB-1 to 7), IIHR farm (DPNSB-8 to 15), IARI farm (DPNSB-16 to 20) and farm of APHU (DPNSB-21 to 28). Among these strains, IARI isolate of DPNSB-18 exhibited the highest chitinase activity (4.65 IU/ml), IIHR isolate of DPNSB-15 produce highest protease activity (0.79 IU/ml), maximum , β-1, 3 glucanase production was noted in *Bacillus* strains viz., DPNSB-14 (IIHR isolate), DPNSB-2 (IIVR isolate) and DPNSB-20 (IARI isolate), range from 0.24 IU/ml to 0.39 IU/ml, cellulase production was made by isolates of IIVR, DPNSB-3 (0.75 IU/ml) and DPNSB-1 (0.60 IU.ml) respectively.

Rodrigues *et al.* (2014) conducted a study an to evaluate the antagonistic activity of a bacterial strain *Bacillus circulans* against *Curvularia lunata*, *Alternaria alternata* and *Cladosporium sp.,* which are important seed and soil borne pathogens distributed throughout the world. It was observed that a clear hydrolytic zone was visible around the bacterial culture in the chitinase plate. This zone indicated that the bacterium was capable of utilizing the substrate chitin. After 48 hrs of incubation, the qualitative screening resulted in clear hydrolytic regions 20 mm in diameter, around the culture colony. Thus the test *bacilli* screened and selected for the production chitinase was capable of degrading the substrate chitin, which is a major structural component of fungi cell walls.

Seleim *et al.* (2014) studied the effect of certain bioagents for the control of bacterial wilt of tomato under greenhouse and field conditions and the effect of these bioagents in induction of some enzyme activity in planta e.g. Peroxidase (PO) and polyphenoloxidase (PPO). Under greenhouse conditions the effect of *Pseudomonas putida* and *P. fluorescens*, and their combination were studied, and found that both of them reduced the disease 60 and 66.67%, respectively and the combination treatment reduced the disease 53.33%. Also under field conditions *P. putida* was the best in reduction of the disease followed by the combination and then *P. fluorescens*.

2.1.1.1.4 Antibiosis

The production of one or more antibiotics is the mechanism most commonly associated with the ability of plant growth-promoting bacteria to act as antagonistic agents against phytopathogens (Glick *et al.,* 2007). The basis of antibiosis, activity of biocontrol based on secretion of molecules that kill or reduce the growth of the target pathogen, has become better understood over the past two decades (Lugtenberg and Kamilova, 2009).

Gupte and Kulkarni (2002) determined the correct levels of the three process parameters identified as the important for maximum production of antifungal antibiotic by the newly isolated strain of *Streptomyces chattanoogensis*. Several abiotic factors such as oxygen, temperature, specific carbon and nitrogen sources and micro-elements have been identified to influence antibiotic production by bacterial biocontrol agents (Raaijmakers *et al.,* 2002).

Girish and Umesha (2005) used plant growth promoting rhizobacteria in managing bacterial canker disease of tomato. The rate of reduction in the bacterial canker disease incidence was directly proportional to the amount of increased level of phenylalanine ammonia lyase (PAL) and total phenol content.

Sultana *et al.* (2004) studied the production of antifungal antibiotic activity of *B. megaterium* and observed that *B. megaterium* antagonized growth of *Dematophora necatrix,* the causative organism of the most destructive disease white root rot of apple.

Lanteigne *et al.* (2012) investigated the antagonistic capacity of PCA, produced by *Pseudomonas* sp. LBUM223, and DAPG and HCN, both produced by *Pseudomonas* sp. LBUM300, on *C. michiganensis* subsp. *michiganensis* under in vitro and in planta conditions. In planta, only *Pseudomonas* sp. LBUM300 was capable of significantly reducing disease development and *C. michiganensis* subsp. *michiganensis* rhizospheric population, suggesting that the production of both DAPG and HCN was involved. In summary, simultaneous DAPG/HCN production by *Pseudomonas* sp. LBUM300 shows great potential for controlling bacterial canker of tomato.

Zhou *et al.* (2012) isolated a bacterial strain J12, from the rhizosphere soil of tomato plants strongly inhibited the growth of phytopathogenic bacteria *Ralstonia solanacearum*. Strain J12 was identified as *Pseudomonas brassicacearum* based on its 16S rRNA gene sequence. J12 could produce 2,4-diacetylphloroglucinol (2,4-DAPG), hydrogen cyanide (HCN), siderophore(s) and protease.

Samvat *et al.* (2014) tested five strains of Pseudomonas against R. solani causing damping off disease in cotton seedlings. The results showed that five isolates of *P. fluorescent* significantly inhibited the growth of *R. solani*. The efficacy of phenazine producing a wild type strain of *P. aureofaciens* was higher than its non-phenazine producing mutant, indicating that phenazine plays an important role in the antagonistic activity of *P. aureofaciens*. The indication is, that production of this antibiotic is a major antagonistic mechanism of this bacterium.

Loganathan *et al.* (2014) observed that plant growth promoting *Bacillus subtilis* (BS2) was found effective against tomato wilt caused by *Fusarium oxysporum* f sp. *lycopersici* under field conditions. Induction of phenyl alanine ammonia lyase (PAL), peroxidase (PO) polyphenol oxidase (PPO), chitinase and phenol was observed in plants treated with PGPR upon challenged with FOL. Induction of PAL was more in PGPR treated plants challenged with FOL and the effect was greater in BS2 (30.53 nmol min-1 mg-1) followed by BA1 (29.10

nmol min-1 mg-1), pathogen inoculated control (22.00 nmol min-1 mg-1) and untreated control (20.00 nmol min-1 mg-1).

2.1.1.1.5 Induced Systemic Resistance

Plant growth-promoting rhizobacteria (PGPR) are among the various groups of plant-associated microorganisms that can elicit plant defenses (van Loon and Glick, 2004). In concert with the terminology used by van Loon and Glick in their recent review of PGPR, we will use the term induced systemic resistance (ISR) for the process whereby treatment of plants with PGPR elicits host defense as indicated by reduction in the severity or incidence of diseases caused by pathogens that are spatially separated from the inducing agent. The event of ISR Induced systemic resistance has been demonstrated in various plants inoculated with different species of rhizobacteria. The ISR occurs when plants previously exposed to biotic and abiotic agents are induced to defense against pathogens, which are spatially separated from the inducer agent (Rathore, 2014).

Induced resistance is a state of enhanced defensive capacity developed by a plant reacting to specific biotic or chemical stimuli. In 1991, the research groups of B. Schippers in Baar and J. W. Kloepper in Auburn, AL, discovered independently that induced systemic resistance (ISR) is a mode of action of plant growth-promoting rhizobacteria (PGPR), in suppressing diseases. The involvement of ISR is typically studied by Bakker *et al.,* (2007) in systems in which the *Pseudomonas* bacteria and the pathogen are inoculated and remain spatially separated on the plant, e.g., the bacteria on the root and the pathogen on the leaf, or by use of split root systems. Since no direct interactions are possible between the two populations, suppression of disease development has to be plant-mediated.

Kloepper *et al.* (2004) studied that *Bacillus* spp. elicit ISR and promote plant growth. First, specific strains of spore-forming *Bacillus* spp. can elicit ISR that results in reduction in disease severity by a broad range of pathogens. ISR is also well documented in the literature using fluorescent pseudomonad PGPR. Second, the same strains of *Bacillus* spp. that elicit ISR typically promote plant growth. With fluorescent pseudomonads, ISR is not as closely associated with

growth promotion. Perhaps this difference indicates some fundamental differences in plant response to *Bacillus* PGPR in comparison to pseudomonad PGPR, but no specific studies comparing multiple aspects of plant response to the two groups of PGPR have been reported.

Masunaka *et al.* (2009) studied the biocontrol agent *Pythium oligandrum* (PO) that can suppress bacterial wilt caused by *Ralstonia solanacearum* (RS) in tomato. The tomato plants were pretreated with sterile water or preinoculated with PO followed by inoculation with RS. In plants that were preinoculated with PO, the movement of RS was suppressed, bacteria appeared to be restricted to the pit of vessels and a reaction similar to that observed in resistant rootstocks. PO was not observed near wound sites or root tips where RS tended to colonize. However, RS colonization was significantly repressed at these sites in PO preinoculated plants. These observations suggest that the induction of plant defense reactions is the main mechanism for the control of tomato bacterial wilt by PO, not direct competition for infection sites.

The plant growth promoting *Pseudomonas* strains, which induced resistance systematically in watermelon to gummy stem rot, are investigated on their induced systemic resistance (ISR) - related characteristics. Their work supports the concept that PGPR can protect plants against the pathogens by inducing defense mechanisms by iron-binding siderophore, HCN and other associates. The plant growth promoting rhizobacteria induced systemic protection against Tomato late blight. Under *in vitro* conditions *P. fluorescens* (ENPF1) and *P. chlororaphis* isolate (BCA) promotes plant growth and induce systemic resistance against stem blight pathogen *Corynespora cassiicola* in *P. amarus* (Saharan and Nehra, 2011).

Investigation was carried out by Mishra *et al.* (2014) to evaluate potent rhizobacterial isolates against ToLCV (Tomato Leaf Curl Virus). The investigations were carried out with potent rhizobacterial isolates alone and, along with elicitor molecules such as chitosan to determine its ability to control ToLCV in tomato. Application of chitosan or the bacterial inoculant alone was not effective to control the severity of ToLCV disease. However, the plants

inoculated with the chitosan based formulation of Pseudomonas sp.(206(4) +B-15+ JK-16) recorded the highest activity of ISR molecules and recorded maximum plant height, total biomass, chlorophyll content, fruit number and yield over the diseased control. The quantification of viral load was done by semi quantitative PCR analysis which revealed the lowest viral load in plants inoculated with both chitosan and Pseudomonas sp. 206(4) + B-15+ JK-16. Hence, suggesting the involvement of ISR in biocontrol mechanism.

2.1.1.2 Biocontrol by PGPR

Bacterial canker disease of tomato has spread into different regions all over the world, causing considerable losses up to 70% of the yield mainly in out-door tomato crop production (Boudyach *et al.,* 2001). Bacterial canker is one of the most difficult tomato diseases to control once it has established in vascular tissues of the crop for long periods and becomes seed-born, control measures used are not sufficient enough any more to eliminate the disease.

Boudyach *et al.* (2001) isolated 178 bacterial strains, antagonistic towards *Clavibacter michiganensis subsp. michiganensis*. The strains were characterized on the basis of the Gram stain, sporulation, fluorescence on King's B medium and physiological tests. All of the strains inhibited C. m. subsp. *michiganensis* on nutrient-broth yeast extract agar (NBYA), with zones of inhibition ranging from 2-30 mm. Twenty-four strains were selected for their ability to colonize tomato roots. Less than a third (7/24) completely colonized the roots of all the seedlings. Only six strains colonized the main and lateral roots of 50% of the seedlings, and eleven colonized < 50% of the seedlings. Eighteen strains were screened in greenhouse pot experiments for control of bacterial canker. Only three of the 18 strains reduced infection when applied as a seed treatment compared to the untreated control. The majority of the strains reduced the infection totally when applied as a seed treatment followed by a root treatment before transplanting compared to the control. This combined treatment was significantly more efficient than the seed treatment alone.

Bashan and Bashan (2002) observed that seed inoculation with *Azospirrilum brasilense* combined with a single streptomycin foliar treatment and

two foliar bactericide applications at 5-day intervals reduced disease severity of bacterial speck caused by *Pseudomonas syringe* pv. tomato in tomato seedlings by over 90% after 4 weeks, and significantly slowed disease development under mist conditions. *A. brasilense* increased the level of salicylic acid in inoculated plants. Treatment of tomato seeds that were artificially inoculated with *P. syringae* pv. tomato*,* with a combination of mild chemothermal treatment, *A. brasilense* seed inoculation, and later, a single foliar application of a copper bactericide, nearly eliminated bacterial leaf speck even when the plants were grown under mist for 6 weeks.

Utkhede and Koch (2004) conducted experiments to determine the effects of treatments on *Clavibacter michiganensis* ssp. *michiganensis in vitro* conditions and on young seedlings inoculated with the pathogen under greenhouse conditions. Treatments with *B. subtilis* and *Trichoderma harzianum*, lysozyme, vermicomposte, *Rhodosporidium diobovatum*, *B. subtilis* applied as a spray at 0.3 g/l, 0.6 g/l, 10 g/l, concentrated, 1×10^9 cfu/ml, and 0.5 g/l, respectively, have the ability to prevent the incidence of bacterial canker of tomato plants caused by *Clavibacter michiganensis* ssp. *michiganensis* under greenhouse conditions.

Girish and Umesha (2005) studied the plant growth promoting rhizobacteria in managing bacterial canker of tomato. Tomato seeds were treated with PGPR strains viz. *Bacillus pumilus* INR7, *Bacillus pumilus* SE34, *Bacillus pumilus* T4, *Bacillus subtilis* GBO3, *Bacillus amyloliquefaciens* IN937a and *Brevibacillus brevis* IPC11 were subjected for seed germination and seedling vigor. Among the PGPR strains tested, only three strains (IN937a, GBO3 and IPC11) which showed enhancement in the seed quality parameters like seed germination and seedling vigour, were further subjected for estimation of one of the defence-related enzymes, Phenylalanine Ammonia Lyase (PAL) with total phenol contents. The same three strains were recorded for maximum disease protection under greenhouse conditions. The rate of reduction in the bacterial canker disease incidence was directly proportional to the amount of increased level of PAL and total phenol content. The results suggested the possible use of PGPR strains in effective management of bacterial canker of tomato.

El-Hendawy *et al.* (2005) studied the effect of two antagonistic strains of *Rahnella aquatilis* on bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* strain 2 in tomato. Tomato seedlings were pretreated, with either strain of *Rahnella aquatilis* before the pathogen, with either through leaves, roots, soil or seeds. In all experiments, seedlings pretreated with *R. aquatilis* showed reduced susceptibility toward *X. c.* pv. *vesicatoria*.

Shanmugam *et al.* (2011) developed a talc-based formulations of plant growth promoting rhizobacterial strain S2BC-2 (*Bacillus atrophaeus*) and strain mixture, S2BC-2 þ TEPF-Sungal (*Burkholderia cepacia*), inhibitory to the growth of *Fusarium oxysporum* f. sp. *gladioli* (FOG) in gladiolus. The results showed that in comparison to the individual strain, the strain mixture recorded maximum spike and corm production of 100 and 150%, respectively with less vascular wilt and corm rot incidences of 73.6 and 54.8% reduction over the pathogen control in greenhouse when inoculated with FOG. In field experiments, the strain mixture recorded less vascular wilt and corm rot incidences of 48.6 and 46.1% mean reduction over the non-bacterised control, and was almost comparable with that of fungicide (51.5 and 47.1%, respectively).

Biological control of chestnut blight was investigated by using 3 hypovirulent isolates of *Cryphonectria parasitica*, *Trichoderma* sp., *Penicillium* sp. and *Bacillus* sp. isolates. Antagonistic microorganisms yielded varying percent inhibition (PI) values in 3 time periods and the highest rate of inhibition (68 %) was obtained from the *Trichoderma* sp. isolate. A *Penicillium* sp. isolate and two *Bacillus* sp. isolates also provided 30 %, 40 % and 31 % disease inhibition, respectively, fifty eight days after the inoculation. Effectiveness of the hypovirulent isolates varied depending on the virulent isolates and the hypovirulent isolate Z - 1 provided 59 % inhibition against the most virulent isolate, while it gave 32 % inhibition against the less aggressive isolate. The other hypovirulent isolate Ba-6 also inhibited the canker development of the virulent isolate by 42 % (Akilli, 2012).

Tolba and Soliman (2012) analysed the *in vitro* antagonistic activity of seventy native bacterial isolates towards plant tumorigenic *Agrobacterium*

tumefaciens causing crown gall in different plants, resulted in a selection of eight potential biocontrol agents. *C. flaccumfaciens* reduced the incidence of crown gall up to 100% in the case of rose shoots and kalanchoe leaves whereas the same antagonist reduced galling of squash fruits to 75%. Likewise, *P. asplenii, P. viridilivd* and *P. polymyxa* reduced the incidence of crown gall up to 100% in the case of kalanchoe leaves and squash fruits, whereas they reduced galling of rose shoots to 66.7%, 55.6% and 44.5% respectively. In the same manner, the two isolates of *P. fragi* reduced galling up to 100% in squash fruits, while it was 88.9% in rose shoots and kalanchoe leaves. Interestingly, *B. megaterium* isolate completely suppressed the gall development in rose shoots, whereas the gall incidence was 100% in kalanchoe leaves and 25% in squash fruits. Bacterial isolates characterized in this study may be considered as potential sources of novel bioactive metabolites as well as promising candidates to develop new biocontrol agents for controlling crown gall disease.

Dairo *et al.* (2012) evaluated two commercially formulated plant growth promoting rhizobacteria (PGPR): equity and trichoshied at two concentration each (0.15%, 0.3%) and (0.5%, 1%) respectively for the control of bacterial wilt disease of tomato caused by *Ralstonia solanacearum,* a soil borne pathogen using two varieties of tomato (Ibadan local and UC82B). Results showed that 0.3% equity and 1% trichoshield recorded the lowest incidence of 68.0%, 44.0%, 54.0% and 48% on Ibadan local and UC82B, respectively. Plant growth was also enhanced by 1% trichoshield recording the highest shoot, root and leaf weight of 5.28 g, 3.52 g and 2.38 g, respectively.

Ramyasmruthi *et al.* (2012) isolated 18 bacterial isolates from the rhizosphere of brinjal, capsicum, chilli and screened them for the production of various PGPR activities. 10 isolates were the most potent chitinolytic bacterial species. These isolates were also found to produce siderophore, IAA, HCN, phosphate solubilisation, NH_3 and catalase. Dual plate assay against few selected soil borne phytopathogens- *Alternaria alternata* OTA36; *Alternaria brassicola* OCA1; *Alternaria brassiceae* OCA3; *Collectotrichum gleosporidose* OGC1 revealed anti-fungal activity by isolate R. The isolate R was identified as

Pseudomonas fluorescens by biochemical test. Chilli seeds inoculated with *Pseudomonas* showed 100% germination index and almost 50% reduction in disease incidence by *C. gloeosporiodes* OGC1.

Shehata *et al.* (2012) conducted a pot and field experiment to evaluate the effect of *Pseudomonas fluorescens* and *Bacillus subtilis*. The pot experiment evaluated the probable suppressive effect of rhizobacteria as bioagents against *Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii* under artifically infested soil. Results showed that co-inoculation of soybean with rhizobacteria led to a significant decrease in pre and post emergence damping-off caused by all pathogens besides enhancing the nodulation. Field experiments carried out in Etay-El Baroaud evaluated the promotive and suppressive disease effects of rhizobacteria on nodulation, plant growth and yield of soybean.

Chakravarty and Kalita (2012) applied the antagonistic strain of *P. fluorescens* as suspension in pot experiment by different methods viz. seed, root, soil, and their integration methods seed+root, root+soil, seed+soil and seed+ root+soil against bacterial wilt of brinjal. The control treatments were inoculated control (only pathogen inoculated) and uninoculated control (neither pathogen nor antagonist inoculated). The percent wilt incidence (PWI) was found to be lowest (33.33%) in root+soil and seed+root+soil treatment of the antagonist. The population dynamics of the pathogen and antagonist in brinjal rhizosphere soil showed that the crop receiving seed+root+soil treatment had the lowest population recovery of the pathogen 26×106 cfu/g (7.33) and correspondingly highest population recovery of the antagonist 179.67×106 cfu/g (8.25). The yield, yield attributes and physiological and biochemical parameters were also found to be best performing in the seed+root+soil treatment of the antagonist suspension indicating its potential as PGPR.

Sundaramoorthy and Balabaskar (2012) tested the strains of *Bacillus subtilis* (EPCO16 and EPC5) and *Pseudomonas fluorescens* (Pf, Py15 and Fp7) individually and in combination for their effectiveness against early blight of tomato incited by *A. solani* under *in vitro* and pot culture conditions. The results revealed that the strains of *Bacillus subtilis* and *Pseudomonas fluorescens* were

compatible. Under *in vitro* conditions the combined application of EPCO16+Pf1 was found to effectively inhibit the mycelial growth of the pathogen and promote the growth of tomato seedlings when compared to application of individual strains of the antagonists. Further, a significant reduction in early blight incidence of tomato under greenhouse conditions was observed due to the combined application of EPCO16+Pf1.

Bakthavatchalu *et al.* (2012) isolated 51 *Pseudomonas* isolates from the rhizospheric soil samples of different crops and were screened initially on the basis of their antagonistic activity, and fifteen fluorescence *Pseudomonas* spp. designated FP1-FP15 were selected for further study. These isolates were then tested *in vitro* for specific PGPR traits. Of the 15 isolates, FP6 was found to be promising for all PGPR attributes. FP6 isolate showed significant antagonistic activity against *Alternaria alternata, A. brassicicola, A. brassicae* and *Collectotrichum gleosporioides*. It produced maximum zone inhibition against *A. alternata* (94.6%) and *P. capsici* (83.1%). Based on the morphological, cultural and biochemical characteristics, the FP6 isolate was identified as *Pseudomonas aerugonisa*. Two secondary metabolites 2,4- Diacetylphloroglucinol (DAPG) and phenazine were identified through thin layer chromatography. Inoculation of cowpea seeds with the FP6 isolate significantly enhanced seed germination, seedling vigor index, plant height, fresh weight and dry weight in comparison with the control. Results of this study provide comprehensive informationon the biocontrol mechanism of the isolate FP6 that can be used as an effective biocontrol agent.

Sen *et al. (*2013) screened tomatoes or wild relatives of tomato for resistance to *Clavibacter michiganensis* ssp. *michiganensis*, to be used for starting breeding programs. They screened 24 different wild accessions of tomato and found several new tolerant sources: *Solanum pimpinellifolium* GI.1554*, S. parviflorum* LA735 and *S. parviflorum* LA2072 and also confirmed the tolerance which was reported previously in *S. peruvianum* LA2157, *S. peruvianum* PI127829, *S. peruvianum* LA385*, S. habrochaites* LA407 and *S. lycopersicum* cv. IRAT L3. Also accessions showing a low disease score still

contained high titers of bacteria as determined by a dilution plating method, using tow selective media.

Tan *et al.* (2013) isolated two tomato root colonizing strains, *Bacillus amyloliquefacien* CM-2 and T-5 and evaluated for their antagonistic activities against pathogenic *Ralstonia solanacearum* (RS) in vitro. Both CM-2 and T-5 strains showed strong biocontrol and growth promotion effects on tomato seedlings. The best biocontrol efficacy was obtained by treating both seedlings and soils with the biocontrol agents. In comparison to the control, the disease incidence was reduced by 70.1 and 79.4% for CM-2 and T-5, respectively. The numbers of colony-forming units of RS in rhizosphere soil were significantly (P < 0.05) decreased as compared to the control. The density of both strains in the rhizosphere soils remained at a high level (_107 CFU/g) during a five-week period. Both strains were recovered from the interior of the stems and roots of plants. They showed positive reactions for ammoina, indole acetic acid and siderophores production, and phosphate solubilizing activity.

Maji and Chakrabartty (2014) isolated and identified five antagonistic PGPRs i.e. *Pseudomonas aeruginosa* T1, *Pseudomonas sp.BH25, Pseudomonas* sp.AM12, *Pseudomonas* sp.AM13 and *Pseudomonas putida*R6 and were assessed for their biocontrol potential against bacterial wilt infected field. Among the strains *Pseudomonas* sp. BH25 was found to be promising to combat the pathogenic effect of *R. solanacearum* Tom5 in bioassays. *Ralstonia solanacearum* Tom5 caused only 40% seedling emergence as compared to 76% in the control, while combination of the antagonist BH25 with the pathogen Tom5 (Tom5:BH25 at 1:10) improved the percentage of the seedling emergence and the value (75%) was almost similar to that of the control. Combination of BH25 with the pathogen also improved the fresh weight, dry weight and vigour index of the seedlings as compared to those in the pathogen treated ones and their values were almost similar to those of the control. Vigour index of the seedlings was reduced from 935 in the control to 237 in the Tom5 treated ones and the value was restored to 878 by combining a 10-fold high concentration of BH25 with the pathogen Tom5 during inoculation.

Achari and Ramesh (2014) isolated 167 bacteria were from the xylem of healthy eggplant, chilli, and *Solanum torvum* Sw. by vacuum infiltration and maceration. These were tested for antagonistic activity against bacterial wilt of eggplant caused by *Ralstonia solanacearum.* Twenty-eight strains inhibited growth of *R. solanacearum* and produced volatile and diffusible antagonistic compounds and plant growth promoting substances *in vitro*. Antagonistic strains XB86, XB169, XB177, and XB200 recorded a biocontrol efficacy greater than 85% against bacterial wilt and exhibited 12 %–22 % increase in shoot length in eggplant in the greenhouse screening.

Shrestha *et al.* (2014) observed the efficacy of lactic acid bacteria against bacterial spot pathogen (Xanthomonas campestris pv. vesicatoria) and their plant growth-promoting activities in pepper (Capsicum annuum L. var. annuum), under greenhouse and field conditions. LABs significantly ($P < 0.05$) reduced bacterial spot on pepper plants in comparison to untreated plants in both the greenhouse and the field experiments. The plant growth-promoting effect of LABs on pepper varied; some strains had a significant effect on growth promotion ($P < 0.05$) compared with untreated plants, while some showed no significant effect in the greenhouse and field experiments. Additionally, LABs were able to colonise roots, produce indole-3-acetic acid (IAA), siderophores and solubilise phosphate. These findings indicate that application of LABs could provide a promising alternative for the management of bacterial spot disease in pepper plants and could therefore be used as a healthy plant growth-promoting agent.

2.1.2 PGPR AS GROWTH PROMOTERS

In recent years, plant scientists have become interested in the study of PGPR due to their potential for improving plant growth and yield. There are several ways in which different PGPR may directly facilitate the proliferation of their plant hosts. They may: (1) provide mechanisms for solubilization of minerals such as phosphorus; (2) fix atmospheric nitrogen, and supply it to the plants; (3) synthesize various phytohormones, including auxins and cytokinins (Pradhan and Shukla, 2005 and Chen *et al.,* 2006).

2.1.2.1 Phosphate solubilization by PGPR

Phosphorus (P) is one of the major essential macronutrients for biological growth and development (Ehrlich, 1990). The concentration of soluble P in soil is usually very low, normally at levels of 1 ppm or less than 1 ppm due to chemical fixation. Phosphate solubilizing microorganisms include different types of microorganisms that convert insoluble phosphatic compounds into soluble forms. Important genera of phosphate solubilizing bacteria are *Bacillus* and *Pseudomonas* (Richardson, 2009). It has been reported that certain strains of *Rhizobium* can also solubilize both organic and inorganic phosphate (Alikhani *et al.,* 2006).

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

Chaiharn *et al.* (2009) isolated 216 phosphate-solubilizing bacteria from different rice rhizospheric soil in Northern Thailand. These isolates were screened *in vitro* for their plant growth-promoting activities such as solubilization of inorganic phosphate, ammonia (NH_3), catalase and cell wall-degrading enzyme activity. The results showed that all the isolates solubilized inorganic phosphate, 77.7% of isolates produced NH_3 and most of the isolates were positive for catalase. They concluded that the isolates exhibited more than two or three plant growth-promoting (PGP) traits, may promote plant growth directly or indirectly or synergistically.

Panhwar *et al.* (2009) conducted an *in vitro* study to determine the solubilization of phosphorus from inorganic phosphates in three broths containing tricalcium phosphate (NBRIP broth), aluminum phosphate (PDYA-AlP broth) and Christmas island rock phosphate (CRIP broth) by using PSB strains isolated from aerobic rice field. Result showed that the PSB strains were able to solubilize P from tricalcium phosphate and Christmas island rock phosphate, but not from aluminum phosphate. These PSB isolates could be efficient biofertilizer for improved P-nutrition in aerobic rice cultivation system.

A phosphate-solubilizing bacterial strain NII-0909 isolated from the Western ghat forest soil in India exhibited the plant growth-promoting attributes of phosphate solubilization, auxin production, 1-aminocyclopropane-1-carboxylate deaminase activity and siderophore production. It was able to solubilize 122.4 µg/ml of phosphate and produce 109 µg/ml of IAA at 30ºC. These results demonstrated that isolates NII-0909 has the promising PGPR attributes to be develop as a biofertilizer to enhance soil fertility and promote the plant growth (Dastager *et al.,* 2010).

Mehta *et al.,* (2010) isolated a strain of *Bacillus circulans* MTCC 8983 from apple rhizosphere. The strain solubilized tri-calcium phosphate and produced substantial amount of soluble phosphorus (957.30 mg/l) in Pikovskaya's (PVK) broth and exhibited the production of indole acetic acid (IAA) (15.13 µg/ml), siderophore (57.80%) and growth inhibition against *Dematophora necatrix* (46.57%). Regression analysis revealed that phosphate solubilization of *B. circulans* was inversely correlated with pH ($r = -0.98$) and positively correlated with growth ($r = 0.98$), siderophore production ($r = 0.99$), IAA ($r = 0.78$) and antifungal antibiotic activity against *D. necatrix* ($r = 0.87$).

Kannapiran and Ramkumar (2011) isolated *Pseudomonas*, *Bacillus*, *Vibrio*, *Micrococcus*, *Flavobacterium*, *Corynebacterium*, *Alcaligenes* and *Enterobacter*. *Pseudomonas* and *Bacillus* were found to solubilize more phosphates than others. Further phosphate solubilizing activity and solubilization index were also monitored. The phosphate solubilizing potential of *Pseudomonas* sp. was confirmed as a proficient solubilizer than others, where P solubilization was 1670 µg/ml associated with reduction of pH. These bacteria were found to be highly adaptive and therefore, can significantly contribute to the phosphate economy of the marine environment.

Sharma *et al.* (2012) isolated and screened a number of rhizospheric microorganisms from the tea plants of Darjeeling hills for the *in vitro* solubilisation of tricalcium phosphate (TCP). Eight isolates were able to solubilize TCP in Pikovskaya's solid and liquid medium and also produced IAA. Amount of phosphate solubilized ranged from 40.62 ± 1.1 to 136.73 ± 1.7 mg/l

and IAA production ranged from 10 - 30 mg/l. Phosphate solubilizing activities of these strains were associated with a drop in the pH of the medium.

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%) 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.* Result showed that mechanism involved in these weathering processes was the capability of isolates to produce siderophores.

Kaur and Sharma (2013) isolated a total of 35 isolates of rhizobacteria from 25 soil samples collected from healthy chickpea rhizospheric locations of Punjab (India). PGPRs (*Pseudomonas* sp.) were screened for growth promotion activities (indole acetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN), siderophore, phosphate (P) solubilization, catalase, antibiotic resistance spectra) and seed germination on water agar medium along with reference strain PGPR LK884 (*Pseudomonas diminuta*). Seventy per cent of isolates showed capacity for P solubilization in the range of 5.08 to 13.45 mg/100 ml. Maximum P-solubilization was noticed with PGPR-3 (13.45 mg/100 ml) followed by PGPR-2 (13.15 mg/100 ml).

Prakash and Kartikeyan (2013) obtained 10 bacterial isolates from *Acorus calamus* rhizospheric soil of Melaiyar and Nagapattinam districts in Tamil Nadu. These bacterial strains were tested on morphological, biochemical and screened for their direct growth promoting activities (IAA production, production of Ammonia and Phosphate solubilization) and indirect growth promoting activities (HCN production, Siderophore production). Phosphate solubilzation was detected in 83% of isolates of *Bacillus* followed by *Azotobacter* (68.47%), *Pseudomonas* (60.56%) and *Azotobacter* (68.47%), *Pseudomonas* (60.56%) and *Azospirillum* (55%). Production of siderophore was detected less frequently than other PGP characteristics. The isolates of *Pseudomonas* spp. were strong siderophore producers (18.22%) followed by *Azospirillum* spp. (16.22%). The production of

HCN was detected for all cultures in less frequently. The *Pseudomonas* spp. were maximum produced (60%), followed by *Bacillus* spp. (45%), *Azopirillum* spp. (20%) and *Azotobacter* spp. (10%).

2.2.1.2 Nitrogen fixation by PGPR

Microbial communities are a main component of ecosystems that play critical roles in the biochemical transformations of elements including nitrogen fixation (Madigan *et al.,* 2000). Therefore, nitrogen that is available to plants grown for many years without N fertilizers is considered to be due to biological fixation (James, 2000). This process catalyzed by nitrogenase enzyme is essential for maintaining fertility in many ecosystems. The ability to fix nitrogen is widely distributed among diverse groups of bacteria and archae, in different ecosystems. However, this distribution is non-random and depends on the habitat characteristics (Zehr *et al.,* 2003). The biological dinitrogen-fixation process provides the major biological source of nitrogen in natural ecosystems. Several groups of symbiotic N_2 fixing bacteria have been identified in soils and flooded systems, such as *Azotobacter*, *Azomonas*, *Beijerinckia*, *Derxia* (aerobic), *Azospirillum*, *Aquaspirillum*, *Thiobacillus*, *Pseudomonas*, *Xanthobacter*, *Rhizobium*, *Methylosinus*, *Mycobacterium* (Microaerobic), *Klebsiella*, *Erwinia*, *Enterobacter*, *Citrobacter*, *Escherichia*, *Bacillus* (Facultative anaerobic) and *Desulfovibrio*, *Desulfotomaculum*, *Clostridium* anaerobic (Zani *et al.,* 2000).

Most studies on associative nitrogen fixation have focused on crops of agronomic interest such as rice or sugar cane (Engelhard *et al.,* 2000 and Steenhoudt and Vanderleyden, 2000), where fertilisers are required for crop growth. Few studies have aimed to understand the role of the associative dinitrogen fixation in nitrogen-limited natural ecosystems (Bagwell and Lovell, 2000). The *nif* H gene was widely used to detect nitrogen-fixing bacteria (NFB) (Zani *et al.,* 2000 and Poly *et al.,* 2001). It encodes for the dinitrogenase reductase, a key enzyme in the nitrogen fixation process.

Free-living nitrogen fixing bacteria were isolated from rhizosphere of seven different plant namely sesame, maize, wheat, soybean, lettuce, pepper and

rice grown in Chunbuk Province, Korea. Strains with nitrogenase activity were identified based on phenotypic and 16S rDNA sequence analysis and concluded that isolates had potential for developing as a plant growth promoting rhizobacteria (Park *et al.,* 2005).

Bacillus megaterium C4 a nitrogen fixer, which was originally isolated from the maize rhizosphere. It has been demonstrated that the bacterium has nitrogenase acitivity and its *nif* H gene was amplified by polymerase chain reaction (Ding *et al.,* 2005). In many diazotroph's nitrogenase activities correspond well to the levels of *nif* H transcription under various conditions (Saito and Minamisawa, 2006).

Chowdhury *et al.* (2009) studied diversity of *nif* H genes derived from rhizospheric soil and roots of an endemic drought tolerant grass, *Lasiurus sindicus.* The study showed that PCR amplification of *nif* H genes using total DNA as template produced a total of 48 *nif* H clones from the rhizosphere soil and root samples and revealed a predominance of *nif* H sequences closely affiliated to *Pseudomonas pseudoalcaligenes*. Thus, this study provided the evidence that *L. sindicus* harbors a diversity of bacteria with potential for nitrogen fixation.

Jahanian *et al.* (2012) observed that shoot length in Artichoke (*Cynara scolymus*) was significantly affected by *Pseudomonas*, N fixing bacteria and their integrated application. It was observed that *P. putida* has the most increasing effect on shoot length (1.4 times more than control). These results demonstrated growth improvement by rhizobacteria on germination and seedling establishment. It was illustrated that means of shoot length in *P. Putida* treatment was about 1.3 times more than treatment without *P. Putida* and 1.8 times more than control.

Naseri and Sharafzadeh (2013) conducted a factorial experiment based on randomized completed block design with three replications to study the effect of Plant Growth Promoting Rhizobacteria (PGPR) on yield and yield components of rapeseed. The factors consisted of three levels of nitrogen fertilizer (100, 150 and 200 kg ha) and bio-fertilizer (non-inoculation, *Azotobacter chroococcumand,*

Pseudomona putida). The results showed that nitrogen rates had significant effects on yield and yield components. Significant increase was observed in all characters with applying bio-fertilizers and increasing nitrogen from 100 to 200 kg ha^{-1}. Applying *Azotobacter* and *Pseudomona* increased yield and yield components by 15.8 and 13.7%, respectively compared with control treatment.

Vasanthabharathi and Jayalakshmi (2014) isolated *P.fluorescens* BCPBMS-1 from the sponge *Callysspongia diffusa*, and tested for its biocontrol and PGP traits. It was observed in their study that the available nitrogen was 6.4 mg/g for uninoculated *P. fluorescens* soil sample whereas 7.2 mg/g was observed with *P. fluorescens* inoculated soil.

Sivasakthi *et al.* (2014) observed that inoculation of pepper with P-solubilizing bacteria significantly reduces the Phytophthora blight and crown blight and increases the yield as compared to untreated control.

2.2.1.3 IAA (Indole-3-Acetic Acid) production by PGPR

Plant growth regulating substances are naturally occurring organic compounds that influence physiological processes in plants. The ability to synthesize phytohormones is widely distributed among plant associated bacteria. Indole-3-acetic acid (IAA) assumed to be the most abundant and widespread auxin that mediates an enormous range of development and growth responses including embryo symmetry establishment, initiation of cell divison, promote vascular differentiation, root initiation and apical dominance. Besides its hormonal functions, indole-3-acetic acid (IAA) is involved in stimulation of ethylene synthesis (Sahasrabudhe, 2011).

Fatima *et al.* (2009) reported that seven strains isolated from the rhizosphere of wheat, showed significant production of IAA, ranged from 5.5 to 30.6 µg/ml. The study revealed that the strains WPR-51, WPR-42 and WM-3 belonging to *Azotobacter* and *Azospirillum* produced IAA ranging from 19.4 to 30.2 µg/ml and possessed phosphate solubilizing ability. These strains positively affected the germination of wheat as well as increased biomass and root shoot length by inhibiting *Rhizoctonia solani* growth when tested in pot experiments.

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

Yeon *et al.* (2010) isolated three hundred and seventy four rhizobacteria from soil rhizosphere or rhizoplane contaminated with petroleum and heavy metals. The isolates were screened for plant growth-promoting trait (PGPT), including indole acetic acid (IAA) productivity, 1-aminocyclopropane -1-carboxylic acid (ACC) deaminase activity and siderophore (s) synthesis ability. They observed that PGPT-possessing rhizobacteria were more abundant in rhizoplane (82%) samples than the rhizosphere soil (75%). Clusturing analysis by principle component analysis showed that rhizoplane was the most important factor influencing the ecological distribution and physiological characterization of PGPT-possessing rhizobacteria.

Mishra *et al.* (2010) conducted a pot culture experiment of *Cicer arietinum* inoculated with ten isolates PGB1, PGB2, PGB3, PGB4, PGB5, PGT1, PGT2, PGT3, PGG1 and PGG2. Isolates PGB4, PGT1, PGT2, PGT3, PGG1 and PGG2 induce the production of indole acetic acid, whereas only PGT3 isolate was able to solubilize phosphorus. Most of isolates resulted in a significant increase in shoot length, root length and shoot and root dry matter of *Cicer arietinum* seedlings. Application of PGPR isolates significantly improves the percentage of seed germination under saline conditions

Ashraf *et al.* (2011) isolated twelve bacterial strains from root and rhizosphere samples of different sugarcane growing areas. Out of these twelve strains, ten strains were identified as *Pseudomonas* and two as *Azotobacter*. All isolates showed IAA production in growth medium containing tryptophan as a precursor. Maximum IAA production (4.49mg/L) was detected in isolate A17 whereas, values for IAA production by nitrogen fixing isolates Azoto1 and

Azoto2 were comparatively low (0.2 and 0.1mg/l respectively). Beneficial effects of inoculation on sugarcane grown in pots were also observed.

Aziz *et al.* (2012) studied the effect of IAA-producing and non-IAA-producing diazotroph *Bacillus cereus* strains on early growth of shallot (*Allium ascalonicum*) and mustard (*Brassica juncea*) plants. Inoculation with IAA-producing *B. cereus* UPMLH1 significantly increased shallot adventitious roots (root number and length) and shoot growth (19 to 54% increment) whereas inoculation of non-IAA-producing *B. cereus* UPMLH24 did not produce significant effect. However, inoculation with IAA-producing and non-IAA-producing strains significantly increased primary roots and shoot growth of mustard plants.

Reetha *et al.* (2014) isolated *Pseudomonas fluorescens* and *Bacillus subtilis* from rhizosphere of Onion and analysed of these bacteria for *in vitro* indole acetic acid production and studying the effect of these bacteria on plant growth of onion Plant. The results originated from both qualitative and quantitative assays of IAA reflected the ability of two tested microorganisms to produce indole compounds. The two tested microorganisms exhibited a pink to red colour with a little variation in intensity. In the quantitative measurements, the highest value of IAA production was obtained by *P. fluorescens* followed by *B. subtilis,* as they produced (15.38±0.537) and (12.67±0.325) respectively.

2.2 GENETIC DIVERSITY OF PGPR

Rhizobacteria establish positive interactions with plant roots, plant growth promoting rhizobacteria (PGPR), play a key role in agricultural environments and are promising for their potential use in sustainable agriculture. An analysis of genotypic and phenotypic characteristics of indigenous rhizobacteria can help to clarify the mechanisms of interaction between them and plant roots. The study of the genetic structures of microbial population is important not only for understanding their ecological role in natural environment but also any biotechnological application, in which it is necessary to predict the fate of genetically engineered micro organisms released in to the environment and to identify the source of epidemic outbreaks of pathogenic bacteria. For successful

functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity of indigenous community, their distribution and behaviour in soil habitats (Hill, 2000).

Biochemical, molecular characterization and growth promoting effects of phosphate solubilizing *Pseudomonas* sp. isolated from weeds grown in salt range of Pakistan was studied. Preliminary identification of bacterial isolate was made on the basis of morphological and biochemical characters and confirmed by partial 16S rRNA gene sequencing. The genetic diversity among the isolates was evaluated by Randomly Amplified Polymorphic DNA finger printing and similarity matrix was measured. The results showed that all the *Pseudomonas* sp. were capable of solubilizing phosphate, produced phytohormones in culture media. He concluded that the strain *Pseudomonas stutzeri* Khsr3 appears to be a potential candidate as bio-inoculant for saline fields (Naz and Banu, 2010).

Aranda *et al.* (2011) determined the structure and diversity of bacterial communities in the rhizosphere and endosphere of oleaster from 11 provinces of Andalusia, southern Spain. The study of 16S rDNA gene sequence indicated that most of the 94 bacterial isolates belong to genera *Bacillus* (56.4%), *Pseudomonas* (27.7%), and *Paenibacillus* (7.4%). Overall, the rhizosphere and endosphere of wild olives were proved as a good reservoir of bacteria antagonists against *V. dahliae*.

Jinsheng *et al.* (2011) studied the diversity of bacteria in Bohai Bay by RFLP analysis of PCR-amplified 16S rDNA gene fragments. A total of 24 bacterial communities were sampled from seawater and sediment of three representative sites in a whole seasonal cycle: spring (April), summer (July), autumn (October), and winter (January). Sequence analyses revealed that 47.5% (48) of clone sequences were similar to those of uncultured marine bacteria in the environment. In addition, bacterial diversity and composition clearly displayed seasonal variety. They observed that more genera were discovered in summer than any other seasons, and some special species appeared only in specific season.

Mulla *et al.* (2013) studied the physiological and molecular characterization of PGPR *Pseudomonas fluorescens* isolated from different agro climatic zones of Karnataka and its effects on the medicinal plant Sarpagandha *(Rauwolfia serpentina)*. *P. fluorescens* strains from 10 different agro climate zones were isolated, identified and confirmed using standard synaptic keys. Molecular diversity of these isolates was characterized by RAPD marker analysis. The genetic relatedness of the 10 isolates was studied and these formed 2 groups based on the phylogenetic tree obtained. The genetic variations however had no relationship with the effect on plant growth and nutrition. It was concluded from the study that the *Pseudomonas fluorescens* isolates of zone-3 and zone-6 showed maximum effect on growth and nutrient content of Sarpagandha compared to uninoculated control.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled "**Characterization of plant growth promoting rhizobacteria and evaluation of their biocontrol potential against tomato bacterial canker**" 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 2012-2014. 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 King's medium B

Protease peptone	:	2.0%
K_2HPO_4 anhydrous	:	0.15%
$MgSO_4.7H_2O$	:	0.15%
Glycerol	:	1.5%
pH	:	7.2

3.1.3 Pikovskaya's (PVK) broth

Glucose	:	1.0%
$Ca_3 (PO_4)_2$	:	0.5%

$(NH_4)_2SO_4$	:	0.05%
KCl	:	0.02%
$MgSO_4.7H_2O$	:	0.01%
$MnSO_4$	:	0.0004%
$FeSO_4$	:	0.0002%
Yeast extract	:	0.05%

3.1.4 Pikovskaya's agar

Pikovskaya's broth + 2.0% agar

3.1.5 Nitrogen free medium (Jensen's medium)

$CaHPO_4$	:	0.1%
K_2HPO_4 anhydrous	:	0.02%
$MgSO_4.7H_2O$	:	0.02%
NaCl	:	0.02%
$FeCl_3$	:	0.01%
Trace elements stock solution	:	1.00 ml

Trace elements stock solution consists of:

Bo	:	0.05%
Mn	:	0.05%
Zn	:	0.005%
Mo	:	0.005%
Cu	:	0.002%
Agar	:	2.00%
pH	:	6.5-7.5

3.1.6 Luria Bertani (LB) agar

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

3.1.7 Chromazurol S (CAS) agar

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

3.1.8 Minimal agar

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

3.1.10 Mineral salt medium

K_2HPO_4	:	0.1%
KH_2PO_4	:	0.34%
NaCl	:	0.045%
$MgSO_4.7H_2O$	:	0.05%
$MnSO_4.7H_2O$	:	0.01%
$CuSO_4.5H_2O$	:	0.0005%
$ZnSO_4.7H_2O$	:	0.00005%
$CaCl_2$	:	0.01%
Glucose	:	1.0%
Agar	:	2.0%

3.1.11 Protease medium

Pancreatic digest of casein	:	0.5%

Yeast extract : 0.25%
Glucose : 0.1%
Skim milk : 0.7 g/10ml (autoclaved separately)
Agar : 2.0%

3.1.12 Starch Agar Medium

Starch (soluble) : 2.0%
Peptone : 0.5%
Beef extract : 0.3%
Agar : 2.0%

3.1.13 Czapek mineral salt agar medium

$NaNO_3$: 0.2%
KH_2PO_4/ K_2HPO_4 : 0.1%
$MgSO_4.7H_2O$: 0.05%
KCl : 0.05%
Carboxymethyl cellulose (CMC): 0.5%
Peptone : 0.2%
Agar : 2.0%

3.1.14 TBA Medium (Tributyrin agar)

Peptone : 0.5%
Beef Extract : 0.3%
Tributyrin : 0.1%
Agar : 1.5%

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 antagonistic activity and its maintenance

Rhizobacterial strains of different crops with multifarious PGP traits were obtained from Microbiology laboratory, Department of Basic Sciences, Dr. Y S Parmar, University of Horticulture and Forestry Nauni, Solan, Himachal Pradesh were used in the study. The cultures were maintained in nutrient agar at 4˚C.

3.3.1.2 Bacterial pathogen and its maintenance

The Bacterial pathogen *Clavibacter michiganensis* was obtained from the department of Plant Pathology, Dr. Y S Parmar, University of Horticulture and Forestry, Nauni, Solan. The culture was maintained on nutrient agar at 4ºC. Sub-culturing was done periodically on same medium at incubation temperature of 28±1ºC.

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 *IN VITRO* SCREENING OF RHIZOBACTERIAL ISOLATES FOR ANTAGONISTIC ACTIVITY AGAINST *Clavibacter michiganensis*

In vitro antagonism studies between rhizobacterial isolates and the pathogenic strains of *Clavibacter michiganensis* were carried out on nutrient agar plates using agar diffusion method (Mitchell and Carter, 2000).

3.5.1 Agar Diffusion Test

Four hundred microlitres of *C. michiganensis* suspension containing 10^8 cfu/ml was spread on nutrient agar plates and four wells of 8mm diameter punched into the agar. In these wells 100µl suspension of each test antagonist (10^8 cfu/ml) was added and the plates incubated at 28˚C for 48h. Inhibition of *C. michiganensis* growth was assessed by measuring the diameter of inhibition zone (mm) after incubation for 48h at 28˚C.

3.6 *IN VITRO* SCREENING OF THE ANTAGONISTIC BACTERIAL ISOLATES FOR MULTIFARIOUS PLANT GROWTH PROMOTING TRAITS

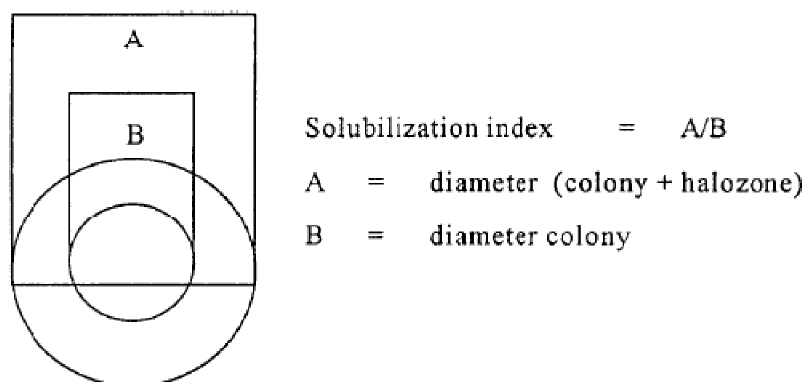
3.6.1 Phosphate solubilization

3.6.1.1 Qualitative estimation of phosphate solubilization (Pikovskaya, 1948)

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 48h. Each treatment was

replicated three times. The bacterial solubilization of phosphorus was exhibited with coloured zones produced around the isolated bacterial colony.

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).



3.6.1.2 Quantitative estimation of P–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.

3.6.3 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 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

3.6.4 SIDEROPHORE PRODUCTION

3.6.4.1 Qualitative estimation of siderophore by Chromazurol-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 colored zone with a yellowish (hydroxamate), pinkish (catecholate) and whitish (carboxylate) colour in the dark blue medium indicated the production of siderophore.

3.6.4.2 Quantitative estimation of siderophore using Chromazurol 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 Chromazurol S (CAS) assay solution (Appendix) 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.

3.6.5 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 (ammended with 5 mM L-tryptophan, 0.065 per cent sodium dodecyl sulphate and 1% glycerol) for 72h at 37°C under shaking 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).

3.6.6 HCN production (Bakker and Schippers, 1987)

The bacterial cultures were streaked on prepoured plates of King's medium B ammended 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.

3.6.7 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 Jensen's medium described in (3.1.5), incubated for 72h and the colonies showing growth were selected.

3.6.8 Cell wall degrading enzyme production

3.6.8.1 Chitinase assay (Robert and Selitrennikoff, 1988)

Preparation of colloidal chitin

Colloidal chitin was prepared by the method (Berger and Reynolds, 1958) given below:

1. Powdered chitin digested overnight with concentrated hydrochloric acid at 4ºC.
2. After digestion step carefully added distilled water and mix thoroughly.
3. Centrifuge and remove the supernatant carefully (the first two-three wash is highly acidic).
4. Continue washing with distilled water until the pH of solution reaches around 4.0
5. Adjust the pH of the colloidal chitin solution by using 2N NaOH (pH around 6-6.5).

6. Add liquid (10ml of chitin in 100 ml media) directly or the chitin suspension in water was centrifuged and the pellet was collected, dried and used at 0.3% in minimal salt media.

The bacterial cultures were spotted on to the prepared minimal agar medium ammended with 0.3% colloidal chitin and the plates were incubated at 30ºC for 7 days. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production.

3.6.8.2 Protease activity (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 with 24h old bacterial culture was done on skim milk agar plate. Plates were incubated at 37ºC for 24-48h and observed for proteolysis i.e. clear zone (diameter, mm) produced around the spot.

3.6.8.3 Amylase activity (Shaw *et al*., 1995)

All bacterial isolates were screened for amylolytic activity by plate assay method. Spot inoculation with 24h old bacterial culture was done on starch hydrolysis agar plate. Plates were incubated at 37ºC for 24-48h. 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.

3.6.8.4 Cellulase activity (Ghose, 1987)

All bacterial isolates were screened for cellulolytic activity by plate assay method. Spot inoculation with 24h old bacterial culture was done on Czapek mineral salt agar medium. Plates were incubated at 37°C for 48-72h. HDTMA was poured on agar plates after incubation. Agar plates were observed for cellulose hydrolysis i.e. clear zone (diameter, mm) produced around the spot.

3.6.8.5 Lipase activity (Kumar *et al.,* 2012)

All bacterial isolates were screened for lipase production by plate assay method. Spot inoculation with 24h old bacterial culture was done on TBA

medium. Plates were incubated at 37°C for 48-72h. Agar plates were observed for clear zone (diameter, mm) produced around the spot.

3.7 ANTIBACTERIAL METABOLITE PRODUCTION

One hundred microlitres of *C. michiganensis* suspension containing 10^8 cfu/ml was spread on nutrient agar plates and four wells of 8 mm diameter punched into the agar plates. Supernatant of 48h old bacterial antagonist culture was added in the wells at different concentrations i.e. 0.25% (v/v), 0.50% (v/v), 0.75% (v/v), 1.00% (v/v). The plates were incubated at 28°C for 48h. Inhibition of *C. michiganensis* growth was assessed by measuring the diameter of inhibition zone (mm) after incubation for 48h at 28°C.

3.8 PHENOTYPIC CHARACTERIZATION OF SELECTED ANTAGONISTIC BACTERIAL ISOLATES

The most efficient bacterial isolates selected on the basis of *in vitro* antibacterial activity and plant growth promoting traits were subjected to morphological, cultural and biochemical characterization 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.

3.8.2 Biochemical characterization

Biochemical characteristics of isolates like catalase, urease, H_2S production etc. and ability to ferment sugars were investigated.

3.9 GENOMIC DNA EXTRACTION BY CONVENTIONAL METHOD (Sambrook *et al.,* 1989)

The efficient antagonist isolates were grown overnight at 37ºC in nutrient broth under shaking condition 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 : Isolamyl alcohol (25: 24: 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 500 µl 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 (50 mg/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: Isoamyl alcohol (25: 24: 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 buffer.

3.9.1 Gel electrophoresis

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

3.10 EVALUATION OF GENETIC DIVERSITY OF ANTAGONISTIC BACTERIAL ISOLATES BY USING RAPD-PCR

Genomic profile of ten bacterial isolates was screened by RAPD-PCR. Initially, five random primers (RBa-1, RBa-2, RBa-3, RBa-4 and RBa-5) were used for RAPD analysis of two antagonist bacterial isolates (representative antagonism from different groups based on plant growth promoting activities). Out of five, four primers that showed maximum polymorphic bands with representative isolates were selected for further RAPD-PCR analysis of ten isolates. The detail of primers used in the study is depicted in table mentioned below.

S. No.	Primers used	Sequence 5' ⟶ 3'
1	RBa-1	AAAACCGGGC
2	RBa-2	ACAGGGCTCT
3	RBa-3	ACAGGGGTGT
4	RBa-5	AGGGGCGGCA

RAPD amplification was carried out on Gradient PCR (Labnet International Inc.). A 25µl reaction mixture contained 19 µl deionized water, 2.5

μl 10 X Taq polymerase buffer, 0.5 μl of 1 U Taq polymerase enzyme, 1μl of 10 mM dNTPs, 1 μl of 100 mM random primer and 1 μl of 50 ng template DNA was used. PCR was programmed as given below:

PCR Conditions (Temperature °C)							
Initial Denaturation	**Denaturation**	**Annealing**	**Extension**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
94ºC	94ºC	35ºC	72°C	94°C	38°C	72°C	72°C
5 min	45 sec	1 min	1.5 min	45 sec	1 min	1 min	10 min
	× 8 cycles			× 35 cycles			

3.10.1 Gel electrophoresis

The isolated DNA was finally suspended in 100 μl of elution buffer and quantified on 1.2 % agarose gel.

3.11. EVALUATION OF SELECTED BACTERIAL ANTAGONISTS FOR THE CONTROL OF TOMATO BACTERIAL CANKER UNDER NET HOUSE CONDITIONS

3.11.1 Growth of Plants

The soil, sand and farmyard manure were mixed in a ratio of 2:1:1 in order to make a potting mixture and the mixture was sterilised by 3 successive autoclave cycles of 1h each at 121°C. 1.5 kg of potting mixture was filled in 12 cm diameter pots. Tomato seeds (variety Solan Lalima) were surface sterilised with 2% sodium hypochloride for 2 min, washed thoroughly with sterilised water and then planted in the pots containing sterilised potting mixture (100 seeds/pot). The plants were maintained in net house at temperatures of 24-28°C and 75-90% relative humidity and seedlings were watered with sterile water when necessary.

3.11.2 Preparation of bacterial pathogen inoculum

The pathogen inoculum was prepared by culturing *C. michiganensis* in nutrient broth for 48h at 28°C (10^8 cfu/ml) and 120 rpm on rotary shaker the liquid culture was used for the pathogen challenge at 4-5 leaved old tomato seedlings.

3.11.3 Preparation of antagonistic strains inoculum

The PGPR inoculums was prepared by culuturing the five best antagonist PGPRs in nutrient broth for 48h at 37°C (10^8 cfu/ml) and 120 rpm on rotary shaker. The liquid culture was used for seedling dip and drenching of tomato seedlings at 4-5 leaves stage.

3.11.4 Biocontrol Experiment

1.5 kg of the sterilized potting mixture, was mixed with 100ml inoculum of *C. michiganensis* (10^8 cfu/ml) and placed in 12 cm diameter pots. One week after incorporation of the pathogen into the soil and one day before transplanting the seedlings, antagonists were incorporated in the soil at a rate of 75 ml per pot at 10^8 cfu/ml. Four weeks old tomato seedlings were root dipped in bacterial suspension of antagonistic bacteria (10^8 cfu/ml) for 60 min and transplanted into pathogen-antagonist mixture soil (Lemessa and Zeller, 2007). Treatments were replicated four times with five plants per pot. Positive control was maintained which was only inoculated with the pathogen and negative control was also maintained without any inoculation of pathogen or antagonist PGPR. Disease ratings were recorded two weeks after pathogen challenge according to the scale given by Shenge *et al.* (2010).

3.11.5. Disease assessment

Disease ratings were recorded two weeks after pathogen challenge according to the scale given by Shenge *et al*. (2010).

0	:	No symptoms
1	:	Trace; one leaf symptomatic
2	:	Slight, two leaves symptomatic
3	:	Moderate, > 2 leaves symptomatic
4	:	Severe, half plant affected
5	:	Very severe, more than half plant affected
6	:	Stunted with extensive collapse
7	:	Dead plant

Per cent Disease Index (PDI):

$$\text{Percent Disease index} = \frac{\sum(rating \times no.of\ plants\ rated)}{Total\ no.of\ plants\ observed\ \times\ highest\ rating} \times 100$$

$$\text{Percent disease incidence} = \frac{\text{No. of diseased plants}}{Total\ no.of\ plants\ observed} \times 100$$

$$\text{Biocontrol efficacy} = \frac{\text{Disease incidence of control – disease incidence of plants treated with antagonist}}{\text{Disease incidence of control}} \times 100$$

3.12 EFFECT OF SELECTED BACTERIAL ANTAGONISTS ON GROWTH PROMOTION OF TOMATO UNDER NET HOUSE CONDITIONS

At the end of experiment (2 months after transplanting) plants including roots were harvested from the pots and fresh weight recorded. Healthy plants were evaluated for the following root and shoot parameters.

3.12.1 Shoot characteristics

3.12.1.1 Shoot length

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

3.12.2 Root characteristics

3.12.2.1 Root length

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

3.13 OPTIMISATION OF CULTURAL CONDITIONS FOR ANTIBACTERIAL METABOLITE PRODUCTION BY THE MOST EFFICIENT BACTERIAL ANTAGONIST

3.13.1 Effect of temperature

Effect of different temperatures was studied for zone of inhibition by the selected bacterial isolate against *C. michiganensis* at different temperatures (30, 35, 45 and 50° C) in order to maintain optimum temperature for further experiments.

3.13.2 Effect of incubation period

Effect of incubation period on zone of inhibition was studied by growing the selected bacterial isolate at different incubation periods (24, 48, 72, 96 and 120h). The incubation time at which maximum zone of inhibition was observed was used for subsequent studies.

3.13.3 Effect of size of inoculum

Inoculum size of different population densities (1%, 2%, 3%, 4%, 5% and 10%) was added to nutrient broth and antagonism was determined. The inoculums size showing maximum antagonism was observed.

3.13.4 Effect of pH on antagonistic activity

Antagonistic activity by the test organism was studied at different pH. The medium was adjusted to various pH (5.0, 6.0, 7.0, 8.0 and 9.0). The optimum pH was observed.

3.14 INTRINSIC ANTIBIOTIC RESISTANCE OF MOST EFFICIENT BACTERIAL STRAIN

Antibiotic resistance pattern of most efficient selected strain was determined by testing their tolerance to intrinsic levels of different antibiotics using disc diffusion method (Bakthavatchalu *et al.,* 2012).

3.15 MOLECULAR CHARACTERIZATION OF MOST EFFICIENT ISOLATE BY 16S rDNA SEQUENCE ANALYSIS

3.15.1 PCR amplification of 16S rDNA

PCR reaction was carried out in 20 µl reaction containing ~50ng of template DNA, 20 p moles 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 100 V 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).

3.15.2 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°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 let it stand for 1 min 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 centre 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.

Sequencing and phylogenetic analysis

Sequencing was done by commercial sequencing facility (Xcleris lab. Ahmadabad). 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 by neighbor-joining algorithm using PHYLIP package (Felsenstain, 1993). The stability of phylogenetic tree was accessed by taking 1000 replications of data set and was analyzed using the programme SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package. Tree was viewed with the help of Tree View from the website http://taxonomy.zoology.gla.ac.uk/rod/treeview.html (Page, 1996).

3.16 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

EXPERIMENTAL RESULTS

The results obtained during the course of this study have been presented in this chapter under following sections:

4.1 *In vitro* screening of bacterial isolates for antagonistic activity against *Clavibacter michiganensis*.

4.2 Comparision of antagonist bacterial isolates from different horticultural crops on the basis of plant growth promoting traits (P-solubilization, siderophore production, growth on N-free medium, IAA production and HCN production).

4.3 Screening of antagonistic bacterial isolates from different horticultural crops for multifarious PGP traits.

4.4 Antibacterial activity of selected antagonistic bacterial isolates against *Clavibacter michiganensis* at different concentrations of cell free supernatant.

4.5 Selection of antagonistic bacterial isolates against *Clavibacter michiganensis* based on similarity coefficient derived from their multifarious PGP traits.

4.6 Phenotypic and metabolic characterization of ten selected antagonistic bacterial isolates.

4.7 Random amplified polymorphic DNA (RAPD) studies.

4.8 Quantitative estimation of plant growth promoting traits by selected antagonistic isolates.

4.9 Net house studies.

4.10 Optimization of cultural conditions for the antagonistic effect of the best selected most bacterial isolate S1 against *C. michiganensis*.

4.11 Molecular identification of most efficient antagonistic bacterial isolate S1 based on 16S rRNA gene sequencing.

4.1 *IN VITRO* SCREENING OF BACTERIAL ISOLATES FOR ANTAGONISTIC ACTIVITY AGAINST *Clavibacter michiganensis*

Bacterial canker is a very contagious and destructive disease of green house as well as field grown tomatoes. But, the biological control of canker, caused by *Clavibacter michiganensis* ssp. *michiganensis* has not been worked out. Consequently, this study was undertaken with the aim of identifying a potential biocontrol agent of the pathogen. A total of 550 rhizobacterial isolates from different horticultural, vegetable crops and medicinal plants grown under mid hills and high hills of Himachal Pradesh were screened for antagonistic activity against *Clavibacter michiganensis.* These crops were from different agroclimatic conditions with respect to altitude and soil pH (Table 1). However, the soil pH did not vary much with the altitude and was almost neutral. Table 1 revealed that out of total, only 40 bacterial isolates showed antagonistic activity by depicting inhibition zone in the range of 3.20 to 12 mm (Plate 1). A total of 8 (20%) isolates were of endorhizospheric origin while the rest 34 (80%) were rhizospheric. Out of these 40 isolates, 20 isolates were isolated from strawberry rhizosphere, 10 from apple and 10 from apricot rhizosphere.

The total antagonistic bacterial isolates (20/40) present in strawberry rhizosphere of district Solan, Shimla and Sirmaur showed 3.2 to 12.0 mm zone of inhibition against *Clavibacter michiganensis*. The status of antagonistic bacterial isolates from strawberry rhizosphere depending on the degree of antagonism was excellent for 15% (3/20) isolates obtained from Shimla, 15% (3/20) were rated very good and rest 70% (14/20) were good.

The antagonistic bacterial isolates (10/40) present in apple rhizosphere of district Shimla, Chamba and Kinnaur showed 3.56 to 10.66 mm range of inhibition zone. The status of antagonistic bacterial isolates from apple rhizosphere depending on the degree of antagonism was excellent for 10% (1/10) isolates obtained from Shimla, 10% (1/10) were very good obtained from Shimla and Chamba, while the rest 80% (8/10) from Shimla, Chamba, Kinnaur were rated good.

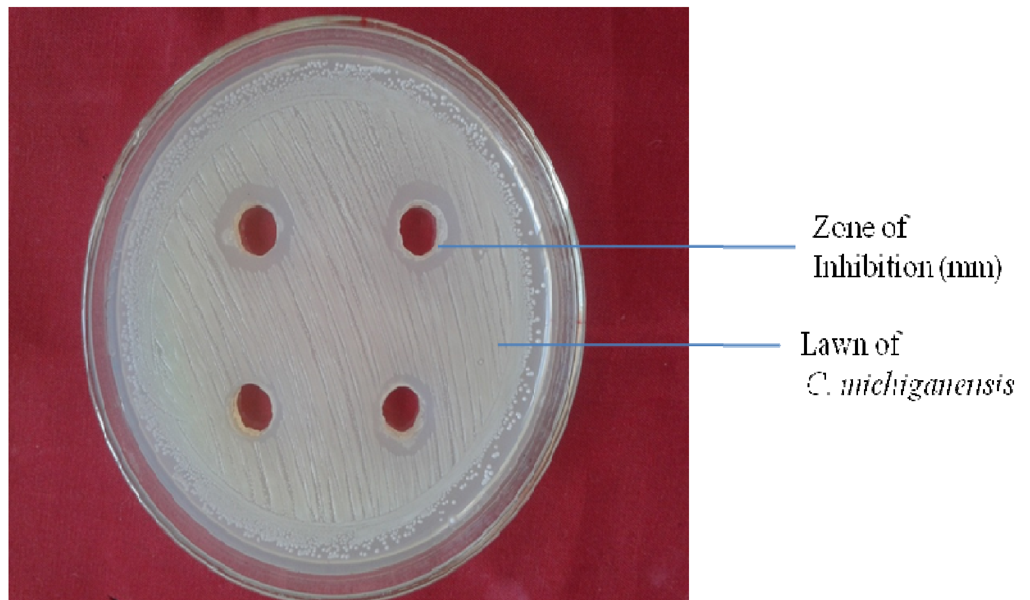


Plate 1. Screening of rhizobacterial isolates for antagonism against *Clavibacter michiganensis* from different horticultural crop rhizosphere

Table 1. Site characterization and screening of rhizobacterial isolates for antagonism against *Clavibacter michiganensis*

Isolate	Origin*	Site of isolation	Source	Altitude (metres above the sea level)	soil pH	Zone of inhibition** (mm)	Degree of antagonism
KU_2 S 1	RS	Kufri, Shimla	Strawberry	2,290	5.7-6.3	10.33	+++
NA(2)	RS	Nauni, Solan	Strawberry	1,502	6.5-7.0	4.50	+
NA(5)	RS	Nauni, Solan	Strawberry	1,502	6.5-7.0	4.90	+
S1	RS	Rohru, Shimla	Strawberry	1,583	5.1-7.2	12.00	+++
$KU_{3(1)}$	RS	Kufri, Shimla	Strawberry	2,290	5.7-6.3	8.67	++
$R_2S_{(1)}$	RS	Rohru, Shimla	Strawberry	1583	5.1-7.2	10.00	+++
$RO_{5(6)}$	ER	Rohru, Shimla	Strawberry	1583	5.1-7.2	3.66	+
$Ra_{34(5)}$	RS	Rajgarh, Sirmaur	Strawberry	1,555	6.2-6.7	3.90	+
NA(6)	RS	Nauni, Solan	Strawberry	1,502	6.5-7	4.30	+
$Ra_{1(3)}$	ER	Rajgarh, Sirmaur	Strawberry	1,555	6.2-6.7	5.00	+
$Ra_{31(5)}$	RS	Rajgarh, Sirmaur	Strawberry	1,555	6.2-6.7	4.50	+
$KU_{3(3)}$	ER	Kufri, Shimla	Strawberry	2,290	5.7-6.3	8.43	++
KU_3	RS	Kufri, Shimla	Strawberry	2,290	5.7-6.3	9.34	++
Na-12 S 1	RS	Nauni, Solan	Strawberry	1,502	6.5-7	3.20	+
$Ra_{2(2)}$	ER	Rajgarh, Sirmaur	Strawberry	1,555	6.2-6.7	4.60	+
$RO_{4(5)}$	RS	Rohru, Shimla	Strawberry	1,583	5.1-7.2	4.70	+
$RO_{2(7)}$	RS	Rohru, Shimla	Strawberry	1,583	5.1-7.2	3.80	+
Na 8	RS	Nauni, Sirmaur	Strawberry	1,583	6.5-7	3.20	+
$KU_{1(5)}$	RS	Kufri, Shimla	Strawberry	2,290	5.7-6.3	3.91	+
$RO_{5(1)}$	ER	Rohru, Shimla	Strawberry	1,583	5.1-7.2	4.80	+
$RG_{1(3)}$	RS	Matiana, Shimla	Apple	2,381	5.5-7.32	10.66	+++
G2(6)	RS	Matiana, Shimla	Apple	2,381	5.5-7.32	4.33	+
RG2(1)	RS	Matiana, Shimla	Apple	2,381	5.5-7.32	4.30	+
R5(2)	ER	Matiana, Shimla	Apple	2,381	5.5-7.32	4.72	+
R3(4)	RS	Matiana, Shimla	Apple	2,381	5.5-7.32	4.99	+
CH_8A	RS	Chamba	Apple	996	7.55-7.6	9.33	++
CK_9A	RS	Chamba	Apple	996	7.55-7.6	4.30	+
KPO_8A	RS	Recong Peo Kinnaur	Apple	4,000	5.7-6.1	3.90	+
ES_3A	RS	Chopal, Shimla	Apple	2,550	5.15-7.32	3.56	+
CH_3B	RS	Chamba	Apple	996	7.55-7.6	4.80	+
$AP_{3(1)}$	RS	Recong Peo, Kinnaur	Apricot	2,290	5.7-6.1	4.10	+
$AP_{2(1)}$	RS	Recong Peo, Kinnaur	Apricot	2,290	5.7-6.1	3.60	+
$AT_{1(1)}$	RS	Tabo, Kinnaur	Apricot	3,280	5.5-5.9	3.89	+
$AG_{1(7)}$	RS	Gaura, Sirmaur	Apricot	932	5.8-6.3	10.33	+++
$AG_{5(1)}$	ER	Gaura, Sirmaur	Apricot	932	5.8-6.3	4.9	+
$AK_{1(4)}$	RS	Kandaghat, Solan	Apricot	1,425	6.6-7.3	4.98	+
$AN_{5(2)}$	ER	Nauni, Solan	Apricot	1,502	6.5-7	4.60	+
$AN_{1(1)}$	RS	Nauni, Solan	Apricot	1,502	6.5-7	4.50	+
$AN_{2(2)}$	RS	Nauni, Solan	Apricot	1,502	6.5-7	4.83	+
$AS_{3(2)}$	RS	Subathu, Solan	Apricot	1,265	6.9-7.3	4.65	+

Origin* :RS- Rhizospheric, ER- Endorhizospheric
Zone of inhibition** = Zone size-well size
+ Good = zone size ranging from 0-5 mm
++Very good = zone size ranging from >5- <10 mm
+++ Excellent = zone size from 10-15 mm

The antagonistic bacterial isolates (10/40) present in apricot rhizosphere of district Kinnaur, Solan and Sirmaur showed average of 3.60 to 10.33 mm diameter of inhibition zone. In contrast, the status of antagonistic bacterial isolates from apricot rhizosphere depending on the degree of antagonism was excellent for 10% (1/10) isolates obtained from Sirmaur, while the status of rest of the isolates was rated good.

Hence, the percentages of antagonistic bacterial isolates showing excellent degree of antagonism has been arranged in order of strawberry (15%) > apple (10%) = apricot (10%).

4.2. COMPARISION OF ANTAGONIST BACTERIAL ISOLATES FROM DIFFERENT HORTICULTURAL CROPS ON THE BASIS OF PLANT GROWTH PROMOTING TRAITS (P-SOLUBILIZATION, SIDEROPHORE PRODUCTION, GROWTH ON N-FREE MEDIUM, IAA PRODUCTION AND HCN PRODUCTION)

Table 2 showed the results of the plant growth promoting traits (PGPT) for the antagonistic bacterial isolates associated with rhizosphere of strawberry, apple and apricot. A total of 40 antagonist bacterial isolates were screened for PGPTs viz. P-solubilization, siderophore production, growth on N free medium, IAA production and HCN production.

Table 2. Characterization of antagonistic bacterial isolates from different horticultural crops possessing plant growth promoting traits (P solubilization, siderophore production and growth on N free medium)

Source		Single traits			Binary traits			Triple traits
	Isolates without any activity	P	S	N	P+S	P+N	S+N	P+S+N
Strawberry	3/20 (15)	3/20 (15)	1/20 (5)	1/20 (5)	4/20 (20)	5/20 (25)	1/20 (5)	2/20 (10)
Apple	0/10 (0)	3/10 (30)	0/10 (0.00)	0/10 (0.00)	3/10 (30)	1/10 (10)	1/10 (10)	2/10 (20)
Apricot	0/10 (0)	1/10 (10)	1/10 (10)	0/10 (0)	2/10 (20)	4/10 (40)	1/10 (10)	1/10 (10)
Total	3/40 (7.5)	7/40 (17.5)	2/40 (5)	1/40 (2.5)	9/40 (22.5)	10/40 (25)	3/40 (7.5)	5/40 (12.5)

Frequencies of antagonistic rhizobacteria possessing plant growth promoting traits vary with respect to crop, are expressed in terms of single, binary

and triple PGP traits. Different combinations of traits were made by incorporating the important traits of P solubilization, siderophore production and N fixation, excluding IAA and HCN production because all the isolates were negative for HCN production, whereas IAA was present in all the isolates. It was observed that combination of binary traits of nitrogen fixation and phosphate solubilization was highest for apricot (40.0%). Rhizobacterial percentages having binary traits of siderophore production and nitrogen fixation was found to be same (10.0%) in apple and apricot, while it was found to be (5.0%) in case of strawberry, whereas the percentages of PGPRs having binary traits of phosphate solubilization and siderophore production was found maximum in apple (30.0%) which was relatively higher as compared to the binary traits shown by rhizobacterial antagonists of other two crops.

Ratios of antagonistic rhizobacteria possesing triple traits of phosphate solubilization, siderophore production and nitrogen fixation were highest in apple (20.0%). However, in case of strawberry and apricot, it was 10.0%. It was also observed that all the antagonistic PGPRs were able to produce substantial amount of IAA, but vary in possessing different combinations of PGP traits like P solubilization, siderophore production and growth on N free medium. Strikingly, it was observed that none of the antagonist was able to produce HCN, which was considered as an important trait for antagonism. Hence, antagonism in the present isolates may be due to some other factors.

Overall percentages of bacterial antagonists for plant growth promoting traits viz. P solubilization, siderophore production and IAA production from strawberry, apple and apricot rhizosphere are presented in Fig. 1. which revealed that fourteen out of twenty bacterial isolates (70.0%) from strawberry rhizosphere were able to solubilise phosphorus and all the isolates (100%) produced IAA, whereas only eight isolates (40.0%) were able to produce siderophore on CAS medium. Nine out of ten isolates (90.0%) from apple rhizosphere were phosphate solubilizers, all (100%) were able to produce IAA, whereas, only six (60.0%) were able to produce siderophore. However, in case of apricot, eight isolates

(80.0%) were phosphate solubilizers, four (40%) were able to produce siderophore and all (100 %) were able to produce IAA.

Frequencies of rhizobacterial antagonists having P-solubilization among three horticultural crops has been decpicted in Fig 1. Percentages of bacteria having phosphate solubilization activity is arranged in the order of apple (90%) > apricot (80%) > strawberry (70%). In total, 80.0% (32/40) were able to solubilize phosphorous. The highest P solubilization was recorded for the isolate from strawberry rhizosphere, while the lowest for the isolate from apricot rhizosphere. In total, 77.5% (31/40) of the isolates were able to solubilize phosphorous.

The ratio of bacterial isolates showing siderophore synthesising ability were in the order of apple (60.0%) > apricot (50.0%) > strawberry (40.0%). The highest siderophore producers were recorded in rhizospheric isolates from apple and lowest were recorded in strawberry and only 45.0% (18/40) of total PGPT possesing isolates were siderophore producers.

4.3. SCREENING OF ANTAGONISTIC BACTERIAL ISOLATES FROM DIFFERENT HORTICULTURAL CROPS FOR THEIR MULTIFARIOUS PGP TRAITS

A total of 40 isolates from the rhizospheres of strawberry, apple and apricot were taken for qualitative detection of multifarious plant growth promoting traits viz. phosphate solubilization, siderophore production, IAA production, growth on nitrogen free medium and hydrogen cyanide production. These isolates were tested for qualitative estimation of P solubilization on PVK medium, siderophore production on CAS medium and growth on nitrogen free medium (Jensen's medium). In addition, the production of various lytic enzymes like amylase, cellulase, lipase, protease and chitinase was also investigated.

4.3.1 Screening of bacterial antagonists from strawberry rhizosphere against *C. michiganensis* for multifarious PGP traits

A total of twenty rhizobacterial isolates, rhizospheric and endorhizospheric, from strawberry rhizosphere were screened for multifarious

plant growth promoting traits viz. phosphate solubilization, siderophore production, IAA production, growth on nitrogen free medium , HCN production and production of different lytic enzymes viz. amylase, cellulase, lipase, protease and chitinase. Results revealed that all the isolates were negative for HCN production however, exhibited concomitant production of other four plant growth promoting activities (Table 3). These isolates were tested for qualitative estimation of P-solubilization (Plate 2) and PSI (Phosphate solubilization index) showed a lot of variation. Maximum PSI was observed with $KU_{3(1)}$ (3.31), with corresponding SE % (Solubilization Efficiency) of 113.25%. Minimum PSI was recorded for $Ra_{1(3)}$ (0.34), with corresponding SE% of 11.33%. Out of twenty, six isolates were unable to solubilize P [NA(2), $RO_{5(6)}$, Ra $_{34(5),}$ Na-12 S 1, Na 8, $KU_{1(5)}$].

The siderophore production of the bacterial isolates were estimated qualitatively on the basis of colored zone on CAS medium (Plate 2). Variation was observed in zone size which ranged between 2.56 mm to 10 mm. Maximum zone was produced by isolate S1 (10 mm) which was significantly higher than all other isolates. Minimum zone (2.56 mm) was produced by isolate R_2 $S_{(1).}$

Bacterial isolates were screened for IAA production in Luria Bertani broth ammended with tryptophan after 72h of incubation. All the isolates (100%) were able to produce IAA. The IAA produced was in the range of 2.00 µg/ml to 35.00 µg/ml. Maximum IAA was produced by the isolate $KU_{3(1)}$ (35.00 µg/ml) and minimum by the isolate $RO_{4(5)}$ (2.00 µg/ml). Whereas, out of twenty, nine (45.0%) antagonists were able to grow on N-free medium (Plate 2).

These twenty isolates were also screened for production of different lytic enzymes viz. amylase, cellulase, lipase, protease and chitinase (Plate 2). Out of twenty isolates, eleven (55%) exhibited amylase activity. A lot of variation was observed in amylase activity ranging from 0.12 to 2.19 E.I. (Enzyme index). Maximum E.I (2.19) was recorded for isolate KU_2 S1 whereas the isolate $RO_{4(5)}$ showed minimum E.I. (0.12).

Table 3. Screening of bacterial antagonists from strawberry rhizosphere for multifarious PGP Traits

Isolate	Phosphate solubilization		Siderophore production (zone size in mm)	Indole-3-acetic acid (µg/ml)	Growth on nitrogen free medium	Amylase	Cellulase	Lipase	Protease	Chitinase
	PSI*	SE%**				E.I.***	E.I.	E.I.	E.I.	E.I.
KU$_2$ S 1	2.77	76.59	3.23	24.00	+	2.19	1.47	1.68	1.80	1.24
NA(2)	0.00	0.00	-	5.00	-	1.80	-	1.68	1.72	1.13
NA(5)	1.33	33.33	-	7.00	-	-	1.30	1.50	1.72	1.25
S 1	2.94	94.16	10.00	27.00	+	1.80	-	1.73	1.62	1.13
KU$_{3(1)}$	3.13	113.25	3.33	35.00	+	-	1.30	1.50	1.72	1.52
R$_2$S$_{(1)}$	2.33	33.33	2.56	23.00	+	-	-	1.53	1.76	1.42
RO$_{5(6)}$	0.00	0.00	2.99	20.00	-	1.66	-	1.63	1.87	1.45
Ra $_{34(5)}$	0.00	0.00	-	3.00	+	1.33	-	-	1.41	1.50
NA(6)	2.33	33.00	-	7.00	-	1.51	-	1.42	1.10	0.56
Ra $_{1(3)}$	0.34	11.33	-	5.00	-	1.31	-	1.12	1.33	1.04
Ra $_{31(5)}$	2.30	30.00	-	6.00	+	-	1.10	-	-	1.50
KU$_{3(3)}$	2.87	87.36	5.00	22.00	+	-	1.50	1.51	1.69	1.22
KU$_3$	2.44	44.32	8.70	24.00	-	-	-	1.60	1.76	0.72
Na-12 S 1	0.00	0.00	-	4.00	-	0.78	1.20	-	-	0.90
Ra $_{2(2)}$	2.22	21.74	-	9.00	+	-	-	1.23	0.21	1.22
RO$_{4(5)}$	2.25	25.00	3.56	2.00	-	0.12	-	-	-	0.80
RO$_{2(7)}$	1.54	32.00	-	5.50	-	0.70	-	-	0.43	0.43
Na 8	0.00	0.00	-	6.20	-	-	-	-	0.80	1.45
KU$_{1(5)}$	0.00	0.00	-	8.00	+	1.12	0.40		-	-
RO$_{5(1)}$	2.21	23.60	-	9.00	-	-	-	1.01	-	1.20

Phosphate solubilization index (**PSI**)* = $\frac{A}{B}$ where A= diameter of colony+halo zone, B= diameter of colony

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

+ Isolates showing positive PGP activity

- Isoltes showing negative PGP activity

E.I.*** = $\frac{A}{B}$

Where, A = Diameter of halo zone, B = Colony diameter

Seven (35%) out of twenty isolates exhibited cellulase activity. Variation in cellulase activity was observed in the range of 0.40 to 1.50 E.I. Maximum cellulase activity was recorded for isolate $KU_{3(3)}$ (1.50 E.I.) and isolate $KU_{1(5)}$ (0.40 E.I.) was recorded with minimum enzyme acttvity. Thirteen (65%) out of twenty isolates exhibited lipase activity. Lipase activity was observed within the range of 1.01 to 1.73 E.I. Maximum lipase activity was recorded for isolate S 1 (1.73 E.I.) and minimum lipase activity for isolate $RO_{5(1)}$ (1.01 E.I.) was recorded. Fifeen (75%) out of twenty isolates exhibited protease activity. Protease activity was observed within the range of 0.21 to 1.87 E.I. Maximum enzyme activity was recorded for isolate $RO_{5(6)}$ (1.87 E.I.) and minimum enzyme activity was recorded for isolate $Ra_{2(2)}$ (0.21 E.I.). Nineteen (95%) out of twenty isolates exhibited chitinase activity. Variation in chitinase activity was observed in the range of 0.43 to 1.52 E.I. Isolate which exhibited maximum chitinase activity was $KU_{3(1)}$ (1.52 E.I.) and isolate that exhibited minimum chitinase activity was $RO_{2(7)}$ (0.43).

4.3.2. Screening of bacterial antagonists from apple rhizosphere against *C. michiganensis* for their multifarious PGP traits

Ten antagonistic bacterial isolates from apple rhizosphere were screened for multifarious plant growth promoting traits. It was observed that all the isolates showing antagonistic activity against the pathogen were negative for HCN production but exhibited concomitant production of other four plant growth promoting traits (Table 4). PSI of bacterial isolates showed a lot of variation. Maximum PSI was observed with isolate CH_8A (2.65) with corresponding SE% of 64.52% and minimum was recorded for isolate KPO_8A (2.12) with corresponding SE% of 17.1%.

The siderophore production of the bacterial isolates were estimated qualitatively on the basis of colored zone on CAS medium. Variation was observed in zone size which ranged between 3.9 mm to 11 mm. Maximum zone was produced by isolate CH_8A (11 mm) which was significantly higher than all other isolates. Minimum zone (3.9 mm) was produced by isolate G2(6).

All the ten (100%) isolates were able to produce IAA in Luria Bertani broth ammended with tryptophan after 72h of incubation. IAA was produced in the range of 1.50 µg/ml to 70.00 µg/ml. Maximum IAA was produced by the isolate $RG_{1(3)}$ (70.00 µg/ml), whereas, minimum IAA production was shown by the isolate RG2(1) (1.50 µg/ml).

Phosphate solubilization was observed in nine (90%) isolates, four isolates (40%) were able to grow in nitrogen free medium (Plate 2), six (60%) isolates were able to produce siderophore on CAS medium and all the ten (100%) isolates were IAA producers.

The isolates from apple rhizosphere were also screened for production of different lytic enzymes viz. amylase, cellulase, lipase, protease and chitinase. Out of ten isolates, six isolates (60%) exhibited amylase activity. A lot of variation was observed in enzyme activity ranging from 0.75 E.I. to 1.92 E.I. Maximum amylase activity was recorded for isolate $RG_{1(3)}$ (1.92 E.I.) which was significantly higher than the enzyme activity recorded for isolate CH_3B (0.75 E.I.) with minimum enzyme index.

Four (40%) out of ten isolates exhibited cellulase activity. Enzyme activity was observed in a range of 0.23 E.I. to 1.1 E.I. Maximum cellulase activity was recorded for the isolate CH_8A (1.1 E.I.), whereas the isolate R5(2) (0.23 E.I.) showed minimum cellulase activity. Five (50%) out of ten isolates exhibited lipase activity. Enzyme activities were observed in the range of 0.78 E.I. to 1.52 E.I. Moreover, maximum and minimum lipase activities were recorded for the isolates $RG_{1(3)}$ (1.52 E.I.) and isolate CH_3B (0.78 E.I.). Five (50%) out of ten isolates exhibited protease activity. On the basis of enzyme index maximum enzyme activity was recorded for isolate $RG_{1(3)}$ (1.81 E.I.) and minimum protease activity was recorded for isolate R5(2) (0.89 E.I.). In case of chitinase enzyme, all the isolates were positive in terms of zone size. Enzyme activity variation was in the range of 0.80 E.I. to 1.55 E.I. Isolate CK_9A exhibited maximum enzyme activity (1.55 E.I.) and minimum enzyme activity was shown by the isolate G2(6) (0.80 E.I.).

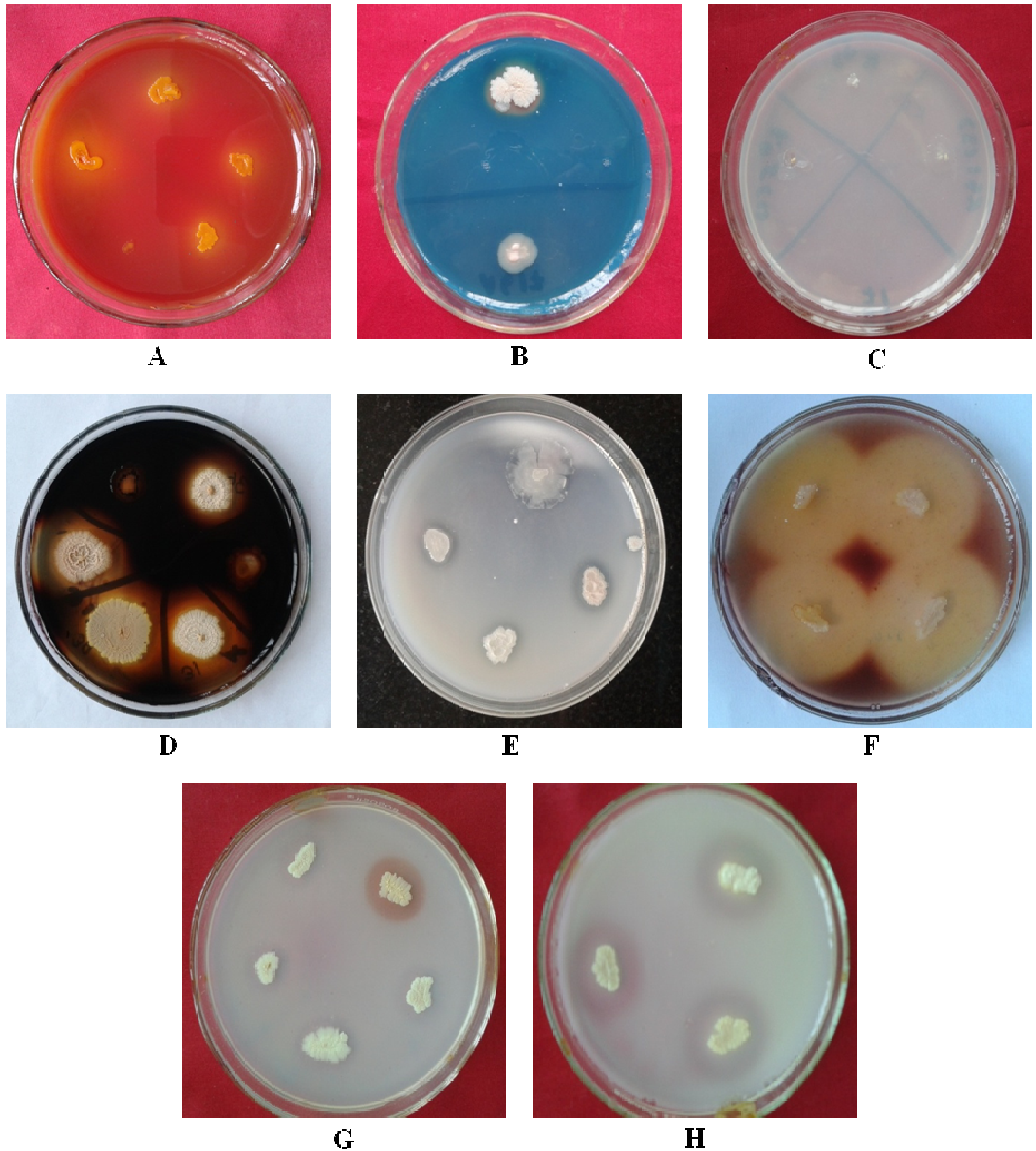


Plate 2: Screening of antagonistic bacterial antagonists from different horticultural crops for multifarious plant growth promoting traits and lytic enzymes production :

A) Phosphate Solubilization	**B) Siderophore production**
C) Growth on Nitrogen free medium	**D) Amylase**
E) Protease	**F) Chitinase**
G) Lipase	**H) Cellulase**

Table 4. Screening of bacterial antagonists from apple rhizosphere for multifarious PGP traits

Isolate	Phosphate solubilization		Siderophore production (zone size in mm)	Indole-3-acetic acid (µg/ml)	Growth on nitrogen free medium	Amylase	Cellulase	Lipase	Protease	Chitinase
	PSI*	SE%**				E.I.***	E.I.	E.I.	E.I.	E.I.
$RG_{1(3)}$	2.62	62.14	7	70.00	+	1.92	-	1.52	1.81	1.26
G2(6)	2.20	20	3.9	3.00	-	1.14	-	-	1.21	0.80
RG2(1)	2.18	18.2	6.1	1.50	-	-	-	1.12	-	0.99
R5(2)	0.00	0.00	4.78	2.00	+	-	0.23	-	0.89	1.34
R3(4)	2.39	38.8	5.2	4.00	-	0.79	0.45	-	-	1.35
CH_8A	2.65	64.52	11	17.00	+	1.51	1.1	1.46	1.73	1.24
CK_9A	2.58	30.1	-	5.50	-	-	-	1.11	-	1.55
KPO_8A	2.12	17.1	-	6.40	+	-	0.78	-	-	1.22
ES_3A	2.57	19.78	-	5.00	-	0.89	-	-	1.33	1.06
CH_3B	2.31	26.3	-	2.00	-	0.75	-	0.78	-	1.13

*, **, *** same as in table 3
+, - same as in table 3

4.3.3. Screening of bacterial antagonists from apricot rhizosphere against *C. michiganensis* for their multifarious PGP traits

Ten antagonistic isolates from apricot rhizosphere were screened for multifarious plant growth promoting traits viz. phosphate solubilization, siderophore production, IAA production, growth on nitrogen free medium, production of hydrogen cyanide and lytic enzymes (Table 5). Results revealed that none of the isolate was able to produce HCN. These isolates were tested for qualitative estimation of P solubilization. Maximum PSI was observed for the isolate $AG_{1(7)}$ (2.65), with corresponding value of SE% of 64.52% and minimum PSI was recorded for isolate $AS_{3(2)}$ (1.21), with corresponding SE% of 24.08%. Two isolates i.e. $AK_{1(4)}$ and $AP_{2(1)}$ were negative for P-solubilization (0.00).

The siderophore production of the bacterial isolates were estimated qualitatively which revealed that zone size ranged between 0.56 mm to 2.58 mm. Maximum zone was produced by isolate $AG_{1(7)}$ (2.58 mm) which was significantly higher than all other isolates and minimum zone (0.56 mm) was produced by isolate $AN_{1(1).}$

IAA was produced by all the ten (100%) isolates with production ranging from 3.00 µg/ml to 40.00 µg/ml. Maximum quantity of IAA was produced by the isolate $AG_{1(7)}$ (40.00 µg/ml) and minimum quantity by the isolate $AN_{5(2).}$

Phosphate solubilization was observed in eight (80%) isolates, six isolates (60%) were able to grow in nitrogen free medium, six isolates (60%) were able to produce siderophore on CAS medium, whereas all isolates (100%) were able to produce IAA.

The isolates from apricot rhizosphere were screened for production of different lytic enzymes i.e. amylase, cellulase, lipase, protease and chitinase. Out of ten isolates, five (50%) exhibited amylase activity. A lot of variation was observed in enzyme activity ranging from 0.65 E.I. to 1.97 E.I. Maximum amylase activity was recorded for isolate $AG_{1(7)}$ (1.97 E.I.) which was significantly higher than the enzyme activity recorded for isolate $AT_{1(1)}$ (0.65

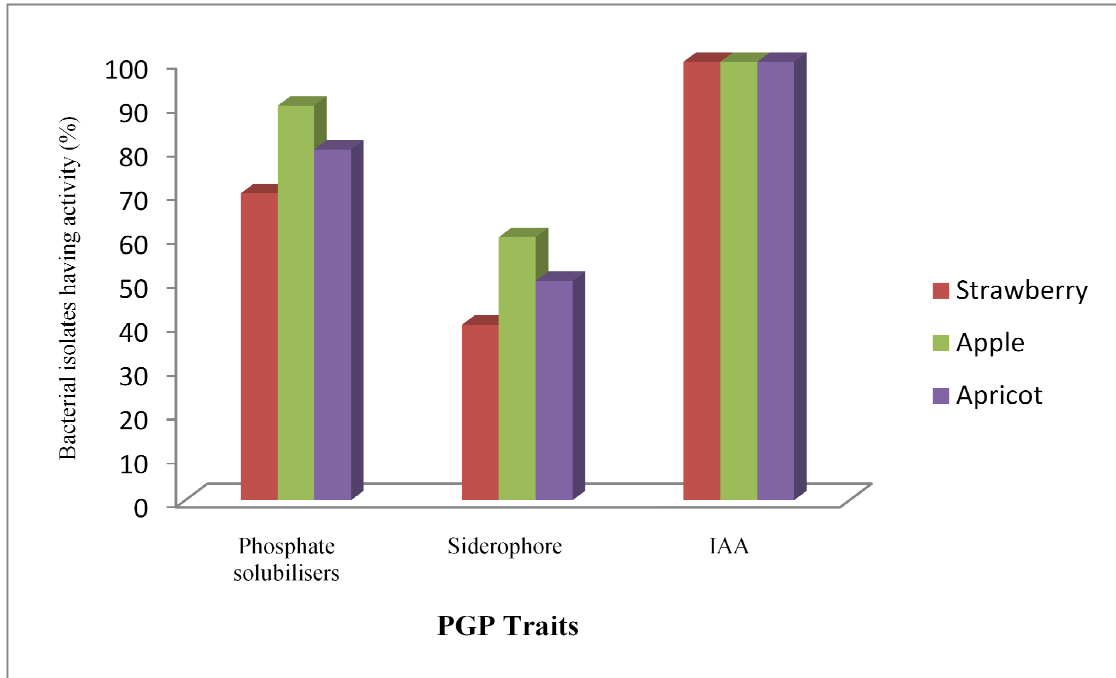


Fig1: Percentages of rhizobacteria from rhizosphere of strawberry, apple and apricot having PGP traits of P-solubilization, siderophore production and IAA production

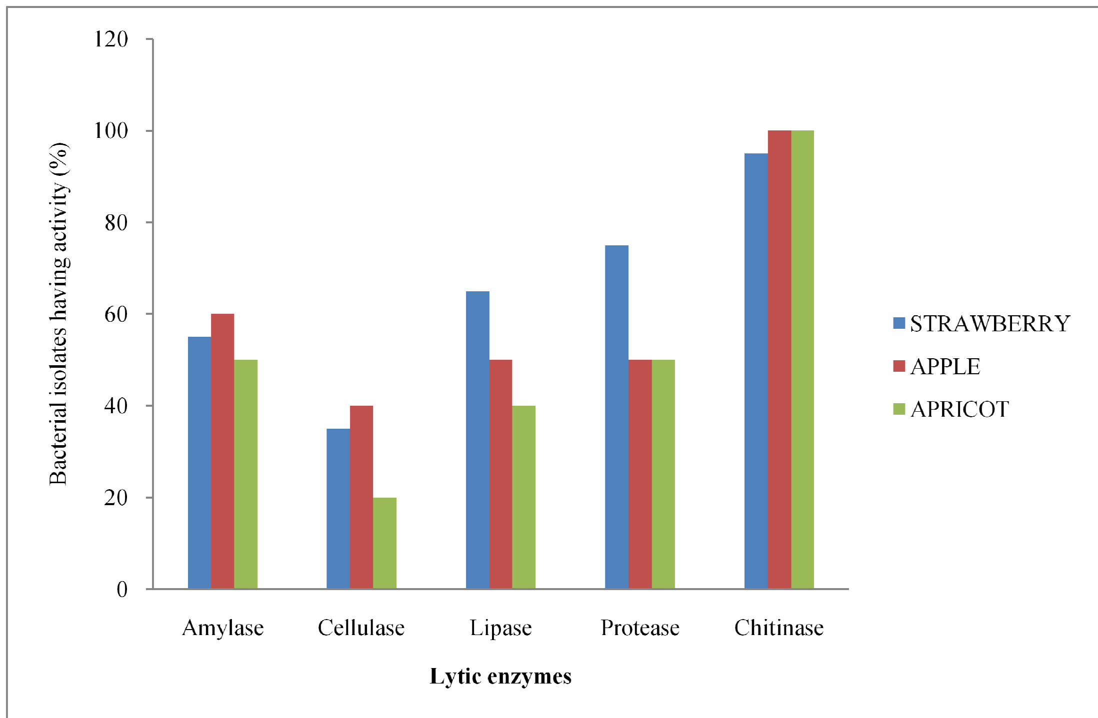


Fig. 2: Percentages bacterial antagonists from different horticultural crops viz. strawberry, apple, apricot for lytic enzymes production

E.I.) with minimum enzyme index. Two (20%) out of ten isolates exhibited cellulase activity. Enzyme activity observed on the basis of enzyme index was 1.32 E.I. for isolate $AT_{1(1)}$ and 0.65 E.I. for $AN_{5(2)}$. Four (40%) out of ten isolates exhibited lipase activity. Enzyme activity was in the range of 0.77 E.I. to 1.14 E.I. Maximum and minimum enzyme activities were also recorded for the isolates $AS_{3(2)}$ (1.14 E.I.) and $AK_{1(4)}$ (0.77 E.I.), respectively. Five (50%) out of ten isolates exhibited protease activity. Enzyme activity varied in the range of 1.15 E.I. to 1.52 E.I. Maximum and minimum enzyme activities were recorded for the isolates $AG_{1(7)}$ (1.52 E.I.) and $AT_{1(1)}$ (1.15 E.I.). All the isolates exhibited chitinase activity. Variation in enzyme activity was in the range of 1.15 E.I. to 1.5 E.I. Isolate $AG_{1(7)}$ exhibited maximum enzyme activity (1.50 E.I.) and minimum enzyme activity was exhibited by the isolate $AN_{1(1)}$ (1.15 E.I.).

Figure 2 shows the overall frequencies of bacterial antagonists for the production of lytic enzymes viz. Amylase, cellulase, lipase, protease and chitinase from three fruit crops i.e. strawberry, apple and apricot. Eleven (55%), out of twenty antagonists from strawberry rhizosphere were able to produce amylase, seven (35%) were cellulase producers, thirteen (65%) produced lipase, fifteen (75%) showed protease activity and nineteen (95%) showed chitinase activity. From the apple rhizosphere, six (60%) out of ten isolates were recorded with amylase activity, four (40%) with cellulase activity, five (50%) with lipase activity, five (50%) showed protease activity and all the ten (100%) showed chitinase activity.

However, five (50%) out of ten isolates from apricot rhizosphere were able to produce amylase, whereas, only two (20%) were able to produce cellulase, four (40%) were recorded with lipase and five (50%) with protease activity and all the ten (100%) produced chitinase.

Percentages of PGPRs having different lytic enzyme activity among different horticultural crops has been depicted in Fig. 2. Percentage of bacteria having amylase activity was arranged in the decreasing order, apple (60%) > strawberry (55%) > apricot (50%). The ratio of bacterial antagonists showing

Table 5. Screening of bacterial antagonists from apricot rhizosphere for multifarious PGP traits

Isolate	Phosphate solubilization		Siderophore production (zone size in mm)	Indole-3-acetic acid (µg/ml)	Growth on nitrogen free medium	Amylase	Cellulase	Lipase	Protease	Chitinase
	PSI*	SE%**				E.I.***	E.I.	E.I.	E.I.	E.I.
$AP_{3(1)}$	2.57	33.10	2.11	10.00	-	-	-	0.98	1.21	1.24
$AP_{2(1)}$	0.00	0.00	2.33	11.00	+	1.24	-	-	-	1.22
$AT_{1(1)}$	2.76	30.56	-	5.00	+	0.65	1.32	-	1.15	1.25
$AG_{1(7)}$	2.65	64.52	2.58	40.00	+	1.97	-	-	1.52	1.50
$AG_{5(1)}$	2.11	28.6	-	6.50	-	1.43	-	-	-	1.30
$AK_{1(4)}$	0.00	0.00	0.91	7.00	-	-	-	0.77		1.26
$AN_{5(2)}$	2.56	41.12	-	3.00	+	-	0.67	0.86	-	1.19
$AN_{1(1)}$	1.68	25.6	0.56	4.00	-	-	-	-	1.30	1.15
$AN_{2(2)}$	2.58	38.23	-	6.00	+	1.2	-	-	-	1.33
$AS_{3(2)}$	1.21	24.08	-	4.50	+	-		1.14	1.17	1.45

*, **, *** = same as in table 3

+, - same as in table

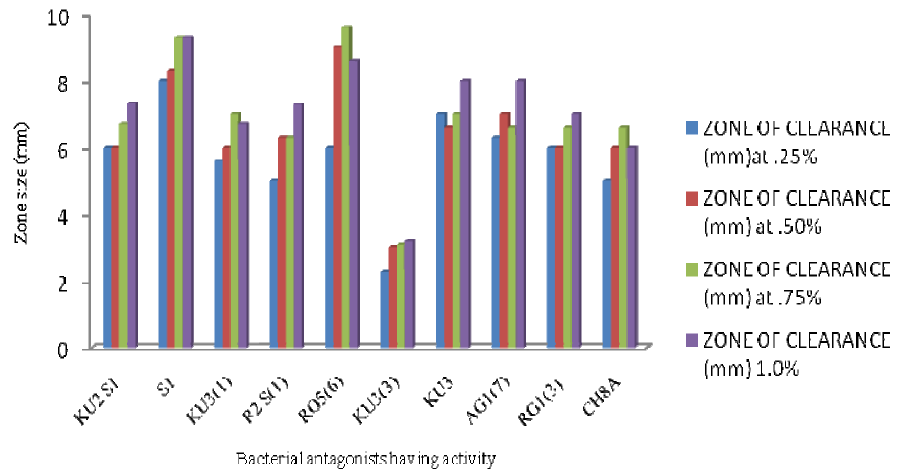


Fig 3: **Antibacterial activity of selected bacterial antagonists in cell free supernatant against *Clavibacter michiganensis***

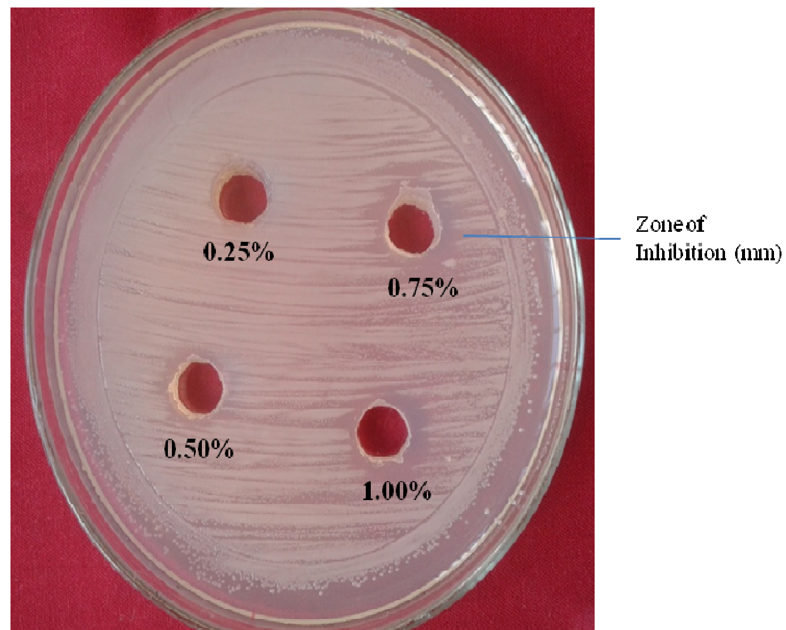


Plate 3. **Evaluation of antibacterial metabolite from cell free supernatant by selected antagonistic bacterial isolates against *Clavibacter michiganensis***

cellulase activity are arranged in the order, apple (40%) > strawberry (35%) > apricot (20%). Lipase activity have also been represented in the decreasing order as strawberry (65%) > apple (50%) > apricot (40%). The order of protease activity was found to be strawberry (75%) > apple (50%) = apricot (50%). However, chitinase activity followed the order of apple (100%).

4.4 ANTIBACTERIAL ACTIVITY OF SELECTED ANTAGONISTIC BACTERIAL ISOLATES AGAINST *C. michiganensis* AT DIFFERENT CONCENTRATIONS OF CELL FREE SUPERNATANT

Fig. 3 revealed that ten bacterial antagonists selected on the basis of their plant growth promoting traits showed variation for antibacterial metabolite production. The *in vitro* antibacterial metabolite production by ten selected bacterial antagonists was tested using the cell free supernatant of 48h old cultures of bacterial antagonists against *Clavibacter michiganensis* at four different concentrations i.e. 0.25% (v/v), 0.50% (v/v), 0.75% (v/v) and 1.00% (v/v) (Fig. 3). Cell free supernatant of seven isolates from strawberry, two from apple and one from apricot rhizospheres were studied for the inhibition against *Clavibacter michiganensis*.

A lot of variation was observed in size of zone of inhibition, ranging from 2.28 mm to 9.6 mm (Plate 3). The maximum antibacterial activity in cell free supernatant at 0.25 per cent concentration (v/v) was recorded for the bacterial antagonist S1 with 8 mm zone of clearance, which increased to 9.3 mm with the increase in concentration upto 0.75%, but with further increase in concentration to 1.00%, zone size remained stable. Following S1, isolate $RO_{5(6)}$, showed the zone of clearance of 6 mm at 0.25% concentration, which increased to 9.6 mm with the increase in concentration to 0.75%. But, with further increase in the concentration to 1.00%, decrease in zone size to 8.6 mm was observed. Whereas, minimum activity was shown by the isolate $KU_{3(3)}$ with zone of clearance of 2.28 mm at 0.25% concentration, which increased subsequently to 3.2 mm with the increase in concentration (v/v) upto 1.00%.

4.5 SELECTION OF ANTAGONISTIC BACTERIAL ISOLATES AGAINST *Clavibacter michiganensis* BASED ON SIMILARITY COEFFICIENT DERIVED FROM THEIR MULTIFARIOUS PGP TRAITS

A total of forty antagonistic isolates from rhizospheres of different horticultural crops viz. strawberry, apple and apricot were screened for multifarious PGP traits. Data generated on the basis of PGP traits were subjected to cluster analysis. Dendrogram was constructed using the unweighted pair-group method with airthmatic mean (UPGMA).

Cluster analysis on PGP traits grouped the forty rhizobacterial antagonists into two main clusters at 52 per cent similarity level (Fig. 4). The upper cluster was divided into two sub clusters at 60 % similarity level. The upper sub cluster consisted of eleven isolates at 73 % similarity level, with three isolates i.e. R_2S1, $Ra_{2(2)}$ and $AS_{3(2)}$ and two isolates i.e. $Ra_{31(5)}$ and $AN_{5(2)}$ showing 100 per cent similarity among them. The lower sub cluster was divided into two groups at 66 per cent similarity level. The upper group was further divided into two sub groups at 68 per cent similarity level. The upper sub group consisted of four isolates at 74 per cent similarity level, with two isolates i.e. NA(2) and $RO_{5(6)}$ showing 100 per cent similarity among them. The lower sub group consisted of eight isolates at 74 per cent similarity level, with isolates KU_3, $AP_{3(1)}$ and $RO_{5(1),}$ CK_9A showing 100 per cent similarity with each other. The lower group was divided into two sub groups at 73 per cent similarity level. At 80 per cent similarity level, the upper sub group consisted of seven isolates, with isolates S1 and $RG_{1(3)}$, and $RO_{2(7)}$ and G2(6) showing 100 per cent similarity among them, while four isolates were present in the lower sub group at 83 per cent similarity level.

The lower cluster consisted of only six isolates with 60 % similarity. One isolate i.e. $KU_{1(5)}$ was present singly, whereas, other five isolates were present in a cluster at 65 % similarity level. This indicated that the isolate $KU_{1(5)}$ was totally different from other five isolates grouped in cluster with respect to PGP traits.

Out of total, ten isolates were further selected on the basis of their antagonistic activity and multifarious PGP traits. Five isolates i.e. KU_2 S1, $KU_{3(1),}$

R_2S1, $KU_{3(3)}$ and CH_8A, which were showing atleast three PGPTs of P-solubilization, siderophore production, growth on N free medium, lytic enzyme production were selected from upper sub cluster, along with inhibition zone ranging from 8.43 to 10.33 mm. However, five antagonistic bacterial isolates i.e. $RO_{5(6)}$, $RG_{1(3)}$, KU_3, S1 and $AG_{1(7)}$, from lower sub cluster were selected for further study. From selected isolates, maximum inhibition zone (12.0 mm) was observed for isolate S1 from strawberry rhizosphere followed by isolates KU_2S1 and $AG_{1(7)}$ (10.33 mm) from strawberry and apricot rhizospheres, respectively.

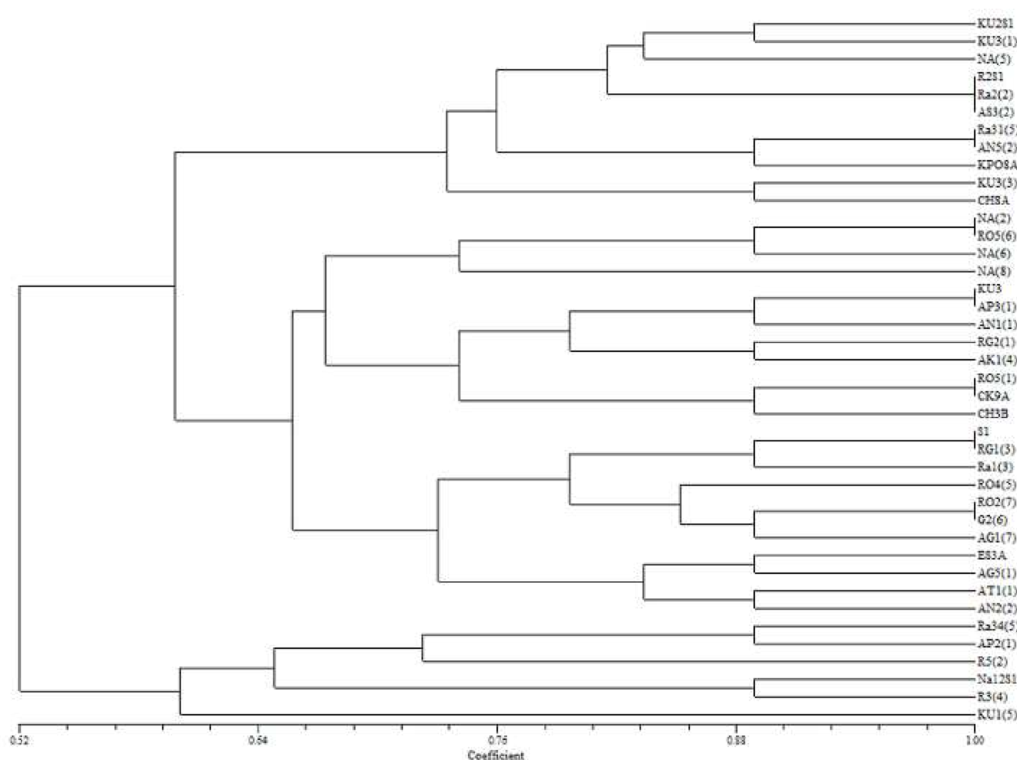


Fig 4. Dendrogram showing relationship among bacterial antagonists based on similarity coefficient derived from their multifarious plant growth promoting traits

4.6 PHENOTYPIC AND METABOLIC CHARACTERIZATION OF TEN SELECTED ANTAGONISTIC BACTERIAL ISOLATES

4.6.1 Morphological characterization of ten selected antagonistic bacterial isolates

The results in Table 6 depicts the colony morphology, Gram's reaction, cell shape and arrangement of selected bacterial antagonists. All isolates were positive for Gram's reaction except one isolate ($RO_{5(6)}$), which was found to be

gram negative. All the antagonists were rod shaped. Most of the isolates occured singly while one isolate i.e. $KU_{3(3)}$ was arranged in chains when observed microscopically. From the results it was revealed that all the selected isolates shared variable morphological features with respect to their colony shape, margin and elevation and appeared as circular, irregular, punctiform, undulate, flat, creamish, raise, curled, entire and convex for different selected antagonists.

Table 6. Morphological characterization of ten selected antagonistic isolates

Isolates	Colony morphology	Gram's Reaction	Shape	Arrangement
KU_2 S 1	Creamish,circular, raised, entire margin	+	Rods	Single
S 1	Creamish, irregular, flat, undulate	+	Rods	Single
$KU_{3(1)}$	Creamish, circular, raised, curled	+	Rods	Single
$R_2 S_{(1)}$	White, circular, convex, entire margin	+	Rods	Single
$RO_{5(6)}$	Creamish, circular, raised, entire margin	-	Rods	Single
$KU_{3(3)}$	White, irregular, flat, undulate margin	+	Rods	Chains
KU_3	Creamish, irregular, flat, undulate margin	+	Rods	Single
$AG_{1(7)}$	White, punctiform, flat, undulate margin	+	Rods	Single
$RG_{1(3)}$	Creamish, irregular, flat, undulate margin	+	Rods	Single
71	Creamish, irregular, flat, undulate	+	Rods	Single

4.6.2 Metabolic fingerprinting of antagonistic PGPRs on the basis of their biochemical tests

Figure 5 showed the dendrogram based on cluster analysis of biochemical characters and carbon utilisation tests (Table 7). Cluster analysis showed that at 74% similarity level ten isolates were divided into two clusters, with lower cluster consisting of only one isolate S1, whereas other nine isolates were present in the upper cluster.

At 81% similarity level the upper cluster was divided into two groups, with upper group containing seven isolates and lower group containing two isolates. The upper group was divided into two sub groups at 83 % similarity

Table 7. Metabolic fingerprinting of ten selected antagonist PGPRs based on biochemical tests

Isolate	Catalase	Urease	Indole Production	Citrate Utilisation	H_2S Production	Ammonia Production	Methyl Red	Sugar				
								Dextrose	Maltose	Fructose	Sucrose	Lactose
KU_2S1	+	-	-	+	-	+	+	+	+	-	-	-
CH_8A	+	+	-	-	+	-	+	-	+	-	-	-
$RG_{1(3)}$	+	-	-	+	-	+	-	-	+	-	-	-
R_2S1	+	-	-	+	-	+	-	-	+	+	-	-
KU_3	+	-	-	+	-	+	+	-	+	+	-	-
$KU_{3(3)}$	+	-	-	+	-	+	-	-	+	+	-	-
$AG_{1(7)}$	+	+	-	+	-	+	+	-	+	+	-	-
$KU_{1(3)}$	+	+	-	+	-	+	+	-	+	-	-	-
$RO_{5(6)}$	+	+	-	+	-	+	+	-	-	-	-	-
S 1	+	-	-	-	-	+	+	-	-	+	-	-

+ Antagonists with activity

- Antagonists without activity

level, whereas the lower group consist of two isolates showing 92% similarity amongst them.

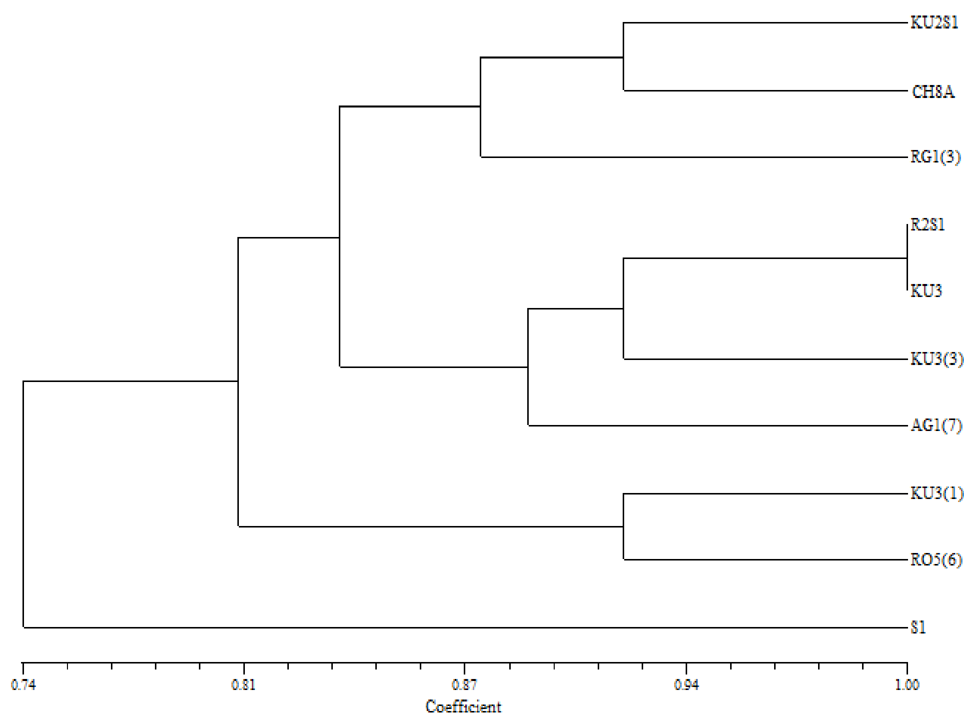


Fig. 5. Cluster analysis of antagonistic bacterial isolates against *C. michiganensis* based on similarity coefficient derived from their metabolic fingerprinting

The upper subgroup further divided the seven isolates into two sub-subgroups. The upper sub-sub group comprised of four isolates. In lower sub-sub group isolates R_2 S1 and KU_3 were showing 100% similarity with each other. These isolates were positive for catalase, citrate utilisation, fructose and maltose fermentation, whereas showed negative results for urease, indole production, H_2S production, methyl red, dextrose, sucrose and lactose fermentation (Table 7).

4.7 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) STUDIES

4.7.1 Extraction of Genomic DNA

Total genomic DNA of selected ten antagonistic bacterial isolates were extracted using conventional method as described in section 3.9. Presence of DNA and its quality was checked by running it on 1.0% agarose gel (Plate 4).

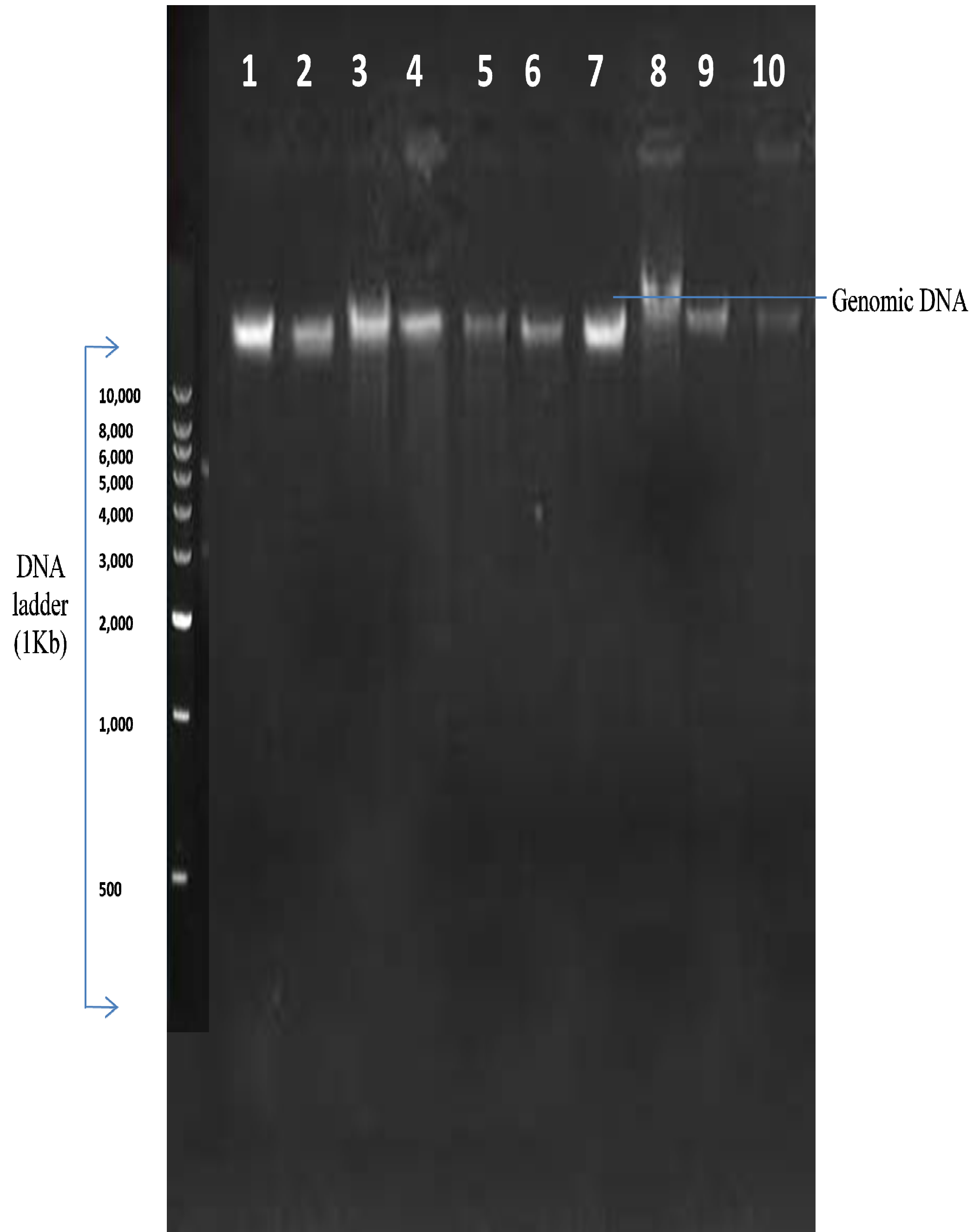


Plate 4 . Genomic DNA of ten selected antagonistic bacterial isolates

Lane 1, KU_2 $S1_1$; Lane 2, $KU_{3(1)}$; Lane 3, S1; Lane 4, $AG_{1(7)}$; Lane 5 $R_2S(1)$, ; Lane 6, $RO_{5(6)}$; Lane 7, CH_8A; Lane 8, KU_3; Lane 9, $KU_{3(3)}$; Lane 10, $RG_{1(3)}$

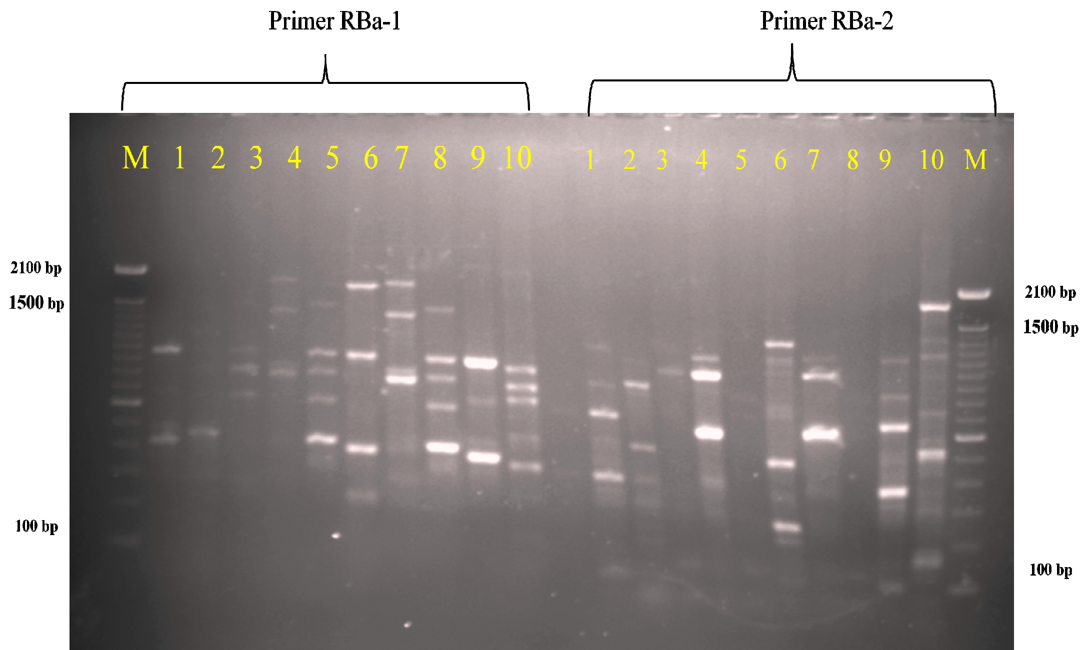


Plate 5a. RAPD profile of antagonist bacterial isolates (1-10) using primers RBa-1, RBa-2

Lane M, DNA marker; Lane 1, KU$_2$ S1$_1$; Lane 2, KU$_{3(1)}$; Lane 3, S1; Lane 4, AG$_{1(7)}$; Lane 5 R$_2$S(1), ; Lane 6, RO$_{5(6)}$; Lane 7, CH$_8$A; Lane 8, KU$_3$; Lane 9, KU$_{3(3)}$; Lane 10, RG$_{1(3)}$

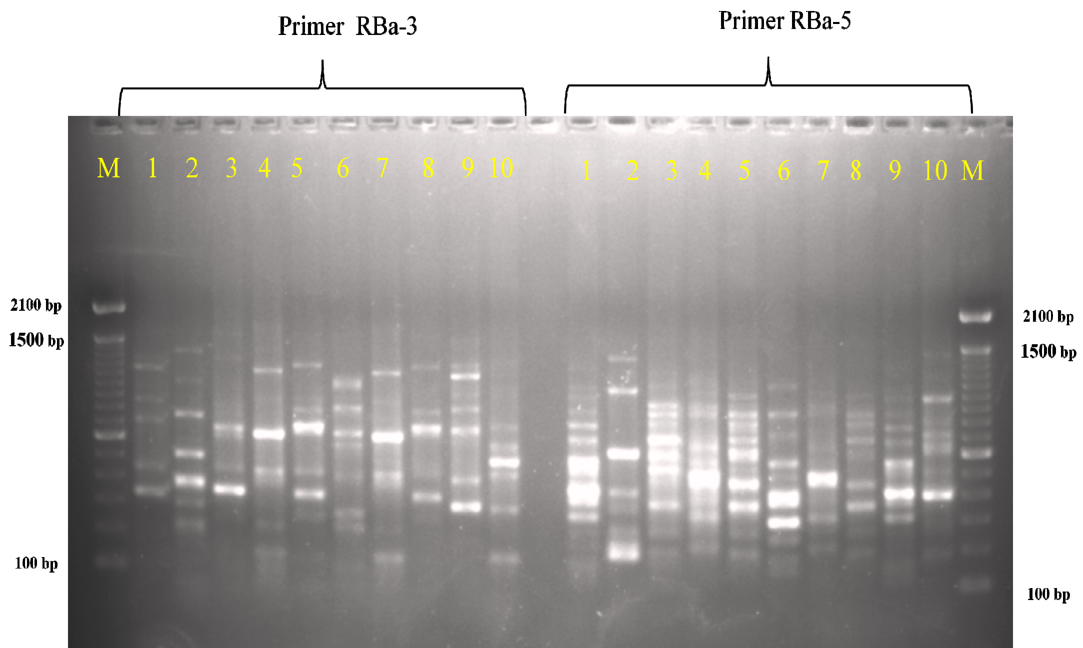


Plate 5b. RAPD profile of antagonist bacterial isolates (1-10) using primers RBa-3 and RBa-5

Lane M, DNA marker; Lane 1, KU$_2$ S1$_1$; Lane 2, KU$_{3(1)}$; Lane 3, S1; Lane 4, AG$_{1(7)}$; Lane 5 R$_2$S(1), ; Lane 6, RO$_{5(6)}$; Lane 7, CH$_8$A; Lane 8, KU$_3$; Lane 9, KU$_{3(3)}$; Lane 10, RG$_{1(3)}$

4.7.2 Random amplified polymorphic DNA (RAPD) studies

Genetic diversity amongst ten antagonistic bacterial isolates selected on the basis of zone of inhibition (mm) against *Clavibacter michiganensis* and their PGP traits was analysed using RAPD-PCR analysis. Five random decamer oligonucleotide primers (RBa-1, RBa-2, RBa-3, RBa-4 and RBa-5) were screened initially with two representative bacterial antagonists. Four primers (RBa-1, RBa-2, RBa-3 and RBa-5) producing best results of amplification (expressed as average number of bands per primer) were further selected for RAPD-PCR analysis of ten antagonistic isolates (Plate 5a and 5b). The distribution of polymorphic bands among ten antagonistic isolates are summarized in Table 8.

The similarity cofficient revealed that the similarity among ten antagonistic bacterial isolates ranged between 60 to 100 per cent.

Table 8. Summary of the RAPD amplified products obtained from selected ten antagonistic bacterial isolates using four primers

Total number of Primer screened	5
Total number of Primer examined	4
Total number of bands amplified from Primer (RBa-5)	77
Total number of polymorphic bands identified	205
Size range of amplified products	110-2100bp
Per centage of total polymorphic bands	100%

The results from Fig. 6 revealed that first major bifurcation divided the ten bacterial antagonists into two main clusters. Upper cluster consisted single isolate $KU_{3(3)}$, which was selected for biocontrol studies under net house. Lower cluster consisted of nine isolates, which further segregated into two sub clusters. Upper sub cluster consisted of three isolates (KU_3, KU_2 S1 and R_2S1), with isolates KU_3 and KU_2 S1 showing 68 per cent similarity amongst them. The isolate KU_3 from upper sub cluster was selected for biocontrol studies under net house conditions.

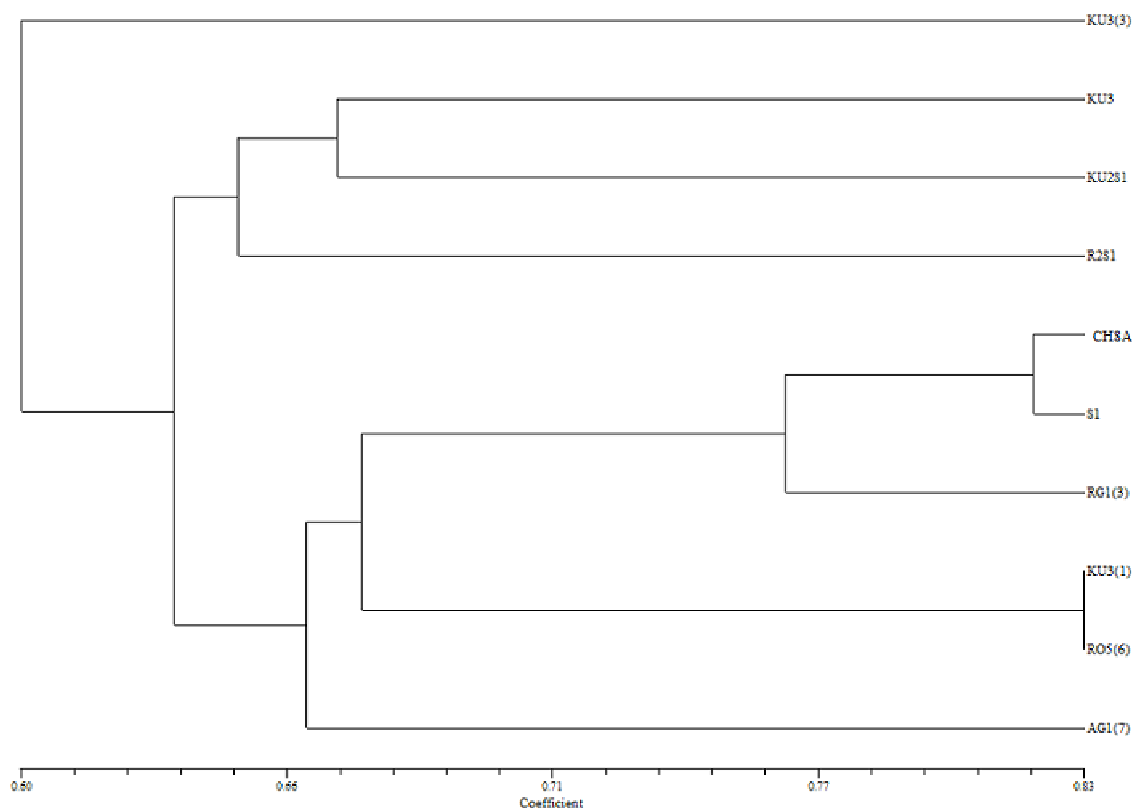


Fig. 6 Dendrogram showing the genetic relationship amongst selected 10 antagonistic isolates based on RAPD analysis

The lower sub cluster comprised of six isolates (CH_8A, S1, $RG_{1(3)}$, $KU_{3(1)}$, $RO_{5(6)}$ and $AG_{1(7)}$), with two isolates i.e. $KU_{3(1)}$ and $RO_{5(6)}$ showing 100 per cent similarity among them. Three isolates (CH_8A, S1 and $RG_{1(3)}$) from the lower sub cluster were also evaluated for biocontrol potential against *Clavibacter michiganensis* under the net house conditions.

4.8 QUANTITATIVE ESTIMATION OF PLANT GROWTH PROMOTING TRAITS BY SELECTED ANTAGONISTIC ISOLATES

Ten selected bacterial antagonists were assessed for quantitative estimation of PGP traits after 72h of incubation at 37°C and pH 7 under shaking conditions (110 rpm) and were compared on the basis of their phosphate solubilizing activity (µg/ml), % siderophore unit and IAA production (µg/ml). A significant variation was observed among the bacterial antagonists for these PGP traits.

4.8.1 Quantitative estimation of P-solubilization by selected bacterial antagonists

Phosphate solubilizing activity of bacterial antagonists was estimated quantitatively on the basis of their P-solubilization (µg/ml) in PVK liquid medium. The results (Table 9) revealed that the individual isolate effectively solubilized the insoluble tri-calcium phosphate in liquid medium. Among all the ten isolates maximum P-solubilization was recorded for isolates $RG_{1(3)}$ (258.00 µg/ml) which was statistically at par with the isolate KU_2 S1 (255.50 µg/ml) and was significantly higher than the phosphate solubilized by all the other isolates, with follwing viable count of 21.50 × 10^7 cfu/ml and pH of 5.09. Minimum P-solubilization was recorded for isolates R_2 $S_{(1)}$ (50.00 µg/ml) which was statistically at par with $KU_{3(3)}$ (52.00 µg/ml).

Maximum viable count (25.00 × 10^7 cfu/ml) was recorded in isolate S1 and maximum decrease in final pH of supernatant (5.00) was also recorded in case of isolate S1. Viable count after 72 hours of incubation ranged between minimum 11.70 × 10^7 cfu/ml (KU_3) to maximum 25.00 × 10^7 cfu/ml (S 1) whereas final pH of supernatant varied from minimum 4.7 (S 1) to maximum 5.19 (KU_3) as compared to initial pH 7.0.

Table 9. Screening of antagonistic bacterial isolates for quantitative estimation of tricalcium phosphate solubilization at 72 h of incubation

Isolates	P-solubilization (µg/ml)	Viable Count (10^7 × cfu/ml)	Final pH of Supernatant
KU_2 S1	255.50	17.80	5.01
S 1	156.00	25.00	4.70
$KU_{3(1)}$	253.00	21.00	5.00
$R_2 S_{(1)}$	50.00	12.50	5.05
$RO_{5(6)}$	55.50	13.90	5.12
$KU_{3(3)}$	52.00	19.80	5.10
KU_3	250.00	11.70	5.19
$AG_{1(7)}$	150.00	20.10	5.02
$RG_{1(3)}$	258.00	21.50	5.09
CH_8A	54.00	15.90	5.01
lsd	7.38	1.55	0.29

4.8.2 Indole-3-acetic acid production by selected bacterial antagonists

The antagonistic rhizobacterial isolates were screened for IAA production in Luria Bertani broth ammended with tryptophan after 72h of incubation. Perusal of data in Table 10 revealed that isolate $RG_{1(3)}$ produced significantly higher concentration of IAA (70.00 µg/ml) after 72 hours of incubation. Final pH of the supernatant ranged between 5.30 ($RG_{1(3)}$) to maximum 5.9 ($KU_{3(3)}$) as compared to initial pH 7.0. Minimum IAA (17.00 µg/ml) was recorded for isolate CH_8A.

Maximum viable count recorded for isolate $AG_{1(7)}$ was 25.70×10^7 cfu/ml. Viable count ranged between minimum 13.20×10^7 cfu/ml in isolate $RG_{1(3)}$ to maximum 25.70×10^7 cfu/ml in isolate $AG_{1(7)}$.

Table 10. Indole-3-acetic acid production by bacterial antagonists in Luria Bertani broth with tryptophan at 72h of incubation

Isolates	Indole-3-acetic acid (µg/ml)	Viable Count ($10^7 \times$ cfu/ml)	Final pH of supernatant
KU_2 S1	24.00	15.60	5.50
S 1	27.00	23.10	5.70
$KU_{3(1)}$	35.00	15.90	5.66
$R_2 S_{(1)}$	23.00	15.50	5.50
$RO_{5(6)}$	20.00	18.20	5.44
$KU_{3(3)}$	22.00	18.40	5.90
KU_3	24.00	18.40	5.77
$AG_{1(7)}$	40.00	25.70	5.80
$RG_{1(3)}$	70.00	13.20	5.30
CH_8A	17.00	14.50	5.39
lsd	1.68	0.95	0.34

4.8.3 Quantitative estimation of siderophore production by selected bacterial antagonists

Quantitative estimation of siderophore using chromazurol S (CAS) liquid assay revealed that isolates S1 and $R_2S(1)$ produced maximum (230.77% siderophore unit) at 72 hours of incubation, with following viable count of 23.80×10^7 and 21.70×10^7 cfu/ml and final pH of 5.20 and 5.30, respectively (Table 11).

Table 11. Siderophore production by bacterial antagonists on CAS medium after 72h of incubation

Isolates	Quantitative estimation (% S.U.)	Viable Count ($10^7 \times$ cfu/ml)	Final pH of supernatant
KU_2 S 1	128.21	12.80	5.64
S 1	230.77	23.80	5.20
$KU_{3(1)}$	128.21	18.10	5.66
$R_2S(1)$	230.77	21.70	5.30
$RO_{5(6)}$	179.49	19.60	5.33
$KU_{3(3)}$	128.21	18.50	5.42
KU_3	128.21	20.80	5.60
$AG_{1(7)}$	179.47	12.70	5.20
$RG_{1(3)}$	179.47	12.90	5.65
CH_8A	128.21	20.20	5.35
lsd	5.51	1.83	0.43

Maximum viable count at 72 hours of incubation corresponded to isolate S 1 (23.80×10^7 cfu/ml). Maximum decrease in final pH of supernatant (5.20) was recorded in case of two isolates i.e. S1 and $AG_{1(7)}$, which otherwise ranged between minimum 5.20 to maximum 5.66 ($KU_{3(1)}$) as compared to initial pH of 7.0.

4.9 NET HOUSE STUDIES

4.9.1 Biocontrol studies

In planta evaluation of best five antagonistic strains against *C. michiganensis* revealed that the tested strains decreased disease incidence of bacterial canker of tomato under net house conditions (Table 12). The canker symptoms in control plants appeared after two weeks since the seedlings were transplanted. But the disease incidence and index were lower as compared to complete outbreak of the disease which came after four weeks of transplantation. Temperature of net house varied between 25-35°C and relative humidity between 50-100% during the course of the experiment .The disease incidence and index were assessed after four weeks since transplantation. All the isolates showed significant decrease in disease incidence over control. Isolate S1 showed minimum per cent disease incidence over control. Isolate S1 showed significant

decrease in disease incidence (30%), as well as maximum biocontrol efficacy (70%), which was significantly higher than control and all other isolates tested under net house conditions. Whereas, maximum disease incidence was recorded with isolate KU_3 (85%) over control with minimum biocontrol efficacy of 15% (Plate 6).

Out of five, the disease index was found to be significantly lower for biocontrol treatment with isolate S1 (28.55%) as compared to control and all other treatments followed by the isolate $KU_{3(3)}$ with disease index of 43.75%.

The population of both antagonist and pathogen in the rhizosphere of tomato showed different levels. Minimum population of pathogen i.e. 7×10^6 cfu/ml and 11×10^6 cfu/ml was found with the isolate S1 and $KU_{3(3)}$. In, addition the antagonist count in the rhizosphere of soil treated with isolate S1 and $KU_{3(3)}$ was 72×10^6 cfu/ml and 64×10^6 cfu/ml, respectively, which was significantly higher than other antagonistic isolates.

Table 12. Biocontrol efficacy of PGPR strains in controlling bacterial canker caused by *Clavibacter michiganensis*

Treatment	Percent Disease Index (%)*	Percent Disease Incidence**	Biocontrol Efficacy (%)***	Bacterial population of pathogen (cfu/ml)	Bacterial population of antagonist (cfu/ml)
CONTROL	82.80 (65.57)	100 (90.00)	00 (0.00)	112×10^6	2×10^7
S 1	28.55 (32.17)	30 (32.90)	70 (57.10)	7×10^6	72×10^6
$KU_{3(3)}$	43.75 (41.38)	50 (45.00)	50 (45.00)	11×10^6	64×10^6
KU_3	77.62 (61.81)	85 (70.08)	15 (19.92)	34×10^6	18×10^6
$RG_{1(3)}$	56.47 (48.75)	65 (54.22)	35 (35.78)	23×10^6	34×10^6
CH_8A	65.75 (54.26)	70 (57.10)	30 (32.90)	28×10^6	26×10^6
lsd	5.71	13.05	13.05	5.32	3.27

Percent disease Index* = $\frac{\Sigma\ (Rating \times No.\ of\ plants\ rated)}{Total\ number\ of\ plants\ observed \times Highest\ rating} \times 100$

Per cent disease incidence** = $\frac{No.\ of\ diseased\ plants}{Total\ no.\ of\ plants\ observed} \times 100$

Biocontrol Efficacy*** = $\frac{Disease\ incidence\ of\ control - disease\ incidence\ of\ antagonist\ treated}{Disease\ incidence\ of\ control} \times 100$

Figure in Parenthesis () are arc sign transformed values

4.9.2 Growth Promotion

4.9.2.1 Effect on shoot Parameters

Data appended in Table 13 illustrated maximum shoot length (49.17 cm) in case of seedlings treated with isolate S1 which was significantly higher than control (26.87 cm) and all other isolates. However, shoot length in case of seedlings treated with isolate $KU_{3(3)}$ was 33.73 cm, which was significantly at par with the shoot length of the isolate $RG_{1(3)}$ (33.50 cm) and was significantly higher than control. Whereas, minimum shoot length was recorded for the isolate CH_8A (28.37 cm), which was significantly lower than all other isolates.

Increase in shoot dry weight was observed with all the isolates. Seedling treatment with isolate S1 resulted in maximum shoot dry weight (15.10 g) which was significantly higher than uninoculated seedlings (9.67 g). Among five isolates, S1 inoculation showed maximum per cent increase in shoot length (73.32%) which was statistically significant than other isolates. Per cent increase in shoot dry weight was found maximum with S1 inoculation (56.15%) which was statistically significant than other isolates. However, minimum per cent increase (17.92%) in shoot dry weight was observed with isolate CH_8A inoculation (Fig. 7, Table 13).

4.9.2.2 Effect on root Parameters

Perusal of Table 13 revealed that seedlings treated with isolate S1 resulted in maximum root dry weight (3.23 g) which was significantly higher than the seedlings raised from uninoculated seeds (1.53 g). Maximum root length (7.43 cm) was observed in case of seedlings treated with isolate S1 which was statistically higher than the seedlings raised from uninoculated control (2.33 cm), $KU_{3(3)}$ (3.17 cm), KU_3 (3.13 cm), $RG_{1(3)}$ (3.32 cm) and CH_8A (3.03 cm). Treatment with isolate isolate S1 showed maximum per cent increase in root length (218.88%) which was statistically significant than other isolates. The maximum per cent increase in root dry weight (111.11%) was observed in S1 inoculation which was statistically significant than other isolates.

Out of five antagonistic PGPRs, isolate S1 used for treating tomato seedlings showed significant control of bacterial canker of tomato caused by *Clavibacter michiganensis* as well as increase in different growth parameters as compared to untreated seedlings kept as control and treated seedlings of different isolates. Therefore, isolate S1 was also characterized by morphological, physiological, biochemical and molecular based characterization.

Table 13. Effect of liquid formulation of selected bacterial isolates on tomato seedlings and their growth promotion under net house conditions

Treatment	Shoot length (cm)	Root length (cm)	Shoot dry weight (g)	Root dry weight (g)
Control	26.87	2.33	8.20	1.53
S 1	49.17	7.43	15.1	3.23
$KU_{3(3)}$	33.73	3.17	14.57	2.46
KU_3	31.90	3.13	13.37	2.40
$RG_{1(3)}$	33.50	3.32	14.30	2.46
CH_8A	28.37	3.03	9.67	2.06
lsd	4.89	0.24	0.39	0.24

4.10 OPTIMIZATION OF CULTURAL CONDITIONS FOR THE ANTAGONISTIC EFFECT OF THE BEST SELECTED BACTERIAL ISOLATE S1 AGAINST *C. michiganensis*

4.10.1 Effect of incubation period on antagonistic activity against *C. michiganensis* by selected bacterial isolate S1

The antagonistic activity was monitored for 24, 48, 72, 96 and 120h in nutrient broth containing 1% inoculum. The results from Fig. 8 revealed that the viable count and antagonistic activity increased with increase in incubation period from 0 to 48h and after that decrease in the antagonistic activity was observed. Maximum antagonistic activity (12 mm) was obtained at 48h of incubation corresponding to maximum viable count (193×10^6 cfu/ml). Minimum antagonistic activity (3 mm) and corresponding viable count (171×10^6 cfu/ml) was recorded at 24h of incubation, whereas, no antagonistic activity was recorded at 96 and 120h of incubation, with corresponding viable count (21×10^6 cfu/ml and 9×10^6 cfu/ml), respectively. Statistical analysis revealed that there was significant difference between the values obtained for all parameters studied at different incubation period.

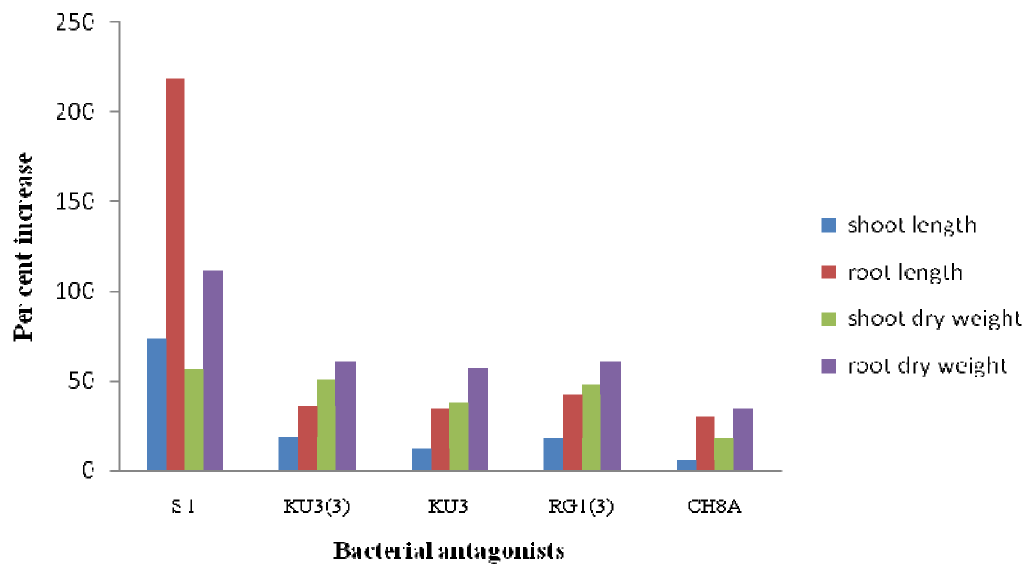
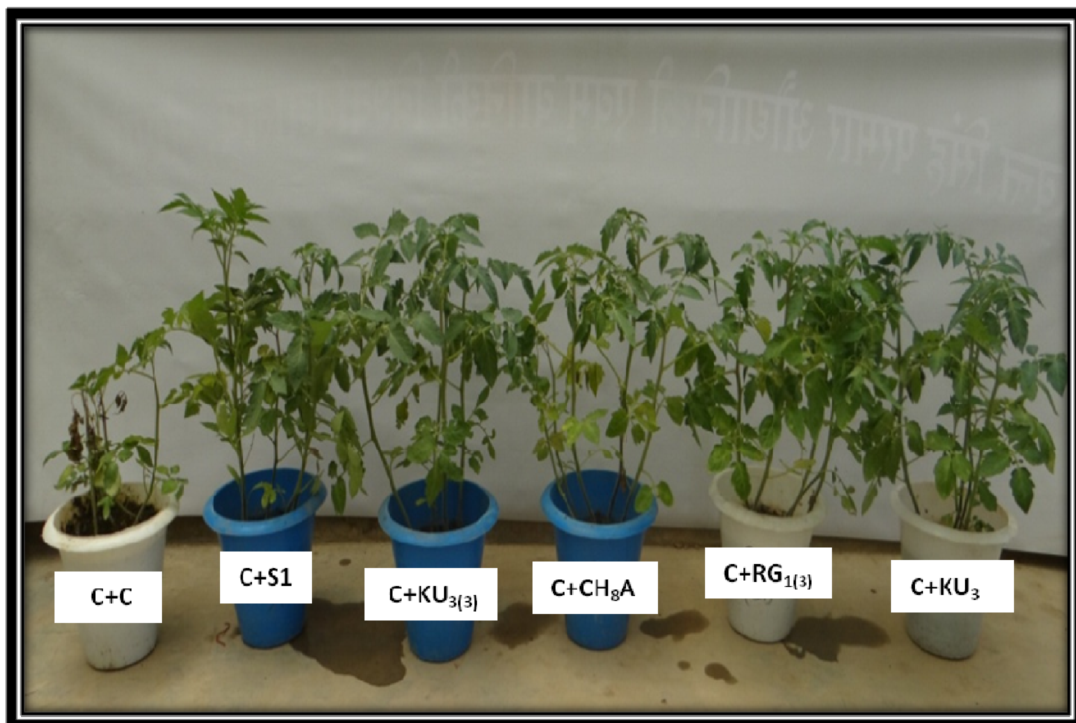


Fig. 7: Per cent increase in plant growth parameters under net house conditions



C+C	=	Treatment with *C. michiganensis*
C+S1	=	Treatment with *Clavibacter* and antagonist S1
C+$KU_{3(3)}$	=	Treatment with *Clavibacter* and antagonist KU3(3)
C+ CH_8A	=	Treatment with *Clavibacter* and antagonist CH8(A)
C+$RG_{1(3)}$	=	Treatment with *Clavibacter* and antagonist RG1(3)
C+KU_3	=	Treatment with *Clavibacter* and antagonist KU3

Plate 6. Biocontrol activity of antagonistic bacterial isolates against *Clavibacter michiganensis* inoculated to tomato plants under net house conditions

4.10.2 Effect of temperature on antagonistic activity against *C. michiganensis* by selected bacterial isolate S1

The effect of different temperatures (30, 35, 45, 50ºC) on antagonistic activity was measures for the potential antagonist S1. Fig. 9 revealed that there was a significant difference between the values obtained at different temperature. The maximum antagonistic activity (13 mm) was observed at 35±2ºC (fig.8) corresponding to viable count (221×10^6 cfu/ml) and minimum (8 mm) at 30±2° corresponding viable count (182×10^6 cfu/ml). At 45˚C and 50ºC no antagonistic activity was observed corresponding to viable count (34×10^6 cfu/ml and 10×10^6 cfu/ml), respectively. Hence, 35±2 °C was optimum for further studies.

4.10.3 Effect of inoculum size on antagonistic activity against *C. michiganensis* by selected bacterial isolate S1

Effect of inoculum size of the isolate on the antagonistic activity was observed after 48 h of incubation and it was revealed from Fig. 10 that at 2% inoculum size antagonistic activity was best (13.5 mm), with viable count of 43×10^7 cfu/ml. But, with further increase in inoculum size decrease in the antagonistic activity was observed. Minimum zone size of 0.3 mm was observed at 4 % inoculum size, corresponding to viable count of 156×10^7 cfu/ml. Further, no antagonistic activity was observed with increase in inoculum size to 5% and 10%, with following viable count of (188×10^7 cfu/ml and 198×10^7 cfu/ml), respectively. Therefore, 2% inoculums size was kept optimum for further studies.

4.10.4 Effect of pH on antagonistic activity against *C. michiganensis* by selected bacterial isolate S1

A study on antagonistic activity of the isolate was also conducted with different pH ranging from 5 to 9. From Fig. 11, it was observed that with the increase in pH of medium (5.0 to 7.0) antagonistic activity increased, but with further increase in pH the antagonistic activity declined. The antagonistic activity was found to be best at pH 7 with the viable count of 101×10^6 cfu/ml whereas the minimum activity was recorded at pH 8 (4 mm) with viable count of 83×10^6 cfu/ml. However, no antagonistic activity was observed at pH 5 and 9, with following viable count of (57×10^6 cfu/ml and 26×10^6 cfu/ml), respectively. Hence, pH 7.0 was kept optimum.

4.10.5 SENSITIVITY OF THE EFFICIENT BACTERIAL ANTAGONIST S1 TOWARDS DIFFERENT ANTIBIOTICS

The efficient bacterial antagonist S1 was tested for its resistance towards different antibiotics viz. amoxycilin, penicillin G, ampicillin, erythromycin E, tetracycline, gentamycin, kanamycin and bacitracin at different concentrations and was studied for intrinsic antibiotic resistance (IAR). The IAR pattern of the strain S1 showed variation and was observed in terms of zone of clearance around the antibiotic disc (Table 14). The IAR pattern of the strain S1 showed variation. However, it was observed that most of the antibiotics used inhibited the growth of isolate S1. But, the strain was found resistant to the antibiotic penicillin G at the concentration of 10 µg/disc (Plate 7).

Table 14. Intrinsic antibiotic resistance of efficient antagonist S1 towards different antibiotics

S. No.	Antibiotic	Concentration (µg/disc)	Zone of Clearance (mm)
1.	Amoxycilin	10	2.33
2.	Penicillin G	10	0.00
3.	Ampicillin	25	0.70
4.	Erythromycin E	15	4.30
5.	Tetracycline	30	1.43
6.	Gentamycin	50	3.33
7.	Kanamycin	5	2.43
8.	Bacitracin	10	0.90

Maximum susceptibility was observed towards erythromycin E (15 µg/disc), with zone size of 4.30 mm. Whereas, the antagonist was least susceptible towards ampicillin (25 µg /disc), for which zone size of 0.70 mm was observed.

4.11 MOLECULAR IDENTIFICATION OF MOST EFFICIENT ANTAGONISTIC BACTERIAL ISOLATE S1 BASED ON 16S rRNA GENE SEQUENCING

Molecular identification of most efficient bacterial antagonist S1 was done using universal 16S rRNA primers. A PCR product of 1350 bp was generated for the antagonistic bacterial isolate (Plate 8) which was further sequenced by commercial sequencing facility (Xcleris Lab, Ahemdabad).

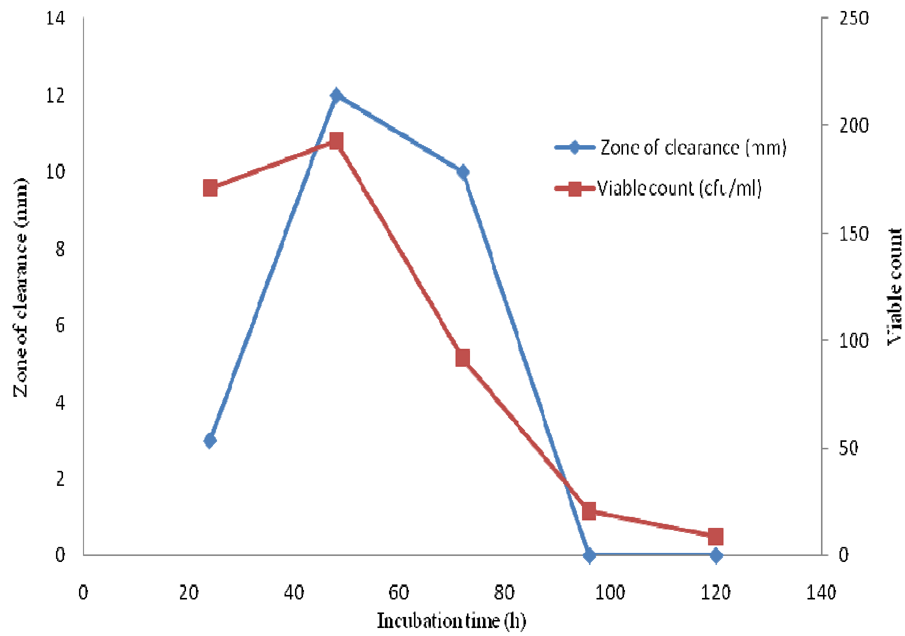


Fig. 8: Effect of incubation time (h) on antagonistic activity of bacterial antagonist S1 against *C. michiganensis*

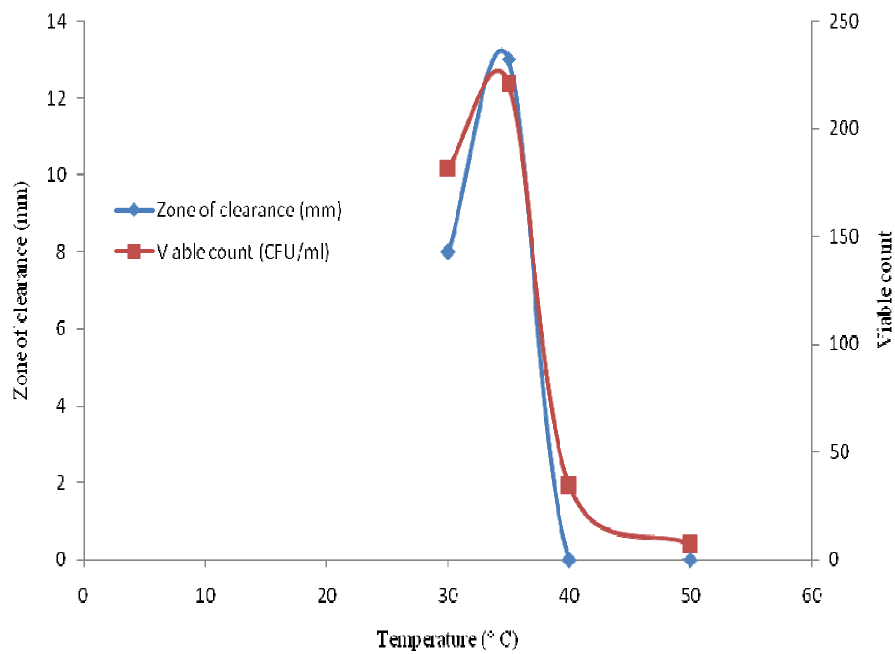


Fig. 9: Effect of temperature (°C) on antagonistic activity of bacterial antagonist S1 against *C. michiganensis*

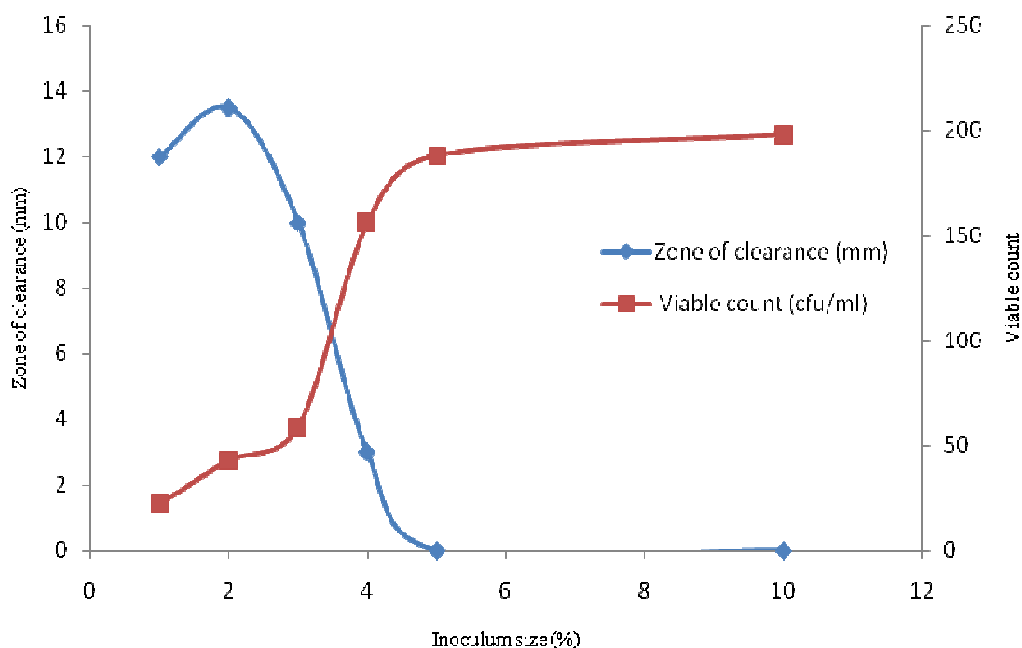


Fig. 10: **Effect of inoculum size (%) on antagonistic activity of bacterial antagonist S1 against *C. michiganensis***

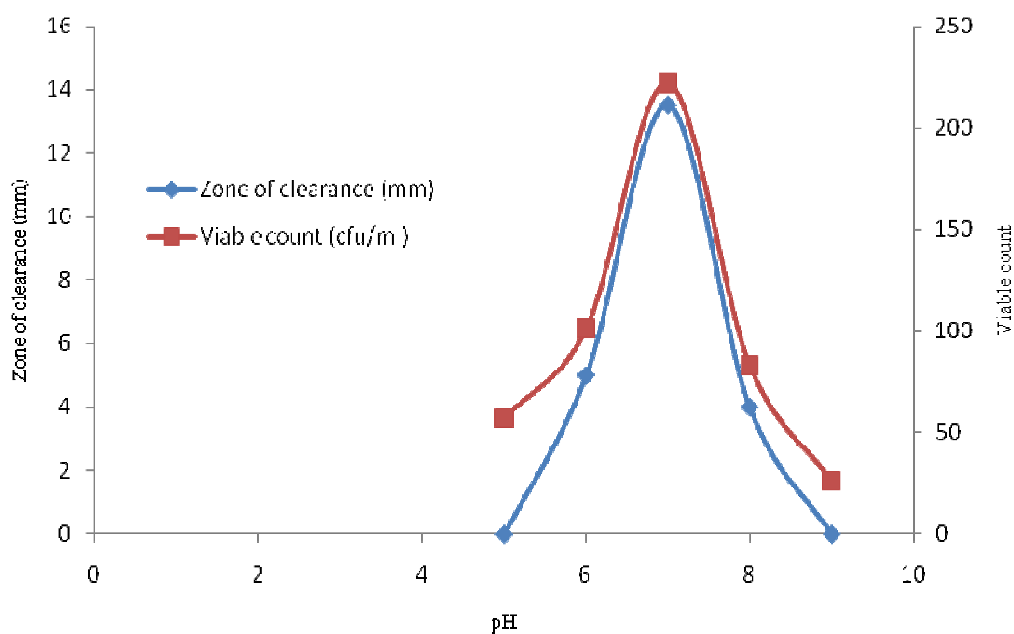
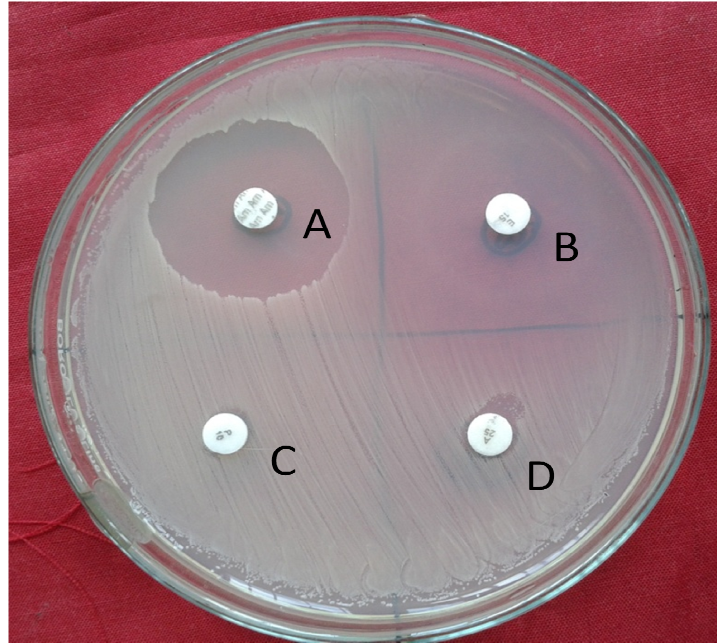


Fig. 11: **Effect of pH on antagonistic activity of bacterial antagonist S1 against *C. michiganensis***



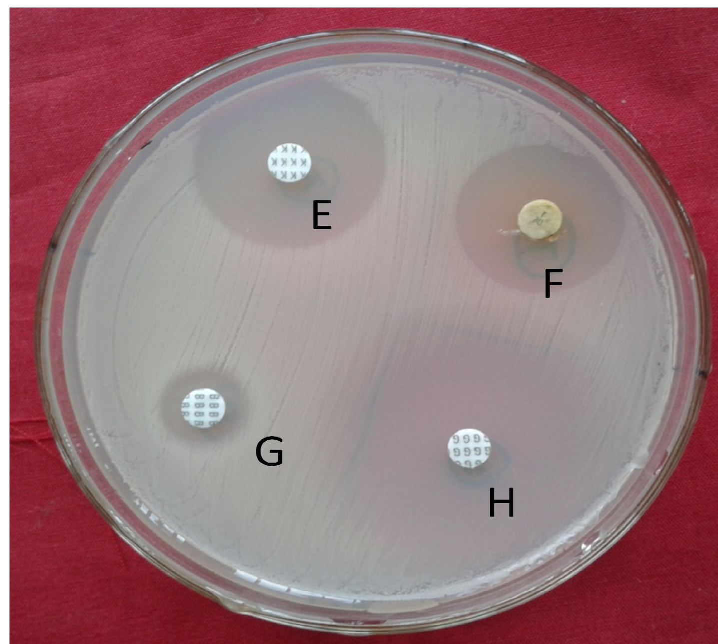


Plate 7 : **Intrinsic antibiotic resistance (IAR) Pattern of most efficient antagonist S1**

A) Amoxycilin	**B) Ampicillin**
C) Penicillin	**D) Erythromycin**
E) Tetracycline	**F) Gentamycin**
G) Kanamycin	**H) Bacitracin**

It was further observed that the sequence consisted of 320 adenines, 297 cytosines, 400 guanines and 253 thymines (Table 15). The genomic DNA G+C content was 54.88%.

The sequence of 16S rRNA gene of S1 was analysed using BLASTn. The partial 16S rRNA gene sequences of strain S1 were determined and aligned to other known *Bacillus* sequences deposited in Gen Bank. The sequence similarity between antagonistic bacterial isolate and other known *Bacillus* strains varied from 90 to 99%. S1 isolate showed 99% similarity with *Bacillus amyloliquefaciens* (EU855192Ba) and (EU855195Ba). The sequence of the strain has been deposited in Gen Bank database as *Bacillus amyloliquefaciens* and assigned the accession number KM658175.

Table 15. Nucleotide base composition in the 16S rRNA gene sequence of S1

Nitrogenous base	Nucleotide count	
	Total	Per cent (%)
Adenine (A)	320	25.19
Thymine (T)	253	19.92
Cytosine (C)	297	21.96
Guanine (G)	400	31.49
G+C	697	54.88
A+T	573	45.12

To trace out the evolutionary patterns of the test isolate and to find out relationship of the same with other selected sequences at NCBI, phylogenetic tree was also constructed using Neighour-Joining (J) method of mathematical averages (UPGMA) among 16S rRNA sequence of S1 (Plate 8) and corresponding sequence of 23 different *Bacillus* spp. Isolate S1 was united with quite high statistical support by the bootstrap method estimates for 1,000 replications and values inferred greater than 50 per cent are only presented in Fig.12. The phylogenetic analysis (Fig. 12) revealed that the isolate S1 was clustered with the type strain *B. amyloliquefaciens*. Based on 16S rRNA gene sequences and phylogenetic positions, the antagonistic bacterial isolate S1 was designated as *B. amyloliquefaciens* strain S1.

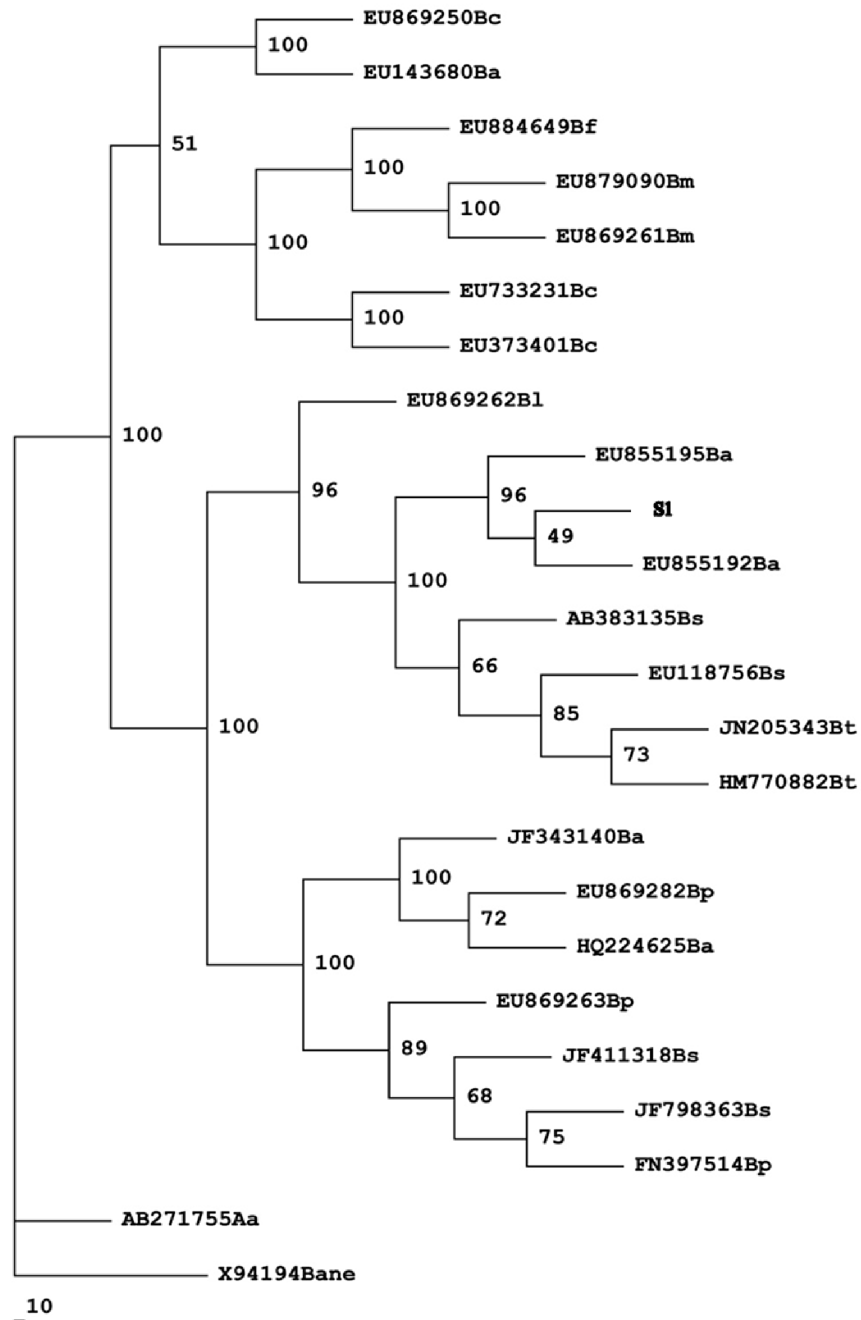
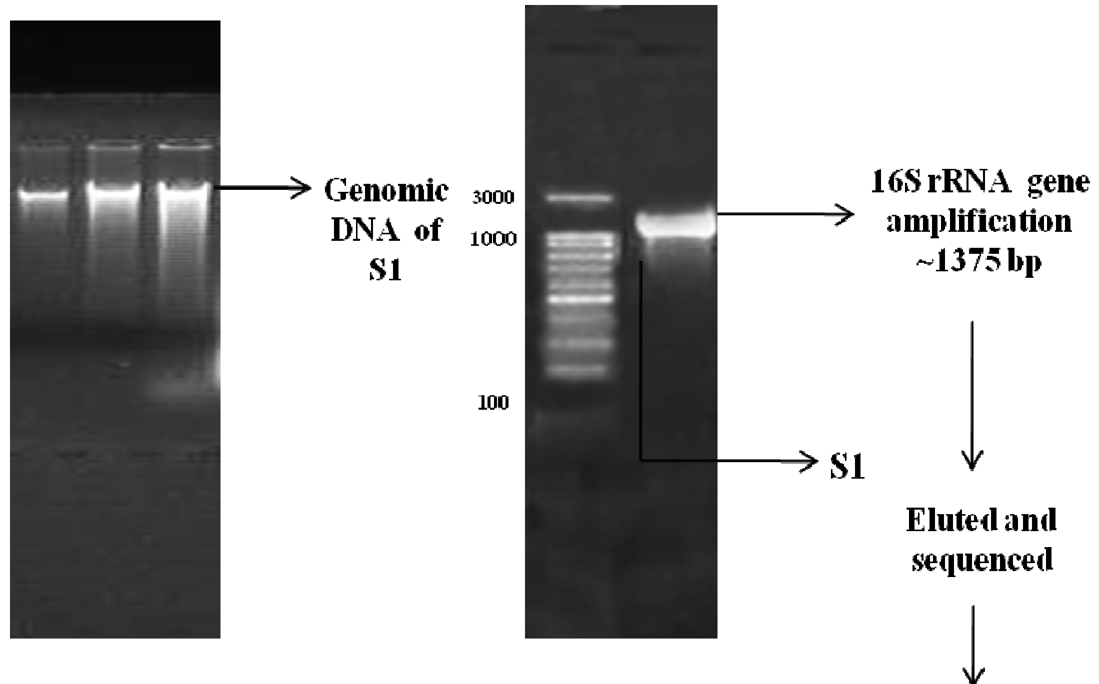


Fig. 12. Neighbour – joining tree based on 16S rDNA gene sequence showing the phylogenetic relationship of isolate S1 with the analysed sequence (Gen Bank accession no. KM658175) isolate



GGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGT
TGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGC
GGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAA
CCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA
ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAG
CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTT
CCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT
GACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGG
CACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCT
GGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTG
TTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGC

↓

Submitted to NCBI and accession number KM658175 is assigned

Plate 8. Molecular identification of selected antagonistic bacterial isolate S1 based on 16S rRNA amplification

Chapter-5

DISCUSSION

The present study was aimed to explore the diversity of PGPRs colonizing the rhizosphere soil and roots of different horticultural crops and medicinal plants grown in natural conditions for evaluating their biocontrol potential against *Clavibacter michiganensis*, causing bacterial canker of tomato. Bacterial canker of tomato is one of the most devastating and contagious disease spread from plant to plant by cultural practices such as transplanting and pruning (Boudyach *et al.*, 2001), causing great economic losses to farmers, as well as their plant growth promoting activity towards the development of microbial inoculants for the crop.

C. michiganensis causing bacterial canker of tomato is a gram positive, non-sporulating seed or soil borne phytopathogen. Some potential control measures to prevent the disease by using agrochemicals like copper and mancozeb and several antibiotics are used frequently by the growers, but their use has limited efficacy when conditions favour the canker development (Soylu et al., 2003). Microbial control agents provide an effective route to eco-friendly plant protection. The induction of plant resistance by using microbial bioagents may be useful method for the reduction of severity of this disease (Girish and Umesha, 2005). In this context, the use of plant growth promoting rhizobacteria as biocontrol agents is an effective alternative to the agro-chemicals.

Bacteria that inhabit the rhizosphere may influence plant growth by contributing to a host plant's endogenous pool of bioactive compounds such as phytohormones, antibiotics, siderophores (Mubarik *et al.*, 2010). Those kind of bacterial group are well-known as Plant Growth Promoting Rhizobacteria (PGPR). PGPR can exhibit a variety of characteristics responsible for influencing plant growth. PGPR are considered to promote plant growth directly or indirectly. Indirect effects are related to production of metabolites, such as antibiotics, siderophores, or HCN, that decrease the growth of phytopathogens and other deleterious microorganisms. Direct effects are dependent on production of plant

growth regulators or improvements in plant nutrients uptake (Wahyudi *et al.*, 2011). In recent years, much attention has been paid to natural methods of crop growing in expectation of moving toward agriculturally and environmentally sustainable development. PGPR promote plant growth due to their abilities in plant growth promotion and disease control, and therefore have the potential to reduce the application of agro-chemicals and maintain biotic diversity in the plant associated bio-community.

5.1 ANTAGONISTIC BACTERIAL POPULATION AGAINST *Clavibacter michiganensis* FROM THE RHIZOSPHERE SOIL AND ROOT ENDOSPHERE OF DIFFERENT HORTICULTURAL CROPS viz. STRAWBERRY, APPLE AND APRICOT

This study showed that strawberry, apple and apricot rhizospheres support a diverse population of bacteria antagonistic to *Clavibacter michiganensis*. Under *in vitro* conditions the screening of rhizobacteria with the putative antagonistic activity is the first step towards selection of candidate PGPR for development of biological control of plant diseases. In the present study, a total of 550 rhizobacterial isolates of different horticultural, vegetable crops and medicinal plants grown under mid hills and high hills of Himachal Pradesh were screened for antagonistic activity against *Clavibacter michiganensis*. An arbitrary conformation was done on the basis of diameter of inhibition zone. These crops were from different agroclimatic conditions with respect to altitude and soil pH. Out of total, only 40 bacterial isolates from the rhizospheres of strawberry, apple and apricot showed antagonistic activity by depicting inhibition zone in the range of 3.20 to 12 mm. 20 % of the isolates were of endorhizospheric origin while the rest 34 (80 %) were rhizospheric. Out of these 40 isolates, 20 isolates were isolated from strawberry rhizosphere, 10 from apple and 10 from apricot rhizosphere (Table 1). However, Boudyach *et al.* (2001) screened 178 bacterial strains which showed inhibitory activity *in vitro* against *C. michiganensis* subsp. *michiganensis* isolated from the rhizosphere of tomato, originating from different sites in the Souss-Massa Valley, Agadir, Morocco. But, our study was reporting the efficient antagonistic isolates against *C. michiganensis* from rhizospheres of different crops viz. strawberry, apple and apricot.

5.2. SCREENING OF ANTAGONISTIC BACTERIAL ISOLATES FOR BIOCONTROL AND PLANT GROWTH PROMOTING TRAITS

The tested bacterial antagonists could simultaneously display binary and triple PGP traits, which may promote plant growth directly, indirectly or synergistically, suggesting that application of PGPR with multiple traits to be more beneficial. The exhibition of multiple PGPR traits by a single strain of PGPR has also been reported earlier in our lab by Mehta *et al*. (2010) and Walia *et al*. (2013). The comparison of percentages of PGPT possessing bacterial antagonists in rhizospheres of different horticultural crops viz. strawberry, apple and apricot showed that 15% of antagonistic bacteria, specifically of strawberry rhizosphere were without any plant growth promoting traits. It was observed that combination of binary traits of nitrogen fixation and phosphate solubilization was highest for apricot (40.0%). Rhizobacterial percentages having binary traits of siderophore production and nitrogen fixation was found to be same (10.0%) in apple and apricot, while it was found to be (5.0 %) in case of strawberry, whereas the percentages of PGPRs having binary traits of phosphate solubilization and siderophore production was found maximum in apple (30.0 %) which was relatively higher as compared to the binary traits shown by rhizobacterial antagonists of other two crops. Ratios of antagonistic rhizobacteria possesing triple traits of phosphate solubilization, siderophore production and nitrogen fixation were highest in apple (20.0 %) and in case of strawberry and apricot, it was 10.0 %. Similar studies were also undertaken in our lab by Mehta (2012) and Sharma (2013).

The PGPR include a diverse group of free living soil bacteria that can stimulate the growth of plants and biocontrol of phytopathogens by several different mechanisms (Jalili *et al.,* 2009). Bacterial plant growth promotion is a well-established and complex phenomenon, and is often achieved by the activities of more than one plant growth promoting traits (PGPTs) exhibited by plant- associated bacteria (Aslantas *et al.,* 2007). The results (Fig. 3, 4 and 5) highlights the plant growth promoting traits of P-solubilizing bacterial isolates associated with rhizosphere of strawberry, apple and apricot. The present study revealed that the rhizosphere of these horticultural crops inhabited a large number

of antagonistic gram-positive bacteria which possessed one or more of the properties frequently associated to the biocontrol and plant growth promotion: P-solubilisation, IAA, siderophore, nitrogen fixation, HCN production. Variation in PGPTs among antagonistic bacterial isolates from different locations was observed under *in vitro* condition, which is attributed due to their individual rhizospheric competencies.

The forty antagonistic isolates were screened for their PGP traits like P-solubilization, siderophore production, IAA production, growth on N free medium and HCN production. These antagonists were also tested for the production of lytic enzymes viz. amylase, cellulase, lipase, protease and chitinase. The screening of bacterial isolates for multifarious PGP traits under *in vitro* and their use as biostimulant and bioprotectant for growth enhancement of different agricultural crops and biocontrol efficacy against different plant pathogens has been reported by Sharma *et al*. (2012).

The production of siderophore, IAA, cell wall lytic enzymes such as amylase, cellulase, protease, lipase and chitinase is considered important for biocontrol (Hameeda *et al*., 2006; Suresh *et al.,* 2010; Ahemad and Khan, 2011 and Wahyudi *et al.,* 2011). In the present study, siderophore production lies in the range between 12.58% to 70.24% siderophore units (Table 3, 4 and 5). The study suggests that the occurrence of higher antagonistic siderphore producers in these crop rhizospheres is of direct significance to plants as it helps in iron sequestering near the roots, especially in iron deficient conditions. In addition, siderophore producing micro organisms protects plants at two levels: first, limiting growth of plant pathogens and secondly triggering plants defensive mechanism (Ramos Solano *et al.,* 2010).

Lytic enzymes have been studied as potential antibacterial agents against bacterial plant pathogens because the enzymes play a key role in the mechanism of parasitic entry in to host cells (Dahiya *et al*., 2006 and Nguyen *et al*., 2008). Production of different lytic enzymes viz. amylase, cellulase, lipase, protease and chitinase was also tested for these antagonists. Overall, 55% of the isolates were able to produce amylase, 32.5 % were cellulase producers, 55 % were lipase

producers, 60% protease producers and 97.5% were chitinase producers. Chakraborty *et al.* (2013) also screened three bacterial antagonists for PGP characteristics (P-solubilization, IAA production, siderophore production, protease and chitinase production). It has been reported that several mechanisms are responsible for suppression of pathogen involving the lytic enzymes that play a key role in biocontrol potential against different plant pathogens (bacterial, fungal and viral). The proposed mechanism to provide a protective effect on the roots through antagonism towards the phytopathogenic bacteria is by producing metabolites such as siderophores, lytic enzymes like amylase, protease, cellulase, lipase and chitinase; plant hormones like auxins and IAA (Amaresan *et al.*, 2011; Neeraja *et al.,* 2010; Maksimov *et al.,* 2011).

HCN production has been shown both as a beneficial and harmful property for plants (Rudrappa *et al.,* 2008 and Selvakumar *et al.,* 2008). Several studies have demonstrated that the production of siderophores, HCN, secondary metabolites and lytic enzymes by bacterial strains are most effective in controlling the plant root pathogens (Nagrajkumar *et al.,* 2004). However, HCN production was reported in none of the isolates, the role of HCN was not expected as these isolates were negative for HCN production which might not be due to variation in growth parameters like temperature, nutrient availability and growth pattern (Singh *et al.,* 2014).

A plant growth- promoting (PGP) effect of antagonistic bacterial isolates may also be related to their ability to solubilize phosphorous and to produce IAA. Phosphorus is one of the major nutrient second to nitrogen required by the plants. Most phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by plants (Pradhan and Sukla, 2005). Thus, P-solubilization is considered as one of the most important attribute of the PGPR (Patel *et al.,* 2008 and Joseph and Jisha, 2009). It is well established fact that improved phosphorus nutrition influences overall plant growth and root development. Phosphate solubilizing bacteria convert the insoluble form of phosphorus to soluble form through acidification, secretion of organic acids or protons (Richardson *et al.,* 2009). The initial screening protocols used for the identification of PSB isolates

in the present study rely on halozone formation by bacterial isolates on PVK agar plates containing tri-calcium phosphate. Some obvious differences in the size of halos (not shown) and phosphate solubilizing index (Table 3, 4 and 5) suggest the existence of antagonistic isolates exhibiting different degree of P- solubilizing efficiencies in the rhizosphere soil and root samples collected. However, 77.5% of the bacterial antagonists were found out to be P-solubilisers.

Bacterial IAA stimulates the root development of host plant, which results in better absorption of water and nutrients from the soil (Ahemad and Khan, 2011). In the present investigation, all the (100%) antagonistic isolates had the ability to produce IAA in the range of 1.50 µg/ml to 70 µg/ml. Similar studies were carried out earlier in our lab by Balnatah (2013) in which the range of IAA produced by PGPR isolated from strawberry rhizosphere varied between 1.00 to 56.00 µg/ml and Sharma (2012) in which twenty six PGPR isolates successfully produced auxin in the range of 15 µg/ml to 95 µg/ml. Gracia *et al*. (2001) reported that IAA is one of the physiologically most active auxins. IAA is common product of L-tryptophan metabolism by general microorganisms including PGPRs inhabiting rhizosphere of various plants which synthesize and release auxin as secondary metabolites because of rich supplies of substrate exuded from roots as compared to non-rhizospheric soil.

5.3 PRODUCTION OF ANTIBACTERIAL METABOLITES IN CELL FREE SUPERNATANT AGAINST *C. michiganensis*

Various mechanisms account for the ability of biocontrol PGPR to control plant pathogens, including competition for iron and other nutrients, production of antibacterial metabolites. The present study revealed that ten bacterial antagonists selected on the basis of their plant growth promoting traits showed variation for antibacterial metabolite production which was tested using the cell free supernatant of 48h old cultures of bacterial antagonists against *Clavibacter michiganensis* at four different concentrations i.e. 0.25 % (v/v), 0.50 % (v/v), 0.75 % (v/v) and 1.00 % (v/v). A lot of variation was observed in size of inhibition zone, ranging from 2.28 mm to 9.6 mm (Table 1). Whereas, zone of inhibition in case of extracts with cells ranged from 3.20 to 12 mm. Similarly,

Cruz-Quiroz *et al.* (2011), demonstrated that cell free extracts exhibited a limited antagonist capacity in comparison of those extracts with cells, which showed an excellent capacity to inhibit the growth of *C. michiganensis, X. axonopodis* and *E. carotovora*, demonstrating the intracellular nature of the bioactive metabolites associated to bacterial growth inhibition. Suppression of bacterial growth by the cell free extract of antagonistic isolates and formation of inhibition zone were presumably due to the metabolites being released from bacteria into the culture medium. Many strains of *B. subtilis* have been reported as potential biocontrol agent against bacterial pathogens and the principal mechanism of this involves the production of different metabolites (Lanteigne *et al*., 2012). In addition, the production of volatile compounds by *B. subtilis* strains antagonise a range of soil-borne plant pathogen including *R. solani* and *Pythium ultimum* (Yari *et al*., 2002). Biratu *et al*. (2013) studied the antagonistic activity of actinobacteria in cell free suspension test, out of 36, 21 of isolates such as isolate Gosu qoras#196-1, Awaros#174-2, Senkeles#132-5, Awaros#183-1 have showed inhibitory activity against *Ralstonia solanacearum* EF Smith which shows that their inhibitory activity comes from secretion of extracellular antimicrobial compounds.

5.4 QUANTITATIVE ESTIMATION OF PLANT GROWTH PROMOTING TRAITS BY SELECTED ANTAGONIST ISOLATES

In the present study, 10 bacterial isolates out of 40 bacterial antagonists were selected on the basis of dendrogram derived from similarity coefficient among PGP traits. All the 10 bacterial isolates were found to solubilize tri-calcium phosphate effectively in liquid medium. Quantitative estimation of P-solubilization among all the isolates was in the range of 50.00 µg/ml to 258.00 µg/ml from the initial value 1000 µg/ml as earlier reported by Chatli *et al.* (2008) where the range was 95.0 µg/ml to 100.6 µg/ml. A significant decline in the pH of the culture medium by strains was observed during mineral phosphate solubilization, which is suggested due to the microbial production of organic acids as reported earlier (Pandey *et al*., 2006). The formation of a clear zone on Pikovskaya's medium (Plate 2) unequivocally suggests the P-solubillizing potential of all bacterial antagonists. The efficiency of P-solubilization was assessed and observed suggesting that bacterial isolate $RG_{1(3)}$ had maximum

activity of P-solubilization (258.00 µg/ml) and IAA (70.00 µg/ml) followed by another isolate KU_2 S1 (255.00 µg/ml and 24.00 µg/ml) indicated their inherent plant growth promoting potential. This is in accordance with earlier studies demonstrating the plant growth promoting activities of such PGPRs (Kloepper *et al*., 2004; Idris *et al*., 2007). As is evident from Table 14, Fig. 10 where considerable per cent increase in root length by five isolates was observed under net house conditions over uninoculated control. It has also been reported in literature that auxins are expressed quantitatively by PGPR and is considered as a major plant phytohormone required for stimulation of root growth (Shahab *et al.,* 2009). Siderophore being a secondary metabolite and participant in biocontrol mechanism, the bacterial cultures may initiate siderophore production over a wide range of incubation period and temperature conditions (Chaiharn *et al*., 2009). Maximum siderophore production (230.77 SU %) was observed for isolate S1 and R_2S1 at pH 7 and 72h of incubation period. Significant lower levels of siderophore were observed in rest of the isolates. However, the maximum siderophore corresponded with maximum drop in final pH of the medium from initial pH of 7 to 5.20 (Table 11). These studies are similar with earlier report on decreased pH with the increase in siderophore production by Chandel (2011), but are in disagreement with studies of Diaz *et al.* (2002) where increase in pH was observed from 7 to 8.5 during the growth period.

5.5 PHENOTYPIC CHARACTERIZATION OF SELECTED BACTERIAL ANTAGONISTS

Use of colony morphotype is a common procedure to select isolates from complex environment such as soil for diversity measurements and/or isolation of dominant species within culturable communities (Haldeman and Amy, 1993 and Lebaron *et al*., 1998). The results in Table 6 depicts the colony morphology, Gram's reaction, cell shape and arrangement of bacterial antagonists. All isolates were positive for Gram's reaction except $RO_{5(6)}$. All the antagonists were rod shaped. Most of the isolates were occurred singly while one isolate was arranged in chain when observed microscopically. In the present study, two or maximum four morphotypes from antagonistic bacterial population was recorded on nutrient agar from different rhizospheres. The diversity among ten selected rhizobacterial

isolates was determined by biochemical tests. The rhizobacterial isolates were categorized into four groups and all the isolates were catalase positive and were tentatively belonged to genus *Bacillus* (Fig. 4). However, Boudyach *et al*. (2001) reported the abundance of Gram-negative bacteria antagonistic to *C. michiganensis* subsp. *michiganensis* (about 70% of strains) on tomato roots.

5.6 METABOLIC AND GENOTYPIC FINGERPRINTING

Traditionally, a search for PGPRs involves screening a large number of isolates and identifying a desired phenotypic trait. Once isolates are purified, the main goal is to keep the maximum genetic diversity in the minimum number of isolates, for further biological assays. This goal may be achieved through PCR-RAPDs, ITS-PCR, AFLP, techniques that define differences at the strain level (Louws *et al*., 1999). RAPD technique was developed as an efficient tool to analyse phylogenetic relationship among and within closely related species (Williams *et al*., 1990). Genetic variability among the isolates can be used to gain precise information about genetic similarities and dissimilarities.

The evaluated phenotypic traits have been previously proposed as good indicators of putative PGPRs (Cattelan *et al*., 1999). However, any phenotypic trait shown *in vitro* reveals that the information is contained within the bacterial genome, but it is not constitutively expressed. Because of this, the phenotypic screening were carried out first, to define genetic differences by PCR- RAPDs afterwards.

The reproducibility of RAPD analysis is known to be highly influenced by experimental conditions. It is therefore essential to optimized the PCR conditions to obtain reproducible and interpretable results before going on routine analysis. The PCR conditions for RAPD analysis were optimized by investigating each factor individually. Girgis *et al.* (2008) also reported the optimization of PCR condition in the similar manner. The sharp and clear amplification products obtained after treatments of DNA with RNase may be the result of inactivation of endogenous endonucleases.

In the present study, ten antagonistic bacterial isolates that tested positive for any of the evaluated PGPTs were analysed by PCR-RAPDs to reduce genetic

redundance while retaining the maximum genetic diversity by selecting genetically different antagonistic isolates. Polymorphism among ten antagonistic bacterial isolates were detected using four random primers that were screened in RAPD analysis for their ability to produce sufficient amplification products. Primer RBa-5 generated sufficient reproducible polymorphism among ten antagonistic bacterial isolates (Plate 5 and 6). Primer RBa-5 generated 77 amplified bands in the size ranging from 100 to 2100 bp (Table 8). The level of 100 per cent polymorphism detected indicate that the primer RBa-5 could be employed in the future for RAPD based genetic diversity studies on Gram positive rod shaped antagonistic bacterial species. The polymorphism in the amplification products may be due to either from changes in the sequence of the primer binding site (*e.g.* point mutations) or from changes which alter the size or prevent the successful amplification of the target DNA (*e.g.* insertation, deletions, inversions) as suggested by Rani *et al.* (1995).

5.7 NET HOUSE STUDIES

In the present work, five isolates were selected on the basis of their *in vitro* antagonistic activity against *C. michiganensis* and PGPTs i.e. P-solubilisation, IAA production, siderophore production, ability to grow on N-free medium and lytic enzyme production. *In planta* evaluation of best five antagonistic strains against *C. michiganensis* revealed that the tested strains decreased disease incidence of bacterial canker of tomato. Minimum disease incidence of bacterial canker was recorded for the isolate S1 i.e. 28.55% as compared to control which was 82.80%, with corresponding biocontrol efficacy of 70% over control (100%). Similar studies were carried out by Girish and Umesha (2005), which showed that three PGPR strains treatment effectively reduced the canker incidence. The minimum disease incidence (44%) was recorded in strain IN937a (*B. amyloliquifaciens*) treated plants as compared to the control (93%). Boudyach *et al*. (2001) also reported the significant reduction in the infection when applied with three strains (HF22, HF142 and HF183) as bioinoculants as compared to untreated control. It has been suggested that the ability of PGPRs to induce disease resistance might be due to some metabolites or certain compounds (Miyazawa *et al*., 1998). Development of induced

resistance in plants is also associated with the coordinate expression of a complex set of PR proteins, so-called 'SAR genes' (Conrath *et al*., 2001). The enzymatic activities of several PR proteins have been identified and include β-1, 3-glucanases (PR-2) and chitinases (PR-3), which possess direct antimicrobial activity by degrading microbial cell wall components (Van Loon, 1997). Some plant chitinases also have lysozyme activity and can therefore hydrolyse bacterial cell walls (Boller *et al*., 1983; Heitz *et al*., 1994). Furthermore, breakdown products of pathogen and/or plant cell wall components released by the activity of these enzymes have been shown to act as elicitors of plant defence responses (Van Loon, 1997). The expression of PR genes and the associated accumulation of PR proteins have been considered as the molecular basis of induced resistance. In a study conducted by Baysal *et al*. (2003) a correlation was also found between induced resistance and accumulation of chitinase. Induction of the PR protein chitinase in ASM-treated tomato leaves coincides with the accumulation of chitinase and glucanase reported by Burketova *et al*. (1999) and Suo & Leung (2001) in other plant–pathogen interactions. Hence, it could be assumed that biocontrol potential of the present isolate S1 might be due to ISR and due to the cumulative effect of certain genes, enzymes and metabolites accumulated by the test strain.

In present work, the data obtained after population density study of antagonist and pathogen, it was revealed that application of strain S1 indeed significantly reduce the pathogen i.e. *C. michiganensis* population in soil, thus, protecting plant roots from pathogen attack. This is in agreement with study of Ding *et al.* (2013), in which strain BIO23- treated plants showed reduction in population of *R. solanacearum* from 1.1×10^7 to 1.3×10^6 cfu/g of soil.

The effect of PGPR treatment on shoot and root parameters was also studied. Most of the isolates consistently increased the different plant attributes such as shoot and root length, dry weight over uninoculated control (Table 14). Maximum increase in shoot length (49.17 cm), shoot dry weight (15.10 g), root length (7.43 cm) and root dry weight (3.23 g) was observed in case of seedlings treated with isolate S1 which was significantly higher than control and all other

isolates. Auxin production is known to stimulate root development, which result in better absorption of water and nutrients from soil and thereby, stimulated the plant growth. Similarly, all the five isolates were auxin producers and showed root development in tomato seedlings, thereby, promoting the growth of entire plant. Romero *et al.* (2003) studied the biocontrol and growth promotional effect of *Azosprillum brasilense* Sp7-mediated plant on the development of bacterial canker of tomato. Their study revealed that *Azospirrilum* treated plants showed 20-30 % control in disease incidence and significant increase in plant height from 7.2 cm in control plants to 9.5 cm in plants treated. The increased growth and biomass in seedlings raised from seeds treated with PGPR may be attributed to the cumulative effect of P-solubilisation, N-fixation and production of plant growth regulators. The increase in growth and biomass yield by the use of *Bacillus* sp. and *Pseudomonas* sp. have been reported for various agricultural crops (Walia *et al.*, 2013; Kundu *et al.*, 2002) and horticultural crops like apple (Mehta, 2012).

4.8 OPTIMISATION AND CHARACTERISATION OF MOST EFFICIENT ANTAGONISTIC BACTERIAL ISOLATE S1

Screening and characterization of antagonistic rhizobacteria is first step to develop bacterial inoculants for application as plant growth promoting rhizobacteria for biocontrol of bacterial canker of tomato. In the present study, 10 antagonistic rhizobacterial isolates were evaluated for their ability to produce antibacterial metabolites. Among 10 antagonistic rhizobacterial isolates, the isolate S1 was found with maximum production of antibacterial metabolite in cell free supernatant. S1 also possessed other PGP traits such as P-solubilisation, IAA, Siderophore and lytic emzyme activity.

The bacterial antagonist S1 was also tested for its resistance towards different antibiotics viz. amoxycilin, penicillin G, ampicillin, erythromycin E, tetracycline, gentamycin, kanamycin and bacitracin at different concentrations and was studied for intrinsic antibiotic resistance (IAR). It was observed that most of the antibiotics used inhibited the growth of isolate S1. But, the strain was found resistant to the antibiotic penicillin G at the concentration of 10 mcg/disc.

Gopalakrishnan *et al.* (2012) has studied the antibiotic resistance pattern of different biocontrol potential bacteria from rice rhizosphere, in which all the isolates were found resistant to ampicillin (>100 ppm; except two isolates) but sensitive (<10 ppm) to chloramphenicol, kanamycin, nalidixic acid, streptomycin (except two isolates) and tetracycline. The ability of the present isolate to grow in the presence of Penicillin (Plate 7) can be used to study the population dynamics of introduced S1 strain in the rhizosphere of tomato seedlings in field. The rhizobacterial isolates that are resistant to high concentration of antibiotics may have survival and competitive qualities required for a good bioinoculant to be used as biocontrol agent. Malleswari (2014) and Kloepper et al. (1980) has also reported that resistance of PGPR to several antibiotics have ecological advantage of survival in rhizosphere when they are introduced as inoculum.

On the basis of results obtained by phenotypic and biochemical characterization the S1 strain was tentatively identified as strain of *Bacillus* sp. The identification of isolate was further confirmed by phylogenetic analysis using 16S rDNA technique. The sequence of 16S rDNA from S1 was analyzed using BLASTn analysis and was found to have 99% homology with *B. amyloliquefaciens* which is used as effective biocontrol agent against bacterial canker (Plate 8). 16S rRNA sequence analysis is allows the identification of bacterial isolates at species level (Naz and Bano, 2010) as well as the prediction of phylogenetic relationships (Pace, 1997). The abundance of *Bacillus* sp. in the rhizosphere and their usage as PGPR and biocontrol agents is in agreement with the previous reports where *Bacillus* sp. have been frequently isolated from different crops (Suarez *et al*., 2011, Turan *et al.,* 2013).

Optimization of cultural conditions is a significant step in production of antibacterial metabolites as it actually determines the incubation period, temperature, inoculums size and medium pH for any microbial culture. Isolate S1 showed maximum zone of inhibition and viable count at 48h of incubation period (Fig. 6). Further increase in the incubation period decreases the zone of inhibition (mm) by the isolates. Isolate S1 showed maximum zone of inhibition (mm) at temperature 35°C and pH 7.0 (Fig. 7 and 9). Inoculum prepared from young culture of 48h old was found most effective may be because the population in

young culture is most nearly uniform in terms of biochemical and physiological properties. The increase in the zone of inhibition (mm) increase up to inoculum size 2 per cent and further increase in inoculums size lead to decrease in the antagonistic activity (Fig. 8).

The present study highlights S1 *Bacillus* sp. with biocontrol potential against *C. michiganensis* and also had plant growth promoting potential and increase growth and for the development of tomato seedlings. A significant feature of the isolate S1 is its ability to produce antibacterial metabolites (9.3 mm), siderophore (230.77 SU%) and lytic enzymes viz. amylase (1.80 E.I.), lipase (1.73 E.I.), protease (1.62 E.I.) and chitinase (1.13 E.I.), that might have participated in its biocontrol efficacy against *C. michiganensis* under net house conditions. The isolate S1 was also found with maximum growth promotional effects under net house conditions due to its inherent multifarious plant PGPTs viz. P- solubilisation (2.94 PSI), IAA production (27.00 µg/ml) and growth on N-free medium. This gains significance since IAA is a growth promoting hormone and siderophore and lytic enzyme production and their release by rhizospheric microbes has been proposed as a possible line of defense against soil borne plant pathogens (Selavakumar *et al*., 2008). The increase in growth and biomass in the seedlings raised from seeds treated with bacterial isolate S1 may be attributed to the cumulative effect on root length, shoot length, root dry weight, shoot dry weight and per cent increase in root and shoot parameters (Table 14). Therefore, S1 was further subjected to morphological, biochemical (Table 6 and 7) and molecular based characterization which confirmed the identity of strain S1 as *Bacillus amyloliquefaciens* (Plate 10). The sequence of the isolate S1 showed maximum similarity with *Bacillus amyloliquefaciens*. The sequence was also deposited in NCBI database. It has been proved as an efficient biocontrol agent and PGPR due to its colonization in the rhizosphere because in literature also it has been reported that *B. amyloliquefaciens* are generally better adapted to colonization of the rhizosphere of plants than other members of *Bacillus subtilis* group and can be considered as distinct ecotype (Reva *et al*., 2004). There are several reports in the literature suggested the use of *Bacillus* sp. as biocontrol and growth promotional agent for various crops (Sivasakthi *et al*., 2014).

Chapter-6

SUMMARY AND CONCLUSION

The specific objectives of the present study were: (i) Screening and characterization of plant growth promoting rhizobacteria (PGPR) for growth promoting and biocontrol traits; (ii) Evaluation of potential antagonist PGPR against *Clavibacter michiganensis* under laboratory and net house conditions.

The present study was aimed to explore the diversity of bacterial communities colonizing the rhizosphere soil and roots of different horticultural and vegetable crops and medicinal plants grown in natural conditions for evaluating their biocontrol potential against *Clavibacter michiganensis,* causing bacterial canker of tomato. The present study is one of the first to explore whether is there any kind of crop specificity associated with antagonism and it was found that antagonism is independent of crop specificity.

The present study revealed that the population of antagonistic bacterial isolates from different agroclimatic conditions was higher in soil rhizosphere than the root endosphere of different crops. Five hundred and fifty isolates were screened for antagonistic activity against *C. michiganensis*. Out of total, only 40 isolates were able to antagonise the pathogen and showed the zone of inhibition ranging from 3.2 to 12.0 mm. These 40 bacterial antagonists were also screened for their PGP traits; P-solubilisation, IAA production, growth on N-free medium, HCN production and production of different lytic enzymes viz. amylase, cellulase, protease, lipase and chitinase. P-solubilisation was observed with 31 (77.50%) antagonists, IAA production by all (100%) the antagonists, whereas siderophore production and growth on N-free medium was detected in 19 isolates (47.50%). However, none of the isolate was able to produce HCN. The frequencies of antagonistic rhizobacteria showing single (30%) and triple traits (20%) was maximum in apple, whereas binary activities were maximum in apricot (40%). However, 15% of the antagonistic isolates from strawberry showed none of the PGP traits.

Ten bacterial antagonists out of forty were selected on the basis of dendrogram derived from similarity coefficient among PGP traits. Maximum P-solubilization was observed with isolate $RG_{1(3)}$ (258.00 µg/ml), maximum siderophore production was exhibited by isolate S1 and R_2S1 (230.77 % S.U.) and isolate $RG_{1(3)}$ showed maximum IAA production with a concentration of 70.00 µg/ml. Production of extracellular enzymes also accounts for antibacterial activity. Maximum amylase activity (2.19 E. I.) was recorded for the isolate KU_2S1, cellulase activity (1.50 E.I.) for isolate $KU_{3(3)}$, lipase activity (1.73 E.I.) for isolate S1, protease activity (1.87 E.I.) for isolate $RO_{5(6)}$ and chitinase activity (1.55 E.I.) by isolate CK_9A.

Antibacterial activity in the cell free supernatant of 48h old cultures at four different concentrations of 0.25%, 0.50%, 0.75% and 1.00% of 10 antagonistic bacterial isolates against *C. michiganensis* was recorded. All the 10 (100%) of the isolates inhibited *C. michiganensis in vitro.* The maximum antibacterial activity in cell free supernatant at 0.25 per cent concentration (v/v) was recorded for the bacterial antagonist S 1 with 8 mm zone of clearance, which increased to 9.3 mm with the increase in concentration upto 0.75 %, but with further increase in concentration to 1.00 %, zone size remained stable.

Five out of 10 antagonists were evaluated for their biocontrol potential against *C. michiganensis* under net house conditions. Minimum disease index of 28.55% was observed with the isolate S1 with maximum biocontrol efficacy of 70% over control and and other isolates. Effect of these antagonists was also evaluated for their growth promotional effects. Plant growth responses were variable with respect to the variability among bacterial isolates. Maximum per cent root length increase (218.88%) and shoot length increase (73.32%) was observed with S1 inoculation. Maximum increase in root (3.23g) and shoot dry weight (56.15%) was also observed for the isolate S1 over control (1.53g, 9.67g respectively) and other isolates. Population dynamics of the inoculated soil was also studied. Maximum population of the pathogen (112×10^6 cfu/ml) was recorded in the control and minimum (7×10^6 cfu/ml) in soil inoculated with the isolate S1. Whereas, bacterial population of the antagonist was maximum in soil

inoculated with the isolate S1 (72×10^6 cfu/ml) and minimum in case of soil inoculated with the pathogen (2×10^7 cfu/ml).

The optimisation of different cultural conditions i.e. incubation period, temperature, inoculums size and pH was also done for the best selected isolate S1. The maximum antagonistic activity against *C. michiganensis* was observed at 35°C temperature after 48h of the incubation period and further increase in these factors led to decrease in the antagonistic activity. Best antagonistic activity was recorded at 2% inoculum size with pH remaining neutral and with any change in these factors antagonistic activity declined.

The isolate was also tested for Intrinsic Antibiotic Resistance (IAR) towards different antibiotics viz. amoxycilin, penicillin G, ampicillin, erythromycin E, tetracycline, gentamycin, kanamycin and bacitracin at different concentrations. IAR pattern of the isolate showed that most of the antibiotics used inhibited the growth of isolate S1. But, the strain was found resistant to the antibiotic penicillin G at the concentration of 10 µg/disc. Maximum susceptibility was observed towards erythromycin E (15 µg/disc).

Among all, isolate S1 was selected for identification by phenotypic and molecular based method as it showed maximum number of multifarious plant growth promoting traits and maximum biocontrol efficacy against bacterial canker of tomato. It was identified as *Bacillus amyloliquefaciens.*

In conclusion, high diversity of antagonistic bacteria in the rhizosphere of different horticultural crops viz. strawberry, apple and apricot was observed. *In planta* evaluation of antagonistic bacterial isolate i.e. S1 identified as *Bacillus amyloliquefaciens* revealed that it reduced the disease index of bacterial canker of tomato to 28.55% over control and other isolates under net house conditions. It showed maximum biocontrol efficacy of 70%. Therefore, this isolate can be used as efficient biocontrol agent against bacterial canker of tomato under net house conditions. Further, more studies are required to harness the potential of this isolate as bioinoculant in agriculture.

Chapter-7

REFERENCES

Achari G A and Ramesh R. 2014. Diversity, biocontrol, and plant growth promoting abilities of xylem residing bacteria from solanaceous crops. *International Journal of Microbiology* 1-14.

Agrawal K, Sharma D K and Jain V K. 2012. Seed-borne bacterial diseases of tomato (*Lycopersicon esculentum* mill.) and their control measures: a review. *International Journal of Food, Agriculture and Veterinary Sciences* **2**(2): 173-182.

Ahemad M and Kibret M. 2014. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *Journal of King Saud University – Science* **26**: 1–20.

Ahemed M and Khan M S. 2011. Functional aspects of plant growth promoting rhizobacteria: recent advancements. *Insight Microbiology* **1**(3): 39-54.

Akhtar A, Hisamuddin, Robab M I, Abbasi and Sharf R. 2012. Plant growth promoting Rhizobacteria: An overview. *Journal of Nautural Products and Plant Resources* **2**(1): 19-31.

Akilli S. 2012. Feasibility of biological control of chestnut blight caused by *Cryphonectria parasitica*, in Marmara region of Turkey. *African Journal of Agricultural Research* **7**(45): 6068-6072.

Alikhani H A, Saleh-Rastin N and Antoun H. 2006. Phosphate solubilization activity of rhizobia native to Iranian soils. *Plant and soil* **287**: 35–41.

Altschul S F, Thomas L M, Alejandro A S, Jinghui Z, Zheng Z, Webb M and David J L. 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programmes. *Nucleic Acids Research* **25**: 3389-3402.

Amaresan N, Jayakumar V, Kumar K and Thajuddin N. 2011. Endophytic bacteria from tomato and chilli, their diversity and antagonistic potential against *Ralstonia solanacearum. Archives of Phytopathology and Plant Protection* **45**(3): 344-355.

Antoun H and Kloepper J W. 2001. Plant growth promoting rhizobacteria. ***In***: Brenner S and Miller J H (eds) Encyclopedia of Genetics. Academic, New York, pp 1477-1480.

Aranda S, Miguel-Borrego M, Rafael M, Diaz-Jimenez and Landa B B. 2011. Microbial communities associated with the root system of wild olives (*Olea*

europaea L. subsp. *europaea* var. *sylvestris*) are good reservoirs of bacteria with antagonistic potential against *Verticillium dahlia*. *Plant and Soil* **343**: 329-345.

Ashraf M A, Rasool M and Mirza M S. 2011. Nitrogen fixation and indole acetic acid production potential of bacteria isolated from rhizosphere of sugarcane (*Saccharum officinarum* L.). *Advances in Biological Research* **5**(6): 348-355.

Aslantas R, Cakmkci R and Sachin F. 2007. Effect of plant growth promoting rhizobacteria on young apple tree growth and fruit under orchard conditions. *Scientia Horticulture* **111**(4): 371-377.

Atlas R M. 1995. Handbook media for environmental microbiology. New York : CRL, Press Bocaratar. pp. 43-371.

Aziz A Z F, Saud H M, Rahim K A and Ahmed O H. 2012. Variable responses on early development of shallot (*Allium ascalonicum*) and mustard (*Brassica juncea*) plants to *Bacillus cereus* inoculation. *Malaysian Journal of Microbiology* **8**(1): 47-50.

Badri D V, Weir T L, van der Lelie D and Vivanco J M. 2009. Rhizosphere chemical dialogues: plant–microbe interactions. *Current Opinion in Biotechnology* **20**: 642-650.

Bagwell C E and Lovell C R. 2000. Persistence of selected *Spartina alterniflora* rhizoplane diazotrophs exposed to natural and manipulated environmental variability. *Applied Environmental Microbiology* **66:** 4625-4633.

Bakker and Schippers. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp. mediated plant growth stimulation. *Soil Biology and Biochemistry* **19**: 451-457.

Bakker P A H M, Pieterse C M J and van Loon L C. 2007. Induced systemic resistance by fluorescent *Pseudomonas* spp. *The American Phytopathological Society* **97**(2): 239-243.

Bakthavatchalu S, Shivakumar S and Sullia S B. 2012. Identification of multi-trait PGPR isolates and evaluation of their potential as biocontrol agents. *Acta Biologica Indica* **1**(1): 61-67.

Balnatah U. 2013. Studies on plant growth promoting rhizobacteria associated with strawberry (Fragaria x ananassa). Nauni, Solan: UHF. Thesis (M.Sc.)-Deptt. Of Basic Sciences, UHF, 2013.

Bashan and Bashan. 2002. Reduction of bacterial speck (*Pseudomonas syringae* pv. *tomato*) of tomato by combined treatments of plant growth-promoting bacterium, *Azospirillum brasilense*, streptomycin sulfate, and chemo-thermal seed treatment. *European Journal of Plant Pathology* **108**: 821–829.

Baysal O, Soylu E M and Soylu S. 2003. Induction of defence-related enzymes and resistance by the plant activator acibenzolar-S-methyl in tomato seedlings against bacterial canker caused by *Clavibacter michiganensis* ssp. *michiganensis. Plant Pathology* **52**: 747-753.

Beattie G A. 2006. Plant-associated bacteria: Survey, molecular phylogeny, genomics and recent advances. ***In***: Gnanamanickam S S (ed) Plant-Associated Bacteria. Springer, Dordrecht, pp. 1-56.

Beneduzi A, Ambrosini A and Passaglia L M P. 2012. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology* **35**(4): 1044-1051.

Benitez T, Rincon A M, Limon M C and Codon A C. 2004. Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology* **7**: 249-260.

Bent A F. 2002. Crop diseases and strategies for their control. ***In***: Plants, genes and Crop Biotechnology Chrispeels M J and Savada D E (eds) 2nd ed. Sudburry, Canada, Jones and Bartlett Publishers, pp 390-413.

Berger L R and Reynolds D M. 1958. The chitinase system of a strain of S*treptomyces griseus*. *Biochemistry Biophysics Acta* **29**: 522-534.

Bhagat D, Sharma P, Sirari A and Kumawat K C. 2014. Screening of *Mesorhizobium* sp. for control of Fusarim wilt in chikpea *in vitro* conditions. *International Journal of Current Microbiology and Applied Sciences* **3**(4): 923-930.

Bhattacharya and Jha. 2012. Plant growth promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiol Biotechnol*. **28**: 1327–1350.

Bholay A D, Jadhav P U, Borkhataria B V, Dhalkari M V. 2012. Fluorescent *Pseudomonads* as plant growth promoting rhizobacteria and their siderophoregenesis. *Journal of Pharmacy and Biological Sciences* **3**(1): 27-32.

Biratu K S, Selvaraj T and Hunduma T. 2013. *In vitro* evaluation of actinobacteria against tomato bacterial wilt (*Ralstonia solanacearum* E F Smith) in West Showa, Ethiopia. *J Plant Pathol Microb.* **4**:1.

Boller T, Gehri A, Mauch F and Vogeli U. 1983. *Phaseolus vulgaris* chitinase in bean leaves: induction by ethylene, purification, properties and possible function. *Planta* **157**: 22–31.

Boudyach E H, Fatmi M, Okhayat O, Benizri E and Aoumar A A B. 2001. Selection of antagonistic bacteria of *Clavibacter michiganensis* subsp. *michiganensis* and evaluation of their efciency against bacterial canker of tomato. *Biocontrol Science and Technology* **11**: 141-149.

Bray R H and Kurtz L T. 1945. Determination of total organic and available forms of phosphorus in soils. *Soil Science* **59**: 39-45.

Budi S W, Van Tuinen D, Arnould C, Dumas-Gaudut E, Gianinazzi-Pearson V and Gianinazzi S. 2000. Hydrolytic enzyme activity of *Paenibacillus* sp. strain B2 and effect of antagonistic bacterium on cell wall integrity of two soil-borne pathogenic fungi. *Applied Soil Ecology* **15**: 191–199.

Burketova L, Tindelárová M and Tindelár L. 1999. Benzothiadiazole as an inducer of β-1,3-glucanase and chitinase isozymes in sugar beet. *Biologia Plantarum* **42**: 279–87.

Calis O, Bayan Y and Celik D. 2012. Characterization of resistant tomato mutants to bacterial canker disease. *African Journal of Biotechnology*. **11**(32): 8070-8075.

Cattelan A J, Hartel P G and Fuhrmann J J. 1999. Screening for plant growth-promoting rhizobacteria to promote early soybean growth. *Soil Science Society of American Journal* **63**: 1670-1680.

Chaiharn M, Chunhalenchanon S and Lunyong S. 2009. Screening siderophore producing bacteria as potential biological control agent for fungal rice pathogens in Thialand. *World Journal of Microbial Biotechnol*ogy **25**: 1919-1928.

Chakraborty U, Chakraborty B N, Chakraborty A P, Sunar K and Dey P L. 2013. Plant growth promoting rhizobacteria mediated improvement of health status of tea plants. *Indian Journal of Biotechnology* **12**: 20-31.

Chakravarty G and Kalita M C. 2012. Biocontrol potential of *Pseudomonas fluorescens* against bacterial wilt of Brinjal and its possible plant growth promoting effects. *Annals of Biological Research* **3**(11): 5083-5094.

Chandel S. 2011. Studies on siderophore producing plant growth promoting rhizobacteria associated with apple cultivars. Nauni, Solan: UHF. Thesis (M.Sc.)-Deptt. Of Basic Sciences, UHF, 2011.

Chatli A S, Beri V and Sidhu B S. 2008. Isolation and characterisation of phosphate solubilizing microorganisms from the cold desert habitat of *Salix alba* Linn. in trans Himalayan region of Himachal Pradesh. *Indian Journal of Microbiology* **48**: 267-273.

Chen Y P, Rekha P D, Arun A B, Shen F T, Lai W A and Young C C. 2006. Phosphate solubilizing bacteria from subtropical soil and their tri-calcium phosphate solubilizing abilities. *Applied Soil Ecology* **34**: 33-41.

Chowdhury S P, Schmid M, Hartmann A and Tripathi A K. 2009. Diversity of 16S rRNA and *nif* H genes derived from rhizosphere soil and roots of an

endemic drought tolerant grass, *Lasiurus sindicus*. *European Journal of Soil Biology* **45**:114-122.

Claus D and Berkley R. 1986. Genus *Bacillus* Cohn. 1872, 174. ***In***: Bergey's manual of systematic bacteriology. Sneath P H A, Nair N S, Sharpe M E and Holt J G, Baltimore M D (*eds*.). USA: Williams and Wilkins. **2**: 1105-1139.

Compant S, Duffy B, Nowak J, Clement C and Barka E A. 2005. Use of plant growth-promoting bacteria for mechanisms of action, and future biocontrol of plant diseases: Principles, Prospects. *Applied Environmental Microbiology* **71**(9): 4951–4959.

Conrath U, Thulke O, Katz V, Schwindling S and Kohler A. 2001. Priming as a mechanism in induced systemic resistance of plants. *European Journal of Plant Pathology* **107**: 113–9.

Cruz-Quiroz R D L, Chavez C, Hernandez M, Rodriguez R, Hernandez D and Aguilar C N. 2011. Antagonist capacity of newly isolated strains of *Pseudomonas fluorescens* against three important phytopathogenic bacteria. *American Journal of Agricultural and Biological Sciences* **6** (2): 267-272.

Dahiya N, Tewari R and Hoondal G S. 2006. Biotechnological aspects of chitinolytic enzymes: a review. *Applied Microbiology and Biotechnology* **71**: 773-782.

Dairo, Paul K, Akintunde and Kehinde J. 2012. Evaluation of plant growth-promoting rhizobacteria for the control of bacterial wilt disease of tomato. *Global Journal of Bio-Science and Biotechnology* **1**(2): 253-256.

Das A J, Kumar M and Kumar R. 2013. Plant growth promoting rhizobacteria (PGPR): An alternative of chemical fertilizer for sustainable, environment friendly agriculture. *Research Journal of Agriculture and Forestry Sciences* **1**(4): 21-23.

Dastager S G, Deepa C K and Pandey A. 2010. Isolation and characterization of novel plant growth promoting *Micrococcus* sp. NII-0909 and its interaction with cowpea. *Plant Physiology Biochemistry* **48**(12): 987-992.

Dave B P, Kena A and Puja H. 2006. Siderophores of halophilic archaea and their chemical characterization. *Indian Journal of Experimental Biology* **44**: 340-344.

Deshwal V K and Kumar P. 2013. Production of plant growth promoting substances by Pseudomonads. *Journal of Academia and Industrial Research* **2**(4): 221-225.

Diaz M E, Villa P and Fries A. 2002. Evaluation of the siderophore production by *Pseudomonas aeruginosa* PSS. *Revista Latioamericana de Microbiologica* **44**: 112-117.

Ding C, Shen Q, Zhang R and Chan W. 2013. Evaluation of rhizosphere bacteria and derived bioorganic fertilizers as potential biocontrol agents against bacterial wilt (*Ralstonia solanacearum*) of potato. *Plant Soil* **366**: 453-466.

Ding Y, Wang J, Liu Y and Chem S. 2005. Isolation and identification of nitrogen-fixing bacilli from plant rhizospheres in Beijing region. *Journal of Applied Microbiology* **99**: 1271-1281.

Dobbelaere S, Vanderleyden J and Okon Y. 2003. Plant growth promoting effects of diazotrophs in the rhizosphere. *Critical Review on Plant Sciences* **22**: 107-149.

Edi Premono M, Moawad A M and Vlek P L G. 1996. Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Science* **11**:13-23.

Ehrlich H L. 1990. Geomicrobial processes. A physical and biochemical overview. ***In***: Geomicrobiology. 2nd ed. Marcel Dekker, pp. 225-231.

El-Hendawy H H, Osman M E, Sorour N M. 2005. Biological control of bacterial spot of tomato caused by *Xanthomonas campestris* pv. *vesicatoria* by *Rahnella aquatilis*. *Microbiological Research* **160**: 343-352.

Engelhard M, Hurek T and Reinhold-Hurek B. 2000. Preferential occurrence of diazotrophic endophytes, *Azoarcus* spp., in wild rice species and land races of *Oryza sativa* in comparison with modern races. *Environmental Microbiology* **2**: 131-141.

Fatima Z, Saleem M, Muhammad Z, Sultan T, Aslam M, Rehman R and Chaudhary M F. 2009. Antifungal activity of plant growth promoting rhizobacterial isolates against *Rhizoctonia solani* in wheat. *African Journal of Biotechnology* **8**(2): 219-225.

Fleming H P, Etchells J L and Costilus R H. 1975. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. *Applied Microbiology* **30:** 1040-1042.

Gerhardson B. 2002. Biological substitutes for pesticides. *Trends in Biotechnology* **20**: 338-343.

Ghose T K. 1987. Measurement of cellulase activities. *Pure and Applied Chemistry* **59**: 257-268.

Girgis M G Z, Khalil Heba M A and Sharaf M S. 2008. *In vitro* evaluation of rock phosphate and potassium solubilizing potential of some *Bacillus* strains. *Australian Journal of Basic and Applied Sciences* **2**(1): 68-81.

Girish N and Umesha S. 2005. Effect of plant growth promoting rhizobacteria on bacterial canker of tomato. *Archives of Phytopathology and Plant Protection* **38**(3): 235-243.

Glick B R, Cheng Z, Czarny J and Duan J. 2007. Promotion of plant growth by ACC deaminase-producing soil bacteria. *European Journal of Plant Pathology* **119**: 329-339.

Gopalakrishnan S, Upadhyaya H D, Vadlamudi S, Humayun P, Vidya M S, Alekhya G, Singh A, Vijayabharathi R, Bhimineni R K, Seema M, Rathore A and Rupela O. 2012. Plant growth-promoting traits of biocontrol potential bacteria isolated from rice rhizosphere. *SpringerPlus* **1**(71): 2-7.

Gordon S A and Paleg L G. 1957. Quantitative measurement of indole acetic acid. *Physiology Plant* **10**: 37-48.

Gracia de Salamone I E, Hynes R K and Nelson L M. 2001. Cytokinin production by plant growth promoting rhizobacteria and selected mutants. *Canadian Journal of Microbiology* **47**: 404-411.

Gray E J and Smith D L. 2005. Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biology and Biochemistry* **37**: 395-412.

Gupte M D and Kulkarni P R. 2002. A study of antifungal antibiotic production by *Streptomyces chattanoogensis* MTCC 3423 using full factorial design. *Letters in Applied Microbiology* **35**: 22-26.

Haas D and Keel C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annual Review on Phytopathology* **41**: 117-153.

Haggag M W. 2010. Role of Entophytic Microorganisms in Biocontrol of Plant Diseases. *Life Science Journal* **7**(2): 57-62.

Haldeman Dana L and Amy Penny S. 1993. Diversity within a colony morphotype: implications for ecological research. *Applied Environmental Microbiology* **59**(3): 933-935.

Hamdali H, Moursalou K, Tchangbedji G, Ouhdouch Y and Hafidi M. 2012. Isolation and characterization of rock phosphate solubilizing actinobacteria from a Togolese phosphate mine. *African Journal of Biotechnology* **11**(2): 312-320.

Hameeda B, Harish Kumar Reddy Y, Rupela O P, Kumar G N and Reddy Gopal. 2006. Effect of aarbon substrates on rock phosphate solubilization by bacteria from composts and macrofauna. *Current Microbiology* **53**: 298-302.

Heitz T, Segond S, Kauffmann S, Geoffroy P, Prasad V, Brunner F, Fritig B and Legrand M. 1994. Molecular characterization of a novel tobacco pathogenesis-related (PR) protein – a new plant chitinase/lysozyme. *Molecular and General Genetics* **245**: 246–54.

Hill G T. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Applied Soil Microbiology* **15**: 25-36.

Idris H A, Labuschagne N and Korsten L. 2007. Screening rhizobacteria for biological control of *Fusarium* root and crown rot of sorghum in Ethiopia. *Journal of Biological Control* **40**(1): 97-106.

Jahanian A, Mohammad Reza Chaichi M R, Rezaei K, Rezayazdi K and Khavazi K. 2012. The effect of plant growth promoting rhizobacteria (PGPR) on germination and primary growth of artichoke (*Cynara scolymus*). *International Journal of Agriculture and Crop Sciences* **4** (14): 923-929.

Jalili F, Khavazi K, Pazira E, Negati A, Asadi Rahmani H, Rasuli Sadaghiani H and Miransari M. 2009. Isolation and characterization of ACC demainase producing *fluorescent Pseudomonads* to elevate salinity stress on Canola (*Brassica napus* L.) growth. *Journal of Plant Physiology* **166**: 667-674.

James E K. 2000. Nitrogen fixation in endophytic and associative symbiosis. *Field Crops Res*earch **65**: 197-209.

Jinsheng S, Guo F, Geng X, Wei J, Li X and Li J. 2011. Seasonal changes and diversity of bacteria in Bohai Bay by RFLP analysis of PCR-amplified 16S rDNA gene fragments. *World Journal of Microbiology and Biotechnology* **27**: 275-284.

Joseph B, Patra R R and Lawrence R. 2007. Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L). *International Journal of Plant Production* **1**: 141-152.

Joseph S and Jisha M S. 2009. Buffering reduces phosphate solubilizing ability of selected strains of bacteria. *World Journal of Agricultural Sciences* **5**(1): 135-137.

Kannapiran E and Ramkumar V S. 2011. Isolation of phosphate solubilizing bacteria from the sediments of Thondi coast, Palk Strait, Southeast coast of India. *Annals of Biological Research* **2**(5): 157-163.

Karnwal A. 2009. Production of indole acetic acid by fluorescent *Pseudomonas* in the presence of L- tryptophan and rice root exudates. *Journal of Plant Pathology* **91**(1): 61-63.

Kaur M and Sharma P. 2013. Screening and characterization of native *Pseudomonas* sp. as plant growth promoting rhizobacteria in chickpea (*Cicer arietinum* L.) rhizosphere. *African Journal of Microbiological Research* **7**(16): 1465-1474.

Kerr A. 1972. Biological control of crown gall: seed inoculation. *Journal Appied Bacteriology* **35**: 493–497.

Kloepper J W, Schroth M N and Miller T D 1980. Effect of rhizosphere colonization by plant growth promoting rhizobacteria on potato development and yield. *Phytopathology* **70**: 1080-1082.

Kloepper J. W, Ryu C-M, and Zhang S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **94**: 1259-1266.

Krewulak K D and Vogel H J. 2008. Structural biology of bacterial iron uptake. *Biochemistry Biophysics Acta* **1778**: 1781-1804.

Kumar A, Kumar A, Devi S, Patil S, Payal C and Negi S. 2012. Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an *in vitro* study. *Recent Research in Science and Technology* **4**(1): 1-5.

Kundu B S, Gera R, Sharma N, Bhatia A and Sharma A. 2002. Host specificity of phosphate solubilizing bacteria. *Indian Journal of Microbiology* **42**: 19-21.

Lanteigne C, Gadkar V J, Wallon T, Novinscak and Filion M. 2012. Production of DAPG and HCN by *Pseudomonas* sp. LBUM300 contributes to the biological control of bacterial canker of tomato. *Biological Control* **102**(10): 967-973.

Lebaron Philippe, Ghiglione Jean-Francois, Fajon Ceèline, Batailler Nicole and Normand Philippe. 1998. Phenotypic and genetic diversity within a colony morphotype. *FEMS Microbiology Letters* **160**: 137-143.

Lemessa F and Zeller W. 2007. Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia. *Biological Control* **42**: 336-344.

Loganathan M, Garg R, Venkataravanappa V, Saha S and Rai A B. 2014. Plant growth promoting rhizobacteria (PGPR) induces resistance against *Fusarium* wilt and improves lycopene content and texture in tomato. *African Journal of Microbiology Research* **8**(11): 1105-1111.

Louws F J, Rademaker J L W and de Bruijn F J. 1999. The three Ds of PCR-based genomic analysis of phytobacteria: Diversity, detection and disease diagnosis. *Annual Review of Phytpathology* **37**: 81-125.

Lugtenberg B and Kamilova F. 2009. Plant-growth-promoting rhizobacteria. *Annual Review on Microbiology* **63**: 541-56.

Madigan M T, Martinko J M and Parker J. 2000. Brock Biology of Microorganisms. Upper Saddle River. Newjersy. Prentice Hall.

Maji S and Chakrabartty P K. 2014. Biocontrol of bacterial wilt of tomato caused by *Ralstonia solanacearum* by isolates of plant growth promoting rhizobacteria. *Australian Journal of Crop Science* **8**(2): 208-214.

Maksimov IV, Abizgil'dina R R and Pusenkova L I. 2011. Plant growth promoting rhizobacteria as alternative to chemical crop protectors from pathogens (Review). *Applied Biochemistry and Microbiology* **47**:333-345.

Malleswari D. 2014. *In vitro* antagonistic activity of diverse bacterial isolates against *Macrophomina phaseolina* (Tassi) Goid. *International Journal of Current Microbiology and Applied Sciences* **3**(5): 755-763.

Marcic S M, Gatermann K H, Frohwitter J, Eichenlaub R, Todorovic B, Rekanovic E and Potocnik I. 2012. Characterization of *Clavibacter michiganensis* subsp. *michiganensis* strains from recent outbreaks of bacterial wilt and canker in Serbia. *European Journal of Plant Pathology* **134**: 697–711.

Martinez-Viveros O, Jorquera M A, Crowley D E, Gajardo G and Mora M L. 2010. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of Soil Science and Plant Nutrition* **10** (3): 293–319.

Masunaka A, Nakaho K, Sakai M, Takahashi H and Takenaka S. 2009. Visualization of *Ralstonia solanacearum* cells during biocontrol of bacterial wilt disease in tomato with *Pythium oligandrum. Journal of General Plant Pathology* **75**: 281–287.

Mazhar S and Hasnain S. 2011. Screening of native plant growth promoting cyanobacteria and their impact on *Triticum aestivum* var. Uqab 2000 growth. *African Journal of Agricultural Research* **6**(17): 3988-3993.

Mehta P, Chauhan A, Mahajan R, Mahajan P K and Shirkot C K. 2010. Strain of *Bacillus circulans* isolated from apple rhizosphere showing plant growth promoting potential. *Current Sci*ence **98**: 538-542.

Mehta P. 2012. Diversity and functions of phosphate solubilizing rhizobacteria associated with apple seedlings. Nauni, Solan: UHF. Thesis (M.Sc.)-Deptt. Of Basic Sciences, UHF, 2012.

Menzies J G and Jarvis W R. 1994. Diseases and Pests of Vegetable Crops in Canada. ***In***: Howard R J, Garland J A and Seaman W L (eds), Bacterial canker. *The Canadian Phytopathological Society and the Entomological Society of Canada,* pp. 341-342.

Mishra M, Kumar U, Mishra P K and Prakash V. 2010. Efficiency of plant growth promoting rhizobacteria for the enhancement of *Cicer arietinum* L. growth and germination under salinity. *Advances in Biological Research* **4**(2): 92-96.

Mishra S, Jagadeesh K S, Krishnaraj P U and Prem S. 2014. Biocontrol of tomato leaf curl virus (ToLCV) in tomato with chitosan supplemented formulations of *Pseudomonas* sp. under field conditions. *Australian Journal of Crop Science* **8**(3): 347-355.

Mitchell J K and Carter W E. 2000. Modeling antimicrobial activity Clorox^{TM} using an agar-diffusion test: a new twist on an old experiment. *Bioscan* **26**: 9-13.

Miyazawa J, Kawabata T and Ogasawara N. 1998. Induction of an acidic isozyme of peroxidise and acquired resisitance to wilt disease in response to treatment of tomato roots with 2-furoic acid, 4-hydroxy benzoic hydrazide or salicylic hydrazide. *Physiological and Molecular Plant Pathology* **52**: 115-126.

Mubarik N R, Mahagiani I, Anindyaputri A, Santoso S and Rusmana I. 2010. Chitinolytic bacteria isolated from chili rhizosphere: Chitinase characterization and its application as biocontrol for whitefly (*Bemisia tabaci* Genn.). *American Journal of Agricultural and Biological Sciences* **5**: 430-435.

Mulla S R, Sandeep C, Salimath P J, Varsha P, Suresh C K and Priyadarsini R I. 2013. Effect of *Pseudomonas fluorescens* isolated from various agro climatic zones of Karnataka on *Rauwolfia serpentine. International Journal of Pharmacy and Pharmaceutical Sciences* **5**(4): 141-146.

Nagrajkumar M, Bhaskaran R and Velazhahan R. 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath of blight pathogen. *Research in Microbiology* **159**: 73-81

Nakouti I and Hobbs G. 2012. Characterization of five siderophore producing actinomycetes from soil samples and the use of antibiotic resistance to differentiate the isolates. *International Journal of Agriculture Sciences* **4**(3): 202-206.

Naseri S and Sharafzadeh S. 2013. Impact of *Azotobacter* on growth and total phenolic content of garden thyme. *Advances in Environmental Biology* **7**(1): 113-115.

Naz I and Bano A. 2010. Biochemical, molecular characterization and growth promoting effects of phosphate solubilizing *Pseudomonas sp.* isolated from weeds grown in salt range of Pakistan. *Plant and Soil* **334**:199-207.

Neeraja C, Anil K, Purushotham P, Suma K, Sarma P, Moerschbacher B M and Podile A R. 2010. Biotechnological approaches to develop bacterial chitinases as a bioshield against fungal diseases of plants. *Critical Review on Biotechnology* **30**:231-241.

Neilands J B and Leong A S. 1986. Siderophores in relation to plant growth and disease. *Annual Review of Plant Physiology* **37**: 187-208.

Nguyen N V, Kim Y J, Oh K T, Jung W J and Park R D. 2008. Antifungal activity of chitinases from *Trichoderma aureoviride* DY-59 and *Rhizopus* microspores VS-9. *Current Microbiology* **56**: 28-32.

Nielson T H, Sorensen D. Tobiasen C, Anderson J B, Christophersen C, Givskov M and Sorensen J. 2002. Antibiotic and lipopeptides produced by fluorescent *Pseudomonas* sp. from sugarbeet rhizosphere. *Applied Environmental Microbiology* **68**: 3416-3424.

Nikolay V, Maria V and Lana N. 2006. Stimulation of phosphate solubilizing and biocontrol activity of microorganism: potential and future trends. *Journal of Applied Microbiology and Biotechnology* **71**(2): 137-144.

Noori M S S and Saud H M. 2013. Potential plant growth-promoting activity of *Pseudomonas* sp. isolated from paddy soil in Malaysia as biocontrol agent. *Journal of Plant Pathology and Microbiology* **3**(2): 2157-7471.

Osorio H, Martinez V, Nieto P A, Holmes D S and Quatrini R. 2008. Microbial iron management mechanisms in extremely acidic environments: Comparative genomics evidencefor diversity and versatility. *BMC Microbiology* **8**:203.

Ouda M S. 2014. Biological control by microorganisms and ionizing radiation. *International Journal of Advanced Research* **2**(5): 314-356.

Pace N R. 1997. A molecular view of microbial diversity and the biosphere. *Science.* **276**: 734-740.

Page R D M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applied Bioscience* **12**: 357-358.

Pal K K and McSpadden G B. 2006. Biological Control of Plant Pathogens. *The Plant Health Instructor* DOI: 10.1094/PHI-A-2006-1117-02.

Pandey A, Trivedi P, Kumar B and Palni L M S. 2006. Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (BO) isolated from a sub-alpine location in the Indian central Himalaya. *Current Microbiol*ogy **53**: 102–107.

Panhwar Q A, Radziah O, Sariah M and Ismail M R. 2009. Solubilization of different phosphate forms by phosphate solubilizing bacteria isolated from aerobic rice. *International Journal of Agriculture and Biology* **11**: 667–673.

Park K H, Lee C Y and Son H J. 2005. Mechanism of insoluble phosphate solubilization *by Pseudomonas fluorescens* RAF15 isolated from ginseng rhizosphere and its plant growth promoting activities. *Letters in Applied Microbiology* **49**: 222-228.

Patel D K, Archana G and Kumar G N. 2008. Variation in the nature of organic acid secretion and mineral phosphate solubilization by *Citrobacter* sp. DHRSS in the presence of different sugars. *Current Microbiology* **56**(2): 168-174.

Pikovskaya R I. 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Mikrobiologiya* **7**: 362-370.

Pitt J I. 2000. Toxigenic fungi: which are important? *Medical Mycolology 38 Suppl* **1**: 17-22.

Podile A R and Kishore G K. 2006. Plant growth-promoting rhizobacteria. ***In***: Gnanamanickam S S (ed) Plant-Associated Bacteria. Springer, Netherlands, pp. 195-230.

Poly F, Ranjard L, Nazaret S, Atlas G F and Monrozier J L. 2001. Comparison of *nif* H gene pools between soils and between soil microenvironments of contrasting properties. *Applied Environmental Microbiology* **67**: 2255-2267.

Pradhan N and Sukla L B. 2005. Solubilization of inorganic phosphate by fungi isolated from agriculture soil. *African Journal of Biotechnology* **5**: 850-854.

Prakash P and Karthikeyan B. 2013. Isolation and purification of plant growth promoting rhizobacteria (PGPR) from the rhizosphere of *Acorus Calamus* grown soil. *Indian Streams Research Journal* **3**(7): 1-13.

Rachid D and Ahmed B. 2005. Effect of iron and growth inhibitors on siderophores production by *Pseudomonas fluorescens*. *African Journal of Biotechnology* **4**(7): 697-702.

Radjacommare R, Kandan A, Nanda K R and Samiyappan R. 2004. Association of the hydrolytic enzyme chitinase against *Rhizoctonia solani* in rhizobacteria treated rice plants. *Journal of Phytopathology* **152**: 365-370.

Ramette A, Frapolli M, Défago G and Moënne-Loccoz Y. 2003. Phylogeny of HCN synthase encoding *hcnBC* genes in biocontrol fluorescent Pseudomonads and its relationship with host plant species and HCN synthesis ability. *Molecular Plant-Microbe Interactions* **16**: 525–535.

Ramos Solano B, Garcia J A L, Villaraco A G, Algar E, Cristobal J G and Manero F J G. 2010. Siderophore and chitinase producing isolates from the rhizosphere of *Nicotiana glauca* Graham enhance growth and induce systemic resistance in *Solanum lycopersicum* L. *Plant and Soil* **334**: 189-197.

Ramyasmruthi S, Pallavi O, Pallavi S, Tilak K and Srividya S. 2012. Chitinolytic and secondary metabolite producing *Pseudomonas fluorescens* isolated from Solanaceae rhizosphere effective against broad spectrum fungal phytopathogens. *Asian Journal of Plant Science and Research* **2**(1): 16-24.

Rani V, Parida A and Raina N S. 1995. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus detoides* marsh. *Plant Cell Reports* **14**: 459-462.

Rathore P. 2014. A review on approaches to develop plant growth promoting rhizobacteria. *International Journal of Recent Scientific Research* **5**(2): 403-407.

Reetha S, Bhuvaneswari G, Thamizhiniyan P and Mycin T R. 2014. Isolation of indole acetic acid (IAA) producing rhizobacteria *of Pseudomonas fluorescens* and *Bacillus subtilis* and enhance growth of onion *(Allim cepa*. L*).* *International Journal of Current Microbiology and Applied Sciences* **3**(2): 568-574.

Reva O N, Dixelius C, Meijer J and Priest F G. 2004. Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *FEMS Microbiology Ecology* **48**: 249-259.

Richardson A E, Barea J M, McNeill A M and Prignent-Combaret C. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil* **321**: 305-339.

Robert W K and Selitrennikoff C P. 1988. Plant and bacterial chitinase differ in antifungal activity. *Journal of General Microbiology* **134**: 169-176.

Rodrigues F, Priadharsini.K, Suja S and Palani P. 2014. *In vitro* antagonism and evaluation of chitinase activity of bacteria–*Bacillus circulans* against

pathogenic fungi in *Vigna unguiculata***.** *International Journal of Applied Biology and Pharmaceutical Technology* **5**(3): 58-64.

Romero A M, Correa O S, Moccia S and Rivas J G. 2003. Effect of *Azospirillum*-mediated plant growth promotion on the development of bacterial diseases on fresh-market and cherry tomato. *Journal of Applied Microbiology* **95**: 832–838.

Rudrappa T, Splaine R E, Biedrzycki M L, Bais H P. 2008. Cyanogenic Pseudomonads influence multitrophic interactions in the rhizosphere. *PloS ONE* **3**: 2073.

Saharan B S and Nehra V. 2011. Plant Growth Promoting Rhizobacteria: A Critical Review. *Life Sciences and Medicine Research*. **21**: 1-30.

Sahasrabudhe M M. 2011. Screening of rhizobia for indole acetic acid production. *Annals of Biological Research* **2**(4): 460-468.

Saito A and Minamisawa K. 2006. Evaluation of nitrogen-fixing ability of endophytic clostridia based on acetylene reduction and reverse transcription-PCR targeting the *nif H* transcript and ribosomal RNA. *Microbes and Environment* **21**: 23-35.

Samalla K, Sessitsch A. and Hartmann A. 2006. The Rhizosphere: ‘soil compartment influenced by the root’. *FEMS Microbiology Ecology* **56**: 165-165.

Samavat S, Asghar Heydari A, Reza H, Zamanizadeh H. R, Rezaee S and Aliabadi A A. 2014. A comparison between *Pseudomonas aureofaciens* (*chlororaphis*) and *P. fluorescens* in biological control of cotton seedling damping-off disease. *Journal of Plant Protection Research* **54**(2): 115-121.

Sambrook J, Fritsch E F and Maniatis T. 1989. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory.

Sarode P D, Rane M R, Chaudhari B L and Chincholkar S B. 2009. Siderophoregenic *Acinetobacter calcoaceticus* isolated from wheat rhizosphere with strong PGPR activity. *Malaysian Journal of Microbiology* **5**(1): 6-12.

Schwyn B and Neilands J B. 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**: 47-56.

Seebold K R, Gitaitis F H, Sanders and Horten T B. 2003. Eeffects of soil solarization and turnip residue on soil fungi, bacteria and yield of sweet onions in Georgia. ***In***: Georgia Onion Research Extension Report 2003. pp. 55-57.

Seleim M A, Abo-Elyousr K A, Mohamed A A A, Al-Marzoky H A. 2014. Peroxidase and polyphenoloxidase activities as biochemical markers for biocontrol efficacy in the control of tomato bacterial wilt. *Journal of Plant Physiology and Pathology* **2**(1): 1-4.

Selvakumar G, Kundu S, Gupta A D, Shouche Y S and Gupta H S. 2008. Isolation and characterization of nonrhizobial plant growth promoting bacteria from nodules of Kudzu (*Pueraria thunbergiana)* and their effect on wheat seedling growth. *Current Microbiology* **56**: 134-139.

Sen Y, Feng Z, Vandenbroucke H, van der Wolf J, Visser R G F and van Heusden A W. 2013. Screening for new sources of resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in tomato. *Euphytica* **190**: 309–317.

Shahab S, Ahmed N and Khan S N. 2009. Indole acetic acid production and enhanced plantgrowth promotion by indigenous PSBs. *African Journal of Agricultural Research* **4** (11): 1312-1316.

Shanmugam V and Kanoujia N. 2011. Biological management of vascular wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycospersici* by plant growth-promoting rhizobacterial mixture. *Biological Control* **57**: 85-93.

Sharma B C, Subba R and Saha A. 2012. *In vitro* solubilization of tricalcium phosphate and production of IAA by phosphate solubilizing bacteria isolated from tea rhizosphere of Darjeeling Himalaya. *Plant Sciences Feed* **2**(6): 96-99.

Sharma K. 2013. Characterisation of plant growth promoting rhizobacteria isolated from the rhizosphere of apricot (*Prunus armeniaca*). Nauni, Solan: UHF. Thesis (M.Sc.)-Deptt. Of Basic Sciences, UHF, 2013.

Sharma R. 2012. Efficiency of plant growth promoting rhizobacteria for the enhancement of tomato growth. Nauni, Solan: UHF. Thesis (M.Sc.)-Deptt. Of Basic Sciences, UHF, 2012.

Sharma T, Navin Kumar N and Rai N. 2012. Isolation, screening and characterization of PGPR isolates from rhizosphere of rice plants in Kashipur region (Tarai region*). Biotechnology International* **5**(3): 69-84.

Shaw F J, Lin P F, Chen C S and Chen C H. 1995. Purification and characterization of an extracellular α- amylase from thermos species. *Botanical Bulletin of Academia Sinica* **36**: 195-200.

Shehata H S, Hamed E R and Eleiwa M E. 2012. Potential for improving healthy and productivity of Soybean by plant growth promoting rhizobacteria. *Australian Journal of Basic and Applied Sciences* **6**(4): 98-107.

Shenge K C, Mabagala R B and Mortensen C N. 2010. Current status of bacterial speck and spot diseases of tomato in three tomato-growing regions of Tanzania. *Journal of Agriculture Extension and Rural Development* **2**(5): 84-88.

Shrestha A, Kim B S and Park D H. 2014. Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper. *Biocontrol Science and Technology* **24**(7): 763-779.

Singh P, Kumar V and Agrawal S. 2014. Evaluation of phytase producing bacteria for their plant growth promoting activities. *International Journal of Microbiology* DOI 10.1155/2014/426483.

Sivasakthi S, Usharani G and Saranraj P. 2014. Biocontrol potentiality of plant growth promoting bacteria (PGPR)- *Pseudomonas fluorescence* and *Bacillus subtilis*: A review. *African Journal of Agricultural Research* **9**(16): 1265-1277.

Slusarski C. 2008. Attempts at biological control of *Clavibacter michiganensis* subsp. *michiganensis* on rockwool-grown greenhouse tomatoes. *Vegetable Research Bulletin* **69**: 125-134.

Soylu S, Baysal O and Soylu M. 2003. Induction of disease resistance by the plant activator, Acibenzolar-S-methyl (ASM), against bacterial canker (*Clavibacter michiganensis* ssp. *michiganensis*) in tomato seedlings. *Plant Science* **165**: 1069-1075.

Steenhoudt O and Vanderleyden J. 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiology Review* **24:** 487-50.

Suarez-Moreno Z R, Caballero-Melledo J, Cautinho B, Mendonca-Previato L, James E K and Venturi V. 2011. Common features of environmental and potentially beneficial plant-associated *Burkholderia* sp. *Microbial Ecology* **10**: 115-121.

Sultana S, Sharma N and Shirkot C K. 2004. Production of antifungal antibiotic by a newly isolated strain of *Bacillus megaterium*. *Journal of Microbial World* **6**(1): 8-15.

Sundaramoorthy S and Balabaskar P. 2012**.** Consortial Effect of Endophytic and Plant Growth Promoting Rhizobacteria for the Management of Early Blight of Tomato Incited by *Alternaria Solani*. *Journal of Plant Pathology and Microbiology* **3**(7): 1-4.

Suo Y and Leung D W M. 2001. Elevation of extracellular β-1,3-glucanase and chitinase activities in rose in response to treatment with acibenzolar-S-methyl and infection by *Diplocarpon rosae*. *Journal of Plant Physiology* **158**: 971–6.

Supraja Y, Reddy R S, Reddy S S and Rani V D. 2011. Plant growth promotion and biocontrol properties of local isolates of *Fluorescent pseudomonads. Journal of Research, ANGRAU* **39**(3): 1-5.

Suresh P, Pallavi P, Srinivas V, Praveen Kumar S, Jeevan Chandra and Ram Reddy S. 2010. Plant growth promoting activities of fluorescent Pseudomonads associated with some crop plants. *African Journal of Microbiology Research* **4**(14): 1491-1494.

Talibi I, Amkraz N, Askarne L, Msanda F, Saadi B, Boudyach E H, Boubaker H, Bouizgarne B and Aoumar A B. 2011. Antibacterial activity of moroccan plants extracts against *Clavibacter michiganensis* subsp. *michiganensis,* the causal agent of tomatoes' bacterial canker. *Journal of Medicinal Plants Research* **5**(17): 4332-4338.

Tan S, Jiang Y, Song S, Huang J, Ling N, Xu Y, Shen Q. 2013. Two *Bacillus amyloliquefaciens* strains isolated using the competitive tomato root enrichment method and their effects on suppressing *Ralstonia solanacearum* and promoting tomato plant growth. *Crop Protection* **43**: 134-140.

Tolba I H and Soliman M A. 2012. Efficacy of native antagonistic bacterial isolates in biological control of crown gall disease in Egypt. *Annals of Agricultural Science* **58**(1): 43–49.

Turan M, Ekinci M, Yildirim E, Guneş A, Karagoz K, Kotan R, Dursun A. 2013. Plant growth-promoting rhizobacteria improved growth, nutrient, and hormone content of cabbage (*Brassica oleracea*) seedlings. *Turkish Journal of Agriculture and Forestry* **38**: 327-333.

Utkhede R and Koch C. 2004. Biological treatments to control bacterial canker of greenhouse tomatoes. *BioControl* **49**: 305-313.

Van Loon L. C. and Glick G. R. 2004. Increased plant fitness by rhizobacteria. ***In***: Molecular ecotoxicology of plants. Sandermann H, (ed.) Springer-Verlag, Berlin. **170**: 177-205.

Van Loon LC. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *European Journal of Plant Pathology* **103**: 753–65.

Vasanthabharathi V and Jayalakshmi S. 2014. Biocontrol and PGPR potential of marine sponge *Callysspongia diffusa* associated *P. fluorescens* BCPBMS-1. *Annals of Experimental Biology* **2**(2): 49-57.

Wahyudi A T, Astuti R P, Widyawati A, Meryandini A and Nawangsih A. 2011. Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria. *Journal of Microbiology and Antimicrobials* **3**(2): 34-40.

Walia A, Mehta P, Chauhan A and Shirkot C K. 2013. Effect of *Bacillus subtilis* strain CKT1 as inoculum on growth of tomato seedlings under net house conditions. *Procedings of National Academy of Sciences section B Biological Sciences.* DOI 10.007/s40011-013-0189-3.

Walker T S, Bais H P, Grotewold E and Vivanco J M. 2003. Root exudation and rhizosphere biology. *Plant Physiology* **132**: 44–51.

Wani P A, Khan M S and Zaidi A. 2007. Co-inoculation of nitrogen fixing and phosphate solubilizing bacteria to promote growth yield and nutrient uptake in chickpea. *Acta Agronomica Hungarica* **55**(3): 315-323.

Williams K J, Kubelik A, Livak K, Fafalski J and Tingey S. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nuclear Agriculture Soil* **18**: 6531-6535.

Yari S D N, Inanlou F, Yari M, Saleh, Farahmand B and Akbarzadeh A A. 2002. Effects of protoplast fusion on endotoxin Production in *Bacillus thuringiensis* sp. (H14). *Iranian Biomedical Journal* **6**(1): 25-29.

Yeon K S, Hong S H, Ryu H W and Cho K-S. 2010. Plant growth promoting trait of rhizobacteria isolated from soil contaminated with petroleum and heavy metals. *Journal of Microbiology and Biotechnology* **20**(3): 587-593.

Zahir A A, Arshad M and Frankenberger W T. 2004. Plant growth promoting rhizobacteria: applications and perspectives in agriculture. *Advanced Agronomy* **81**: 97–168.

Zani S, Mellon M T, Collier J L and Zehr J P. 2000. Expression of *nif* H genes in natural microbial assemblages in lake George, New York, detected by reverse transcriptase PCR. *Applied Environmental Microbiology* **66**: 3119-3124.

Zehr J P, Jenkins B D, Short S M and Steward G F. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environmental Microbiology* **5**: 539-554.

Zhou T, Chen D, Li C, Sun Q, Li F, Shen Q and Shen B. 2012. Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. *Microbial research* **167**: 388-394.

Dr. Y. S. PARMAR UNIVERSITY OF HORTICULTURE AND FORESTRY
NAUNI-173230, SOLAN (H.P.)
DEPARTMENT OF BASIC SCIENCE

Title of Thesis	:	**"Characterization of plant growth promoting rhizobacteria and evaluation of their biocontrol potential against tomato bacterial canker"**
Name of Student	:	Swati Gautam
Admission Number	:	F-2012-24-M
Name of Major Advisor	:	Dr. C. K. Shirkot
Major field	:	Microbiology
Minor field(s)	:	i) Biotechnology ii) Biochemistry
Year of award of Degree	:	2014
Number of pages in Thesis	:	**125**
Number of words in Abstract	:	265

ABSTRACT

Bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* is known to cause significant economic losses to tomato production worldwide. Biological control has been proposed as an alternative to current chemical containment methods, which are often inefficient and may leave adverse effects on the environment. However, only little headway has so far been made in developing biocontrol strategies against *C. michiganensis* subsp. *michiganensis*. To address this knowledge gap, we investigated the antagonistic capacity of different PGPRs having multifarious plant growth promoting traits (P-solubilization, IAA production, siderophore production, lytic enzyme activity, and ability to fix atmospheric nitrogen) on *C. michiganensis* under *in vitro* and *in planta* conditions. PGPRs are highly diverse and in this study we focus on rhizobacteria as biocontrol agents. Their effects can occur via local antagonism to soil-borne pathogens or by induction of systemic resistance against pathogens throughout the entire plant. Several substances produced by antagonistic rhizobacteria have been related to pathogen control and indirect promotion of growth in many plants, such as siderophores, lytic enzymes and antibiotics. Induced systemic resistance (ISR) in plants resembles pathogen-induced systemic acquired resistance (SAR) under conditions where the inducing bacteria and the challenging pathogen remain spatially separated. Five antagonistic bacterial isolates were tested for biocontrol potential against *C. michiganensis* under net house conditions. Isolate S1 showed maximum reduction in disease incidence and disease index along with maximum increase in plant growth parameters. The isolate was identified as *Bacillus amyloliquefaciens* by 16S rDNA sequencing. Therefore, strain S1 had considerable biocontrol potential as well as plant growth promoting ability and could be used as a potential biocontrol agent against bacterial canker of tomato.

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

Countersigned

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

APPENDIX-I

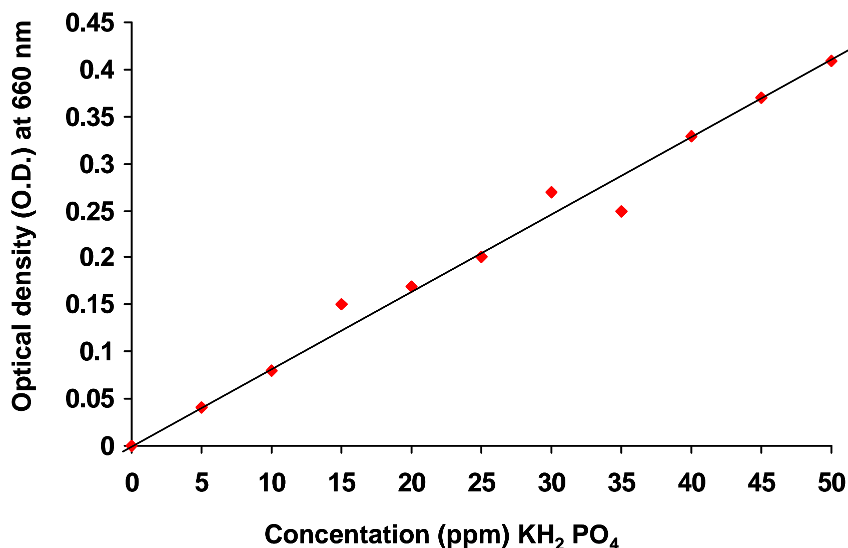
1.1 Preparation of standard curve (5-50 µg/ml) for P-estimation

(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	0.04
0.2	25	10	0.08
0.3	25	15	0.15
0.4	25	20	0.17
0.5	25	25	0.20
0.6	25	30	0.27
0.7	25	35	0.27
0.8	25	40	0.33
0.9	25	45	0.37
1.0	25	50	0.41



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

Stock solution (100 ppm):

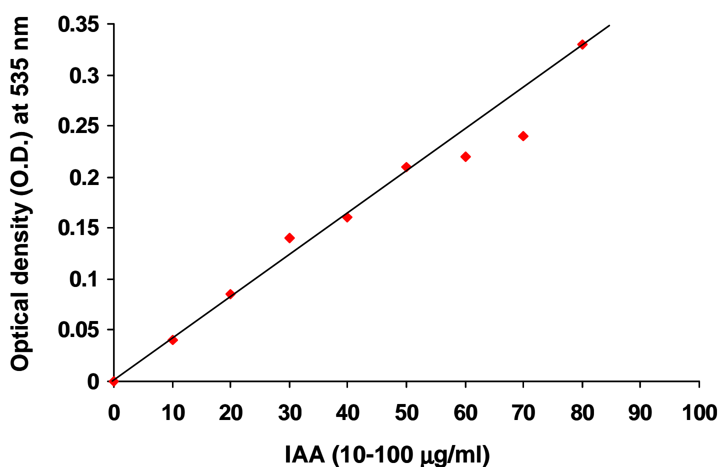
10 mg of IAA (99.0% pure) was dissolved in 50 ml distilled water and the final volume was made to 100 ml in a volumetric flask.

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	0.04
0.6	2.4	3	2	20	0.08
0.9	2.1	3	2	30	0.14
1.2	1.8	3	2	40	0.16
1.5	1.5	3	2	50	0.21
1.8	1.2	3	2	60	0.22
2.1	0.9	3	2	70	0.24
2.4	0.6	3	2	80	0.33
2.7	0.3	3	2	90	0.36
3.0	0	3	2	100	0.41

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.



APPENDIX II

Anova 1 : Quantitative characterization of PGP traits of selected antagonistic bacterial isolates

Source	d.f.	Mean sum of square (MSS)								
		P solubilisation	Viable count	Final pH	Siderophore production	Viable count	Final pH	Indole acetic acid production	Viable count	Final pH
Treatment	**9**	2.70×10^4	63.13	0.01	5347.4	47.95	0.10	217.20	46.18	0.11
Error	**18**	18.77	0.82	0.03	10.48	0.61	0.06	0.97	0.31	0.04

Anova 2 : Efficacy of PGPR strains in controlling tomato bacterial canker

Source	d.f	Mean sum of square (MSS)				
		Disease incidence	Disease index	Biocontrol efficacy	Population of pathogen	Population of antagonist ($\times 10^4$ cfu/g)
Treatment	**5**	1587.1	632.81	1587.1	5015.0	2372.4
Error	**15**	77.21	14.786	77.21	8.94	3.38

Anova 3 : Effect of seedling treatment with liquid formulation of selected bacterial isolates on growth parameters in net house

Source	d.f	Mean sum of square (MSS)			
		Shoot length	shoot dry weight	Root length	Root dry weight
Treatment	**5**	176.79	24.92	10.242	0.93
Error	**15**	7.56	4.83	0.02	0.02

CURRICULUM VITAE

Name	:	Swati Gautam
Father's Name	:	Mr. D. L. Gautam
Date of Birth	:	23 June, 1993
Sex	:	Female
Marital Status	:	Unmarried
Nationality	:	Indian

Educational Qualifications:

Certificate/ Degree	**Class/Grade**	**Board/University**	**Year**
Matriculation	First	CBSE	2007
10+2	First	HPBSE	2009
B.Sc. (Microbiology)	First	Panjab university	2012

Whether sponsored by some state/ Central Government/Univ./SAARC : Nil

Scholarship/stipend/fellowship, any other financial assistance received during study period : Nil

Conference/Proceeding attended:

1. Presented poster in National Conference on Perspective and Trends in Plant Sciences and Biotechnology.

(Swati Gautam)