

**STUDIES ON THE ROLE OF ANTIFUNGAL METABOLITES
PRODUCING NATIVE STRAINS OF *Pseudomonas* SPECIES
TO OVERCOME REPLANT PROBLEM OF APPLE**

Thesis

by

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*Submitted in partial fulfilment of the requirements
for the degree of*

MASTER OF SCIENCE

in

MICROBIOLOGY



**COLLEGE OF FORESTRY
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CERTIFICATE-I

This is to certify that the thesis entitled, “**Studies on the role of antifungal metabolites producing native strains of *Pseudomonas* species to overcome replant problem of apple**” 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 Kalpana Mehta (F-2009-04-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.

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CERTIFICATE-II

This is to certify that the thesis entitled, “**Studies on the role of antifungal metabolites producing native strains of *Pseudomonas* species to overcome replant problem of apple**” submitted by **Ms Kalpana Mehta (F-2009-04-M)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE in 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.

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ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my Major advisor Dr (Mrs.) Mohinder Kaur for the continuous support of my M.Sc study and research, for her patience, motivation, enthusiasm and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis.

Besides my advisor, I would like to thank the rest of my thesis advisory committee: Dr R.C. Rana, Dr Girish Sharma, Dr Rajesh Kaushal and Dr Neerja Rana for their encouragement, insightful comments, and extensive discussions in my work.

My warm Thanks are due to Dr P.K. Mahajan for their kind support and guidance in statistical analysis.

I owe profound debt to Dr C.K. Shirkot Professor and Head Dept of Basic Sciences for his thoughtful assistance and encouragement during the course of investigation.

My special graditude is due to my Mummy, Papa and di Sweety for their loving support. Without their encouragement & understanding it would have been impossible for me to finish this work. I am thankful to Mr. Amod Dutt for his tangible but regular unspoken support for my M.Sc. study.

I thank to my junior Manorma and seniors - Shweta, Sapna & Ranjna Sharma for the stimulating discussions, making our lab. convivial place to work and for all the fun we have had during our trial visit to Rohru. Also I thank my friends Sweety and Prateek, the Charming gardeners, who make my soul blossom in every moment. I wish to extend my warmest thanks to my seniors Himani, Sheetal and Pankaj for their all time love and support.

I would like to express my appreciation to laboratory and office staff of the Deptt. of Basic Sciences for their kind help.

Above all, I thank Almighty God for giving me the strength to complete this research work.

Needless to say, errors and omissions are solely of mine.

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LIST OF ABBREVIATIONS

%	: percent
%I	: percent inhibition
°C	: degree celsius
µg	: Microgram
µl	: Microlitre
ARD	: Apple replant disease
bp	: base pair
C.D.	: Critical difference
CAS	: chrome azurol-S
cfu	: colony forming unit
cm	: centimeter
df	: degree of freedom
dia	: diameter
DNA	: deoxyribonucleic acid
g	: grams
GA	: Gibberellic acid
h	: hour
HDTMA	: Hexadecyltrimethyl Ammonium bromide
IAA	: Indole acetic acid
L	: litre
M	: molar
MEA	: malt extract agar
mg	: milligram
min	: minutes
ml	: milliliter
mm	: millimeter
mM	: millimolar
MSS	: Mean sum of square
N	: normal

NA	: nutrient agar
nm	: nano meter
NTP	: Nucleotide phosphate
O.D.	: Optical density
PCR	: polymerase chain reaction
PDA	: Potato dextrose agar
PGPR	: Plant growth promoting rhizobacteria
PPM	: Pigment production mediaa
PSB	: phosphate solubilizing bacteria
PSE	: phosphate solubilizing efficiency
PVK	: Pikovskaya
RNA	: ribonucleic acid
rpm	: revolutions per minute
SU	: siderophore unit
UV	: Ultra violet
V	: Volts
v/v	: volume by volume
YMA	: Yeast malt agar
RE	: Relative error

Chapter-1

INTRODUCTION

The poor growth and development of fruit trees that occurs after replanting on a site that previously supported the same or closely related species is termed as replant problem. In apple, replant problem is widespread and has been documented in all the major fruit growing regions of the world (Traquair 1984). This problem is becoming very serious problem in horticultural crops and is often encountered in establishing new orchards on old sites (Mai and Abawi, 1981). Apple replant problem is characterized by uneven growth of young trees, small roots system, discolored roots and reduced productivity. Poor growth of newly planted trees is common in replanted orchards. The disease is a complex syndrome also called specific replant disease (SRD) that affects young trees in replant sites and reduces growth survival and yield of replanted trees. The economic impacts of the soil borne disease complex are serious. Huge losses in gross income over ten years could result from failure to control replant disease (Mai and Abawi, 1981).

A replant problem in apple orchards appears to be related to soil and rhizosphere microflora (Catska *et al.*, 1982). It is a complex disease syndrome and its etiology is still unclear (Yao *et al.*, 2006). In most of the replant sites, abiotic and biotic factors have been implicated in SRD (Merwin *et al.*, 2001). Soil bacteria, plant parasitic nematodes, fungi, actinomycetes, oomycetes especially *Phytophthora* and *Pythium* individually or together are causative agent of the replant disease (Mazzola and Gu, 1998; Mai and Abawi, 1981; Catska *et al.*, 1982; Merwin *et al.*, 2001; Rumberger *et al.*, 2007).

Methyl bromide (MB) has been the fumigant used most widely to control this disease. But alternative to methyl bromide, cultural methods are needed because methyl bromide is phased out due to environmental problems (Yao *et al.*, 2006).

Several soil microorganisms are known to improve the plant growth directly through nutrient mobilization and production of plant hormones and indirectly through suppression of plant pathogens or by inducing systemic resistance in plants. The soil microorganisms that enhance nutrient availability to plants include nitrogen fixers, P solubilizers, auxins and gibberellins producers, which have been used to develop biofertilizers. Other soil microorganisms, which possess the ability to improve plant growth indirectly through suppression of disease causing organisms or by inducing systemic resistance in plants, have also been developed as bioinoculants.

Many soil microorganisms possess multiple beneficial traits of nutrient mobilization, production of plant growth promoting substances (PGP) and biocontrol ability. Such organisms have a greater role in sustaining agricultural production. Plant growth promoting rhizobacteria (PGPR), a group of root associated bacteria, intimately interact with the plant roots and consequently influence plant health and soil fertility. They offer an excellent combination of traits useful in disease control and plant growth promotion.

Several studies have shown that rhizosphere bacterial communities may suppress root infections by soil-borne pathogens. Some bacteria are associated with roots of crop plants and exert beneficial effect on the host and are collectively known as plant growth promoting rhizobacteria (Glick, 1995; Kloepper *et al.*, 1989). Some of these rhizobacteria not only benefit from the nutrients secreted by the plant roots, but also beneficially influence the plant in a direct or indirect way resulting in stimulation of its growth.

Amongst the PGPRs, fluorescent pseudomonads have emerged as the largest and potentially the most promising group of PGPR with their rapid growth, simple nutritional requirements, ability to utilize diverse organic substrates and mobility. Fluorescent pseudomonads produce highly potent broad spectrum antifungal molecules against various phytopathogens, thus acting as effective biocontrol agents. They are well equipped as primary root colonizers. Through several mechanisms *viz.*, production of antibiotics (Gutterson *et al.*,

1988), siderophores (Kloepper *et al.*, 1980), HCN (Defago *et al.*, 1990) and competition for space and nutrients (Elad *et al.*, 1987), They inhibit soil borne plant pathogens. They could serve as promising bioinoculants for agricultural system to increase productivity since the action of such bacteria is highly specific, ecofriendly and cost-effective. However, selecting a potential strain with multiple beneficial traits for production of inoculants from natural biodiversity hot spots assumes significance.

PGPR's especially fluorescent *Pseudomonas* sp. can directly promote the growth of plants usually by production of phytohormones and/or by increasing the available nutrients through production of secondary metabolites like lytic enzymes, phosphate solubilization, and siderophores and/or as biocontrol agents that are able to protect plant from infections by phytopathogenic organisms through production of antibiotics, siderophores, HCN, ammonia and cell wall degrading enzymes (Kloepper *et al.*, 1989; Kloepper, 1993; Glick, 1995;). The large scale application of PGPR's to crops as inoculants would be attractive as it would substantially reduce the use of chemical fertilizers, pesticides and methyl bromide etc. which often pollute the environment and reduce the fertility of soil.

Plant growth promoting rhizobacteria can competitively colonize plant roots and stimulate plant growth and/or decrease the incidence of plant diseases. They can be exploited as biological pesticides to decrease the use of chemical pesticides in agriculture. Some *Pseudomonas* sp. especially fluorescent pseudomonads, are particularly suitable to be used as agricultural biocontrol agents because they can produce large amounts of secondary metabolites to protect plants from phytopathogens and stimulate plant growth.

Pseudomonas sp. produce antifungal antibacterial compounds like antibiotics, iron chelating siderophores, cyanide and enzymes such as glucanase, cellulolytic and chitinolytic enzymes. These compounds have the activity by which *Pseudomonas* strains inhibits the fungal growth through damaging cell walls. Antibiotic production by fluorescent sp. is recognized as an important feature in plant disease suppression. The ability of *P. fluorescens* to suppress

soilborne fungal pathogens depends on their ability to produce antibiotic metabolites such as pyoluteorin, pyrrolnitrin, phenazine 1- carboxylic acid and 2, 4-diacetylphloroglucinol (Maurhofer *et al.*, 1995).

Production of antibiotics by several strains of fluorescent pseudomonads has been recognized as a major factor in suppression of root pathogens. Howell and Stipanovic (1979) first established the importance of antibiotic production in the suppression of seedling pathogens using *Pseudomonas fluorescens* Pf-5. A number of disease suppressive antibiotic compounds have been characterized including N-containing heterocycles such as phenazines, pyrrole type antibiotics, pyro-compounds and indole derivatives. 2,4-diacetylphloroglucinol (DAPG) has been identified as a broad spectrum antibiotic, exhibiting antifungal, antibacterial, antihelminthic and phytotoxic activities. Raaijmakers and Weller (1998) demonstrated that root associated fluorescent Pseudomonads with the capacity to produce DAPG were the key components in biological control of *Gaeumannomyces graminis tritici* that caused take all disease in wheat. DAPG was also a major determinant in the biological control activity of *Pseudomonas fluorescens* against black root rot of tobacco caused by *Thielaviopsis basicola* and that of *Pseudomonas fluorescens* F113 against damping off of sugarbeet caused by *Pythium ultimum*. According to Picard *et al.*, (2000) spatial and temporal selection in the rhizosphere was responsible for fluctuation in the population of DAPG producers and induction of DAPG producers in exudates of older plants.

Fluorescent pseudomonads are ubiquitous bacteria that are common inhabitants of the rhizosphere and are the most studied group within the genus *Pseudomonas*. They can be visually distinguished from the other *Pseudomonas* species of soil by their ability to produce water soluble yellow green pigments. They comprise of *Pseudomonas aeruginosa*, the type species of the genus, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. putida* and the plant pathogenic species *P. cichorii* and *P. syringae* (Dwivedi and Johri, 2003).

The isolation, identification and characterization for production of antifungal activities by indigenous plant growth promoting fluorescent *Pseudomonas* species from specific soil and specific plant type and the mass multiplication is necessary in selecting and developing useful plant growth promoting agents for field applications.

Therefore, the present study '**Studies on the role of antifungal metabolites producing native strains of *Pseudomonas* species to overcome replant problem of apple**' was undertaken. The present investigation addressed the hypothesis using the following objectives:

- i) Characterization of selected potential strains for production of antifungal metabolites.
- ii) Effect of selected strains on growth promotion and establishment of apple seedlings in replant sites.

Chapter-2

REVIEW OF LITERATURE

Apple replant disease (ARD) is distributed worldwide and is often encountered in establishing new orchards on old sites (Yao *et al.*, 2006). Symptoms include death of fine feeder roots, stunted growth above- and below-ground, and reduced fruit yields (Rumberger *et al.*, 2007). Replant disease, also called “soil sickness“, causes a significant problem in fruit orchards in the temperate climate zone. It may increase because of intensification of fruit production, which is usually followed by replanting orchards in the same place (Szczygieł & Zepp, 1998). Replant disease is one of the components of replant problem, which is caused by biotic and abiotic factors. In 1968, a work was published calling attention to the connection between fluorescent bacteria from *Pseudomonas* genus (Utkhede and Smith, 1994; Rutkowski *et al.*, 2000a) and *Bacillus subtilis* with a limited growth of apple tree roots as a result of root top necrosis. Actinomycetes might be the reason for apple tree replantation disease (Utkhede and Smith, 1994). Abiotic factors that cause replant problem are phytotoxins, nutrient imbalance, low or high pH, soil structure and drainage, and lack or excess of moisture (Utkhede and Smith, 1994). According to Szczygieł (2003), the limitation of negative results of replantation disease can be exerted by rational fertilization and irrigation.

Colonisation of apple roots by arbuscular mycorrhizae (AM) has been reported to be associated with healthy trees (Trappe *et al.*, 1973), efficient nutrient uptake (Covey *et al.*, 1981) and enhanced plant growth (Plenchette *et al.*, 1981). Earlier observations of apple roots have shown significantly lower mycorrhizal infection in SARD soils (Caruso *et al.*, 1989; Catska and Taube-Baab 1994) and alleviation of disease symptoms by inoculation with AM fungi (Catska, 1994). The colonization of feeder roots by AM was compared between SARD and non-SARD soils with different treatments in two pot experiments.

Bacteria that colonize roots effectively are termed “Rhizobacteria” (Schroth and Hancock, 1982). Root colonization is the process where bacteria survive on seeds, multiply in spermosphere in response to seed exudates rich in carbohydrates and amino acids (Kloepper *et al.*, 1989), attach on to the root surfaces (Suslow, 1982) and colonize the developing root system. Thus, colonization of roots is an active process and not a transitory relation between bacteria and roots in the soil. PGPR are free-living bacteria and some of them invade the tissues of living plants and cause unapparent and symptomatic infections (Sturz and Nowak, 2000) when applied to seeds or crops, enhance the growth of the plant or reduce the damage from soil-borne plant pathogens (Kloepper *et al.*, 1980). Somers *et al.*, (2004) classified PGPR as biofertilizers (increasing the availability of nutrients to plant), phytostimulators (plant growth promoting, usually by the production of phytohormones), rhizoremediators (degrading organic pollutants) and biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites).

Bashan and Holguin (1998) proposed the division of PGPR into two classes: biocontrol-PGPR and PGPR. When the effect of PGPR on plants was demonstrated, the concept of PGPR began to gain importance. A large number of bacterial strains have been isolated, screened (Bertrand *et al.*, 2001), and were evaluated for plant growth promotion (Bashan, 1998; Bent *et al.*, 2001). Several research groups have randomly screened rhizosphere bacteria for antagonistic activity against nematodes (Becker *et al.*, 1988 and Spiegel *et al.*, 1991). Isolates of *Agrobacterium radiobacter*, *Bacillus subtilis*, *Pseudomonas* spp. that are familiar as antagonists of soil borne bacterial and fungal diseases of plants, also have potential as biological control agents for nematodes.

Bacteria that establish positive interaction with plant roots are called plant growth promoting rhizobacteria (PGPR). PGPR play an important role in phytostimulation, phytoremediation and biofertilization. The prime beneficial traits of PGPR include production of plant hormones, siderophores, exopolysaccharides, bacteriocins, fixation of atmospheric nitrogen, mineral phosphate solubilization and antibiotic resistance. They are also known to

provide protection to plants against diseases by suppressing deleterious and pathogenic microorganisms. Plant growth promoting rhizobacteria have a significant role on plant growth and development in two different ways viz., indirectly or directly. The indirect promotion of plant growth occurs when bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism by one or more mechanisms.

The direct promotion of plant growth by PGPR generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment (Glick, 1995 and Glick *et al.*, 1999). Plant growth benefits due to the addition of PGPR include increase in germination rates, root growth and yield including grain, increase in leaf area, chlorophyll content, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought and salt stress, shoot and root weights and delayed leaf senescence (Lucy *et al.*, 2004).

Van Peer and Schippers (1988) documented increased root and shoot fresh weight of tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strains. Several studies have showed that growth promotion effects were seen early in plant development and these subsequently translated into higher yields (Hoffmann-Hergarten *et al.*, 1998 and Polyanskaya *et al.*, 2000).

Glick (1995) reported that *Pseudomonas* make up a dominant population in soil and rhizosphere and exert growth promoting influence on a variety of plant species on account of their strong competitive behaviour, colonization potential and sustainability. Seed bacterization with these organisms has emerged as a powerful technology to enhance plant growth and yield, besides providing protection against diseases. Raji and Lekha (2003) reported that seed bacterization with *Pseudomonas fluorescens* following the wet and dry methods enhanced root length of seedlings by 26.33 and 16.33 per cent respectively. Plant height was also increased in dry and wet method of seed treatment respectively under pot culture condition. Krishnakishore *et al.*, (2003) reported that the seed

bacterization with plant growth promoting rhizobacteria and phylloplane bacteria promoted growth and yield in groundnut.

2.1 FLUORESCENT *PSEUDOMONADS*

The genus *Pseudomonas* encompasses arguably the most diverse and ecologically significant group of bacteria on the planet. Fluorescent pseudomonads are gram negative, rods shaped, and chemoheterotrophic bacteria with polar flagella and are characterized by the yellow green iron chelating low molecular weight siderophores called pyoverdines or pseudobactins that fluoresce under UV light (O'Sullivan and O' Gara, 1992). They are common inhabitants of rhizosphere and are the most studied group within the genus *Pseudomonas*. They can be visually distinguished from the other *Pseudomonas* species by their ability to produce water soluble yellow green pigments. They comprise of *Pseudomonas aeruginosa*, the type species of the genus, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. putida* and the plant pathogenic species *P. cichorii* and *P. syringae* (Dwivedi and Johri, 2003). Fluorescent pseudomonads are well recognized as plant growth promoting rhizobacteria, phosphate solubilizers and as biocontrol agents against plant pathogens.

The occurrence and distribution of fluorescent pseudomonads have been studied by many workers. Johri *et al.*, (1997) reported fluorescent pseudomonads as rapid and aggressive root colonizers of cereals, pulses, oilseeds and vegetable crops. Rao *et al.*, (1999) isolated fluorescent pseudomonads from the rhizoplane and rhizosphere of pea and soybean plants. *Pseudomonas glumae* EM85, a potent biocontrol agent for phytopathogenic *Rhizoctonia solani* of cotton was isolated from maize rhizosphere by Pal *et al.*, (2000). Tripathi and Johri (2002) isolated and characterized fluorescent pseudomonads from rhizoplane and rhizosphere of pea and wheat plants. Suryakala *et al.*, (2004) isolated *Pseudomonas fluorescens* from rhizosphere of rice, wheat, pigeon pea, and ground nut and chilli crops.

Two fluorescent pseudomonad strains viz., RRLJ134 and RRLJ04 were isolated from tea growing soils in Dooars region of West Bengal and rhizoplane of rice from Gujarat, respectively by Mishra *et al.* (2005). *Pseudomonas*

populations showing antagonistic properties towards *Thielaviopsis basicola*, causal agent of black root rot of tobacco were reported in the tobacco rhizosphere by Ramette *et al.*, (2006).

The genus *Pseudomonas* encompasses arguably the most diverse and ecologically significant group of bacteria on the planet. They are typically gram-negative, chemoheterotrophic and motile rods with polar flagella as defined by Palleroni *et al.*, (1973). Members of this genus are found in large numbers in all the major natural environments *viz.* terrestrial, freshwater and marine and they also form intimate associations with plants and animals. This universal distribution suggests a remarkable degree of physiological and genetic adaptability (Spiers *et al.*, 2005).

All fluorescent pseudomonads fall into one of the five 'ribonucleic acid homology groups' as defined by rRNA-DNA complementation experiments. The G + C content ranges from 58 to 68 per cent. Most of the plant beneficial pseudomonads are quite heterogenous in that they comprise a collection of non-enteric, gram-negative strains that are generally aerobic, non-fermenting and motile (Dwivedi and Johri, 2003). They appear to be ubiquitous, but references to ecological speciation of some of the species can be found in literature. The presence of *Pseudomonas fluorescens* in the rhizosphere of plants was also reported later (Bashan, 1986). Fluorescent pseudomonads are well recognised as plant growth promoting rhizobacteria, P-solubilizers and as biocontrol agents against plant pathogens. Many studies have been conducted on the diversity of fluorescent pseudomonads.

Plant growth promoting rhizobacteria (PGPR) were first defined by Kloepper and Schroth (1978) as the soil bacteria that colonize the roots of plants by following inoculation on to seed and that enhance plant growth. PGPRs enhance plant growth by direct and indirect means, but the specific mechanisms involved have not been well characterized (Glick, 1995 and Kloepper, 1993). Direct mechanisms of plant growth promotion by PGPR can be demonstrated in the absence of plant pathogens or other rhizosphere microorganisms, while

indirect mechanisms involve the ability of PGPRs to reduce the deleterious effects of plant pathogens on crop yield. PGPRs have been reported to directly enhance plant growth by a variety of mechanisms, *viz.*, fixation of atmospheric nitrogen that is transferred to the plants, production of siderophores that chelate iron and make it available to the plant roots, solubilization of minerals such as phosphorous and synthesis of phytohormones (Glick, 1995). Direct enhancement of mineral uptake due to increase in specific ion fluxes at the root surface in the presence of PGPR has also been reported (Bashan and Levanony, 1991; Bertrand *et al.*, 2000). PGPR strains may use one or more of these mechanisms in the rhizosphere. Molecular approaches using microbial and plant mutants altered in their ability to synthesize or respond to specific phytohormones have increased our understanding of the role of phytohormone synthesis as a direct mechanism of plant growth enhancement by PGPRs (Glick, 1995; Persello-Carticaux *et al.*, 2003). PGPRs that synthesize auxins and cytokinins or those that interface with plant ethylene synthesis have been identified (Persello-Carticaux *et al.*, 2003). The indirect means by which PGPRs enhance plant growth is through suppression of phytopathogens by a variety of mechanisms.

These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize anti-fungal metabolites such as antibiotics, fungal cell wall lysing enzymes or hydrogen cyanide, which suppress the growth of fungal pathogens. The ability to successfully compete with pathogens for nutrients or to exclude specific niches on the root and the ability to induce systemic resistance in plants are the other mechanisms (Bloemberg and Lugtenberg, 2001).

2.2. ANTIFUNGAL ACTIVITY

Tripathi and Johri (2002) studied the biocontrol potential of fluorescent pseudomonads against *Colletotrichum dematium*, *Rhizoctonia solani* and *Sclerotium rolfsii* *in vitro*, as well as *in vivo* biocontrol action in maize against sheath blight caused by *Rhizoctonia solani*. Some isolates were having multiple disease control potential while some others showed biocontrol potential against a specific pathogen indicating that the fluorescent pseudomonads are diverse with

respect to their biocontrol potential. Seventeen isolates of fluorescent pseudomonads collected from ten rubber growing areas of Kerala were assessed *in vitro* for antagonism against five pathogens of rubber, viz., *Colletotrichum acutatum*, *Corynespora cassiicola* (leaf pathogen), *Phytophthora meadii* (leaf and stem pathogen), *Corticium salmonicolor* (stem pathogen) and *Phellinus noxius* (root pathogen). The isolates were found to antagonize the growth of all the five pathogens (Joseph *et al.*, 2003).

Suryakala *et al.*, (2004) reported that *Pseudomonas fluorescens* isolated from different crop rhizosphere were antagonistic to fungal pathogens like *Fusarium oxysporum*, *Alternaria sp.* and *Colletotrichum capsicii*.

Mishra *et al.*, (2005) reported that two strains of fluorescent pseudomonads viz., RRLJ134 and RRLJ04 isolated from tea rhizosphere and rhizoplane of rice respectively showed significant *in vitro* antibiosis against *Fomes lamoensis* and *Ustilina zonata* causal agents of brown root rot and charcoal stump rot of tea respectively. Application of these strains resulted in significant suppression of both the diseases under gnotobiotic and nursery conditions.

Biological control of plant diseases is gaining attention due to increased pollution concerns because of pesticides use for crop protection and development of pathogen resistance (Wisniewski and Wilson, 1992). The use of environmental friendly microorganisms has proved useful in plant-growth promotion and disease control in modern agriculture (Weller, 1988). Kravchenko *et al.*, (2002) averred that PGPR inoculation is a promising agricultural approach that plays a vital role in crop protection, growth promotion or biological disease control (Dilantha *et al.*, 2006).

The *Pseudomonas sp.* are well known for their involvement in the biocontrol of several plant pathogens (Antoun and Prevost, 2005). The selection of effective strains of particular bacteria is of prime importance for the biocontrol of plant pathogens. Isolation of bacteria from pathogen suppressive soils may increase the chances of finding effective strains (Cook and Baker, 1983). To get

effective isolates, isolation of bacteria should be made from the same environment in which they will be used (Weller, 1988). The ability to colonize roots and resistance against antibiotics are the other parameters to screen effective strains (Siddiqui *et al.*, 2005).

A collection of 905 bacterial isolates from the rhizospheres of healthy avocado trees was obtained and screened for antagonistic activity against *Dematophora necatrix*, the cause of avocado *Dematophora* root rot (also called white root rot). A set of eight strains was selected on the basis of growth inhibitory activity against *D. necatrix* and several other important soil borne phytopathogenic fungi. After typing of these strains, they were classified as belonging to *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. The eight antagonistic *Pseudomonas* spp. were analysed for their secretion of hydrogen cyanide, hydrolytic enzymes, and antifungal metabolites. *P. chlororaphis* strains produced the antibiotic phenazine-1-carboxylic acid and phenazine-1-carboxamide. Upon testing the biocontrol ability of these strains in a newly developed avocado-*D. necatrix* test system and in a tomato-*F. oxysporum* test system, it became apparent that *P. fluorescens* PCL1606 exhibited the highest biocontrol ability. The major antifungal activity produced by strain *P. fluorescens* PCL1606 did not correspond to any of the major classes of antifungal antibiotics produced by *Pseudomonas* biocontrol strains. This compound was purified and subsequently identified as 2-hexyl 5-propyl resorcinol (HPR). To study the role of HPR in biocontrol activity, two Tn5 mutants of *P. fluorescens* PCL1606 impaired in antagonistic activity were selected. These mutants were shown to impair HRP production and showed a decrease in biocontrol activity. This is the first report of a *Pseudomonas* biocontrol strain that produces HPR in which the production of this compound correlates with its biocontrol activity. (Cazorla *et al.*, 2006).

Pseudomonas fluorescens BNM296 and *Bacillus amyloliquefaciens* BNM340, protected plants against the damping-off caused by *Pythium ultimum* and were able to increase the seedling emergence rate after inoculation of soybean seeds with each bacterium. (Le´on *et al.*, 2009).

Bhatia *et al.*, (2005) reported that bacterization of sunflower seeds with fluorescent pseudomonads PS I and PS II reduced the incidence of collar rot by 69.80 and 56.90 per cent respectively in *Sclerotium rolfsii* infested soil. In a study conducted on the suppression of *Verticillium* wilt involving eight isolates of *Pseudomonas fluorescens* and *Pseudomonas putida*, Mercado-Blanco *et al.*, (2004) observed effective control of *Verticillium* wilt by all the isolates under controlled conditions.

Fluorescent pseudomonads are also known to be effective against bacterial pathogens. The bacterial wilt of tomato caused by *Ralstonia solanacearum* has been reported to be suppressed by fluorescent pseudomonads (Jagadeesh, 2000). Gent and Schwartz (2005) reported that *Pseudomonas fluorescens* strain A 506 reduced severity of *Xanthomonas* leaf blight in field experiments.

A strain of *Pseudomonas fluorescens* showed antagonistic property against *Rhizoctonia solani* (Howell and Stipanovic, 1979). A number of *Pseudomonas fluorescens* strains isolated from the rhizosphere of potato plants were reported to be antagonistic to *Rhizoctonia solani* *in vitro* and effectively reduced stem canker under laboratory conditions (Chand and Logan, 1984). Rangeshwaran and Prasad (2000) reported that *Pseudomonas putida* completely inhibited *S. rolfsii* in dual culture. Petri plate assay revealed that the *Pseudomonas* sp. exhibited antifungal activity against the plant pathogens, *Pythium ultimum*, *Rhizoctonia solani*, *Phytophthora capsici*, *Botrytis cinerea* and *Fusarium oxysporum* (Kim *et al.*, 2000). *Pseudomonas fluorescens* was reported to be antagonistic to *Aspergillus flavus*, *Sclerotium rolfsii* and *Aspergillus niger* *in vitro* (Pal *et al.*, 2001). Tripathi and Johri (2002) studied *in vitro* biocontrol potential of fluorescent pseudomonads recovered from rhizoplane and rhizosphere of pea and wheat against *Colletotrichum dematium*, *Rhizoctonia solani* and *Sclerotium rolfsii* and found them to restrict the growth of all the three pathogens but were most effective against *Rhizoctonia solani*. Fluorescent pseudomonad isolates tested against *Phytophthora capsici* on potato dextrose

agar (PDA) and carrot agar (CA) by dual culture technique was found to inhibit the growth of pathogen in 5-7 days (Anith *et al.*, 2002). Pseudomonads suppress soil-borne fungal pathogens by producing antifungal metabolites such as pyoluteorin, pyrrolnitrin, phenazines, and 2,4-di-acetyl phloroglucinol (Deepti and Johri, 2003).

Out of 45 isolates of fluorescent pseudomonads isolated from the rhizosphere of sunflower, 26 had antagonistic effects on the sclerotia of *S. sclerotiorum* (Behboudi *et al.*, 2005). Fluorescent pseudomonads were selectively isolated from black pepper (*Piper nigrum*) roots and screened for volatile and non-volatile metabolite production and inhibition of growth of *Phytophthora capsici*, the causal organism of foot rot disease.

Six isolates of fluorescent pseudomonads obtained from pathogen suppressive soil of a pigeonpea (*Cajanus cajan*) field were reported to have biocontrol potential against wilt disease complex in both laboratory and screen house (Siddiqui *et al.*, 2005).

Gupta *et al.*, (2001) isolated a fluorescent pseudomonad (GRC2) from potato rhizosphere and showed antibiosis against two major plant pathogens viz., *Macrophomina phaseolina* and *Sclerotium sclerotiorum*. Bare root dip treatment or soil drench with *Pseudomonas aeruginosa* strain IE-6S and *Pseudomonas fluorescens* CHA0 significantly suppressed *Rhizoctonia solani* of tomato (Siddiqui and Shaukat, 2002).

2.2.1 Antifungal metabolites

Antifungal metabolites been recognized as a major factor in suppression of root pathogens. These toxic or antimicrobial metabolites mainly include the antibiotics, bacteriocins, hydrocyanic acid (HCN) and several other extracellular metabolites. Antibiotic production is recognized as an important factor in determining the disease suppression potential of fluorescent *Pseudomonas spp.* Fluorescent pseudomonads produce different kinds of compounds like phenazine, pyocyanin, 2, 4-diacetyl phloroglucinol, pyoluteonin, pyrrolnitrin and tropolone

(O' Sullivan and O'Gara, 1992). Some of these compounds like tropolone have a broad spectrum of activity against many bacterial and fungal pathogens which may not be desirable always because they may eliminate many beneficial organisms (Lindberg, 1981).

Production of pyoverdinin and pyrrolnitrin type antimicrobial metabolites exhibiting both antifungal and antibacterial activity by *P. aeruginosa* was reported by Kumar *et al.*, (1997). Involvement of antimicrobial metabolites in suppression of wilt pathogen of lentil, *Fusarium oxysporum* was reported under *in vitro* conditions by Rao *et al.* (1999).

Pal *et al.*, (2000) reported that fluorescent pigments and antifungal antibiotics produced by *Pseudomonas glumae* were responsible for biocontrol of *R. solani*. Ahmadzadeh *et al.*, (2004) studied the production of antimicrobial metabolites by fluorescent pseudomonads and observed 15 out of 19 isolates to produce 2 –4 diacetyl phloroglucinol. Production of 2 –4 diacetyl phloroglucinol by *Pseudomonas* sp. isolated from tobacco rhizosphere was also recorded by Ramette *et al.* (2006).

Production of antibiotics by several strains of fluorescent pseudomonads has been recognized as a major factor in suppression of root pathogens. Howell and Stipanovic (1979) first established the importance of antibiotic production in the suppression of seedling pathogens using *Pseudomonas fluorescens* Pf-5. A number of disease suppressive antibiotic compounds have been characterized including N-containing heterocycles such as phenazines, pyrrole type antibiotics, pyro-compounds and indole derivatives. 2,4-diacetylphloroglucinol (DAPG) has been identified as a broad spectrum antibiotic, exhibiting antifungal, antibacterial, antihelminthic and phytotoxic activities. Raaijmakers and Weller (1998) demonstrated that root associated fluorescent pseudomonads with the capacity to produce DAPG were the key components in biological control of *Gaeumannomyces graminis tritici* that caused take all disease in wheat. DAPG was also a major determinant in the biological control activity of *Pseudomonas fluorescens* against black root rot of tobacco caused by *Thielaviopsis basicola*

and that of *Pseudomonas fluorescens* F113 against damping off of sugarbeet caused by *Pythium ultimum*. According to Picard *et al.*, (2000) spatial and temporal selection in the rhizosphere was responsible for fluctuation in the population of DAPG producers and induction of DAPG producers in exudates of older plants. Fe³⁺ and sucrose have been reported to increase the levels of DAPG production in *Pseudomonas fluorescens* F113, whereas its production in *Pseudomonas fluorescens* Pf-5 and CHAO was stimulated by glucose (Nowak *et al.*, 1994).

Pyrrolnitrin was identified as a broad spectrum antifungal metabolite produced by many fluorescent and non-fluorescent strains of the genus *Pseudomonas* (Kirner, 1998). This highly active metabolite has been primarily used as a clinical antifungal agent for treatment of skin mycoses against dermatophytic fungi, particularly members of the genus *Trichophyton*. A phenyl pyrrol derivative of pyrrolnitrin has been developed as an agricultural fungicide. Schnider-Keel *et al.*, (1995) reported that pyrrolnitrin actively persists in soil for at least 30 days. It did not readily diffuse and was released only after lysis of host bacterial cells. This property of slow release facilitated protection against *Rhizoctonia solani* as the cells died. Chang *et al.*, (1981) reported that the biocontrol agent, *Pseudomonas fluorescens* BL915 contained four gene clusters involved in the biosynthesis of antifungal molecule, pyrrolnitrin, from the precursor tryptophan.

Weinberg (1970) reported that *Pseudomonas fluorescens* produced PCA (Phenazine-1-carboxylic acid), whereas *Pseudomonas aureofaciens* 30-84 not only produced PCA but also lesser amounts of 2-hydroxyphenazines.

The major phenazine derived by *Pseudomonas aeruginosa* was pyocyanin (1- OH-5-methyl phenazine). Smirnov and Kiprianova (1990) showed that bacterization of wheat seeds by *Pseudomonas fluorescens* strains 30-84 and 2-79 provided primary protection against *Gaeumannomyces graminis tritici* on account of release of phenazine. Almost all phenazines exhibited broad spectrum activity against bacteria and fungi.

Cronin *et al.*, (1997) reported that DAPG produced by fluorescent *Pseudomonas* species has got antiviral properties in addition to its antifungal activity and is also known to inhibit the growth of soft-rotting bacteria and cyst nematodes of potato.

Antifungal secondary metabolites produced by six strains of rice associated *Pseudomonas* sp. were determined in four different media. Pigment production medium (PPM), potato dextrose agar (PDA), yeast malt (YM), and king's medium B (KMB), were used to determine best medium for plate assay of these strains against *Rhizoctonia solani*, the rice sheath blight pathogens. Large inhibition zones were obtained on PPM, KMB, and YM using two strains of *P. cepacia*. The other four strains showed varying degree of inhibition zones in the media tested. Their antifungal metabolites were isolated and compared with metabolites from reference strains and known compounds by regular and reversal phase thin- layer chromatography. *Pyrrolnitrin* was present in the cell extracts from the two *P. cepacia* strains. *Phenazine*-1-carboxylic acid and *pyocyanin* were produced by two strains of *P. aeruginosa*, a strain of *P. putida*, in-b-1821, produced 2,4-diacetylphloroglucinol. Several unidentified compounds were produced by another strain of *P. fluorescence* (Rosales *et al.*, 1995).

Production of antibiotic in several strains of fluorescent *Pseudomonads* has been recognized as a major factor in suppression of root pathogens. A number of disease suppressive antibiotic compounds have been characterized including N-containing heterocycles such as phenazines, 2,4-diacetylphloroglucinol, pyrrolnitrin, pyoluteorin. A broad spectrum antibiotic, Phl is phenolic molecules produced by many fluorescent *Pseudomonads* and exhibit antifungal, antibacterial, antihelminthic, and phytotoxic activities (Harrison *et al.*, 1993). In addition it shows herbicidal activity resembling 2,4-dichlorophenoxyacetic acid (2,4 D). Phl is a polyketide synthesized by condensation of three molecules of acetyl CoA with one molecule of malonyl CoA to produce the precursor monoacetylphloroglucinol (MAPG), which is subsequently transacetylated to generate DAPG.

2.3 PLANT GROWTH PROMOTING ACTIVITIES

2.3.1 Phosphate Solubilisation

Several heterotrophic and chemoautotrophic bacteria, fungi and actinomycetes have the capacity to solubilize insoluble mineral phosphates. Many genera of bacteria such as *Achromobacter*, *Agrobacterium*, *Bacillus*, *Pseudomonas*, *Serratia* and several others have been reported to solubilize varying quantities of phosphorus depending on the efficiency of the strains. The most efficient and dominant solubilizers belong to genera *Bacillus* and *Pseudomonas* (Gaur, 1990). Gupta *et al.*, (1994) recorded solubilization of 13.2 per cent tricalcium phosphate in broth medium by *Bacillus licheniformis*. De Frietas *et al.*, (1997) reported *B. polymyxa* and *X. maltophila* isolated from field grown plants to solubilize Carolina rock phosphate to the extent of 19.9 per cent and 22.5 per cent respectively.

Dave and Patel (1999) recorded solubilization of 11 to 72 per cent TCP in broth medium by pseudomonads isolated from rhizosphere and non-rhizosphere soils. Vazquez *et al.*, (2000) studying on phosphate solubilizing capacity of bacteria from mangroves, observed greatest solubilization by *Vibrio proteolyticus* after 18 to 24 hours of incubation which corresponded roughly to the logarithmic growth phase of bacteria.

Kundu *et al.*, (2002) observed five *Bacillus* sp. isolated from rhizosphere of different crop plants to solubilize tri-calcium phosphate in the range of 3.87 to 11.83 mg per 50 ml broth. Dave and Patel (2003) examined the impact of different carbon and nitrogen sources on phosphate solubilization by *Pseudomonas fluorescens* and found glucose and galactose as the best carbon sources and ammonium sulphate as the best nitrogen source.

Haque and Dave (2005) recorded P solubilization efficiency of 26 *Pseudomonas* cultures which varied between 14–88 µg per ml of broth medium. Anu and Kundu (2005) observed that among 76 *Pseudomonas* strains isolated from chickpea rhizosphere, phosphate solubilization efficiency varied from 22 to

248 µg per ml in liquid medium while they were developing and characterizing lacZ marked strains of phosphate solubilizing bacteria.

The biological process of conversion of unavailable /fixed form of inorganic phosphorous into primary orthophosphate (H_2PO_4^-) and secondary orthophosphate (HPO_4^{2-}) has been termed as Mineral Phosphate Solubilization (Goldstein, 1986). Involvement of microorganisms in the solubilization of insoluble phosphate was first shown by Stalstorm (1903). Since then lot of work has been done on the isolation, enumeration, efficiency screening, mechanisms of solubilization and crop response to their inoculation. The solubilization of the precipitated calcium phosphate on agar medium has been used as the criteria for isolation and enumeration of Mineral Phosphate Solubilizing (MPS) microorganisms (Sperber, 1957).

Among the groups of mineral phosphate solubilizing bacteria (MPSB), the pseudomonads assume importance since they are the most common and frequently occurring group in the rhizosphere and are capable of utilizing a wide array of compounds as carbon and energy sources. They are also known to have wide range of plant growth promotional activity by virtue of nutrient mobilization, Phosphate solubilization, production of plant hormones and biocontrol potential.

Illmer and Schinner (1992) isolated *Pseudomonas* sp. and *Pencillium* sp. from forest soils and found them to solubilize high amounts of insoluble inorganic phosphates. The occurrence of phosphate solubilizing bacteria in soils of Marathwada region of Maharashtra state was studied by Bilolika *et al.*, (1996) where in they found predominance of *Pseudomonas* in soils of Aurangabad district. Nahas (1996) studied 31 bacteria for their ability to solubilize rock phosphate and calcium phosphate in culture medium and reported that *Pseudomonas cepacia* had the highest solubilizing activity.

Neelam and Meenu (2003) reported high tricalcium phosphate solubilizing ability of *Pseudomonas* sp. (TP2) isolated from rhizosphere of field grown *Trigonella*. Gupta *et al.*, (2002) described *Pseudomonas* species as a

potent phosphate solublizer while they were developing heavy metal resistant mutants of phosphate solubilizing *Pseudomonas* sp. NBR14014 and their characterization. Das *et al.*, (2003) examined the tricalcium phosphate solubilizing activity of *Pseudomonas fluorescens* and their cold-tolerant mutants and reported that the cold-tolerant mutants were more efficient than their respective wild type counterparts for P-solubilization at low temperatures.

Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation (Kucey *et al.*, 1989; Ponmurugan and Gopi, 2006). *Pseudomonas* sp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop (Son *et al.*, 2006). Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma *et al.*, 2005).

Increased solubilization of fixed soil phosphates and applied phosphates ensuring higher crop yields has been reported on inoculation of phosphate-solubilizing bacteria including *Pseudomonas*, *Bacillus*, *Rhizobium*, *Micrococcus*, *Flavobacterium*, *Burkholderia*, *Achromobacter*, *Erwinia*, and *Agrobacterium* (Rodriguez and Fraga, 1999; Trivedi and Sa, 2008). Several *Pseudomonas* species have been reported among the most efficient phosphate-solubilizing bacteria and as important bio-inoculants due to their multiple biofertilizing activities of improving soil nutrient status, secretion of plant growth regulators, and suppression of soil-borne pathogens (Rodriguez and Fraga, 1999; Botelho and Mendonça-Hagler, 2006; Gulati, *et al.*, 2008; Vyas *et al.*, 2009).

Neelam and Meenu (2003) reported high tricalcium phosphate solubilizing ability of *Pseudomonas* sp. (TP2) isolated from rhizosphere of *Trigonella*. Das *et al.*, (2003) examined the tricalcium phosphate solubilizing activity of *Pseudomonas fluorescens* and their cold-tolerant mutants and reported that the cold-tolerant mutants were more efficient than their respective wild type counterparts for P-solubilization at low temperatures.

Gupta *et al.*, (2002) described *Pseudomonas* species as a potent phosphate solubilizer while they were developing heavy metal resistant mutants of phosphate solubilizing *Pseudomonas* NBRI 4014.

2.3.2 Siderophores

Siderophores are low molecular weight iron-binding ligands which can bind to ferric ion and make it available to the producer microorganism (Neilands, 1981a). Soil pseudomonads generally produce fluorescent, yellow-green, water soluble siderophores. They can be either pyoverdins or pseudobactins. The production of these siderophores has been linked to the disease suppression potential of certain fluorescent pseudomonads. Fluorescent siderophores that have very strong affinity for ferric ions are secreted during growth under low iron conditions. The resulting ferric siderophore complex is unavailable to the other organisms but the producing strain can utilize this complex via very specific receptor in the outer cell membrane (Hemming, 1986). Kumar *et al.*, (1997) demonstrated that siderophores of fluorescent. *In vitro* suppression of plant pathogens by siderophores of fluorescent pseudomonads was also reported by Manwar *et al.*, (2000). Tripathi and Johri (2002) reported production of siderophores as one of the mechanisms for *in vitro* inhibition of *Colletotrichum dematium*, *Rhizoctonia solani* and *Sclerotium rolfsii* by fluorescent pseudomonads. Suryakala *et al.*, (2004) observed that siderophores produced by *Pseudomonas fluorescens* were antagonistic to fungal pathogens like *Fusarium oxysporum*, *Alternaria* sp. and *Colletotrichum capsicii*. Production of iron chelating hydroxamate type siderophores by *P. fluorescens* NCIM 5096 and *P. putida* NCIM 2847 was reported by Sayyed *et al.*, (2005).

The role of siderophores produced by fluorescent pseudomonads in plant growth promotion was first reported by Kloepper *et al.*, (1980). The siderophores of fluorescent pseudomonas were later reported to be implicated in the suppression of plant pathogens (Bakker *et al.*, 1986; Becker and Cook, 1988).

When grown under low iron conditions, fluorescent pseudomonads produce yellow green fluorescent siderophores of the pyoverdine type (Demange

et al., 1987). Competition for iron between pathogens and siderophores of fluorescent pseudomonads has been implicated in the biocontrol of wilt diseases caused by *Fusarium oxysporum* (Kloepper *et al.*, 1980a), damping off of cotton caused by *Pythium ultimum*. Siderophore production by a plant growth promoting fluorescent *Pseudomonas* species RBT 13 effective against several fungal and bacterial pathogens has been demonstrated (Dileep and Dubey, 1993).

Manwar *et al.*, (2000) isolated siderophore producing pseudomonads SC1 and SC4 from soil and the amount of siderophores produced were calculated to be 57 and 50 per cent siderophore units respectively which exerted killing effect on plant deleterious fungi and also promoted growth of wheat plants. Plant growth promoting rhizobacteria belonging to fluorescent pseudomonads produced extracellular siderophores when grown in casamino acid medium under iron deficiency and was found to be of trihydroxamate type pyoverdines forming hexadentate ligands with Fe^{3+} ions (Suryakala *et al.*, 2004).

2.3.3 HCN production

HCN inhibits the electron transport thereby the energy supply to the cell is disrupted leading to the death of the organism. It inhibits proper functioning of enzymes and natural receptors by reversible mechanism of inhibition (Corbett, 1974). It is also known to inhibit the action of cytochrome oxidase (Gehring *et al.*, 1993).

HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens (Defago *et al.*, 1990). Fluorescent *Pseudomonas* strain RRS, isolated from Rajanigandha, a flowering plant produced HCN and the strain improved seed germination and root length (Saxena *et al.*, 2003)

Kremer and Souissi (2001) reported that approximately 32 per cent of bacteria from a collection of over 2000 isolates were cyanogenic, evolving HCN from trace concentration to >30 n moles/mg cellular protein. They also opined that cyanogenesis was predominantly associated with pseudomonads and was enhanced when glycine was provided in the culture medium.

Ramette *et al.*, (2003) reported that HCN synthase is encoded by three biosynthetic genes in fluorescent pseudomonads. Paul *et al.*, (2005) studied antagonistic mechanisms of fluorescent pseudomonads against *Phytohthora capsicii* in black pepper and reported many isolates to produce HCN which limited the growth of *P. capsicii*. Ten fluorescent pseudomonads isolated from rhizosphere of sunflower, potato, maize and groundnut were shown to enhance plant growth and suppress the collar rot of sunflower where in one of the mechanism of suppression of the disease was by HCN production (Shivani *et al.*, 2005). *Pseudomonas* populations producing HCN were reported in the rhizosphere of tobacco in Swiss soils suppressive to *Thielaviopsis basicola*, causal agent of black root rot of tobacco by Ramette *et al.*, (2006).

Ten isolates of fluorescent *Pseudomonas* isolated from rhizosphere of sunflower, potato, maize and groundnut were shown to enhance plant growth and suppress the collar rot of sunflower where in one of the mechanism of suppression of the disease was by HCN production (Shivani *et al.*, 2005. Ramette *et al.*, (2003) reported that hydrogen cyanide (HCN) a broad-spectrum antimicrobial compound involved in biological control of root diseases, is produced by many plant associated fluorescent *Pseudomonas* and HCN synthase is encoded by three biosynthetic genes . However, little is known about the diversity of these genes in fluorescent *Pseudomonas* sp. and other bacteria.

2.3.4 Protease activity

Ellaiah *et al.*, (2002) isolated 30 different categories of bacteria producing protease from seven protein rich soil samples collected from Vishakapatnam. Among these, nine categories showed good proteolytic activity, seven showed moderate, eight showed very poor and six categories showed negligible activity. Out of twelve halophilic bacterial strains from the soils of desert regions of Rajasthan, four were found to be good producers of extracellular alkaline protease (Parul *et al.*, 2003). The results indicated that addition of yeast extract increased the protease production by seven fold whereas the L-ketoglutarate

repressed the exoenzyme production, suggesting that exoenzyme is controlled by a mechanism similar to catabolite repression.

The physiological and nutritional factors that regulate protease synthesis in *Clostridium sporogenes* were studied by Clive *et al.*, (1990) in batch and continuous cultures. Production of enzyme was at the end of active growth in batch cultures. Thus, protease production was inversely related to growth rate. Further, many nutrients added as energy sources strongly repressed protease activity. It was concluded that protease production by *Clostridium sporogenes* was strongly influenced by the amount of energy available to the cells, with the highest levels of protease synthesis under energy limiting conditions.

The replacement of glucose by any other carbon sources reduced drastically the production of enzyme by the mutant. Casein as nitrogen source supported better protease production than any other nitrogen source. The enzyme production had reached maximum at 72 hours (Meenu *et al.*, 2000).

Kumar *et al.*, (2002) reported production of extra cellular protease by the two mesophilic soil bacteria viz., *Bacillus* sp. (S4) and *Pseudomonas* sp. (S22). Wheat bran and soybean meal were the best carbon and nitrogen sources for production of proteases.

2.3.5 Chitinases

Chitinases are PR-proteins which hydrolyze chitin, major cell wall component constituents for 3 to 10 per cent of higher fungi and cuticle of peritrophic membrane in insects. Chitinase cleave a bond between C1 and C4 of two consecutive N-acetyl glucosamine either by endolytic or exolytic mechanisms. A large number of plant chitinases have been purified and characterized which are endochitinases with molecular weights ranging from 25 to 36 kDa. The production of chitinases in plants has been suggested to be a part of their defense mechanism against fungal pathogens (Schlumbaum *et al.*, 1986). Increased chitinase activity in tobacco and maximum activity in cucumber have been observed as a result of systemic resistance by fluorescent *Pseudomonas*

against *P. syringae* pv. *tabaci* (Schneider and Ullrich, 1994), Nandakumar *et al.* (2001) and Nayar (1996) found early and higher induction of chitinase in *P. fluorescens* Pf1 treated rice plants.

Radjacommaré *et al.*, (2004) reported the induction of 28 and 35 kDa chitinase isoforms in rice plants treated with *P. fluorescens* and challenge inoculated with sheath blight pathogen *Rhizoctonia solani*.

2.4 PLANT GROWTH REGULATORS VIZ. AUXINS, GIBBERELLINS AND CYTOKININS

It was investigated that the plant growth regulators (PGR) like gibberellins, kinetins and indole acetic acid (IAA) were detected by thin layer chromatography (TLC) and other conventional methods in culture filtrates of *Azotobacter chroococcum* strains. A few strains produced all the three plant growth regulators (PGRs) while other produced one or two of these. The production of these PGRs was further confirmed by bioassays. Kloepper *et al.*, (1989) observed that the soil microorganisms that colonize root and promote plant growth represent a subset of rhizosphere bacteria called plant growth promoting rhizobacteria, can produce direct or indirect effects on host plants. Indirect effects are those related to enhance the production of metabolites such as antibiotics, siderophores, or HCN, which increase plant growth by decreasing the activities of pathogens or deleterious microorganisms. PGPR can produce direct effects on plant growth by producing metabolites such as plant growth regulators (PGRs) that directly promote plant growth or by facilitating nutrient uptake by the plant (Kloepper, 1993 and Glick, 1995). Information on microbial production of plant growth regulators (PGRs) and their effect on plant growth has been limited and focused primarily on ethylene and auxins (Glick, 1995). Production of cytokinins by plant pathogenic bacteria such as *Agrobacterium*, *Pseudomonas* species is well known (Anith *et al.*, 1999).

Soil microorganisms have the ability to synthesize various metabolites which can affect the soil properties. The growth and chemical composition of plants and their health is considered as one of the most important factors of soil

fertility (Vancura and Jandera, 1986). Production of cytokinins by plant pathogenic bacteria such as *Agrobacterium* and *Pseudomonas* spp. is well known (Anith *et al.*, 1999). However, PGPR including *Azotobacter*, *Azospirillum*, *Rhizobium*, *Bacillus* and *Pseudomonas* species have been reported to synthesize cytokinins in pure culture (Anith *et al.*, 1999).

2.4.1 Auxins

Patten and Glick (2002) studied that many plant associated bacteria synthesizes the phytohormones. Indole-3-acetic acid produced by phytopathogenic bacteria mainly by indole acetamide pathway. To determine whether bacterial IM enhances root development in host plants, the *ipdc* gene that encodes indole pyruvate decarboxylase was isolated from the plant growth promoting bacterium *Pseudomonas putida* GR 12.2 for which it is deficient. It was investigated the production of 1AA under different culture conditions by *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*, *Rhodocyclus gelatinosus* and *Rhodocyclus tenicis*. Indole acetic acid production increased in the presence of tryptophan. L-glutamic acid was the best nitrogen (NAA) or kinetin, PGPR did not affect callus biomass. However, when growth regulators were included in the growth medium and vitamins (Thiamine HCl nicotinic acid, Pyridoxine HCl, and myoinositol), inoculation with strain *Pseudomonas putida* significantly increased the callus biomass (Maruyama, 1986).

2.4.2 Gibberellins

Fusarium species strains were screened out to select the potential strains for the production of gibberellins from different strains of *Fusarium* sp. which was done spectrophotometrically and showed the presence of variable amount of gibberellins Srivastava, *et al.*, (2003). Range of gibberellins production varied between 0.66 to 600 mg/g dry weights of the mycelium. Most of the strains produced 50-180 mg/g gibberellins on mycelium dry weight basis. Thin layer chromatography and high performance liquid chromatographic separation confirmed the presence of GA₃, GA₄ and GA₁. Bottini *et al.*, (1989) observed that the gibberellic acid production *in vitro* by *Azospirillum* sp. and effects of *Azospirillum* sp. on infected plants were studied. GA₁ and GA₃ were

characterized by capillary gas chromatography, mass spectrometry from antibiotic culture of *Azospirillum lipoferum* and *Azospirillum brasilense* (Janzen *et al.*, 1992). In addition, the bacteria have also been shown to metabolize exogenous gibberellic acid GA₅. The optimal cultural parameter for gibberellic acid (GA₃) production by *Pseudomonas* sp. isolated from wastes of processed olive affected by physiological conditions such as incubation period, pH of growth media, incubation conditions and incubation temperature. The highest level of gibberellic acid (GA₃) (250.06 mg/l) production was obtained in nutrient broth when the bacterial culture was incubated at 30°C for 72 h at pH 7 on a rotary shaker and in dark conditions. (Karakoc and Aksoz, 2006)

2.4.3 Cytokinins

Garden *et al.*, (1992) reported the production of plant growth hormone auxins from *Pseudomonas syringae* strain isolated from the olive and oleander. They concluded that amount of auxins produced by the bacteria could be adapted to the sensitivity of the host plant to this hormone. In other words, olive cells might need less auxins (and possibly cytokinins) than oleander to start division. Bright red pigment characteristics of *Serratia marcescens* similar pigment have been isolated from the many prokaryotes including *Pseudomonas magnesorubra*, *Vibrio psychroerythrus* and *Alteromonas rubra* (Gauthier, 1976) which have common tripyrrole structure and differ from one organism to another in the nature of substituent attached to the nucleus.

Plant growth promoting rhizobacteria (PGPR) *Pseudomonas putida* strains were tested for the growth promotion of soybean callus in a tissue culture bioassay. When grown on a nutrient medium without naphthalene acetic (NAA) acid or kinetin, PGPR did not affect callus biomass. However, when growth regulators were included in the growth medium and vitamins (Thiamine HCl nicotinic acid, Pyridoxine HCl, and mesoinositol) omitted inoculation with strain *Pseudomonas putida* significantly increased the callus biomass (Maruyama, 1986).

2.5 ROOT COLONIZATION

The term root colonization includes colonization of the root (internal or surface) as well as the rhizosphere soil by the introduced bacteria. The bacteria are probably not restricted to either location (Bahme and Schroth, 1987).

Ahmad and Baker (1987) used the term rhizosphere competence to describe the ability of biocontrol agents to grow and function in rhizosphere. Rhizosphere competence might also be thought of as the relative root colonizing ability of a strain. Xu and Gross (1986) applied *Pseudomonas putida* W4P63 to potato seed pieces and monitored its population and that of *Erwinia carotovora* on roots in the field. Population of W4P63 ranged between 10⁴ and 10⁵ cfu (colony forming units)/g root, while the population of *E. carotovora* on these roots was only 10 per cent of that on roots with no W4P63.

Szczech and Shoda (2006) demonstrated that the total number of *Bacillus subtilis* RB14-C on the roots of plants grown from coated seeds was significantly lower than on the roots of plants grown in soil mixed with the bacteria. Process of root colonization Howie *et al.*, (1987) hypothesized that colonization of wheat roots by *P. fluorescens* occurs in two phases. In Phase I, the bacteria attach to and are then transported on the elongation root tip and in phase II, the bacteria spread locally and proliferate to the limits of the niche in competition with indigenous organisms and survive. Phase I begins as the introduced bacteria on seeds or seed come into contact with the emerging roots. Some kind of attachment of the cells to the root surface may be essential for initiation of phase I and would also assure priority access to root exudates (Bull, 1987; James *et al.*, 1985). As the root elongates, some of the bacteria are carried along with the root tip, while others are left behind as a source of inoculation on older portions of the root (Weller, 1988).

Dupler and Baker (1984) reported that *P. putida* N-1R colonized radish rhizosphere less efficiently when the bacteria were added to a biologically active soil than when the same soil was air dried prior to the test to reduce microbiological activity. Competition would have been greater in the former soil.

Factors affecting root colonization Transport of bacteria along with the elongating root (Phase I) did not require percolating water (Howie *et al.*, 1987). Nevertheless, such water enhanced bacterial movement (Bahme and Schorth, 1987).

Channels left by old roots or worms (Loper *et al.*, 1984) and possible gap at the root soil interface created by the diurnal shrinking and swelling of roots might allow more rapid downward movement of bacteria in water than that occurred along roots in a more uniform soil matrix (Parke *et al.*, 1986).

Howie *et al.*, (1987) revealed that the colonization of wheat roots by *P. fluorescens* 2-79 was greater at the rhizosphere pH of 6-6.5 than at 7.0 or above, possibly because of less competition from indigenous rhizosphere bacteria at the more acid pH.

Root colonization by the introduced bacteria is also affected by host genotype. Weller (1988) has demonstrated considerable differences among wheat cultivars in their ability to support root colonization by 2-79 and the population was 100-fold greater on the most supportive cultivars.

Plant genotypes also influenced the quantity and composition of the rhizosphere microflora (Azad *et al.*, 1985), possibly through difference in root exudates (Curl and True Love, 1986). Manipulating host genotypes may offer some opportunity to improve the efficiency or consistency of root colonization by the introduced bacteria. Indigenous microorganisms may also enhance root colonization by the introduced bacteria. The population of indigenous gram-negative bacteria of *Pseudomonas* spp. (Brown, 1981) and of applied *P. fluorescens* (Bull, 1987) was larger on roots infected by *G. graminis* var. tritidis than on healthy roots.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled “Studies on the role of antifungal metabolites producing native strains of *Pseudomonas* species to overcome replant problem of apple” was carried out in the Department of Basic Sciences, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan (H.P). The materials used and methodology adopted is described in this chapter.

3.1 ISOLATION OF FLUORESCENT *Pseudomonas* SP. FROM RHIZOSHERE SOIL OF APPLE ORCHARDS OF DISTRICT SHIMLA

3.1.1 Collection of soil sample

Apple orchards affected with replant problem was selected at Maggota, district Shimla for the collection of soil samples. Different composite Rhizosphere soil samples were taken from normal and replant site, around few to 10 cm apart and 15-30 cm deep from different plants randomly.

3.1.2 Isolation and identification

Ten grams soil sample added to 90 ml sterile water blank was allowed to vortex. Double dilutions were made using 9.9 sterile water blank. 0.1 ml from each dilution of each sample was spreaded over prepoured 15 – 20 ml Kings’ B medium for *Pseudomonas* sp. and nutrient agar for total bacteria, respectively. Plates were kept for incubation for 28 – 48 h at 28 ± 2°C.

The most predominant fluorescent *Pseudomonas* isolates were identified on basis of colony morphology, characteristics, production of fluorescent pigment, biochemical and physiological tests as per their genera lay down in Bergey’s manual of Systematic Bacteriology and were confirmed from the department of Basic Science, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP). The colonies were streaked for purification on King’s B medium. All the isolates assumed to be fluorescent *Pseudomonas*

species were maintained at 4°C on nutrient agar and in 20% glycerol stocks and subcultured periodically on the same medium at 28 ± 2°C.

Composition of the media (Atlas, 1995)

Nutrient Agar

Components	grams/litre
Beef extract	3.0
Peptone	5.0
NaCl	5.0
Agar	20.0
pH	7.0

King's B Medium

Peptone	20.0
Dipotassium hydrogen phosphate (K ₂ HPO ₄)	1.5
Magnesium sulphate (MgSO ₄ .7H ₂ O)	1.5
Glycerol	15.0
Agar	20.0
pH	7.2

3.1.3 Used reagents and Chemicals:

Various reagents and chemicals used in the present studies were procured from Merck and Hi-Media. Most of the chemicals used were of analytical grade.

3.2 IDENTIFICATION OF EXPECTED ISOLATES OF FLUORESCENT *Pseudomonas* SP. ISOLATED FROM NORMAL AND REPLANT SITES OF APPLE BY MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL METHODS.

3.2.1 Colony morphology and pigment production (King *et al.*, 1954):

Colony morphology (form, elevation, margin, opacity, surface) and the production of pigment was checked on King's B medium after incubation for 24 h. at 28 ± 2°C.

3.2.2 Gram staining: Four different reagents in the order of Crystal violet(primary stain), iodine solution (mordant), alcohol (decolorizing) and safranin (counter stain) were used to identify and classifying bacteria.

- 3.2.3 Spore staining:** It was done using malachite green heating the slides for 5 minutes followed by counter stain safranin for 30 sec.
- 3.2.4 Catalase test:** On a clean glass slide, a loopful of culture growth was taken and emulsified with three to four drops of hydrogen peroxide. Immediately presence of effervescence/air bubbles indicates positive results.
- 3.2.5 Oxidase test:** A small piece of filter paper was soaked in 1% aqueous solution of tetra-methyl-p-phenylenediamine hydrochloride. A loopful of fresh young growth from culture plate was scraped with edge of a clean glass slide and rubbed on impregnated filter paper. Development of blue color within 10-15 seconds shows positive oxidase test and delayed reaction shows color within 10-60 seconds.
- 3.2.6 Denitrification test:** For denitrification test, semisolid nitrate agar medium containing potassium nitrate was used.

Semisolid Nitrate Medium

Components	grams/litre
Beef extract	3.0
Peptone	5.0
NaCl	5.0
Potassium nitrate	2.0
Agar	1.0

Semisolid nitrate medium was inoculated with all *Pseudomonas* sp. isolates along with control i.e. non nitrate medium. All tubes were placed in ice bath for 10-15 minutes or until medium was completely chilled. Cold made semisolid medium firmer so that overlay agar would stay on top. Then the nutrient agar was transferred aseptically as agar overlay in each tube of chilled medium. This was poured so that it gently ran down the tube and slowly layered over the top of nitrate medium. Each tube was waited to set in agar until overlay agar had solidified. All tubes were incubated at $28 \pm 2^{\circ}\text{C}$ for 48 h. Presence of gas in nitrate containing media (cracking of media) was evidence for the complete reduction of nitrate to nitrogen gas.

3.2.7 Gelatin liquification test

Nutrient gelatin medium deep stabs in tubes were prepared and stab inoculation was made from each *Pseudomonas* isolates. Uninoculated deep tube was kept as a control. All inoculated tubes and uninoculated tube were incubated at $28 \pm 2^{\circ}\text{C}$ for 4-7 days. After incubation, the tubes were placed in refrigerator at 4°C for 15 minutes. Liquification of nutrient gelatin medium in inoculated tubes show positive test for gelatin hydrolysis and those tubes that remain solid show negative reaction for gelatin liquefaction.

Nutrient Gelatin Agar Medium

Components	grams/litre
Peptone	5.0
Beef extract	3.0
Gelatin	12.0

3.2.8 Lecithinase activity

The lecithinase activity was observed by preparing nutrient agar supplemented with 1% NaCl and 10% (v/v) egg yolk emulsion. This medium was poured in petriplates. These prepoured and dried plates were inoculated by streaking with a culture of *Pseudomonas* sp. isolates and incubated for 1-4 days at $28 \pm 2^{\circ}\text{C}$. Lecithinase activity results in the formation of opaque zones around the streaked bacterial growth.

3.2.9 Arginine Hydrolysis

Arginine broth was inoculated with isolated *Pseudomonas* strains and was given incubation with control tube at 28°C for 4 days. 1ml of Nessler's reagent was added to the incubated culture. The development of an orange to brown color indicates the presence of ammonia from arginine the pale yellow color in control indicates its absence.

3.3 GROWTH AT 4°C , 25°C , 37°C AND 42°C

Sterilized 10 ml nutrient broth in test tubes was inoculated with 0.5 ml of overnight culture of each *Pseudomonas* sp. isolates. The tubes were incubated at

4°C, 25°C, 37 °C and 41°C, the growth was observed after 24 h, 48 h and 72 h separately in terms of optical density at 540 nm.

3.4 RESISTANCE OF FLUORESCENT *Pseudomonas* ISOLATES TO SOME SELECTED ANTIBIOTICS, CHEMICALS AND COMMONLY USED FUNGICIDE BY FARMERS.

Well diffusion method was used to check resistance of isolates to antibiotics, chemicals and fungicide. About 20 ml of nutrient agar was poured into sterile petriplates. The plates were swabbed with overnight culture of test isolates of *Pseudomonas* strains. With the help of cork borer wells were made on agar medium under sterile conditions. Different concentration i.e. 25, 50 and 100 ppm of different antibiotics, chemicals and fungicide were added into well and incubated for 24 h at 28 °C. Zone of inhibition of growth was measured and expressed as mm dia. of clear zone around the well after incubation at 28 °C for 24h.

3.5 PLANT GROWTH PROMOTING POTENTIAL OF ISOLATES OF FLUORESCENT *Pseudomonas* sp. FOR MULTIFARROUS ACTIVITIES

3.5.1 Antifungal activity

Antifungal activity of each test strain of *Pseudomonas* sp. isolate against different indicator fungal pathogens viz., *Dematophora* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp. and *Sclerotium* sp. was checked by well plate assay method (Vincent, 1947). 72 h old culture bit of indicator fungi were placed on one side of pre-poured malt extract agar (MEA) plates. On the other side of the plate, well was cut with the help of sterile cork borer. 100µl of 72 h old cell free supernatant of each isolate was added to each well (9 mm dia). Plates were incubated at $28 \pm 2^\circ \text{C}$ for 7 days and observed for inhibition of growth of mycelia produced around the well. For control, culture bit of indicator fungi was kept in the center of malt extract agar plate and incubated at $28 \pm 2^\circ \text{C}$ for 7 days and observed for inhibition of mycelia growth around the well. Antifungal activity expressed in terms of percent inhibition (%I) of fungal mycelia as calculated from the equation:

$$\% I = \frac{C-Z}{C} \times 100$$

Where,

Z = Growth of mycelia in treatment
C = Growth of mycelia in control (each indicator test fungi)

Malt Extract Agar Medium

Components	grams/litre
Malt extract powder	10.0
Agar	10.0
pH	7.0

3.5.2 Phosphate solubilizing activity

For estimation and screening of phosphate solubilizing capacity of *Pseudomonas* sp. isolates, both qualitative and quantitative methods were used.

3.5.2.1 Estimation by Plate assay (Qualitative)

Pikovskaya's agar plates with known amount of inert phosphorous source tricalcium phosphate (TCP) were prepared. 100µl of 72 h old cell free supernatant of each bacterial isolate was added to each well already cut on Pikovskaya's agar with the help of sterile cork borer. Plates were incubated at 28 ± 2° C for 48h and observed for pinkish/orange zone produced around the well. Phosphate solubilizing activity expressed as mm diameter of yellow zone produced around the well (9mm dia) after incubation at 28 ± 2° C for 48h.

Pikovskaya's Agar Medium

Components	grams/litre
Glucose	10.0
Tricalcium phosphate Ca ₃ (PO ₄)	5.0
Ammonium sulphate (NH ₄) ₂ SO ₄	0.5
Magnesium sulphate (MgSO ₄ .7H ₂ O)	0.2

Manganese sulphate (MnSO ₄)	0.1
Ferrous sulphate (FeSO ₄)	0.0001
Yeast extract	0.5
Bromocresol purple	0.1
Agar	20.0
pH	7.2

3.5.2.2 Quantitative estimation.

Quantitative estimation of phosphate solubilization was done by spectrophotometric method (Dickman and Bray's 1940, Bray and Kurtz 1945 and Olsen *et al.*, 1954) by using Pikovskaya's broth. 100µl PVK broth was inoculated with 0.5ml of 48h old culture of each *Pseudomonas* isolates and was incubated at $28 \pm 2^\circ \text{C}$ for 72 h under shake conditions. Supernatants were collected after centrifugation at 10,000 rpm for 20 min at 4°C . To 1 ml supernatant in a 25 ml volumetric flask, 5 ml ammonium molybdate reagent (15 gm of ammonium molybdate in 400 ml distilled water and 342 ml of conc. HCL, total volume was made up to 1L) was added along with shaking followed by 1 ml of working solution of chlorostannous acid (*i.e.* 0.5 ml of stock solution was added to 65.5 ml H₂O to make final 66 ml). Stock solution (40%) was made by dissolving 10 gm SnCl₂.H₂O in 25 ml concentrated HCl. Total volume of reaction mixture was made to 25 ml with distilled water. Blue color intensity of the solution was measured at 660 nm after ten minutes using red filter. Corresponding amount of soluble phosphorous was calculated from standard curve of potassium dihydrogen phosphate (KH₂PO₄; 0-10 µg/ml). Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization, which in turn represents µg/ml of soluble orthophosphate as calibrated from the standard curve of KH₂PO₄ (10-100 µg/ml).

3.5.3 Siderophore Activity

3.5.3.1 Production of Siderophores

King's B medium was used for the production of siderophore with slight modifications. (Meyer and Abdallah, 1978). 0.5 ml of 18 h old inoculum of each

Pseudomonas sp. isolate was added to 100 ml of King's B medium in 250 ml Erlenmeyer flasks. The flasks were incubated at $28 \pm 2^\circ\text{C}$ for 72 h under shake conditions (90 rpm) on rotary shaker. The cell free culture supernatant was harvested by centrifugation at 10,000 rpm for 20 minutes at 4°C and stored at 4°C .

3.5.3.2 Estimation by plate assay (Qualitative)

Siderophores production was detected by chrome azurol-S (CAS) plate assay with slight modifications (Schwyn and Neilands, 1987). 72 h old culture bit of each test bacterium *i.e.*, *Pseudomonas* sp. isolate was placed on pre-poured chrome azurol-S (CAS) agar plates and/or 100 μl of 72 h old cell free culture supernatant of each *Pseudomonas* sp. isolate was added to each well already cut on pre-poured CAS plate with the help of sterilized cork borer. Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48 h and observed for production of yellow/orange zone around the bit/well. Production of siderophores was expressed in terms of mm diameter of yellow/orange zone produced around the bit/well at $28 \pm 2^\circ\text{C}$ in 48 h.

3.5.3.3 Chrome azurol-S Agar Medium

- A. CAS Solution: 60.5 mg of chrome azurol-S (CAS) dye was dissolved in 50 ml of distilled water and this was mixed with 10 ml iron solution (1mM (1.5 ml) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ mixed in 10mM HCl). This solution was added to Hexadecyltrimethyl Ammonium bromide (HDTMA) (72.9 mg/40 ml distilled water).
- B. 750 ml of nutrient agar (composition as described in section 3.2.1.2) was mixed with 1,4 piperazinediethane sulphonic acid (30.24 gm) and pH was adjusted to 6.8 with NaOH (6.0 gm) and it was autoclaved separately. After autoclaving, it was mixed with 100 ml of CAS solution.

3.5.3.4 Estimation by Liquid assay (Quantitative)

For quantitative estimation of siderophores, chrome azurol-S (CAS) liquid assay method was used. Seventy two hour old cell free culture supernatant of each test *Pseudomonas* isolate grown in 100 ml King's B medium and incubated

at 28 °C for 72h under shake condition. 0.5 ml of cell free supernatant was mixed with 0.5 ml Chrome azurol solution (1.5 ml of 1 mM FeCl₃, 6H₂O in 10 mM HCl + 7.5 ml of 2 mM CAS stock solution dissolved in 50 ml of Hexadecyltrimethyl Ammonium bromide (HDTMA), add 30 ml piperazine (pH 5.6) solution into it and final volume was made to 100 ml with distilled water), 10 µl shuttle solution (0.2 M 5-sulfosalicylic acid) was added. Color intensity of the solution was recorded at 630 nm against reference after 10 minutes at room temperature. Siderophore production was observed in terms of reduction in blue color as per cent siderophore units. (% SU).

$$\% \text{ SU} = \frac{\text{Ar}-\text{As}}{\text{Ar}} \times 100$$

Ar = Absorbance of reference at 630 nm
As = Absorbance of supernatant at 630 nm

3.5.4 Lytic enzymes

3.5.4.1 Protease Activity

All *Pseudomonas* sp. isolates were screened out for production of proteolytic activity by well plate assay (Kaur *et al.*, 1989) on skim milk agar plates. 100µl of 48 h old cell free supernatant of each *Pseudomonas* sp. isolate was added to each well already cut on the prepoured skim milk plates. These plates were then incubated at 28±2°C for 48 h and observed for clear zone production around the well. Proteolytic activity was expressed in terms of mm diameter of clear zones produced around the well after incubation at 28±2°C for 48 h .

3.5.4.2 Skim milk agar

1. **Nutrient agar:** 1% of skim milk was prepared and autoclaved separately and then mixed with previously autoclaved, melted and cooled nutrient agar before pouring.
2. 1.5 gm of agar was dissolved in 90 ml of tris buffer (0.05 M, pH 7.0) and autoclaved. 10% of skim milk (*i.e.*, 10 gm for 100 ml of distilled water) was prepared and autoclaved separately. 10ml of skim milk stock added to 90ml cooled melted agar before pouring the plates.

3.5.4.3 Quantitative assay

72h old cell free culture supernatants were used as the crude enzyme source. Protease activity was assayed by the method of Morrihara, (1964) using 2.0% casein in 0.05 M Tris buffer pH 7.0 as a substrate. Casein solution 1ml was incubated with an equal volume of suitable diluted enzyme preparation (supernatant or partially purified preparation) at 37°C. After 5 min the reaction was terminated by the addition of 3 ml of 5% of Trichloroacetic acid.

The mixture was centrifuged at 5000 rpm for 10 min at 4°C and the supernatant was assayed for protein concentration by use of Folin phenol method and the change in the optical density was measured at 660 nm.

3.5.4.4 Chitinase

All isolates of *Pseudomonas* sp. were screened out for chitinase activity by well plate assay on chitin agar plates 1% of colloidal chitin was added to nutrient agar medium 100µl of 72 h old cell free supernatant of each bacterial isolate was added to each well already cut on the prepoured chitin agar plate with the help of sterile borer. Plates were incubated at 28±2°C for 5 days and observed for clear zone produced around the well.

3.5.5 HCN production

For the production of hydrogen cyanide (HCN), the *Pseudomonas* sp. isolates were screened out according to Baker and Schippers (1987). The bacterial cultures were streaked on prepoured plates of King's B media amended with 1.4g/l glycine. Whatman No. 1 filter paper strips were soaked in 0.5% picric acid followed by 2% sodium carbonate, were placed in the lid of each Petri plate. Petri plates were then closed and sealed with parafilm and were incubated at 28±2°C for 1-4 days. Uninoculated plate was also kept as a control for comparison of results. Plates were observed for change of color of filter paper from yellow (-) to orange brown further to dark brown (++ or ++++) after growth of test organisms.

3.5.6 Ammonia production

For the detection of ammonia production, the method of Lata & Saxena (2003) was used. *Pseudomonas* sp. isolates were grown in 5ml of peptone water in tubes. Tubes were incubated at $28\pm 2^{\circ}\text{C}$ for 4 days. After 4 days, 1ml of Nessler's reagent was added to each tube. Presence of very light brown color (+) indicates small amount of ammonia production and light brown (++) to orange brown color (+++++) indicates large amount of ammonia production

Peptone Water

Components	grams/litre
Peptone	10.0
Sodium Chloride (NaCl)	5.0
pH	7.0

Nessler's Reagent

Potassium iodide (KI)	5 g/5 ml
Mercuric chloride (HgCl_2)	2 g/35 ml
Sodium hydroxide (NaOH)	20 ml (5N)

Saturated solution of mercuric chloride was slowly added to KI until the precipitates began to form. Then NaOH was added and entire solution was diluted to 100 ml with distilled water. This reagent was stored in dark bottle.

3.6 Plant Growth Regulators (Auxins, Gibberellins and Cytokinins)

All *Pseudomonas* isolates from rhizosphere soil of apple were screened out for the production of plant growth regulators viz. auxins, gibberellins and cytokinins.

3.6.1 Production of PGR's

All test organisms were grown in 100ml nutrient broth for 72 h. at $28 \pm 2^{\circ}\text{C}$ under shake conditions (100 rpm). Supernatants were prepared by centrifugation at 10,000 rpm for 20 minutes and were stored at 4°C .

3.6.2 Auxins

Quantitative measurement of auxins was done by colorimetric method with slight modifications [Gorden and Paleg, \(1957\)](#). To 2ml of supernatant 2-3 drops of orthophosphoric acid were added followed by 4 ml of Salper reagent (1 ml of 0.5 M FeCl_3 in 50 ml of 30 % HClO_4 ; prepared fresh). This mixture was incubated for 60 minutes in dark. Absorbance was measured at 535 nm. Concentration of auxins was estimated from calibration curve using indole acetic acid (IAA) as standard (10-100 $\mu\text{g/ml}$).

3.6.3 Gibberellins

The gibberellins were estimated colorimetrically by the method of [Holbrook *et al.*, \(1961\)](#) with slight modifications. To 15 ml of supernatant, 2 ml of zinc acetate reagent (21.9 g zinc acetate + 1 ml of glacial acetic acid and volume was made upto 100 ml with distilled water) was added. After 2 minutes, 2 ml of potassium ferrocyanide (10.6% in distilled water) was added and was centrifuged at low speed (2000 rpm) for 15 minutes. To 5 ml of supernatant 5 ml of 30 per cent HCl was added and mixture was incubated at 20°C for 75 min. For blank 5 ml of 5 per cent HCl was used. Absorbance was read at 254 nm and concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA_3 , Hi-media) as standard (100-1000 $\mu\text{g/ml}$).

3.6.4 Cytokinins

Radish cotyledons expansion bioassay test was employed ([Letham, 1971](#)) for assay of cytokinins like substances. The radish seeds were germinated in total darkness for 48 h at 28°C. After removing the seed coat, 12 smaller cotyledons were transferred to each sterilized Petri dishes containing the test solution, control (water) & standard solution on filter paper strips. Plates were incubated at 25°C under fluorescent light for 3 days. Then cotyledon were blotted, dried and weighed. The bioassay response (final weight-initial weight) was expressed as increase in weight. Concentration of cytokinins present in the extract was calculated of by preparing standard curve by using kinetin as standard (1- 10 $\mu\text{g/ml}$ and 10-100 $\mu\text{g/ml}$).

3.7 MOLECULAR IDENTIFICATION OF *Pseudomonas* sp. ISOLATES

3.7.1 16S rRNA Gene Sequencing

3.7.1.1 Isolation of genomic DNA

Genomic DNA from the *Pseudomonas* sp. isolates names An-6-shr, An-3-cha, Ar-Bag, An-M-Mag, An-1-Mag, An-5-Mag, An-6-Mag were isolated according to manufacturer's (B.GeNei) specifications using DNA isolation kit. Following steps involved in it:

1. 1 ml of each *Pseudomonas* isolate's 24 h. old culture grown in nutrient broth was centrifuged at 6000 rpm for 10 minutes.
2. Each bacterial pellet was resuspended in 180 µl of lysis buffer1.
3. 20µl of proteinase K was then added to each mixed thoroughly by vortexing and incubated at 55°C for 1-3 h.
4. 4 µl of RNase A (100mg/ml) was added to each, mixed by vortexing and incubated at room temperature for 5 minutes.
5. 200 µl of lysis buffer 2 was added to each supernatant, mixed thoroughly by vortexing and incubated at 70°C for 20 minutes.
6. Then 200 µl of absolute ethanol (99.9%) was added to each and mixed thoroughly by vortexing.
7. The GeNei PureTM columns were kept in 2ml collection tubes and then the sample-ethanol mixtures were added to each respective column.
8. They were centrifuged at 11,000 rpm for 5 minutes. Collection tubes with flow through were then discarded.
9. One volume of wash buffer 1 was diluted with three volumes of absolute ethanol just before use.
10. The GeNei PureTM columns were then kept in fresh 2ml collection tubes and 500µl of diluted wash buffer 1 was added to each column. Then they were spinned at 11,000 rpm for 1 minute. Collection tubes with wash sample were discarded.
11. One volume of wash buffer 2 was diluted with three volumes of absolute ethanol just before use.

12. The GeNei Pure™ columns were then kept in fresh 2ml collection tubes and 500µl of diluted wash buffer 2 was added to each column. Then they were spinned at 11,000 rpm for 3 minutes. Wash fractions were discarded and collection tubes were retained for the next step.
13. The empty GeNei Pure™ columns were spinned at 11,000 rpm for 2 minutes. Collection tubes were then discarded.
14. The GeNei Pure™ columns were opened and placed in new 1.5 ml vials (sterile), incubated for 2 minutes at 70°C dry bath to ensure complete removal of ethanol.
15. Required amount of elution buffer was taken in a 1.5 ml vial (sterile) and prewarmed in a dry bath set at 70°C for 5 minutes. 200 µl of elution buffer was used for one preparation.
16. The GeNei Pure™ columns were placed in fresh 1.5 ml vials and 200 µl of prewarmed elution buffer was added to each column, incubated at room temperature for 5 minutes and centrifuged for 1-2 minutes to elute the DNA.
17. Eluted DNA samples were stored at -20°C.

3.7.1.2 Amplification of genomic DNA

Amplification of genomic DNA was carried out using *Pseudomonas* specific oligonucleotide primer sequences: 20-mer FP-1 and 18-mer RP-1 in MJ according to Widmer *et al.*, (1998) Mini BIO-RAD personal thermal cycler-100 (PTC-100). These specific primers used to find out their taxonomic affiliation to *Pseudomonas* sp. of group *sensu stricto* that produce fluorescent pigment and belong to group-1 that comparizes of *Pseudomonas aeruginosa*, *P. Putida*, *P.fluorescens* and *P. syringae*.

Primers	Sequence 5'→ 3'
FP-1 (20-mer)	GGTCTGAGAGGATGATCAGT
RP-1 (18-mer)	TTAGCTCCACCTCGCGGC

3.7.1.3 Reagents:

1. Taq DNA polymerase (5U/µl) (GeNei™)
2. Taq buffer A (10X) containing 15 mM MgCl₂ (GeNei™)

3. Deoxynucleotide triphosphate (dNTPs) mixture: 100 mM of each dNTP (GeNeiTM)
4. Genomic DNA (25 ng/μl)
5. Random primers (GeNeiTM)

3.7.1.4 Procedure:

The PCR reaction mixture of 25 μl contained:

1. 18.8 μl sterilised distilled water
2. 0.20 μl Taq DNA polymerase
3. 2.50 μl Taq buffer A
4. 1.50 μl dNTPs mixture
5. 0.50 μl forward specific primer (100 pM/μl)
6. 0.50 μl reverse specific primer (110 pM/μl), and
7. 1.0 μl genomic DNA

The PCR amplification was carried out in MJ Mini BIO-RAD personal thermal cycler-100 (PTC-100) with a total of 35 cycles. Each cycle consisted of:

- One minute denaturation at 94°C
- Two minutes annealing at 55°C and
- Two minute extension at 72°C

All the PCR samples were given 5 minutes pre-amplification at 95°C and 10 minutes post-amplification at 72 °C.

3.7.2 Electrophoresis of amplified DNA (Felsenstein, 1993)

The electrophoresis of the amplified DNA was carried out in 1.2% agarose gel stained with ethidium bromide (0.5 mg/ml), under submerged conditions using 1×TAE buffer as tray buffer. To each PCR amplified sample, 3 μl of 6 X loading dye was added. 100-10,000 bp DNA ladder marker was used as standard and the gel was run at 110 V until the loading dye reached the gel front. The amplified DNA was viewed under the UV trans-illuminator and the image was taken through BIO-RAD gel documentation system using quantity one

software and saved in computer. Molecular weight of amplified bands of all *Pseudomonas* sp. isolates under study was analysed using Quantity one software itself.

3.7.3 Gel elution

The desired amplified bands of only two *Pseudomonas* sp. isolates An-6-shr, An-3-cha were cut down with the help of sterile cutter and their amplified bands were kept in sterile eppendorf tubes at 4°C. These gel pieces were then eluted or purified with gel elution kit (GeNei™).

3.7.4 DNA sequencing and sequence analysis

For DNA sequencing, purified and eluted amplified DNA products of two selected *Pseudomonas* sp. isolates An-6-shr and An-3-cha were sent to Bioserve Private limited, India under refrigerated conditions using gel packs and thermocol box. DNA sequencing was performed at the scientific solutions (Bioserve Private limited, India). For sequence determination of the Ps-PCR products, a generally applicable sequencing strategy was developed.

3.7.5 NCBI Blast

Similarity searches of the Genbank database were performed with BLAST and of the RDP database was performed with SIMILARITY_RANK and CHIMERA_CHECK (Maidack *et al.*, 1996).

For phylogenetic analysis, sequences were manually aligned to the alignment retrieved from RDP by us; the multiple alignment routine clustering was determined by unweighed pair group with mathematical averages (UPGMA) analysis of pairwise genetic distance values. Phylogenetic trees were deduced.

3.8 STANDARDIZATION OF MEDIA FOR MASS MULTIPLICATION AND HIGH PRODUCTION OF ANTIFUNGAL ACTIVITIES

3.8.1 Effect of different media and time of incubation on production of antifungal activities and growth:

Effect of different media and two times of incubation, on the production of antifungal were studied by growing selected *Pseudomonas* sp. isolates. They

were grown in different media broth i.e Nutrient broth, yeast malt (YM), pigment production media (PPM) and 1/5 M523 media and King's B medium. In each case 0.5 ml of inoculum of overnight grown culture of each *Pseudomonas* isolates was used to inoculate 100 ml of each media in a 250 ml Erlenmeyer flask. Flasks were incubated for two times of incubation i.e 48 h and 72 h under shake conditions (100 rpm). Growth was observed as absorbance at 540 nm. Cultures were centrifuged at 10,000 rpm for 20 minutes at 4°C and cell free culture supernatants were separated and stored at 4°C in the form of small aliquots.

3.8.2 Antifungal activity

For antifungal activity, 72 h old culture bit of indicator test fungi viz., *Dematophora* sp., and *Phytophthora* sp., *Pythium* sp., and *Sclerotium* sp. was placed on one side of prepoured malt extract agar (MEA) plates with the help of sterile well cutter and inoculating needle. On the other side of the plate, well was cut with the help of sterile cork borer and 100µl of 48h and 72 h old cell free supernatant of each bacterial isolates was added to each well. Plates were incubated at $28 \pm 2^\circ \text{C}$ for 7 days and observed for inhibition of growth of mycelia produced around the well. For control, culture bit of indicator fungi kept in the center of malt extract agar plate and incubated at $28 \pm 2^\circ \text{C}$ for 7 days. Antifungal activity expressed in terms of percent inhibition (% I) of fungal mycelia according to [Vincent, \(1947\)](#):

$$\% I = \frac{C-Z}{C} \times 100$$

Where, Z = Growth of mycelia in treatment
 C = Growth of mycelia in control

3.8.3 Effect of different media and temperature of incubation on the production of antifungal activity and growth

Effect of different media and two temperatures, on the production of antifungal activity were studied by growing selected *Pseudomonas* sp. isolates. They were grown in different media viz. broth nutrient broth, yeast malt (YM), pigment production media (PPM) and 1/5 M523 media and King's B medium. In

each case 0.5 ml of inoculum of overnight grown culture of *Pseudomonas* was used to inoculate 100 ml of each media in a 250 ml Erlenmeyer flask in duplicate. Flasks were incubated at two temperatures of incubation i.e. 25°C and 37°C under shake conditions (100 rpm). Growth was observed as absorbance at 540 nm. Cultures were centrifuged at 10,000 rpm for 20 minutes at 4°C and cell free culture supernatants were separated and stored at 4°C in small aliquots.

3.8.4 Antifungal Activity

For antifungal activity, 72 h old culture bit of indicator test fungi viz., *Dematophora* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp., and *Sclerotium* sp. was placed on one side of prepoured malt extract agar (MEA) plates with the help of sterile well cutter and inoculating needle. On the other side of the plate, well was cut with the help of sterile cork borer. 100µl of 48 h old cell free supernatant of each *Pseudomonas* isolates was added to each well. Plates were incubated at 28°C for 7 days and observed for inhibition produced around the well. For control, culture bit of indicator fungi kept in the center of malt extract agar plate and incubated at 25°C for 7 days and observed for inhibition around the well. Antifungal activity expressed in terms of percent inhibition (% I) of fungal mycelia according to Vincent (1947):

$$\% I = \frac{C-Z}{C} \times 100$$

Where, Z = Growth of mycelia in treatment
C = Growth of mycelia in control

Ingredients	Type of Medium (g/l)				
	Yeast Malt	1/5M23	PPM	NB	KMB
Yeast extract	3.0	0.8	-	-	-
Malt Extract	3.0	-	-	-	-
Peptone	5.0	-	20.0	5	20
Glucose	10.0	10.0	-	-	-
Sucrose	-	2.0	-	-	-
Casein Hydrolysate	-	1.6	-	-	-
NaCl	-	-	5.0	5	-
KNO ₃	-	-	1.0	-	-
K ₂ HPO ₄	-	0.4	-	-	1.5
MgSO ₄ .7H ₂ O	-	0.06	-	-	1.5
Glycerol	-	-	20	-	15
Beef Extract	-	-	-	3	-
pH	7.0	7.0	7.2	7.0	7.2

3.9 EXTRACTION AND EVALUATION OF ANTIFUNGAL METABOLITES. (Rosales *et al.*, 1995)

3.9.1 Production

0.5ml of overnight inoculum of each test *Pseudomonas* isolates used to inoculate 150ml of KMB broth and incubated at 30°C for 48h on rotatory shaker (100rpm)

↓
The culture was centrifuged at 3500 rpm for 7 min

↓
Pellet was suspended in 150 ml PPM broth and incubated on rotatory shaker at 30°C for 4 days

3.9.2 Extraction

150ml cultures were acidified with conc. HCl (pH 4)

↓
Pigments were extracted by treating the culture with 150ml of benzene

↙
Aqueous phase 60ml (lower layer)

↓
Extracted with 60 ml chloroform

↓
extracted metabolite (Pyocyanin)

↓
Dried fractions was dissolved in methanol (1ml)

↓
Extract was evaluated by thin layer chromatographic analysis for R_f (Solvent system used isopropanol/ammonia/water(8:1:1))

↓
Plates were viewed under UV light and afterwards sprayed with DSA (yellow spots)

↘
benzene phase 90 ml (upper layer)

↓
evaporated on water bath at 65°C

↓
extracted metabolite(phenazine)

↓
Dried fractions was dissolved in methanol (1ml)

↓
Extract was evaluated by thin layer chromatographic analysis for R_f (Solvent system used isopropanol/ammonia/water (8:1:1))

↓
Plates were viewed under UV light and sprayed with DSA (green spots)

Expected color of spot :

Under UV = light blue

After spray with DSA = light yellow and light green

3.9.3 Evaluation of extracted metabolites for antifungal activity

Antifungal activity of extract from each test strain of *Pseudomonas* sp. isolate against different indicator fungal pathogens viz., *Dematophora* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp. was checked by well plate assay method (Vincent, 1947). 72 h old culture bit of indicator fungi were placed on one side of prepoured malt extract agar (MEA) plates. On the other side of the plate, 100µl of extract of each isolate was added to each well (9 mm dia.) cut with the help of sterile cork borer. Plates were incubated at $28 \pm 2^\circ \text{C}$ for 7 days and observed for inhibition of mycelial growth produced around the well. For control, culture bit of indicator fungi was kept in the center of malt extract agar plate and incubated at $28 \pm 2^\circ \text{C}$ for 7 days and observed for inhibition of mycelial growth around the well. Antifungal activity expressed in terms of percent inhibition (%I) of fungal mycelia as calculated from the equation:

$$\% I = \frac{C-Z}{C} \times 100$$

Where,

Z = Growth of mycelia in treatment
C = Growth of mycelia in control (each indicate test fungi)

3.9.4 Evaluation of extracted metabolites for PGP activities:

3.9.4.1 Phosphate solubilization (Pikovskaya, 1948)

Pikovskaya's agar plates with known amount of inert phosphorous source (tricalcium phosphate/TCP) were prepared. 100µl of extract of each *Pseudomonas* isolate was added to each well already cut on agar with the help of sterile cork borer. Plates were incubated at $28 \pm 2^\circ \text{C}$ for 48h and observed for pinkish/orange zone produced around the well. Phosphate solubilizing activity expressed as mm diameter of yellow zone produced around the well (9mm dia) after incubation at $28 \pm 2^\circ \text{C}$ for 48h.

3.9.4.2 Siderophore

Siderophores production was detected by chrome azurol-S (CAS) plate assay with slight modifications (Schwyn and Neilands, 1987). 100 µl of extract of

each *Pseudomonas* sp. isolate was added to each well already cut on prepoured CAS plate with the help of sterilized cork borer. Plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 48 h and observed for production of yellow/orange zone around the well. Production of siderophores was expressed in terms of mm diameter of yellow/orange zone produced around the well at $28 \pm 2^{\circ}\text{C}$ in 48 h.

3.9.4.3 Lytic enzymes

3.9.4.3.1 Protease (Kaur *et. al.*, 1989)

100µl of extract of each *Pseudomonas* sp. isolate was added to each well already cut on the prepoured skim milk plates. These plates were then incubated at $28 \pm 2^{\circ}\text{C}$ for 48 h and observed for clear zone production around the well. Proteolytic activity was expressed in terms of mm diameter of clear zones produced around the well after incubation at $28 \pm 2^{\circ}\text{C}$ for 48 h .

3.9.4.3.2 Chitinase

100µl of extract of each bacterial isolate was added to each well already cut on the prepoured chitin agar plate with the help of sterile borer. Plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 5 days and observed for clear zone produced around the well.

3.10 FIELD EXPERIMENT APPLICATION OF BEST SELECTED TWO *Pseudomonas* ISOLATES AND THEIR CONSORTIUM ON GROWTH OF APPLE PLANT IN REPLANT SITES OF ORCHARD AT MAGGOTA

3.10.1 Collection of root stock

Seventy plants of apple were procured from Navbahar, Shimla. These plants were selected to study the effect of best selected *Pseudomonas* isolates on the growth, establishment of plants, soil parameters, root colonization and PGP activities of soil extracts in replant site of Maggota (Distt. Shimla).

The two best strains of fluorescent *Pseudomonas* sp. viz., An-6-shr and An-3-cha was selected on the basis of their high production of different plant growth promoting activities *in vitro* viz., antifungal, siderophores, phosphate

solubilizing, proteolytic, ammonia, HCN production, auxins, gibberellins and cytokinin.

3.10.2 Preparation of inoculum

The two selected *Pseudomonas* sp. isolates An-6-shr and An-3-cha were grown in nutrient broth at $28 \pm 2^\circ\text{C}$ for 72 h (adjusted to two inoculum densities i.e full and half ($8 \times 10^8 \text{cfu/ml}$ and $4 \times 10^8 \text{cfu/ml}$)) and used for the treatment of roots of plant and soil of pits. Two concentrations (full and half concentration) of each culture were used along with control. Ten plants of each treatment were planted in replant site after the treatment of their roots.

The plants were treated with liquid culture of *Pseudomonas* suspension by dipping their roots in liquid inoculum for 30 minutes before planting them in replant site pits. The soil of pits was treated two times with each inoculum before plantation of apple plants. The cyclic treatment for successive 8 – 9 months were given at monthly interval in rhizosphere of plants by adding 100 – 150 ml of inoculum to the basin of plants. Results were compared with uninoculated control. Effect on plant parameters like percentage survival, plant height, number of nodes/leaves and soil parameters like NPK content of soil, soil pH and moisture content were studied.

3.10.3 Survival percentage

Survival percentage was calculated as the number of plants planted and the number of plants survived expressed in terms of percentage survival.

3.10.4 Plant parameters

3.10.4.1 Plant height

The height of the plant was measured with the help of measuring tape from the base to the tip of the plant and was expressed in centimeters (cm).

3.10.4.2 Number of Nodes

The data of number of nodes was recorded irrespective of their size.

3.11 ROOT COLONIZATION BY INTRODUCED BACTERIA

3.11.1 Determination of total microbial count and total fluorescent *Pseudomonas* count

The total microbial and *Pseudomonas* count was determined from the rhizosphere soil of plants after every month of the bacterial treatment. Ten grams of rhizosphere soil was shaken vigorously in 90 ml sterile water blank in 250 ml Erlenmeyer flask for 30 minutes. Dilutions were made using 9.9 ml sterile water blanks to give hundred fold dilution series from 10^{-2} to 10^{-8} for each soil sample. 0.1 ml of each diluted sample was added to each prepoured nutrient agar and King's B agar amended with antibiotics. Plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 24-48 hr.

3.11.2 Determination of multifarrous PGP activities in rhizosphere soil extract

Different plant growth promoting activities i.e Phosphate solubilizing, siderophore, proteolytic, HCN, ammonia and antifungal activities were checked in rhizosphere soil extract after 3 and 9 month of plantation.

3.12 SOIL PARAMETERS

3.12.1 Soil pH

The pH of the soil is determined by a pH meter. 10 gram of soil is weighed out in a 100 ml beaker, and 40 ml of distilled water is added to it. The soil water suspension was stirred at least four times with in a period of half an hour. This time is required for the soil and water to attain equilibrium. After half an hour, the soil water suspension was again stirred and the pH was measured with a pH meter.

3.12.2 Soil Moisture

Soil moisture is determined by the gravimetric method. Soil water content is determined by drying a known mass of moist soil sample in an oven at 105°C . This method involves three independent measurements viz., mass of wet soil, mass of dry soil and mass of container. The dry mass of the soil is taken after

drying it in an oven at a temperature of 105°C till the mass becomes constant .

The percent moisture present is calculated as follows:

$$\text{Percent moisture in soil} = \frac{\text{Loss in weight}}{\text{Oven dry weight of soil}} \times 100$$

3.12.3 Determination of available nitrogen, phosphorus and potassium (N, P, K) in rhizosphere soil Nitrogen (Subbiah and Asija, 1956).

3.12.3 Nitrogen:

3.12.3.1 Reagents:

- KMnO_4 solution (0.32 %): 3.2 gm of KMnO_4 dissolved in distilled water and made up the volume to 1 litre with distilled water.
- NaOH solution (2.5 %): 25 g of NaOH pellets dissolved in distilled water and make up the volume to 1 litre with distilled water.
- 0.02N H_2SO_4
- 0.02N NaOH
- Methyl red indicator (0.15 %): 0.15 gm of methyl red powder dissolved in small amount of alcohol and make up the volume to 100 ml with distilled water.

3.12.3.2 Procedure

Ten gram of soil sample was added to Kjeldahl distillation flask and along with 100 ml of KMnO_4 (0.32 %) added to it and 100 ml of 2.5 % of NaOH solution was added to the assembly. Pieces of paraffin wax were added to avoid excess frothing. After that 25 ml of 0.02N H_2SO_4 pipetted out in 100 ml conical flask and then added 2-3 drops of methyl red indicator and the end of delivery tube was dipped in to the conical flask. Hot plate was switched on to distill ammonia gas. Approximately 30 ml of distillate collected in H_2SO_4 (0.02N). The excess of H_2SO_4 in the conical flask was titrated against 0.02N NaOH and end point was noted with the colour changes from pink to yellow. Final volume of NaOH used was noted.

3.12.3.3 Observations and Calculations:

- a) Weight of soil taken = 10gm
- b) Volume of 0.02N H₂SO₄ taken = 25 ml
- c) Volume of 0.02N NaOH used (titrate value) = X ml
- d) Volume of 0.02N acid used for NH₃ absorption = (25-X) ml
(1 ml of 0.02N H₂SO₄ = 0.00028 g of N or 0.28 mg N)
- e) Percentage of available N = $\frac{(25-X) \times 0.00028 \times 100}{10}$ = e
- f) Available N in the soil (ppm) = percentage of available N (e) x 10,000 = f
- g) Available N in the soil (kg/ha) = f x 2.24

3.12.4 Phosphorus: (Olsen *et al.*, 1954)

3.12.4.1 Reagents:

- 0.5 M NaHCO₃: 42.0 gm of sodium bicarbonate (NaHCO₃) dissolved in distilled water and the pH of the solution adjusted to pH 8.5 with dilute NaOH Solution and the final volume made up to 1 litre with distilled water.
- Darco-G 60 (activated charcoal)
- Dickman and Bray's reagent: (1.5 % solution of ammonium molybdate in conc. HCl): 15 gm of ammonium molybdate dissolved in 300 ml of warm distilled water and 342 ml of concentrated HCl added and made final volume 1 litre with distilled water.
- Stannous chloride (SnCl₂·2H₂O) solution: (Stock 40%): 10 gm of SnCl₂·2H₂O dissolved in 25 ml of concentrated HCl. Stored in brown bottle.
- Working Solution: 0.5 ml of the Stock solution diluted to 66 ml distilled water. Solution prepared fresh before use.
- Standard P solution (50 ppm): 0.219 gm of potassium dihydrogen phosphate (KH₂PO₄) dissolved in distilled water and diluted to 1 litre with distilled water.
- From this 5 ppm working solution prepared by diluting ten times.

3.12.4.2 Procedure:

One gram of soil was transferred to 100 ml conical flask, followed by the addition of a pinch of Darco-G 60 and 20 ml of 0.5N Sodium bicarbonate. The contents were shaken for 30 minutes on an electric shaker and suspension was made. Suspension was filtered through whatman no. 1 filter paper and 5 ml of filtrate was pipetted out in 25 ml volumetric flask. To 5 ml of the extract, 5 ml ammonium molybdate was added. The mixture was thoroughly shaken so as to avoid direct contact to $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ with concentrated ammonium molybdate. Finally 1.0 ml of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ working solution was added and made final volume 25 ml with distilled water. Solution was kept for 10 minutes at room temperature. Blue color intensity was measured on spectronic-20 at a wavelength of 660 nm using red filter. An appropriate blank was kept using all the reagents except soil.

3.12.4.3 Observations and Calculations:

a.	Weight of soil taken	=	1gm
b.	Volume of 0.5M NaHCO_3 solution added	=	20 ml
c.	First dilution	=	20 times
d.	Volume of filtrate taken for color development	=	5 ml
e.	Final volume made	=	25 ml
f.	Second dilution	=	5 times
g.	Total dilution	=	$20 \times 5 = 100$ times
h.	Absorbance as read from colorimeter	=	X
i.	ppm of P as read from standard curve	=	A
j.	ppm of available P in the given soil	=	$A \times 100 = B$
k.	Kg/ha of available P in the given soil	=	$B \times 100 \times 2.24$
l.	Kg/ha of available P_2O_5 in soil	=	$B \times 100 \times 2.24 \times 2.29$

3.12.5 Potassium (Merwin *et al.*, 2001)

3.12.5.1 Reagents:

- 1N solution of neutral ammonium acetate (pH 7.0): 77.09 gm of ammonium acetate (NH_4OAc) dissolved in distilled water and dilute to 1 litre.

- Standard solution of K (1000 ppm): Dissolve 1.907 gm of KCl in distilled water and dilute to 1 litre.
- Standard solution of K (50 ppm): Dilute the 1000 ppm solution by 20 times.
- Filter paper (Whatman No. 1)

3.12.5.2 Procedure:

1 gm of soil weighed in 150 ml of conical flask. 5 ml of neutral ammonium acetate solution was added and the contents were vigorously shaken on electric shaker for 5 minutes. The contents of the conical flask were filtered through whatman no.1 filter paper and filtrate was fed into atomizer of the flame photometer and readings were noted. Flame photometer already set with standards of 5 ppm and 10 ppm of K solution.

3.12.5.3 Observations and calculations:

a	Weight of the soil	= 1 gm
b	Volume of the extract added	= 5 ml
c	Dilution	= 5 times
d	Readings indicated by the flame photometer	= X
e	ppm of K as read from the standard curve against X	= Y
f	Available K (ppm) in the soil	= $Y \times 5 = Z$
g	Kg/ha of available K in the given soil	= $Z \times 2.24$

Chapter-4

EXPERIMENTAL RESULTS

4.1 ISOLATION, ENUMERATION AND COMPARISON OF BACTERIAL POPULATION AND FLUORESCENT *Pseudomonas* POPULATION IN NORMAL AND REPLANT SITE OF APPLE AT MAGGOTA (SHIMLA)

A random survey was conducted to isolate the fluorescent *Pseudomonas* sp. from rhizosphere of apple orchard in replant and normal sites of orchards of Shimla district (Maggota). Soil samples from these sites were collected and were used for the isolation of fluorescent *Pseudomonas* sp. and total culturable bacterial species using King's B media and nutrient agar respectively. The attempts yielded seven bacterial isolates from twelve samples (Table 1). They were isolated from rhizosphere soil sample by standard serial dilution and pour plate method using King' B medium and nutrient agar respectively after incubation at $28\pm 2^{\circ}\text{C}$ for 48h.

Table 1. Collection of rhizosphere soil samples from normal and replant sites of Maggota.(Shimla District)

District	Name of site	Total no. of composite soil sample		No of isolates An Ar		Symbolic designation To isolates
		A _r	A _n			
Shimla	Maggota	5	7	6	1	An-Mag, Ar-Mag

*Sample from apple (A) of Normal sit A_n & pit in replant site A_r, Mag stands for Maggota.

The results showed that there was a significant difference in the population density of fluorescent *Pseudomonas* sp. as compared to the total bacteria present in rhizosphere soil of replant pit and normal rhizosphere soil of apple plants. Total bacterial count were maximum in rhizosphere of normal site of the apple plants as compared to total counts present in rhizosphere of replant site (Table 2) .Similarly, the results also showed that maximum counts of fluorescent *Pseudomonas* sp. were present in soil samples collected from normal rhizosphere sites of apple plants as compared to counts presents in rhizosphere of replant sites. Total bacteria and total fluorescent *Pseudomonas* sp. on nutrient agar and King's B agar were counted respectively and expressed in terms of

Table 2. Population density of total bacterial and total fluorescent *Pseudomonas* species present in rhizospheric soil of apple in normal & replant site of orchards at Maggota (Shimla district)

Type of site	Soil sample	Viable count of total bacteria (NA)*				cfu/gm of soil at 10^{-6}	Viable count of fluorescent <i>Pseudomonas</i> sp on (KM)*				cfu/gm of soil at 10^{-4}
		10 ⁻²	10 ⁻⁴	10 ⁻⁶	10 ⁻⁸		10 ⁻²	10 ⁻⁴	10 ⁻⁶	10 ⁻⁸	
Replant	Ar-BAG	102	21	4	-	21	22	6	-	-	22
	Ar-1-BARI	180	60	10	-	60	-	-	-	-	-
	Ar-2-BARI	17	2	-	-	2	-	-	-	-	-
	Ar-NAL	210	90	12	-	90	-	-	-	-	-
	Ar-KOTI	5	1	-	-	1	-	-	-	-	-
Normal	An-M-Mag	180	45	11	-	45	9	2	-	-	9
	An-M ₁ -Mag	>300	42	6	-	42	16	4	-	-	16
	An-M ₂ -Mag	>300	110	80	6	110	12	-	-	-	12
	An-M ₃ -Mag	>300	150	15	-	150	10	-	-	-	10
	An-M ₄ -Mag	280	115	62	3	115	12	-	-	-	12
	An-M ₅ -Mag	>300	126	14	-	126	6	2	-	-	6
	An-M ₆ -Mag	268	180	20	-	224	12	4	1	-	12

*NA = Nutrient agar ; KMB = King's B agar ** 2 & 4 dilutions were used to calculate total bacteria cfu/g of soil in 10^{-4} dilution on NA. *** 2& 4 dilution used to calculate fluorescent *Pseudomonas* sp. cfu/g of soil in 10^{-2} dilution on modified KMB amended with antibiotics

colony forming unit per ml of each dilution. Finally population density count data (Table 2) from each dilution (10^{-4}) were converted to log cfu/g of soil using 2-4 dilutions in case of total bacteria and 10^{-2} in case of total *Pseudomonas* sp. population. Significant differences in rhizosphere bacterial communities were observed between old and replant pit and soil of normal tree rhizosphere (Table 2). Total culturable bacterial mesophilic population in replant site was far less as compared to bacterial population in soil from normal site. Trends in number of total culturable fluorescent *Pseudomonas* were more distinct. The densities of fluorescent *Pseudomonas* sp. in replant site were rare or very less as compared to normal site. Two already isolated strains were also included in the study and were used for field trials. Out of these two strains one strain An-6-shr is a native strain of apple of same area (Rohru, Shimla District) and second strain An-4-cha is from normal site of apple growing in orchards of another district of Himachal Pradesh.

4.2 MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS FOR IDENTIFICATION OF BACTERIAL ISOLATES

Five bacterial isolates showing fluorescent pigment production and colony characteristics like *Pseudomonas* sp. were selected out of total twelve bacterial isolates and subjected to detailed morphological, physiological and biochemical studies (Table 3a-3c). In colony morphological studies (Table 3a), they were found to show irregular form, flat elevation, transparent and smooth surface. Ar-Bag and An-6-Mag were found to show undulate whereas rest isolates were entire. The pigment produced by the An-6-shr and An-3-cha was fluorescent green. Ar-Bag produced yellow green while rest of the isolates produced bluish green pigment on King's B medium. In physiological characterization (Table 3c), all isolates except Ar-Bag were found to liquify gelatin and gave opaque zone in lecithinase test. An-6-shr, An-3-cha, An-1-Mag, and An-6-Mag have the ability to produce gas (cracking of agar medium) indicating denitrification test positive. Only An-6-shr and An-3-cha showed chitin hydrolysis on chitin agar plate and β -glucanase positive and all isolates produced dark brown to orange brown color in arginine hydrolysis showing

positive results. All isolates were found to be gram negative rods with no ability to form endospores and the oxidase and catalase test in respect of these isolates were positive (Table 3b). On the basis of the fluorescent pigment production along with other morphological, biochemical, physiological tests and growth maximum at specific temperature all isolates were found to belong to group of fluorescent *Pseudomonas* sp. The two strains An-6-shr and An-3-cha already isolated were also included for detailed investigation and field trials. Both strains are native strains of apple growing in orchards of Himachal Pradesh. One strain (An-6-shr) is a native strain of apple of Shimla distt. of Rohru area i.e Sharontha. This place is very close to Maggota. Second strain An-3-cha is from another District (Chamba) of Himachal Pradesh. Both these strains were selected due to high production of multifarrous PGP activities *in vitro*. Even in this study they were again showing high production of PGP and antifungal activities.

Table 3. Identification of isolates of fluorescent *Pseudomonas* sp. of apple from Maggota

3a. Morphological characteristics

Isolates	Colony Morphological characteristics					Pigment production (color)
	Form	Elevation	Margin	Opacity	Surface	
An-6-shr	Irregular	Flat	Entire	transparent	Smooth	Fluorescent green
An-3-cha	Irregular	Flat	Entire	transparent	Smooth	Fluorescent green
Ar-Bag	Irregular	Flat	undulate	Transparent	Smooth	Yellow green
An-M-Mag	Irregular	Flat	Entire	Transparent	Smooth	Bluish green
An-1-Mag	Irregular	Flat	Entire	Transparent	Smooth	Bluish green
An-5-Mag	Irregular	Flat	Entire	Transparent	Smooth	Bluish green
An-6-Mag	Irregular	Flat	undulate	Transparent	Smooth	Bluish green

Table 3 b: Biochemical characteristics

Isolates	Oxidase (blue color)	Catalase (effervescence)	Gram staining +/-	O/F	Spore Staining +/-	citrate	Indole production	Methyl red	Voges proskaur
An-6-shr	+	+	-	+/-	-	+	-	-	-
An-3-cha	+	+	-	+/-	-	+	-	-	-
Ar-Bag	+	+	-	+/-	-	+	-	-	-
An-M-Mag	+	+	-	+/-	-	+	-	-	-
An-1-Mag	+	+	-	+/-	-	+	-	-	-
An-5-Mag	+	+	-	+/-	-	+	-	-	-
An-6-Mag	+	+	-	+/-	-	+	-	-	-

- Indicates negative

Table 3 c : Physiological characteristics

Isolates	Denitrification (gasproduction)	Gelatin liquification	Lecithinate (opaquezone)	Chitinase (Clear zone)	β -glucanase (clear zone)	Arginine Hydrolysis
An-6-shr	+	+	+	+	+	+++++
An-3-cha	+	+	+	+	+	+++++
Ar-Bag	-	-	-	-	-	+++
An-M-Mag	-	+	+	-	-	+++
An-1-Mag	+	+	+	-	-	+++++
An-5-Mag	-	+	+	-	-	+++
An-6-Mag	+	+	+	-	-	+++

(+) = positive ; (-) = negative ; ++ = light brown; +++ = dark brown ; +++++ = orange brown

4.3 OPTIMUM GROWTH CHARACTERISTICS OF *Pseudomonas* ISOLATES AT FOUR OPTIMUM TEMPERATURES

All the isolates were subjected to grow in King's B broth and were incubated at different temperatures of 4°C, 25°C, 37°C and 42°C to study optimum growth temperature (Table 4) i.e. optical density at 540 nm upto 72 h. An-M-Bag, An-1-Mag, An-5-Mag, An-6-Mag were able to grow over wide range of temperature 4°C -37°C but maximum optimum growth was observed at 25°C and almost no growth at 42°C . These isolates may be related to *Pseudomonas fluorescens* on the basis of temperature optimization of growth. Whereas An-6-shr and An-3-cha showed the ability to grow maximally at temperature ranging 25°C, 37°C & 42°C. Both of these isolates showed growth at 42°C hence they may belong to *Pseudomonas aeruginosa*. Ar-Bag showed the optimum growth at 25°C and 37°C only i.e. mesophilic range and almost no growth at 4°C & 42°C and may be related to some other strain of *Pseudomonas putida*.

Table 4. Growth at different optimum temperature

<i>Pseudomonas</i> isolates	Growth (absorbance at 540 nm)											
	4°C			25°C			37°C			42°C		
	0 hr	48 h	72 h	0 h	48 h	72 h	0 hr	48 h	72 h	0 h	48 h	72 h
An-6-shr	0.002	0.002	0.003	0.006	0.031	0.101	0.004	0.089	0.176	0.001	0.099	0.108
An-3-cha	0.001	0.002	0.007	0.002	0.054	0.078	0.001	0.053	0.127	0.002	0.101	0.132
Ar-Bag	0.001	0.003	0.002	0.003	0.066	0.108	0.002	0.021	0.117	0.002	0.002	0.009
An-M-Mag	0.002	0.011	0.029	0.001	0.049	0.099	0.001	0.010	0.037	0.003	0.005	0.006
An-1-Mag	0.002	0.016	0.022	0.003	0.052	0.094	0.001	0.010	0.037	0.001	0.002	0.003
An-5-Mag	0.002	0.012	0.031	0.001	0.051	0.102	0.003	0.008	0.045	0.002	0.002	0.003
An-6-Mag	0.001	0.014	0.023	0.001	0.049	0.109	0.002	0.011	0.028	0.001	0.004	0.006

4.4 Resistance of fluorescent *Pseudomonas* isolates to some antibiotics and chemicals

The organisms were subjected to check their intrinsic resistance to commonly used different antibiotics (Kanamycin, Streptomycin and Penicillin)

and chemicals (HgCl₂, crystal violet and naphthalene) and fungicide (bavistin) analysis by disk plate assay on nutrient agar medium at 28 °C for 24 h. All the isolates were found resistant for naphthalene, penicillin and bavistin upto higher concentration of 100 ppm and also resistant upto 50ppm to crystal violet and 25 ppm to mercuric chloride (Table 5). All the isolates showed resistance to crystal violet upto 25ppm. Only one or two strains showed resistance to kanamycin and streptomycin upto 25-50 ppm (Ar-Bag and An-M-Mag). Three isolates (An-M-Mag, An-5-Mag & An-6-Mag) showed resistance to HgCl₂ upto 25ppm only. The results indicating towards the possibility of carrying resistance marker plasmids against these antibiotics and chemicals by some strains.

Table 5. Growth inhibition of isolates(resistance) on nutrient agar produced by different concentration of antibiotic or chemicals at 28°C after 24 hr incubation

<i>Pseudomonas</i> isolates.	HgCl ₂ (ppm.)			Mean	Crystal violet (ppm.)			Mean
	25	50	100		25	50	100	
An-6-shr	-	18	23	13.67	-	-	-	0
An-3-cha	-	19	22	13.67	-	-	10	3.33
Ar-Bag	10	29	31	23.33	-	22	26	16.00
An-M-Mag	-	33	35	22.67	-	13	19	10.67
An-1-Mag	13	34	35	27.33	-	17	23	13.33
An-5-Mag	-	34	36	23.33	-	27	28	18.33
An-6-Mag	-	30	33	21.00	-	21	24	15.00
Mean	3.28	28.14	30.71			0	14.29	18.57
CD_{.05} T				.830				.688
I				.543				.450
T*I				1.43				.841
<i>Pseudomonas</i> isolates	Kanamycin(ppm)			Mean	Streptomycin (ppm)			Mean
	25	50	100		25	50	100	
An-6-shr	-	-	-	0	-	-	-	0
An-3-cha	-	-	-	0	-	-	-	0
Ar-Bag	-	-	11	3.667	-	17	20	12.33
An-M-Mag	21	24	27	24.00	-	11	17	9.33
An-1-Mag	16	21	26	20.67	12	18	23	17.67
An-5-Mag	22	24	26	24.00	13	20	27	20.00
An-6-Mag	21	24	27	24.00	11	16	20	15.67
Mean	11.43	13.29	16.57		5.14	11.71	15.29	
CD_{.05} T				.830				.748
I				.543				.49
T*I				1.01				1.25

- Indicates no zone

* Inhibition of growth of *Pseudomonas* isolates expressed as mm dia of clear zone produced by 100 µl of different concentration (25-100ppm) chemical, antibiotic and pesticide around the well(9mm) on nutrient agar lawned with growth of test bacteria at 28°C after 24 h incubation.

** All the strains showed intrinsic resistance against naphthalene, penicillin and bavistin at all conc. 25-100ppm

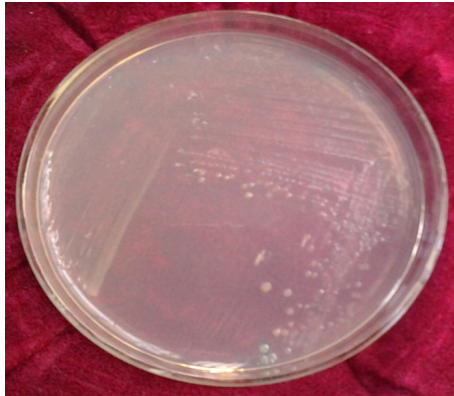


Plate 1. Colony morphology and pigment production shown by strain of *Pseudomonas* sp. An-M-Mag

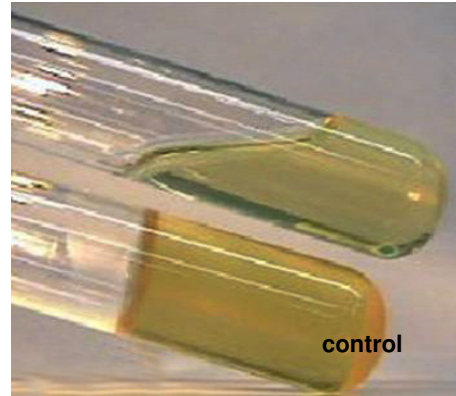


Plate 2 Gelatin liquification by *Pseudomonas* isolate An-6-shr with control

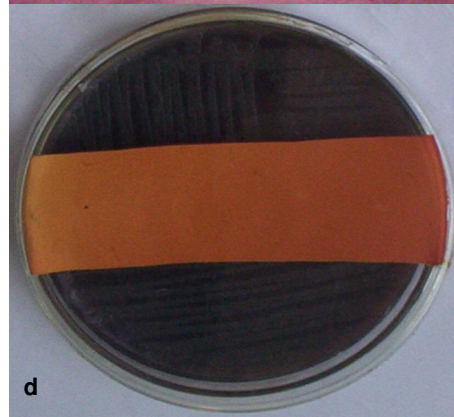
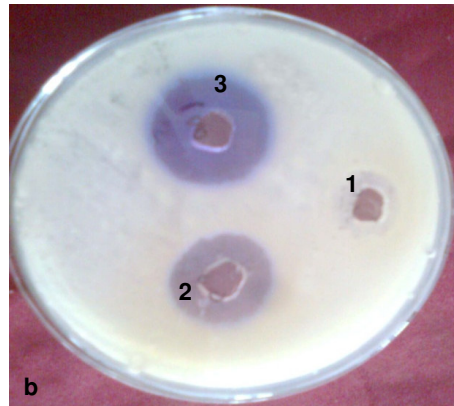
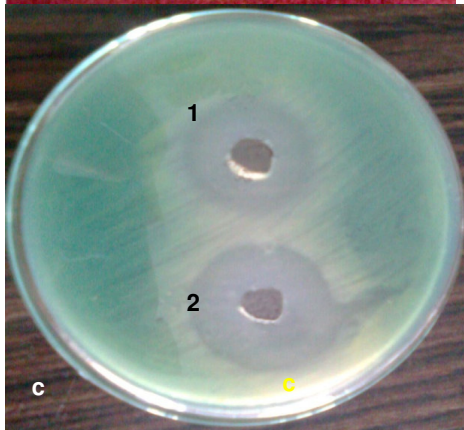
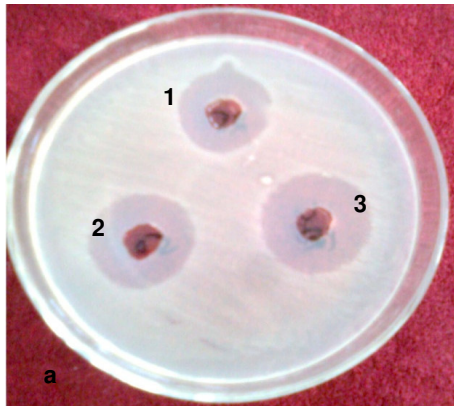


Plate 3 Growth inhibition of *Pseudomonas* isolate in different concentration (1 =25ppm, 2:=50ppm, 3=100 ppm) a) An-M-Mag by $HgCl_2$ b) An-1-Mag by crystal violet c) An-3-cha by streptomycin d) HCN activity shown by An-3-cha

4.5 PGP POTENTIAL OF *PSEUDOMONAS* ISOLATES FOR PRODUCTION OF MULTIFARROUS ACTIVITIES

4.5.1 Phosphate solubilizing ability:

All isolates were tested for their ability to solubilize insoluble inorganic Tricalcium phosphate (TCP) on Pikovskaya's agar. All strains were able to solubilize TCP as it was detected by formation of yellow zone on PVK medium by 100µl of 72h old supernatant of culture. 57% isolates showed Phosphate solubilizing activity on PVK plates in the range of 20-26 mm dia of yellow zone while 43% of isolates showed Phosphate solubilization in 14-19mm dia of yellow zone. The phosphate solubilization was also estimated by quantitative assay in Pikovskaya's broth. All the isolates tested for the production of phosphate solubilizing activity in terms of inorganic tricalcium phosphate solubilization in Pikovskaya's broth *in vitro* and results were expressed as µg/ml of soluble phosphate (Pi). The maximum solubilization of inorganic TCP was shown by An-6-shr and An-3-cha (65-70 µg/ml) while other isolates showed phosphate solubilization between 40 to 55 µg/ml (Ar-Bag, An-M-Mag, An-1-Mag and An-5-Mag).

4.5.2 Siderophore :

Production of siderophores by *Pseudomonas* isolates was estimated by Chrome Azurol S blue agar (CAS) and liquid assay. All the 7 isolates of *Pseudomonas* sp. showed yellow to orange coloured zone around the well after 24 hours of incubation, which primarily indicated the ability of the isolates to produce siderophores (Table 7) . Five isolates out of total seven isolates showed production of siderophore in the range of 14-17mm dia while other showed slightly less production i.e. 10-12 mm dia on CAS agar plates. Siderophore production was also quantitated by liquid assay in terms of reduction in blue color as per cent siderophore units (% SU). All the isolates showed high production of siderophore units in CAS medium i.e in the range of 78.43-90.80%. Three isolates i.e An-6-shr, An-3-cha and An-5-Mag are the high producers of siderophore activity i.e upto 90% SU. Two of them are also being used in field trials at replant site of apple at Maggota.

Table .6: PGP potential of fluorescent *Pseudomonas* isolates for production of multifarrous activities.

<i>Pseudomonas</i> isolates	PGP Activities							
	Phosphate Solubilizing (yellow zone)		Siderophores (Orange pinkish)		Proteolytic (Clear zone)		Ammonia	HCN
	^{1*} mm dia	^{2*} soluble Pi(µg/ml)	*mm dia	3*%SU	*mm dia	4*Units(µg/ml)	Change of color (yellow to brown)	
An-6-shr	23	75	12	90.80(72.36)	11	48	+++++	+++
An-3-cha	26	70	14	89.29(70.83)	13	49	+++++	+++
Ar-Bag	20	50	17	86.56(68.45)	11	46	+++	+++++
An-M-Mag	14	40	10	78.48(62.31)	12	50	+++	+++
An-1-Mag	19	55	16	86.36(68.29)	14	87	+++	+++
An-5-Mag	24	40	15	90.00(71.58)	13	74	+++	+++++
An-6-Mag	16	65	17	85.95(67.95)	11	90	+++	+++
CD _{.05}	2.38	2.81	1.75	1.75(1.52)	2.09	3.10		

1* Phosphate solubilizing, siderophores and proteolytic activities expressed in terms of mm. diameter of yellow , pinkish/orange and clear zone produced around the well by 100µl of supernatant on PVK , Chrome azurol-S (CAS) and skim milk agar at 28° C after 48h incubation.

2* Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization which in turn represents µg/ml of soluble inorganic phosphate(Pi) in supernatant as calibrated from the standard curve of KH₂PO₄ (100-1000 µg/ml).

3* %Siderophore units i.e reduction in blue color of chrome azurol-S reagent as calculated using following equation:

$$\%SU = \frac{Ar-As}{Ar} \times 100$$

Ar = Absorbance of reference solution at 630 nm; As = Absorbance of test solution at 630 nm

Values in parentheses are arc sine transformation

4* One protease unit expressed as the amount of enzyme that is required to solubilize casein substrate in Tris buffer 0.05M, pH 8.0 under standard assay conditions which is represented in terms of µg/ml of protein concentration as calibrated from standard curve by lowry's method.

5* Production of HCN and ammonia were expressed as change of color of paper strip on King's B media amended with glycine and of culture broth after addition of Nessler's reagent (yellow(-) to brown(++++)) respectively after growth of test isolates.

4.5.3 Proteolytic enzymes:

The production of proteases was estimated by skim milk agar assay and activity expressed in terms of mm diameter of clear zone produced by 100µl of 72h old supernatant around the well on agar at 28⁰C after 24h incubation. All the isolates of fluorescent *Pseudomonas* sp. showed proteolytic activity in the range of 11-14 mm dia. of clear zone. Quantitatively maximum production of proteases was showed by three isolates An-1-Mag. An-5-Mag and An-6-Mag in the range

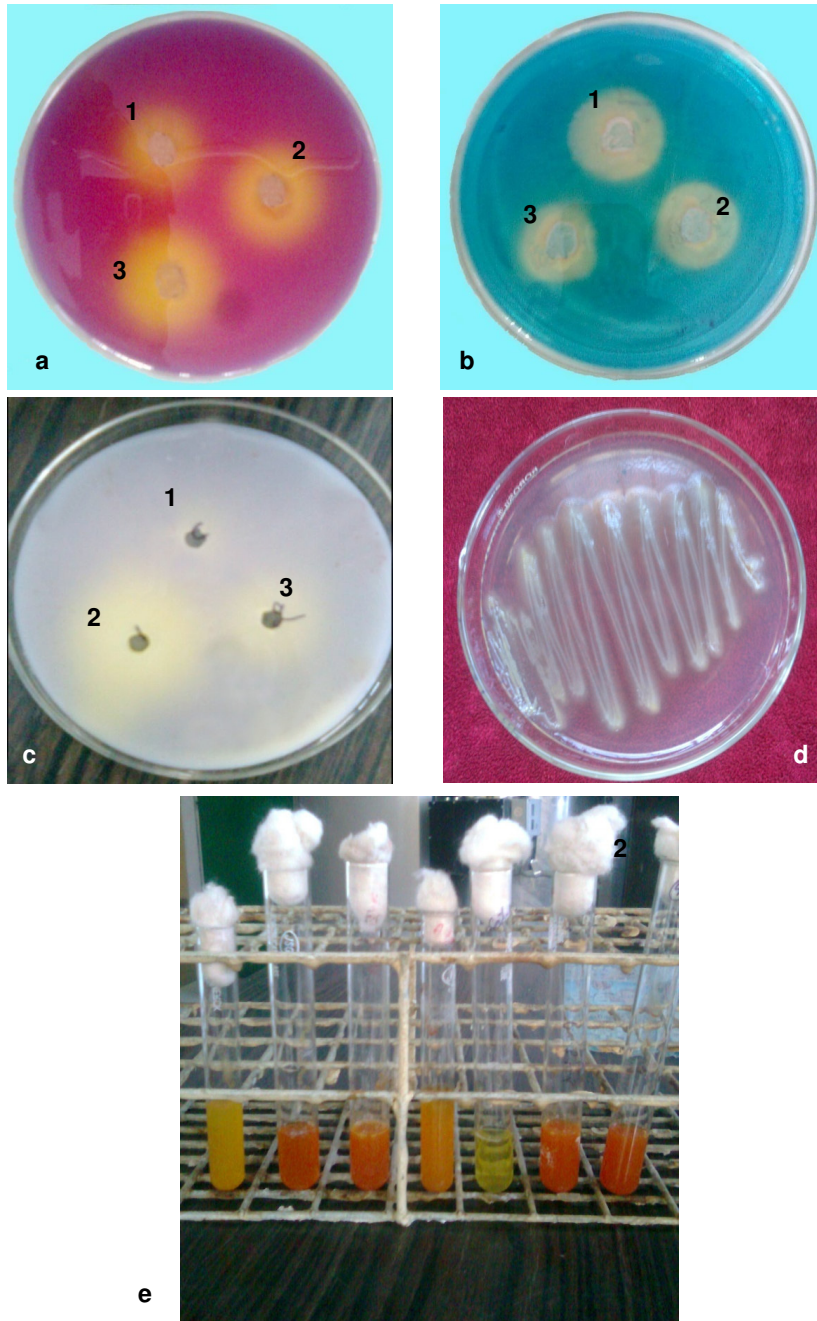


Plate 4 a) Phosphate solubilization b) siderophore production c) proteolytic activity shown by (1)An-6-shr, (2)Ar-Bag and (3)An-M-Mag

d) Lecithinase test shown by An-6-shr

e) Ammonia production shown by Ar-ag, An-3-cha, An-6-shr, An-M-Mag, An-6-Mag, An-1-Mag and An-5-Mag after addition of Nessler's reagent

of 74-90 units and slightly less production of proteases (46-50 units) was shown by another four isolates.

4.5.4 HCN :

All the isolates of *Pseudomonas* sp. also showed HCN production on King's B medium amended with glycine. Out of seven isolates of *Pseudomonas* sp. maximum production of HCN (+++++) was shown by Ar-Bag and An-5-Mag . Other isolates produced slightly less orange brown color (+++).

4.5.5 Ammonia :

The production of ammonia in peptone water after inoculation with test isolate and after 4 days of incubation at 30° C was observed by using Nessler's reagent. Color change in the broth tubes after growth from yellow to brown after addition of Nessler's reagent showed production of ammonia that was soluble in the media. Two isolates i.e An-6-shr and An-3-cha showed high production of ammonia (+++++) while other five isolates showed slightly less production of ammonia (+++).

4.6 Potential For Production of Plant Growth Regulators Viz. Auxins, Gibberellins and Cytokinins

All the strains of *Pseudomonas* species isolated from rhizosphere soil of apple were screened out for the production of plant growth regulators viz., auxins, gibberellins and cytokinins by their specific spectrophotometer and bioassay method by using 72 h old culture supernatant. Production of each growth regulator was expressed in terms of concentration (µg/ml). The result (Table 8) showed that all the strains of *Pseudomonas* species produce plant growth regulators.

The production of growth regulators viz., auxins, gibberellins and cytokinins varied among all the bacterial isolates from apple .The maximum production of auxins shown by *Pseudomonas* strain An-M-Mag , An-6-shr & An-3-cha (21-22 µg/ml), followed by *Pseudomonas* isolates Ar-Bag , An-1-Mag (14-15µg/ml). An-5-Mag, and An-6-Mag (9 to 11 µg/ml) showed slightly less

production of auxins. All isolates differed statistically and significantly from each others in terms of production of auxins.

Maximum gibberellins production was observed in three *Pseudomonas* isolates An-6-shr followed by An-3-Cha and Ar-Bag(63-75µg/ml) followed by An-5-Mag(50µg/ml) and by An-M-Mag, An-1-Mag and An-6-Mag (34-39µg/ml).

Cytokinins production by all strains of *Pseudomonas* sp. was measured indirectly by radish cotyledons bioassay method. Maximum production of cytokinins was shown by two *Pseudomonas* isolate An-3-cha, An-M-Mag (80µg/ml) followed by three isolates An-6-shr, An-Mag and An-6-Mag(40-60µg/ml). Two isolates (Ar-Bag and An-5-Mag) showed less production i.e upto 20µg/ml.

Table 7. PGP potential of isolates of fluorescent *Pseudomonas* sp. for production of plant growth regulators

<i>Pseudomonas</i> isolates	Plant Growth regulator(µg/ml)		
	*Auxin	**Gibberellins	***Cytokinins
An-6-shr	21	75	40
An-3-cha	21	67	80
Ar-Bag	14	63	20
An-M-Mag	22	37	80
An-1-Mag	15	34	60
An-5-Mag	11	50	20
An-6-Mag	9	39	40
CD _{.05}	2.37	2.81	2.88

Production of plant growth regulators viz. auxins*, gibberellins** and cytokinins*** expressed in terms of concentration (µg/ml) of each produced in supernatant as calibrated from their respective standard curve of indole acetic acid (10-100 µg/ml), gibberellic acid (GA₃) (10-100 µg/ml) and kinetin (100-1000 µg/ml).

4.7 PGP POTENTIAL FOR PRODUCTION OF ANTIFUNGAL ACTIVITIES.

4.7.1 Antifungal activity against:

All the *Pseudomonas* sp. isolates were characterized for the production of antifungal activity by well plate assay on MEA against five indicator test fungi (Table 8) viz., *Dematophora* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp.,

Sclerotium sp. Antifungal activity expressed in terms of mm dia of clear zone of mycelial growth produced by 100µl of cell free cultured supernatant (72h. at 28°C) which in turn also expressed as percent inhibition (%I) of growth of mycelia as compared to control.

(a) *Dematophora* sp.

Antifungal activity against plant pathogen *Dematophora* sp. had been shown by four fluorescent *Pseudomonas* sp. isolates. The maximum percent growth inhibition (40.0 %I) was shown by *Pseudomonas* sp. isolates i.e. An-3-cha followed by An-6-shr (37.14%I) Two isolates An-5-Mag & An-M- Mag showed slightly less inhibition of mycelia growth of *Dematophora* sp.(31.42-34.28%I).

(b) *Fusarium* sp.

Out of seven, only four isolates of fluorescent *Pseudomonas* sp. showed antifungal activity against *Fusarium* sp. in the range of 34.21-50.0%I. The maximum percent growth inhibition was shown by An-3-cha (50.0 %) and An-6-shr (44.73 %) as compared to another two isolates Ar-Bag & An-M-Mag that showed slightly less inhibition of mycelia of *Fusarium* sp. (both 40.73 %).

(c) *Pythium* sp.

Out of seven, only three of fluorescent *Pseudomonas* sp. isolates showed antifungal activity against *Pythium* sp. in the range of 17.94- 28.20%I inhibition of mycelia. The maximum percent inhibition of mycelia growth was shown by An-M-Mag (28.20 %I) followed by An-6-shr (25.64%I) and the minimum was shown by An-3-cha (17.94%I).

(d) *Phytophthora* sp.

Antifungal activity against plant pathogen *Phytophthora* sp. had been shown by two of fluorescent *Pseudomonas* sp. isolates. The percent inhibition of mycelia growth was shown by An-6-shr (14.2 %I) and An-3-cha (32.14 %I).

Table. 8: PGP potential of fluorescent *Pseudomonas* isolates for production of antifungal activities against different indicator fungi.

<i>Pseudomonas</i> isolates	Antifungal activities (mm dia and % inhibition of growth mycelia)									
	<i>Dematophora</i> sp.		<i>Fusarium</i> sp.		<i>Pythium</i> sp.		<i>Sclerotium</i> sp.		<i>Phytophthora</i> sp.	
	mm	% I**	mm	% I**	mm	% I**	mm	% I**	mm	% I
Control	35	0.0	38	0.0	39	0.0	37	0.0	28	0.0
An-6-shr	22	37.14(37.74)	21	44.73(41.97)	29	25.64(25.15)	19	48.64(44.22)	24	14.2(22.07)
An-3-cha	21	40(39.20)	19	50(45)	32	17.94(18.43)	22	40.54(39.54)	19	32.14(34.52)
Ar-Bag	-	0	25	34.21(35.78)	-	0	-	0	-	-
An-M-Mag	23	34.28(35.83)	25	34.21(35.63)	28	28.20(26.25)	-	0	-	-
An-1-Mag	-	0	-	0	-	0	-	0	-	-
An-5-Mag	24	31.42(33.96)	-	0	-	0	27	27.02(31.30)	-	-
An-6-Mag	-	0	-	0	-	0	-	-	-	-
CD₀₅	2.56	5.25(4.42)	2.88	6.59(4.60)	1.62	4.15(3.28)	1.14	3.09(1.86)	3.01	6.627(8.084)

- shows no activity.

.**Antifungal activity expressed in terms of mm diameter of clear zone of growth of mycelia (bit) on Malt extract agar (MEA) which in turn also expressed as percent inhibition (% I) of mycelial growth of indicator test fungi by well plate assay method at at 28°C for 5 days

Percent inhibition (%I) = C-T/ T× 100

Where, C: Growth of mycelia in control. T: Growth of mycelia in test.

(e) ***Sclerotium* sp.**

Three of the isolates showed antifungal activity against *Sclerotium* sp. in the range of 27.02 -48.64 percent inhibition of mycelia growth. Maximum percent inhibition shown by An-6-shr (48.64%I) followed by An-3-cha (40.54%) and An-5-Mag (27.02%).

Out of them total seven isolates, two were also used (An-6-shr and An-3-cha) for application in field trial at replant site of orchard at Maggota. Both of them showed broad spectrum against all the five important fungal pathogens of replant disease. An-6-shr showed inhibition of mycelia growth of different five pathogens in the range of 14.2 to 48.64%I. Similarly An-3-cha showed inhibition activity in the range of 17.94 to 50.00%I. One another strain An-M-Mag also showed antifungal activity against three pathogens in the range of 28.2 to 34.21%I.

4.8 Molecular identification of *Pseudomonas* sp. Isolates

Seven isolates of fluorescent *Pseudomonas* viz., An-6-shr, An-3-cha, Ar-Bag, An-M-Mag, An-1-Mag, An-5-Mag, An-6-Mag were used for their genotypic identification by 16S rRNA gene analysis using group specific designed primers.

4.8.1 Isolation and quantification of genomic DNA:

Genomic DNA from all the selected seven *Pseudomonas* sp. was isolated by standard method observed for purity and sharpness of bands by agarose gel electrophoresis using 1.2% agarose under standard conditions. DNA sample was judged by computing 260/280 ratio.

4.8.2 Amplification of genomic DNA

Amplification of genomic DNA was carried out using selective specific oligonucleotide primers for fluorescent *Pseudomonas* group-1 FP-1(20mer) and RP-1 (18mer) in MJ Mini BIO-RAD personal thermal cycler-100 (PTC).

Primers	Sequence 5' 3'→
FP-1 (20-mer)	GGTCTGAGAGGATGATCAGT
RP-1 (18-mer)	TTAGCTCCACCTCGCGGC

4.8.3 Sequence analysis

The sequences of 16s rRNA gene were used to estimate relationships among bacteria and other isolate of *Pseudomonas* sp. to identify unknown bacteria upto genus and species level. 16s rRNA gene analysis was done using group specific primers of sequences FP-1 (20 mer) and RP-1 (18 mer) specific for fluorescent *Pseudomonas* group. The specific primers were used to find out their taxonomic affiliation to fluorescent *sensu stricto* (i.e. subclass of gamma proteobacteria) that produce fluorescent pigment and belong to group-1 that comprises mainly of *P.aeruginosa*, *P. putida* and *P. fluorescens*. Two strains were sequenced from the Bio-serve company. These two strains were also used to for field experiment at Maggota (Shimla) to study their effect on growth promotion and establishment of apple plants in replant sites of orchard. The results showed that two strains were found to belong to *sensu strict* group of fluorescent Pseudomonads group (Plate 5).

Sequences of two *Pseudomonas* sp. isolates (An-6-shr, An-3-cha) and similar searches in the GenBank databases in NCBI-BLAST revealed that the sequences were of predominantly *Pseudomonas* origin. The isolate An-6-shr showed 98% homology with An-6shr strain showed 98% similarity with *Pseudomonas aeruginosa* NEB 98 with accession number (GU121495.1) similarity with *P.putida* and *P. fluorescence*. Similarly An-3-cha showed 98% with *Pseudomonas aeruginosa* strain NBAII AFP-5 16S ribosomal RNA gene, partial sequence acc no HM439971.1 and 92% similarity with *P.putida* and *P. fluorescence*. The results were also presented in Fig. 2& 4.

An-6-Shr with forward primer (FP-1)

TCATAAACGGTCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAA
GCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGT
TGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACATTACCAACAGAATAAGCAC
CGGCTAAATTCGAGCCCAACCGGGTAATATGAAGGGAGGCCCGTTCATCCGAA

An-6-Shr with reverse primer (RP-1)

TTTCCGACCTTGTAGCCGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCAT
CCCCACCTTCCTCCGTTTGTACCCGCGAGTCTCCTTAGAGTGCCCCAGGAGGTGCTGG
TAACCTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCAACATCTCACGACACGAGC
TGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
AGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAACCACATG
CTCCACCGCTTGTGCGGGCCCCGTCATTCATTGAGTTTAAACCTTGCGGCCGTACTC
CCAGGCGGTGCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAACGGCT
AGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCT
TTCGACCTCAGTGTCAGTATCAGTCCAGGTGGTTCGCCTTCGCCACTGGTGTTCCTTCCT
ATATCTACACATTTACCGCTACACAGGAAATTCACCATTGTCTACGTTCTCTA

The isolate An-6-shr showed 98% homology with *Pseudomonas aeruginosa* NEB 98 with accession number (GU121495.1)

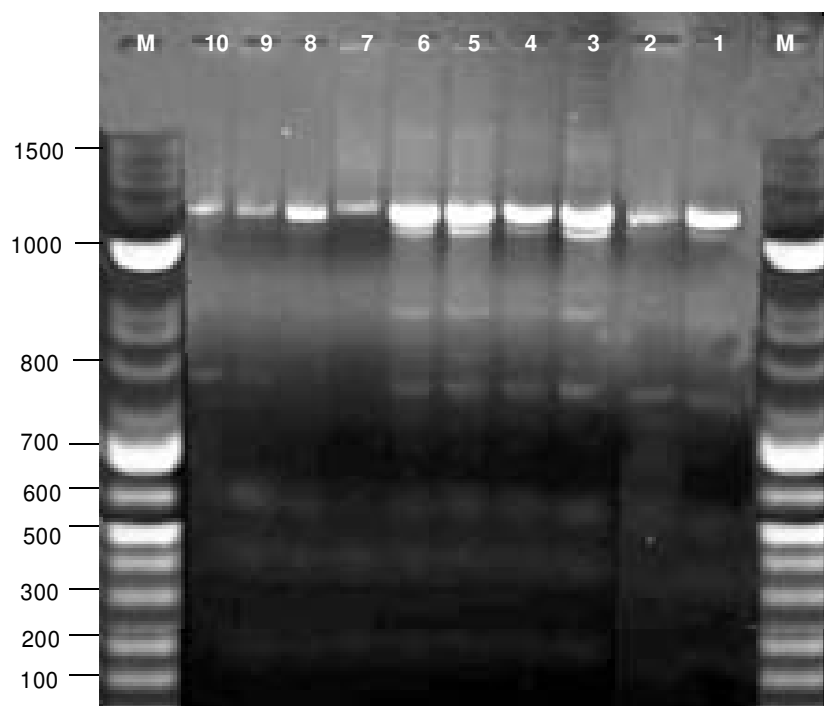


**Plate 5: 16s rRNA of *Pseudomonas* strains (1,4) An-6-Mag
(2,7) An-M-Mag (3) An-3-cha (4) An-5-Mag (5,6) An-6-shr
(8) Ar-Bag (9,10) An-1-Mag**

Fig 1 Phylogenetic tree view of the *Pseudomonas* sequences from GenBank in relation to isolates An-6-shr The unrooted phylogenetic tree is the result of UPGMA cluster analysis

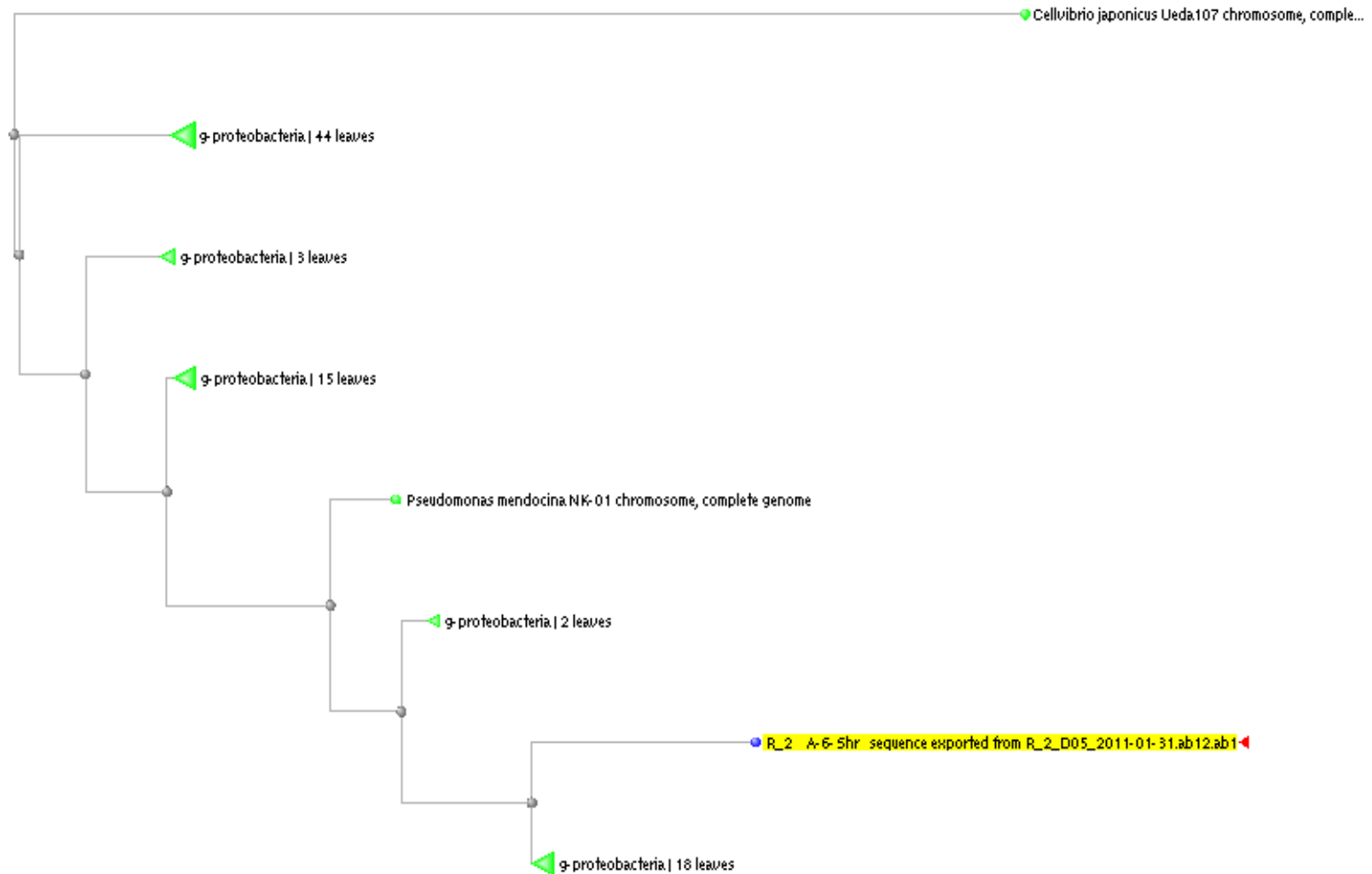
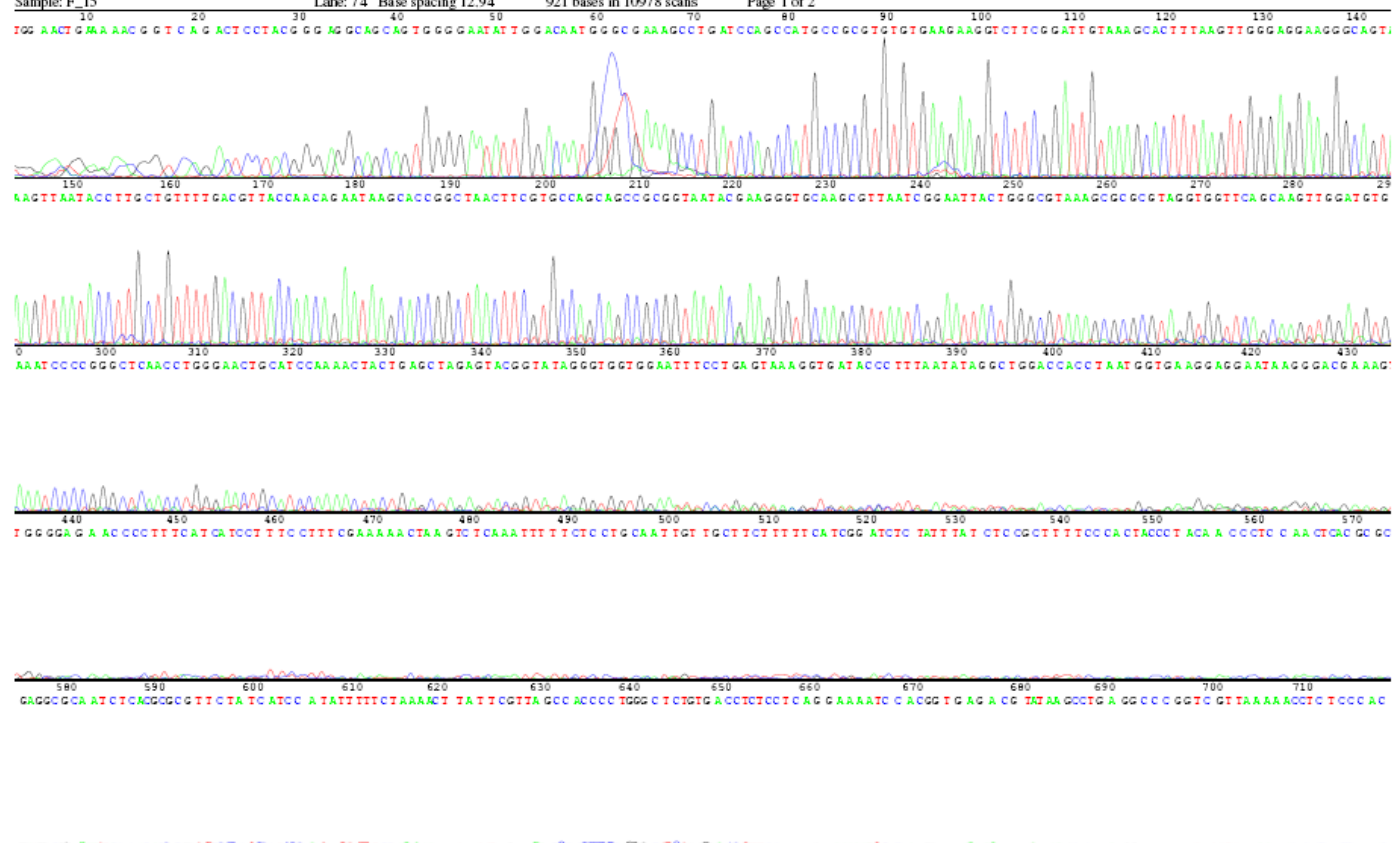


Fig 2 Sequence alignment of *Pseudomonas* isolate An-6-Shr using BLAST

Sequences producing significant alignments						
Accession	Description	<u>Max score</u>	<u>Total score</u>	<u>Query coverage</u>	<u>E value</u>	<u>Max ident</u>
HQ697283.1	<i>Pseudomonas aeruginosa</i> strain P21 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ393865.1	<i>Pseudomonas aeruginosa</i> strain PSBI3-1 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ393863.1	<i>Pseudomonas</i> sp. PSBORB-1 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ457019.1	<i>Pseudomonas aeruginosa</i> strain R2 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ457018.1	<i>Pseudomonas aeruginosa</i> strain DSE2 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ457014.1	<i>Pseudomonas aeruginosa</i> strain HSD1 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ270549.1	<i>Pseudomonas aeruginosa</i> strain GPSD-59 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ658761.1	<i>Pseudomonas aeruginosa</i> strain nsm 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ641264.1	<i>Pseudomonas</i> sp. E11(2010) 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ641262.1	<i>Pseudomonas</i> sp. E9(2010) 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ658764.1	<i>Pseudomonas aeruginosa</i> strain FB3 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ658763.1	<i>Pseudomonas aeruginosa</i> strain FB5 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%
HQ658762.1	<i>Pseudomonas aeruginosa</i> strain FB2 16S ribosomal RNA gene, partial sequence	1027	1027	99%	0.0	98%

Chromas sequence of *Pseudomonas* isolate An-6-Shr

File: F_15_D10_2011-02-09.ab1 F15(do).ab1 Run Ended: Feb 9, 2011, 12:26:23 Signal G:123 A:87 T:91 C:82
Sample: F_15 Lane: 74 Base spacing 12.94 921 bases in 10978 scans Page 1 of 2



AN-3-Cha with forward primjer (FP)

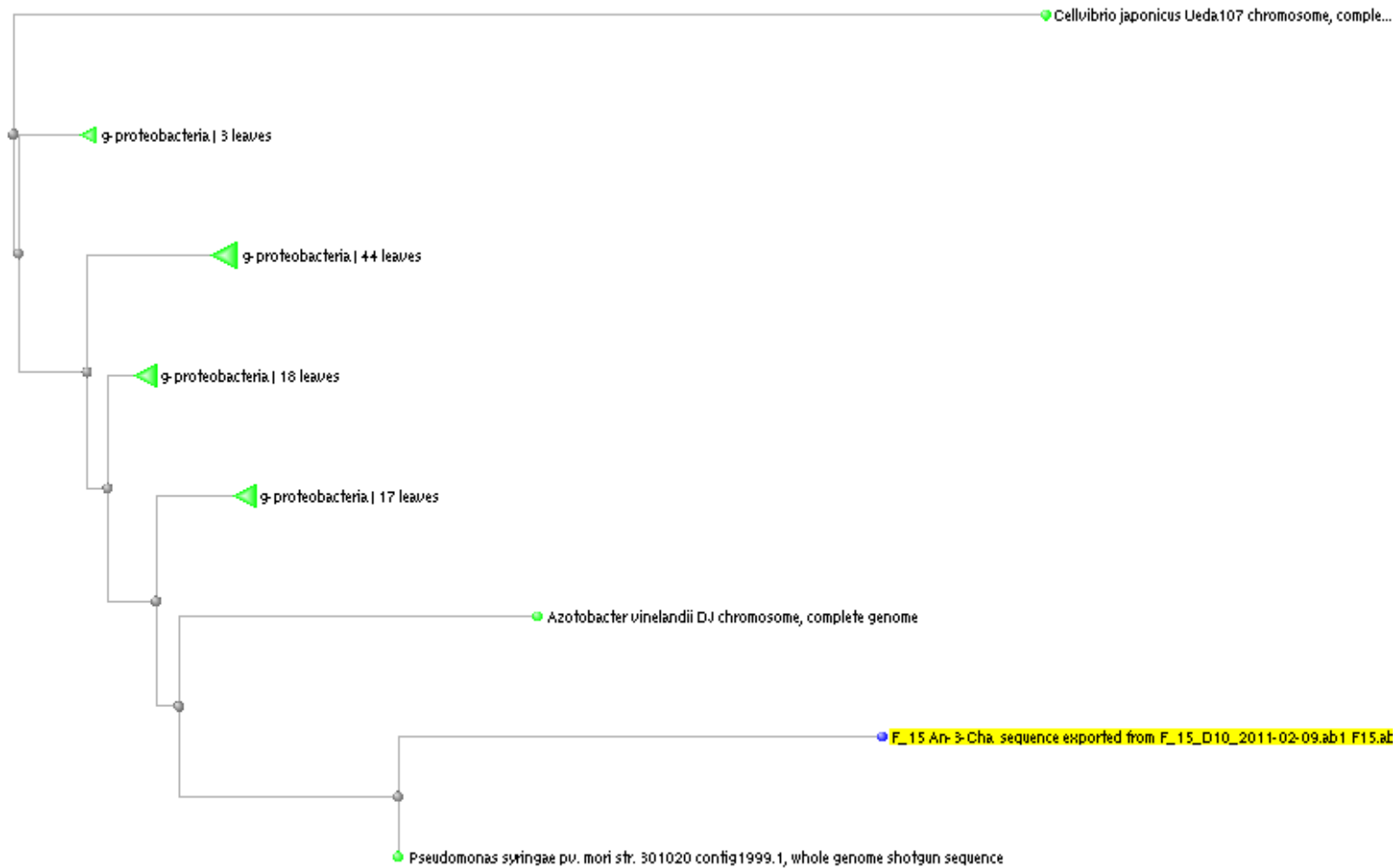
TGGAAC TGAAAAACGGTCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG
G
GCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACT
TTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAAT
AAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATC
G
GAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGG
G
CTCAACCTGGGAACTGCATCCAAACTACTGAGCTAGAGTACGGTATAGGGTGGTGGAAAT
TTCCTGAGTAAAGGTGATACCTTTAATATAGGCTGGACCACCTAATGGTGAAGGAGGAA
TAAGGGACGAAAGTGGGGAGAACCCCTTTCATCATCCTTTCCTTTCGAAAACTAAGTCT
CAAAATTTTCTCCTGCAATTGTTGCTTCTTTTTCATCGGATCTCTATTTATCTCCGCTTT
TCCCACTACCCTACAACCCTCCAACCTCACGCGCGAGGCGCAATCTCACGCGCGTTCTATC
ATCCATATTTTCTAAAACCTTATTCGTTAGCCACCCCTGGGCTCTGTGACCTCTCCTCAG
GAAAATCCACGGTGAGACGTATAAGCCTGAGGCCCGGTCGTAAAAACCTCTCCCACGAC
GCACCTTTTCCGCTCTCCATAGTCGGACTTTTCCCTTCAGAGGGGGAGAGGCCCAATTCC
CACCTCCAAAAAAGAAAGAGACCCCTCCAATTCTATTTTATAGGACAAACAAAAACCTT
CCTCCCACTTATTTCCCATATCCAACGCCTTATTCTAAAAAAAAGCGTTCCAGGGATGCG
CCTTCCTTATATTCCCGACGT

AN-3-Cha with reverse primer (RP)

TTTTTTGTCCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTACCCGGCAGTCTCCTTAGAGTGCCACCCGAG
GTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCAACATCTCACGA
CACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATC
TCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTA
CCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGC
CGTACTCCCCAGGCGGTCGACTTATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCC
AACGGCTAGTCGACATCGTTTACGGCGTGGAATACCAGGGTATCTAATCCTGTTTGCTCC
CCACGCTTTCGCACCTCAGTGTCACTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTT
CCTTCCTATATCTACGCATTTACCGCTACACAGGAAATCCACCACCCTCTACCGTACT
CTAGCTCAGTAGTTTTGGATGCAGTTCCAGGTTGAGCCCGGGGATTTACATCCAACCTT
GCTGAACCACCTACGCGCGCTTTTACGCCCAGTAATTCGATTAACCGCCTGCACCCTTC
GTATTACGCGGCTGCTGGCAACGAAAGTTAGCCGGCTGCTTTATTCTGTTGGTAACGTT
CAAAACAGCAGAGGTATTTATCTGTACTG

AN-3-Cha showed 98% homology with *Pseudomonas aeruginosa* strain NBAl AFP-5
16S ribosomal RNA gene, partial sequence acc no HM439971.1.

Fig 3 Phylogenetic tree view of the *Pseudomonas* sequences from GenBank in relation to isolates An-6-shr The unrooted phylogenetic tree is the result of UPGMA cluster analysis



Chromas sequence of *Pseudomonas* isolate An-3-Cha

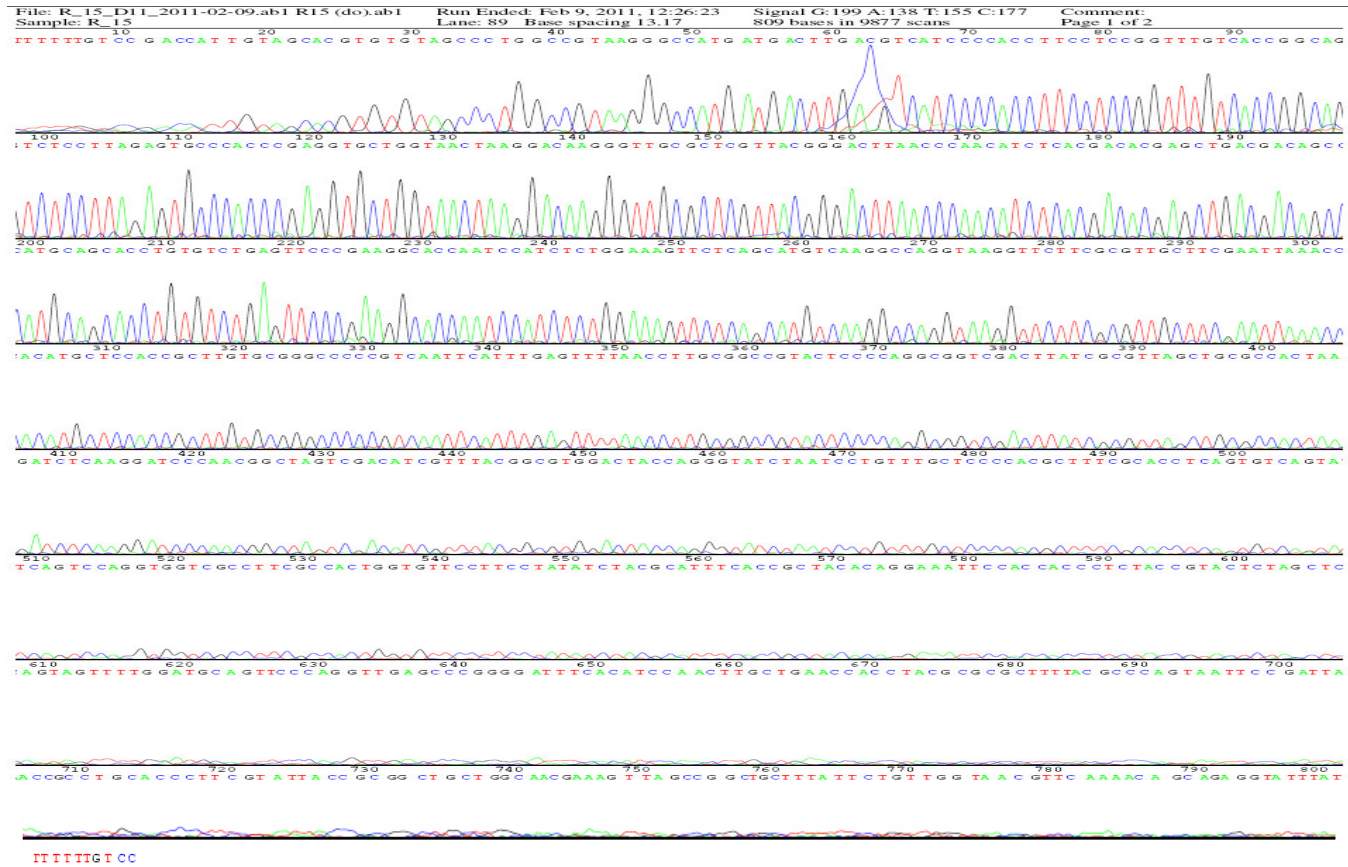


Fig 4 Sequence alignment of *Pseudomonas* isolate AN-3-Cha using Blast

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ143612.1	<i>Pseudomonas aeruginosa</i> strain RHH13 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%
HQ537785.1	<i>Pseudomonas aeruginosa</i> strain zxy0926 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%
HQ697286.1	<i>Pseudomonas aeruginosa</i> strain P43 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%
HQ697285.1	<i>Pseudomonas aeruginosa</i> strain P28 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%
HQ697284.1	<i>Pseudomonas aeruginosa</i> strain P22 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%
HQ697283.1	<i>Pseudomonas aeruginosa</i> strain P21 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%
HQ457018.1	<i>Pseudomonas aeruginosa</i> strain DSE2 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%
HQ457014.1	<i>Pseudomonas aeruginosa</i> strain HSD1 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%
HQ270549.1	<i>Pseudomonas aeruginosa</i> strain GPSD-59 16S ribosomal RNA gene, partial sequence	1408	1408	99%	0.0	98%

4.9 Standarization of cultural conditions for production of antifungal activity and mass multiplication of inoculums.

In all the five different media viz., *i.e.*, Nutrient Broth (NB), Yeast Malt (YM), Pigment production media(PPM), 1/5M,23 and King's B media (KMB) showed (Table 9) maximum growth of test bacteria and maximum production of antifungal activity after 72h of incubation at 28°C under shake conditions. The results revealed that the order of the efficiency for the growth on different media is of order:

YM (0.99 to 1.91 O.D) and 1/5M23 (0.89 to 1.01 O.D) > KMB (0.62 to 0.83 O.D)>NB (0.61 to 0.90 O.D)>PPM (0.49 to 1.01 O.D).

Maximum growth of all the strains of *Pseudomonas* sp. in terms of optical density at 540nm was observed in YM and 1/5M23 medium followed by 0.99 to 1.9 O.D and 1.01 to 1.63 O.D. respectively after 72 h of its incubation.

Table 9 Effect of time of incubation (48h and 72h) and different media on growth of *Pseudomonas* isolates of apple

<i>Pseudomonas</i> strains	Incubation time	NB	YMA	PPM	1/5M23	KMB
An-6-shr	48h	0.65	1.31	0.30	1.31	0.69
	72h	0.79	1.91	0.49	1.50	0.83
An-3cha	48h	0.79	0.99	0.39	1.13	0.56
	72h	0.85	1.39	0.50	1.42	0.70
Ar-Bag	48h	0.41	1.01	0.41	0.89	0.42
	72h	0.61	1.51	0.51	1.01	0.63
An-M-Mag	48h	0.81	1.29	0.49	1.14	0.59
	72h	0.99	1.61	0.61	1.63	0.80
An-1-Mag	48h	0.73	0.99	0.51	1.14	0.49
	72h	0.90	1.48	0.71	1.63	0.62
An-5-Mag	48h	0.76	1.14	0.19	1.11	0.56
	72h	0.89	1.31	1.01	1.41	0.78
An-6-Mag	48h	0.71	1.41	0.30	1.20	0.63
	72h	0.90	1.61	0.51	1.41	0.80

4.10 Effect of time of incubation (48h and 72h) and different media on production of antifungal activity against different fungal pathogens at 28°C under shake conditions (100rpm).

4.10.1 Against *Dematophora* sp.

An-6-shr and An-3-cha showed the maximum production of antifungal activity against *Dematophora* sp. after 72h of incubation. The production of antifungal activity take place in all the five media i.e. nutrient broth (NB), yeast malt (YM), Pigment production medium (PPM), 1/5M23 and King's B broth (KMB). Out of seven isolates An-6-shr showed highest production of antifungal activity after 72h in KMB and NB (both 42.4%I) followed by PPM and 1/5M23 (both 27.3 to 27.5%I) and YM (19.3%I). The production of antifungal activity after 48h was slightly less but the order of medium for high production of activity was almost same:

An-6-shr: NB (39.2%I)> KMB(19.4%I)> 1/5M23 (18.8%I)> YM(16.6%I).

Strain An-3-cha also showed highest production of inhibiting activity against *Dematophora* sp. after 72h of incubation and order of media for highest production of activity was as follows:

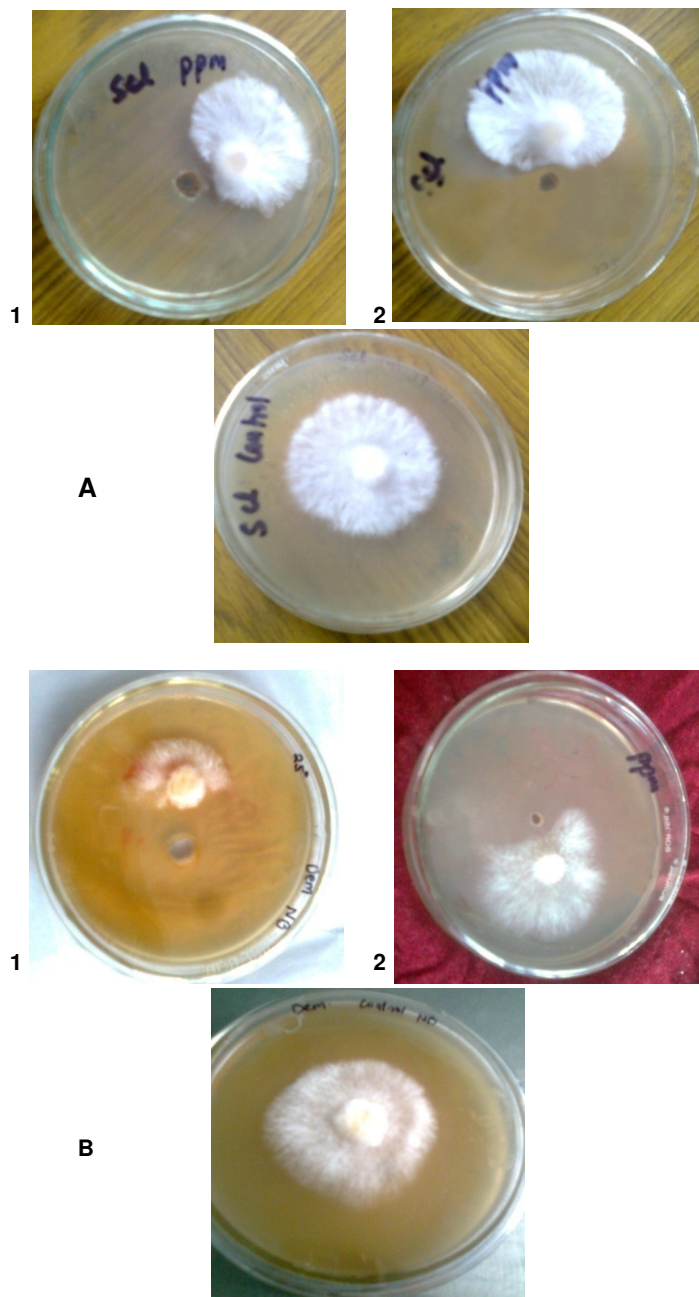


Plate 6 Antifungal activity of *Pseudomonas* strains (1) An-M-Mag (2) An-3-cha against
(A) *Sclerotium* sp (B) *Dematophora* sp. and control

Table 10. Effect of time of incubation (48h and 72h) and different media on production of antifungal activity against different fungal pathogens at 28°C under shake conditions (100rpm).

Table 10 Against *Dematophora* sp.

Dematophora sp. (mm dia. & % inhibition of growth of mycelia)																						
Pseudomonas Strains	Antifungal activity (48h.)											Antifungal activity 72h.										
Medium	NB		YMA		PPM		1/5M23		KMB		Mean	NB		YMA		PPM		1/5M23		KMB		Mean
	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I		mm	%I	mm	%I	mm	%I	mm	%I	mm	%I	
Control	28	0.0	30	0.0	36	0.0	32	0.0	32	0.0		33	0.0	32	0.0	33	0.0	40	0.0	34	0.0	
An-6-shr	17	39.29 (38.80)	25	16.6 (24.01)	29	19.4 (26.12)	26	18.8 (25.62)	21	34.3 (35.87)	25.69 (30.08)	19	42.42 (40.83)	29	19.3 (12.65)	24	27.3 (31.47)	29	27.5 (31.61)	19	42.4 (41.29)	30.08 (32.57)
An-3-cha	17	39.29 (38.80)	15	37.5 (37.64)	21	41.7 (40.19)	17	46.8 (43.19)	16	43.3 (40.32)	45.55 (42.44)	20	39.4 (38.87)	20	50.0 (45)	19	42.4 (40.64)	21	47.5 (43.57)	20	50.0 (45)	41.70 (40.21)
Ar-Bag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0
An-M-Mag	-	-	-	-	25	15.15 (22.84)	-	-	24	25.0 (29.97)	11.11 (12.70)	-	-	-	-	28	30.5 (33.53)	-	-	19	44.11 (42.61)	11.85 (12.89)
An-1-Mag	-	-	24	12.5 (20.60)	28	22.2 (28.09)	-	-	-	-	8.44 (10.92)	-	-	28	20.0 (20.6)	26	23.2 (23.52)	-	-	-	-	2.499 (4.121)
An-5-Mag	15	36.42 (42.95)	-	-	-	-	-	-	17	45.8 (43.19)	18.65 (17.23)	21	46.4 (44.5)	-	-	-	-	-	-	18	46.4 (43.42)	16.69 (16.08)
An-6-Mag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	29	12.1 (20.29)	-	-	-	-	2.427 (4.057)
Mean		16.96 (17.22)		12.38 (13.65)		13.26 (18.28)		9.373 (9.830)		22.31 (22.00)			17.93 (16.68)		8.451 (10.84)		16.85 (16.46)		10.71 (10.74)		25.23 (23.79)	

CD_{.05}

T
I
T*I

0.995
0.841
2.22

1.01
0.856
2.26

An-3-cha: KMB and YM (50.0%I) > 1/5M23 (47.5%I) > PPM(42.4%I) > NB (39.4%I).

Another three strains i.e. An-M-Mag, An-1-Mag and An-5-Mag also showed maximum production of antifungal activity after 72h but in two media only. An-M-Mag showed production in PPM (23.2%I) and YM (20.0%) while An-5-Mag showed production NB and KMB (both 46.4%I). One isolate i.e. An-6-Mag showed production of inhibiting activity in PPM (12.1%) only after 72h of incubation and did not showed production after 48h.

4.10.2 Against *Fusarium* sp. :

In all the media maximum antifungal activity was produced by all the *Pseudomonas* strains after 72h of incubation as compared to 48h of incubation at 28°C. Out of seven, two strains i.e. An-6-shr and An-3-cha produced antifungal activity in all the five media in the range of 20.5 to 42.1%I and 24.3 to 48.7%I respectively. The results showed that after 72h of incubation as compared to 48h the efficiency of a medium for supporting the production of antifungal activity against *Fusarium* sp. in the following order in case of

An-6-shr: KMB and 1/5M23 (41.4 to 42.1%I) > yeast malt (28.6%I) > NB(24.3%I) > PPM (20.5%I).

While in case of An-3-cha, the trend was slightly different. It produced maximum antifungal activity in the following order:

An-3-cha : KMB (48.7%I) > 1/5M23 (44.7%I) > YMA (38.1%I) > PPM (33.6%I) > NB (24.3%I).

Another two strains Ar-Bag and An-M-Mag showed production of antifungal activity in three media only in the order i.e.by Ar-Bag KMB and NB (both 31.7%I) > yeast malt (23.1%I) and by An-M-Mag; KMB (48.7%I) > NB (26.8%I) > yeast malt (21.4%I). Though the order of media for supporting the production of activity was same but the activity in terms of inhibition of mycelia growth varies among both strains. Another two organism i.e. An-1-Mag and An-

Table 11 Against *Fusarium* sp.

	Antifungal activity against <i>Fusarium</i> sp. (mm dia. & % inhibition of growth of mycelia)																						
<i>Pseudomonas</i> Strains	Antifungal activity (48h)											Antifungal activity (72h.)											
Medium	NB		YMA		PPM		1/5M23		KMB		Mean	NB		YMA		PPM		1/5M23		KMB		Mean	
	mm	%I	mm	%I	mm	%I	mm dia	%I	mm	%I		mm	%I	mm	%I	mm	%I	mm	%I	mm	%I		
<i>control</i>	35	0.0	35	0.0	39	0.0	30	0.0	38	0.0	29.55 (32.68)	41	0.0 (29.55)	42	0.0 (32.31)	44	0.0 (26.88)	23	0.0 (40.45)	41	0.0 (40.07)	31.39 (33.85)	
An-6-shr	22	22.1 (40.45)	27	22.9 (28.54)	32	17.9 (25.02)	20	33.3 (35.24)	26	31.5 (34.16)	29.55 (32.68)	31	24.3 (29.55)	30	28.6 (32.31)	35	20.5 (26.88)	22	42.1 (40.45)	24	41.4 (40.07)	31.39 (33.85)	
An-3-cha	25	22.2 (35.78)	23	34.3 (35.83)	26	33.3 (35.25)	18	40 (39.22)	23	39.5 (38.90)	36.25 (37.00)	31	24.3 (29.55)	26	38.1 (38.11)	38	33.6 (21.61)	21	44.7 (41.97)	21	48.7 (44.28)	33.91 (35.11)	
Ar-Bag	27	28.9 (32.48)	27	22.9 (28.54)	-	-	-	-	27	28.9 (32.52)	16.15 (18.71)	28	31.7 (34.26)	32	23.1 (30.61)	-	-	-	-	28	31.7 (34.26)	17.87 (19.82)	
An-M-Mag	29	23.7 (29.10)	30	14.3 (28.64)	-	-	-	-	25	34.2 (35.78)	16.34 (18.70)	30	26.8 (31.17)	33	21.4 (27.54)	-	-	-	-	21	48.7 (44.27)	19.39 (20.60)	
An-1-Mag	--	-	-	-	-	-	-	-	27	28.9 (32.52)	5.785 (6.504)	-	-	-	-	-	-	-	-	25	39.01 (38.64)	7.801 (7.772)	
An-5-Mag	-	-	25	28.6 (32.30)	-	-	-	-	-	-	5.715 (6.460)	-	-	29	30.9 (33.76)	-	-	-	-	-	-	6.180 (6.752)	
An-6-Mag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0	
Mean		18.42 (19.69)		18.92 (21.98)		7.321 (8.609)		10.47 (10.64)		23.29 (24.84)			15.32 (17.79)		20.71 (23.19)		4.870 (6.927)		12.40 (11.77)		29.94 (28.79)		

CD₀₅

T

I

T*I

1.48

1.25

3.26

0.821

0.694

1.95

5-Mag showed production of activity in one medium only i.e. in KMB 39.01%I and in yeast malt 30.9%I respectively. Overall maximum *Pseudomonas* isolates showed production of activity in two media against *Fusarium* sp. i.e. King's B media and yeast malt after 72h of incubation period at 28°C.

4.10.3 Against *Phytophthora* sp. :

Results in Table 12 showed that the maximum isolates produced maximum antifungal activity against *Phytophthora* sp. after 72h of incubation as compared to 48h of incubation. An-6-shr and An-3-cha produced the antifungal activity in all the media in the range of 16.6 to 50.0%I. The results showed that the efficiency of a medium for supporting the production of antifungal activity against *Phytophthora* sp. by An-6-shr after 72h was of the following order:

KMB and 1/5M23 (34.2%I) > NB (18.8%I) > YM(16.6%I) > PPM (15.4%I) while in case of An-3-cha is: KMB (50.0%I) > YM (38.9%I) > NB(37.5%I) > 1/5M23 (33.4%I).

An-3-cha : KMB (50.0%I) > YM (38.9%I) > NB (37.5%I) > 1/5M23(33.4%I)

It did not show the production in PPM medium. Two strains Ar-Bag and An-M-Mag did not show any antifungal activity against *Phytophthora* sp. in any of the media studied. An-1-Mag showed the production of activity in the two media only i.e. in the efficiency order for supported the production of the strain was 1/5M23 (30.9%) > YM (28.1%). Another strains An-5-Mag and An-6-Mag showed the production of activity in one medium only i.e. 1/5M23 in the range of 28.5 to 28.6%I. Over all maximum isolates showed production of antifungal activity against *Phytophthora* sp. i.e. five has supported in 1/5M23 medium in the range of 28.5 to 35.7%I.

4.10.4 Against *Pythium* sp.

The effect of media on the production of antifungal activity against *Pythium* sp. produced by fluorescent *Pseudomonas* sp. was maximum after 72h of incubation as compared to 48h. Two strains An-6-shr and An-3-cha produced antifungal activity in all the media in the range of 22.2 to 50.0%I (An-6-shr) and

Table 12 Against *Phytophthora* sp.

<i>Pseudomonas</i> Strains	Antifungal activity against <i>Phytophthora</i> sp. (mm dia. & % inhibition of growth of mycelia)																					
	Antifungal activity (48h)											Antifungal activity (72h.)										
	NB		YMA		PPM		1/5M23		KMB		Mean	NB		YMA		PPM		1/5M23		KMB		Mean
Medium	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I		mm	%I	mm	%I	mm	%I	mm	%I	mm	%I	
control	28	0.0	30	0.0	36	0.0	29	0.0	28	0.0		32	0.0	36	0.0	39	0.0	42	0.0	32	0.0	
An-6-shr	24	14.2 (22.07)	25	15.7 (24.03)	32	11.1 (19.37)	23	20.6 (26.98)	19	17.4 (31.22)	17.98 (19.23)	26	18.8 (25.62)	27	16.65 (23.22)	33	15.4 (23.05)	27	35.7 (36.68)	21	34.27 (35.88)	23.97 (28.89)
An-3-cha	19	32.14 (34.52)	21	30 (33.19)	0	0	21	27.6 (31.66)	18	21.7 (33.70)	24.15 (26.61)	20	37.5 (37.75)	22	38.9 (35.50)	-	-	28	33.4 (35.27)	16	50 (45.04)	30.94 (30.71)
Ar-Bag		-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0
An-M-Mag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0
An-1-Mag	-	-	25	16.6 (24)	-	-	23	20.6 (26.98)	-	-	7.458 (10.20)	-	-	23	28.12 (32.01)	-	-	29	30.9 (33.78)	-	-	11.81 (13.16)
An-5-Mag	-	-	-	-	-	-	25	13.3 (21.69)	-	-	2.756 (4.337)	-	-	-	-	-	-	30	28.5 (32.29)	-	-	5.710 (6.457)
An-6-Mag	-	-	-	-	-	-	24	17.2 (24.50)	-	-	3.440 (4.901)	-	-	-	-	-	-	30	28.6 (32.30)	-	-	5.713 (6.459)
Mean		6.627 (8.084)		9.044 (11.60)		1.586 (2.767)		14.27 (18.83)		8.324 (9.275)			8.037 (9.053)		11.08 (12.96)		2.198 (3.293)		22.44 (24.33)		12.06 (11.56)	

CD₀₅

T

I

T*I

1.41

1.19

2.42

0.884

0.747

1.97

20.0 to 45.6%I (An-3-cha). The results showed that the efficiency of a medium for supporting the production of antifungal activity against *Pythium* sp. was of the following order:

An-6-shr :KMB (50.00%I) > 1/5M23 (36.1%I)> YM and NB (28.2%I) > PPM (22.2%I)

An-6-cha : KMB (45.6%I) > 1/5M (25.00%I) >NB (23.9%I) > YM and PPM (both 20-21%I).

Three strains i.e. Ar-Bag, An-5-Mag and An-1-Mag showed production of inhibiting activity against *Pythium* sp. in one medium only. Another one strain i.e. An-M-Mag showed production in four media in the following order : KMB (48.6%I)> NB (32.6%I)> 1/5M23 (27.8%I)> YM (18.8%I). While after 48h it showed production in two media only. Overall production of antifungal activity by maximum strains i.e. four was observed in YM media.

4.10.5 Against *Sclerotium* sp. :

Production of antifungal activity against *Sclerotium* sp. in all the media studied was maximum after 72h of incubation at 28°C as compared to production after 48h. The effect of media on the production of antifungal activity produced by *Pseudomonas* sp. revealed two strains that were also used in field experiment i.e. An-6-shr and An-3-cha showed the production of antifungal activity in all the media i.e. in the range of 25.7 to 50.0% I and 26.3 to 47.1%I respectively. Results showed that the efficiency of a medium for supporting the production of antifungal activity against *Sclerotium* sp. was of the following order i.e.

An-6-shr: NB (50.0%I)> YM (36.8%I) > PPM (35.6%I)>KMB(34.6%I)> 1/5M23 (25.7%I)

An-3-cha: NB and 1/5M23(both 47.1 to 46.4%I)>PPM(40.0%I)> KMB(38.7%I)>YM(26.3%I).

Another strain An-M-Mag produced activity in four media in the order YM (36.8%I)>1/5M23 (37.2%I)> KMB (33.3%I)> PPM (13.3%I) and An-1-Mag showed production of antifungal activity in three media while other strain showed production in one or two media.

Table 13 Against *Pythium* sp.

	Against <i>Pythium</i> sp. (mm dia. & % inhibition of growth of mycelia)																					
<i>Pseudomonas</i> Strains	Antifungal activity (48h)											Antifungal activity (72h)										
	NB		YMA		PPM		1/5M23		KMB			NB		YMA		PPM		1/5M23		KMB		
	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I		mm	%I	mm	%I	mm	%I	mm	%I	mm	%I	
Control	42	0.0	30	0.0	29	0.0	30	0.0	42	0.0		45	0.0	32	0.0	45	0.0	36	0.0	46	0.0	
An-6-shr	32	23.8 (29.18)	19	26.9 (31.21)	23	20.7 (27.01)	20	33.3 (35.24)	28	33.3 (35.25)	27.61 (31.58)	33	28.2 (32.09)	23	28.1 (32.00)	35	22.2 (28.10)	23	36.1 (36.92)	23	50 (45.00)	32.93 (34.82)
An-3-cha	36	14.3 (22.16)	17	21.6 (36.02)	25	13.8 (15.70)	23	23.3 (28.48)	28	33.3 (35.25)	23.76 (28.72)	35	23.9 (29.25)	25	21.9 (27.85)	36	20 (26.52)	27	25 (29.96)	25	45.6 (42.49)	27.27 (31.22)
Ar-Bag	-	-	-	-	-	-	-	-	-	-	0	-	-	27	15.6 (22.70)	-	-	-	-	-	-	2.987 (4.539)
An-M-Mag	29	30.9 (33.78)	-	-	-	-	-	-	23	44.1 (42.04)	15.16 (15.18)	31	32.6 (34.81)	26	18.8 (25.62)	-	-	26	27.8 (31.79)	19	48.6 (49.98)	27.56 (28.44)
An-1-Mag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	39	13.3 (21.37)	-	-	-	-	2.667 (4.274)
An-5-Mag	-	-	21	19.2 (25.93)	-	-	-	-	-	-	3.843 (5.186)	-	-	24	25 (29.97)	-	-	-	-	-	-	5.00 (5.994)
An-6-Mag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0
Mean		9.859 (12.16)		11.53 (13.31)		4.92 (6.958)		8.01 (9.102)		15.93 (16.08)			12.11 (13.74)		15.53 (19.14)		7.930 (10.86)		12.69 (14.10)		22.04 (19.64)	

CD_{0.05}

T

I

T*I

0.918

0.776

1.26

0.867

0.773

1.94

Table 14 Against *Sclerotium* sp

	Antifungal activity against <i>Sclerotium</i> sp. (mm dia. & % inhibition of growth of mycelia)																					
<i>Pseudomonas</i> Strains	Antifungal activity (48h)											Antifungal activity (72h.)										
Medium	NB		YMA		PPM		1/5M23		KMB		Mean	NB		YMA		PPM		1/5M23		KMB		Mean
	mm	% I	mm	% I	mm	% I	mm dia	% I	mm	% I		mm	% I	mm	% I	mm	% I	mm	% I	mm	% I	
Control	32	0.0	32	0.0	37	0.0	35	0.0	33	0.0		34	0.0	38	0.0	45	0.0	43	0.0	26	0.0	
An-6-shr	16	50(45)	21	34.4 (35)	25	22.4 (34.69)	26	25.5 (30.44)	21	25 (27.20)	33.57 (35.24)	17	50 (45)	24	36.8 (37.36)	28	35.6 (23.20)	32	25.7 (30.35)	17	34.61 (36.02)	32.12 (34.38)
An-3-cha	17	26.8 (29.20)	24	25 (29.97)	29	21.6 (27.67)	16	44.2 (52.44)	19	30.1 (34.56)	35.98 (36.57)	18	47.1 (43.32)	28	26.3 (30.84)	27	40 (39.23)	23	46.4 (42.98)	18	30.76 (33.66)	38.12 (38.00)
Ar-Bag	-	-	-	-	-	-	29	17.1 (19.40)	-	-	3.425 (4.880)	-	-	25	34.2 (35.76)	-	-	32	25.6 (30.37)	-	-	11.95 (13.23)
An-M-Mag	-	-	28	12.5 (20.60)	29	21.6 (27.67)	27	22.8 (28.52)	-	-	11.39 (15.36)	-	-	24	36.8 (37.36)	35	22.2 (28.09)	33	23.2 (28.79)	-	-	16.45 (18.850)
An-1-Mag	-	-	25	21.9 (27.85)	31	16.21 (20.70)	19	45.7 (42.54)	22	31.25 (33.57)	23.01 (25.61)	-	-	23	39.5 (38.92)	39	13.3 (21.37)	27	37.2 (37.57)	24	33.3 (35.23)	24.66 (26.62)
An-5-Mag	-	-	-	-	-	-	26	25.7 (30.44)	21	33.3 (35.67)	11.94 (13.22)	-	-	-	-	-	-	32	25.6 (30.38)	27	15.6 (23.21)	8.243 (10.72)
An-6-Mag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0
Mean		13.84 (12.60)		13.39 (16.33)		13.12 (16.25)		27.33 (29.11)		17.54 (19.20)			13.87 (12.62)		24.80 (25.75)		13.01 (15.98)		26.23 (28.63)		16.33 (18.30)	

CD_{.05}

T

I

T*I

1.074

0.907

2.4

0.973

0.822

2.17

From all the results it is concluded that the maximum production of antifungal activity against any of the indicator fungal pathogen was after 72h of incubation under shake conditions in any of the media. The extent of production of antifungal activity by test isolates depends upon the individual production potential of strain. Also the choice of media which supported maximum production of activity also depends upon the internal potential of the strain.

4.11 Effect of temperature of incubation (25°C and 37°C) and different media on growth of *Pseudomonas* isolates of apple in different media.

All seven isolates were grown in five different media viz. NB, YM, PPM, 1/5M23 and KMB and incubated at two times of incubation i.e. 25°C & 37 °C. The order of maximum growth in all the media was more at 25°C as compared to 37°C. The order of medium that induce the maximum growth at 25°C was in the following order i.e. YM (0.42 to 1.4 O.D) and 1/5M23 (0.91 to 1.2 6O.D)>KMB (0.51 to 0.85 O.D)>NB (0.43 to 0.85 O.D)>PPM (0.30 to 0.81O.D).

<i>Pseudomonas</i> strains	Temperature (°C)	NB	YM	PPM	1/5M23	KMB
An-6-shr	25	0.75	1.24	0.38	1.26	0.85
	37	0.60	0.99	0.29	1.01	0.99
An-3cha	25	0.85	1.04	0.40	1.15	0.73
	37	0.62	1.01	0.39	1.00	0.85
Ar-Bag	25	0.43	0.42	0.49	0.91	0.51
	37	0.24	0.41	0.41	0.89	0.51
An-M-Mag	25	0.85	1.27	0.51	1.19	0.77
	37	0.04	0.81	0.49	0.99	0.45
An-1-Mag	25	0.77	1.08	0.59	1.19	0.77
	37	0.12	0.19	0.51	0.99	0.45
An-5-Mag	25	0.77	1.11	0.81	1.01	0.81
	37	0.04	0.99	0.89	0.86	0.61
An-6-Mag	25	0.73	1.42	0.30	1.21	0.61
	37	0.04	0.99	0.25	0.91	0.41

4.12 Effect of temperature of incubation (25°C & 37°C) and different media on the production of antifungal activity against different fungal pathogens after 72h under shake conditions(100rpm).

4.12.1 Against *Dematophora* sp.

An-6-shr showed maximum production of antifungal activity in all media in the range of 22.2 to 42.8%I at 25°C and 12.9 to 40.0 %I at 37°C i.e. it showed more production at 25°C as compared to 37°C after 72h of incubation. The order of efficiency of media to support the production of antifungal activity against *Dematophora* sp. by An-6-shr was in the following order :

An-6-shr at 25°C i.e. NB (42.8%I)> 1/5M23 (26.3%I)>KMB (25.9%I) > PPM (24.3%I)>YM (22.2%I)

An-6-shr at 37°C it was NB (40.0%I)>KMB (37.8%I)>PPM (21.8%I)>YM (18.7%I)>1/5M23(12.9).

From this efficiency order it can be observed that the production was more at 25°C as compared to production at 37°C. Similarly in case of An-3-cha the efficiency of media to support production of antifungal metabolite was in following order :

An-3-cha at 25°C 1/5M23 (52.6%I)>YM (50.2%I)> PPM (41.6%I)>NB (35.7%I)> KMB (22.2 %I)

An-3-cha at 37°C 1/5M23 (48.3%I)>YM (47.0%I)> NB (33.3%I)> PPM and KMB (31.2 and 31.4%I).

In this case also production potential of organism was also more at 25°C as compared to 37°C. Two *Pseudomonas* isolates Ar-Bag and An-M-Mag did not show activity against *Demarophora* sp. at either of the temperature i.e. 25 or 37°C. Two other strains i.e. An-1-Mag and An-5-Mag showed production of antifungal activity in two different media i.e. YM and PPM and NB and KMB respectively. But again the production was more at 25°C as compared to 37°C Only one strain An-6-Mag showed production of antifungal activity against

Table 15 **Against *Dematophora* sp.**

Table 15 **Against *Dematophora* sp.**

Dematophora sp. at 25°C in PPM medium and didn't show any production at 37°C. It may be due to that the combination of temperature and some components of medium may have some inducing effect on the particular organism for production of inhibition activity at 25°C.

4.12.2 Against *Fusarium* sp.

An-6-shr and An-3-cha showed maximum production of antifungal activity against *Fusarium* sp. in the entire medium at 25°C and 37°C after 72h. An-6-shr showed production of antifungal activity at 25°C in the range of 20.0 to 41.6% and at 37°C in the range of 15.3 to 34.4% in all the five media. The efficiency of media to support the production in case of An-6-shr was in the following order:

An-6-shr 25°C : 1/5M23 (41.6%I)> YM (28.8%I)>KMB (26.2%I)>NB (22.5%I)> PPM (20.0%I)

An-6-shr 37°C: 1/5M23 (34.4%I)> YM (21.8%I)> KMB(19.4%I)> NB (17.6%I)>PPM (15.3%I).

This efficiency order clearly indicate the production of antifungal activity was more at 25°C as compared to 37°C. While in case of An-3-cha media order was to support production was in the order following:

An-3-cha at 25°C: 1/5M23 and KMB (47.2 to 47.6%I)> YM (42.1%I)> NB (27.5%I)> PPM (20.0%I)

An-3-cha at 37°C: 1/5M23 (41.3%I)> YM (34.3%I)> KMB (27.7%I)> PPM (10.2%I)> NB (8.82%I).

An-M-Mag showed the production of antifungal activity in three media at 25°C i.e. in NB, YM,PPM and KMB in the range of 23.6 to 33.3%I while the same strain showed production in three media at 37°C in the range of 16.7 to 26.4%I. But YM medium did not support the production of activity against *Fusarium* sp. at 37°C .

Table 16 **Against *Fusarium* sp.**

Antifungal activity against <i>Fusarium</i> sp. (mm dia. & % inhibition of growth of mycelia)																						
Pseudomonas Strains		Antifungal activity (25°C)										Antifungal activity (37°C)										
Medium	NB		YM		PPM		1/5M23		KMB		Mean	NB		YM		PPM		1/5M23		KMB		MEAN
	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I		mm	%I	mm	%I	mm	%I	mm	%I	mm	%I	
control	40	0.0	38	0.0	45	0.0	36	0.0	42	0.0		32	0.0	32	0.0	39	0.0	29	0.0	36	0.0	
An-6-shr	31	22.5 (28.06)	27	28.8 (32.51)	36	20 (26.54)	21	41.6 (40.20)	31	26.2 (30.77)	27.79 (31.62)	28	17.64 (24.77)	25	21.8 (27.84)	33	15.3 (23.01)	19	34.4 (35.92)	29	19.4 (26.12)	21.74 (27.53)
An-3-cha	29	27.5 (31.61)	22	42.1 (40.43)	36	20 (26.54)	19	47.2 (43.39)	22	47.6 (43.62)	36.87 (37.12)	31	8.82 (17.07)	21	34 (35.800)	35	10.2 (18.53)	17	41.3 (40.000)	26	27.7 (31.76)	24.45 (28.63)
Ar-Bag	26	35 (36.24)	26	31.5 (34.16)	-	-	-	-	-	-	13.31 (14.08)	-	-	-	-	-	-	-	-	-	-	0
An-M-Mag	27	32.5 (34.75)	29	23.6 (29.08)	30	33.3 (35.24)	-	-	28	33.3 (35.24)	24.55 (26.86)	25	26.4 (30.92)	-	-	32	17.9 (25.020)	-	-	30	16.7 (24.02)	12.20 (15.99)
An-1-Mag	-	-	-	-	30	33.3 (35.24)	-	-	28	33.3 (34.15)	13.32 (14.09)	-	-	-	-	32	17.9 (24.99)	-	-	30	16.7 (24.05)	6.913 (9.808)
An-5-Mag	-	-	26	31.5 (34.05)	-	-	-	-	-	-	6.307 (6.831)	-	-	-	-	-	-	-	-	-	-	0
An-6-Mag	-	-	-	-	-	-	28	25.6 (29.15)	-	-	0	-	-	-	-	-	-	-	-	-	-	0
Mean		16.74 (18.67)		22.53 (24.33)		15.23 (17.65)		12.70 (11.94)		20.06 (20.70)			7.546 (10.39)		8.014 (9.091)		8.767 (13.08)		10.83 (10.85)		11.50 (15.14)	
CD _{.05} T											0.907											1.01
I											0.766											0.859
T*I											2.02											2.27

4.12.3 Against *Phytophthora* sp.

An-6-shr showed maximum production in all media in the range of 23.6 to 39.5%I at 25°C and 13.8 to 30.21 %I at 37°C i.e. it showed more production at 25°C as compared to 37°C after 72h of incubation. The order of efficiency to support the production of antifungal activity against *Phytophthora* sp. was in the following order :

An-6-shr at 25°C : 1/5M23(39.5%I)> PPM(37.8%I)> NB(32.23%I)>YM (26.31%I)> KMB(23.6)

An-6-shr at 37°C : NB (30.21%I)> YM(25.8%I)> 1/5M23 (17.2%I)> KMB(13.8%I)

PPM did not support for the activity against *Phytophthora* sp. From this efficiency order it can be observed that the production was more at 25°C as compared to production at 37°C. Similarly in case of An-3-cha the efficiency of media to support the production of metabolite was in following order at

An-3-cha at 25°C: YM(48.7%I)> 1/5M23 (41.8%I)> NB(39.28)> PPM (37.7%I)> KMB (26.3%I)

An-3-cha at 37°C : YM(41.9%I)> PPM (35.9%I)> NB (32.6%I)> 1/5M23 (24.1%I)>KMB (13.8%I).

In this case as production potential of organism was also more at 25°C as compared to 37°C. Ar-Bag did not show activity against *Phytophthora* sp. at either of the temperature i.e. 25°C or 37°C. Two other strains i.e. An-M-Mag and An-6-Mag showed production in one media only i.e. PPM and 1/5M23 respectively. But again the production was more at 25°C as compared to 37°C. One strain An-1-Mag showed production of antifungal activity against *Phytophthora* sp. at 25°C in three medium whereas the same strain did not support for the production of antifungal activity in YM media at 37°C.

Table 17 **Against *Phytophthora* sp**

Antifungal activity against <i>Phytophthora</i> sp. (mm dia. & % inhibition of growth of mycelia)																							
<i>Pseudomonas</i> Strains	Antifungal activity (25°C)										Antifungal activity (37°C)												
Medium	NB		YMA		PPM	1/5M23		KMB		Mean	NB			YMA		PPM		1/5M23		KMB		Mean	
	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I		mm	%I	mm	%I	mm	%I	mm	%I	mm	%I		
control	43	0.0	38	0.0	45	0.0	43	0.0	38	0.0		38	0.0	32	0.0	39	0.0	29	0.0	36	0.0		
An-6-shr	30	32.23 (33.33)	28	26.31 (30.83)	28	37.8 (37.93)	26	39.5 (38.95)	29	23.6 (29.08)	31.50 (34.03)	25	30.21 (35.78)	23	25.8 (32.000)	-	0	24	17.2 (24.410)	31	13.8 (21.80)	18.67 (22.80)	
An-3-cha	17	39.28 (42.38)	20	48.7 (43.74)	28	37.7 (37.92)	25	41.8 (40.28)	28	26.3 (30.81)	39.81 (39.02)	28	32.6 (46.49)	18	41.9 (41.030)	25	35.9 (36.80)	22	24.1 (29.340)	31	13.8 (21.76)	33.91 (35.09)	
Ar-Bag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-		
An-M-Mag	-	-	-	-	24	46.6 (43.05)	-	-	-	-	9.32 (8,610)	-	-	-	-	21	46.1 (42.78)	-	-	-	-	9.227 (8.556)	
An-1-Mag	-	-	29	23.6 (29.09)	24	31.4 (34.07)	27	37.2 (37.58)	-	-	18.45 (20.15)	-	-	-	-	21	46.2 (42.80)	25	13.7 (21.67)	-	-	11.99 (12.90)	
An-5-Mag	23	46.51 (42.99)	-	-	-	-	29	32.6 (34.79)	23	29.5 (36.90)	23.04 (22.94)	25	34.21 (35.760)	-	-	-	-	26	13.3 (18.58)	25	35.9 (36.760)	16.07 (18.22)	
An-6-Mag	-	-	-	-	-	-	28	34.8 (36.19)	-	-	6.973 (7.237)	-	-	-	-	-	-	24	20 (26.53)	-	-	4.00	
Mean		17.44 (16.95)		13.97 (14.81)		21.94 (21.85)		26.56 (26.83)		12.29 (13.83)			17.28 (16.86)		10.18 (10.430)		18.31 (17.480)		12.19 (17.220)		9.077 (11.48)	5.305	
CD _{.05} T										0.9768										1.11			
I										0.825										0.943			
T*I										2.18										2.49			

4.12.4 Against *Pythium* sp.:

Effect of temperature of incubation on production of antifungal activity against *Pythium* sp. after 72h under shaking condition was more at 25⁰C as compared to 37⁰C . An-6-shr and An-3-cha showed production of antifungal activity against *Pythium* sp. in all the media 25⁰C & 37⁰C after 72h but it was more at 25⁰C. An-6-shr showed production at 25⁰C in the range of 15.8 to 42.4%I and at 37⁰C in the range of 24.4-35.7%I . In all the five media the efficiency of media to support the production was in the following order

An-6-shr at 25⁰C: 1/5 M 23(42.4%I)> NB(37.7%I)>YM (31.4%I) > PPM (28.6%I) > KMB (15.8%I).

An-6-shr at 37⁰C : 1/5 M 23(35.7%I)> NB(30.0%I) >YM (26.6%I)> PPM(25.0%I) >KMB(24.4%I).

While in case of An-3-cha media order to support production was in the following order :

An-3-cha at 25⁰C : NB(40%I)> YM (34.2%I)> 1/5 M 23(27.3%I) > & PPM(26.1%I) > KMB(18.4%I)

An-3-cha at 37⁰C : NB(37.5%I) > YM(33.3%I) >1/5 M 23(25%I) >KMB(17.1%I) >PPM(14.3%I) .

An-M –Mag showed production of antifungal activity in three media at 25⁰C i.e. in NB, YM and KMB in the range of 26.2-33.3%I while the same strain showed the production in two media only at 37⁰C i.e. NB and PPM in the range of 28.6-22.5 %I indicating that the strain did not support the antifungal activity in YM media . Another strain An-5-Mag showed the production of antifungal activity in YM media and at 25⁰C only while An-6-Mag did not show the production of antifungal activity in any of the media.

4.12.5 Against *Sclerotium* sp.

An-6-shr showed maximum production in all media in the range of 15.3 to 42.8%I at 25⁰C and 5.2 to 37.5 %I at 37⁰C i.e. it showed far more production

Table 18 Against *Pythium* sp.

Antifungal activity against <i>Pythium</i> sp. (mm dia. & % inhibition of growth of mycelia)																									
<i>Pseudomonas</i> Strains	Antifungal activity (25°C)											Antifungal activity (37°C)													
Medium	NB		YMA		PPM		1/5M23		KMB		Mean	NB		YMA		PPM		1/5M23		KMB		Mean			
	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I		mm	%I	mm	%I	mm	%I	mm	%I	mm	%I				
control	45	0.0	35	0.0	42	0.0	33	0.0	38	0.0		40	0.0	30	0.0	28	0.0	28	0.0	31	0.0				
An-6-shr	28	37.7 (37.92)	24	31.4 (34.09)	30	28.6 (32.30)	19	42.4 (40.62)	32	15.8 (23.38)	31.19 (33.66)	28	30 (33.24)	22	26.6 (31.040)	21	25 (29.94)	18	35.7 (36.70)	31	24.4 (29.56)	28.35 (32.10)			
An-3-cha	27	40 (39.21)	23	34.2 (35.80)	31	26.1 (30.75)	24	27.3 (31.46)	31	18.4 (25.38)	29.21 (32.52)	25	37.5 (37.76)	20	33.3 (35.25)	24	14.3 (22.09)	21	25 (31.26)	34	17.1 (24.37)	25.82 (30.140)			
Ar-Bag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0			
An-M-Mag	30	33.3 (35.26)	25	28.5 (32.27)	31	26.2 (30.75)	-	-	-	-	17.61 (19.66)	31	22.5 (28.29)	-	-	20	28.6 (32.28)	-	-	-	-	10.21 (12.12)			
An-1-Mag	-	-	-	-	31	26.2 (30.75)	-	-	-	-	5.233 (6.150)	-	-	-	-	20	28.6 (32.28)	-	-	-	-	5.713 (6.456)			
An-5-Mag	-	-	26	25 (30.28)	-	-	-	-	-	-	5.091 (6.056)		-	-	-	-	-	-	-	-	-	0			
An-6-Mag	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0			
Mean		15.87 (16.05)		17.09 (18.92)		15.29 (17.79)		9.952 (10.30)		4.888 (6.965)			12.87 (14.18)		8.567 (9.4700)		13.77 (16.66)		8.95 (9.707)		5.920(7.705)				
CD _{.05} T												0.802												0.898	
I												0.678												0.759	
T*I												1.79												2	

at 25°C as compared to 37°C after 72h of incubation. The order of efficiency to support the production of antifungal activity against *Sclerotium* sp. by An-6-shr was in the following order :

An-6-shr at 25°C : NB (42.8%I)> YM (36.8%I)> KMB (33.3%I) > 1/5M23 (26.2%I)> PPM (15.3%I).

An-6-shr at 37°C NB (37.5%I) > KMB (32.3%I)> 1/5M23(24.3%I)>YM (21.2%I)> PPM (15.3%I).

From this efficiency order it can be observed that the production was more at 25°C as compared to production at 37°C. Similarly in case of An-3-cha the efficiency of media to support production of metabolite was in following order :

An-3-cha at 25°C : YM (50 %I)> 1/5M23 (47.6%I)> KMB (36.7%I)> NB (15.7%I)>PPM (13.3%I)

An-3-cha at 37°C : 1/5M23 (46.6%I)>YM (36.3%I)> KMB (32.3%I)> NB (17.6%I) and PPM (7.8%I).

In this case also production potential of organism was also more at 25°C as compared to 37°C. Ar-Bag showed the activity against *Sclerotium* sp. in two media at 25°C and did not support for any media at 37°C. Two other strains i.e. An-M-Mag and An-I-Mag showed production of antifungal activity in four media at 25°C i.e. YM, PPM, 1/5M23 and KMB but again the production was more at 25°C as compared to 37°C. Another one strain An-6-Mag did not show the production of antifungal activity against *Sclerotium* sp. at both the temperatures in any of the media.

Table 19 **Against *Sclerotium* sp.**

Antifungal activity against <i>Sclerotium</i> sp. (mm dia. & % inhibition of growth of mycelia)																							
<i>Pseudomonas</i> Strains		Antifungal activity (25°C)										Antifungal activity (37°C)											
Medium	NB		YMA		PPM		1/5M23		KMB		Mean	NB		YMA		PPM		1/5M23		KMB		Mean	
	mm	% I	mm	%I	mm	% I	mm	% I	mm	%I		mm	% I	mm	%I	mm	% I	mm	% I	mm	% I		
control	28	0.0	38	0.0	45	0.0	42	0.0	30	0.0		24	0.0	33	0	38	0.0	37	0.0	31	0.0		
An-6-shr	16	42.8 (40.87)	24	36.8 (37.34)	38	15.3 (23.12)	31	26.2 (30.77)	20	33.3 (35.25)	30.93 (33.47)	15	37.5 (37.72)	26	21.2 (27.38)	36	5.2 (12.93)	28	24.3 (29.52)	21	32.3 (34.60)	24.10 (28.43)	
An-3-cha	32	15.7 (23.35)	19	50 (45.00)	39	13.3 (22.08)	22	47.6 (43.62)	19	36.7 (37.26)	32.86 (34.26)	28	17.6 (24.76)	21	36.3 (37.06)	35	7.8 (16.15)	19	46.6 (44.22)	21	32.3 (33.60)	28.54 (31.36)	
Ar-Bag	-	-	29	23.6 (29.08)	-	-	31	26.2 (30.77)	-	-	9.968 (11.97)	-	-	-	-	-	-	-	-	-	-	-	
An-M-Mag	-	-	26	31.5 (34.15)	35	22.2 (28.09)	32	23.8 (29.16)	21	30 (33.19)	21.50 (24.92)	-	-	-	-	-	-	29	21.6 (27.67)	24	22.6 (28.35)	8.84 (11.20)	
An-1-Mag	-	-	26	31.5 (34.15)	35	26.7 (29.09)	26	38.1 (38.09)	24	20 (26.52)	22.66 (25.57)	-	-	21	36.3 (37.06)	-	-	21	43.2 (41.09)	28	9.6 (17.94)	17.84 (19.22)	
An-5-Mag	-	-	-	-	-	-	30	28.6 (32.30)	-	-	5.713 (6.459)	-	-	-	-	-	-	28	24.3 (29.52)	-	-	4.861 (5.903)	
An-6-Mag	-	-	-	-	-	-	-	-	-	-	0(0)	-	-	-	-	-	-	-	-	-	-	0	
Mean		8.371 (9.175)		24.79 (25.67)		10.80 (14.63)		27.20 (29.24)		17.14 (18.89)			7.867 (8.926)		13.41 (14.40)		1.871 (4.1530)		23.15 (24.57)		13.83 (16.50)		
CD _{.05}	T											1.05											1.1
	I											0.888											0.93
	T*I											2.35											2.46

4.13 Thin layer chromatographic analysis and evaluation of plant growth promoting activities of extracted antifungal metabolites of *Pseudomonas* strains

Broad spectrum antifungal metabolites produced by test isolates of fluorescent *Pseudomonas* species i.e phenazine and pyocyanine were tried to be extracted by standard methods using solvent systems i.e isopropanol/ ammonia/ water (8:1:1). The extracts were evaluated for R_f value and color of spots on TLC plates. The extracted and air dried metabolites (expected pigment Phenazine or Pyocyanine) dissolved in minimum quantity of methanol. TLC pattern was observed for detection of yellow and light green spots after spray with DSA. The chromatographic properties of metabolites were compared with their standard literature value of the reference metabolites.

The results showed that (Table 13) the R_f value of the extracted metabolites from all the four isolates of *Pseudomonas* sp. ranged between 0.49-0.53 and the color of spots were blue under UV light and was light yellow and light green when observed after spray with DSA.

Table 20 Thin layer chromatographic analysis of extracted antifungal metabolites on from Isolates of *Pseudomonas* sp. and their evaluation of PGP activities

<i>Pseudomonas</i> isolates	Extract	R _f	Color of spot	Phosphate solubilising mm dia	Siderophore* mm dia	Proteolytic* mm dia	Chitinase mm dia
An-6-shr	Chloroform	.53	Light green	-	-	-	-
	Benzene	.50	Absorb UV	-	13	17	-
An-3-cha	Chloroform	.53	Light green	-	-	-	-
	Benzene	.50	Absorb UV	-	16	14	-
An-M-Mag	Benzene	.50	Absorb UV	-	12	13	-
An-1-Mag	Benzene	.49	Absorb UV	-	16	12	-
CD_{.05}	Solvent system isopropanol/ammonia/water (8:1:1)		Spraying reagent used was diazotised sulphanilic acid(DSA)		1.45	1.45	

- indicates negative

* PGP activities expressed in terms of mm. diameter of yellow , pinkish/orange and clear zone produced around the well (9mm) by 100µl of extract on PVK , Chrome azurol-S (CAS) and skim milk agar at 28° C after 48 h incubation.

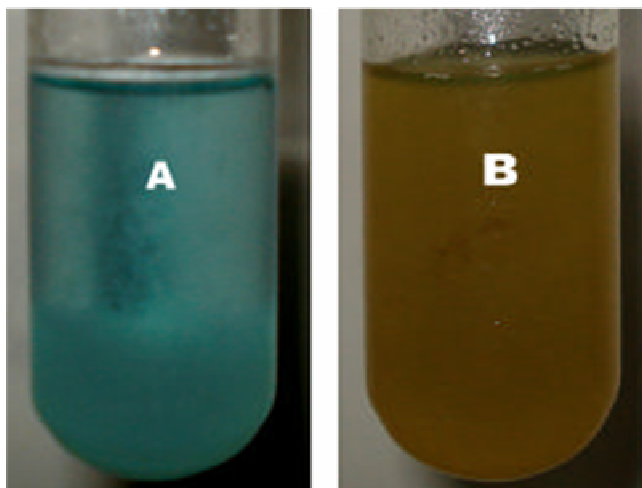


Plate 7: Color of pigment of *Pseudomonas* isolate An-6-shr after extraction (A) chloroform (B) Benzene

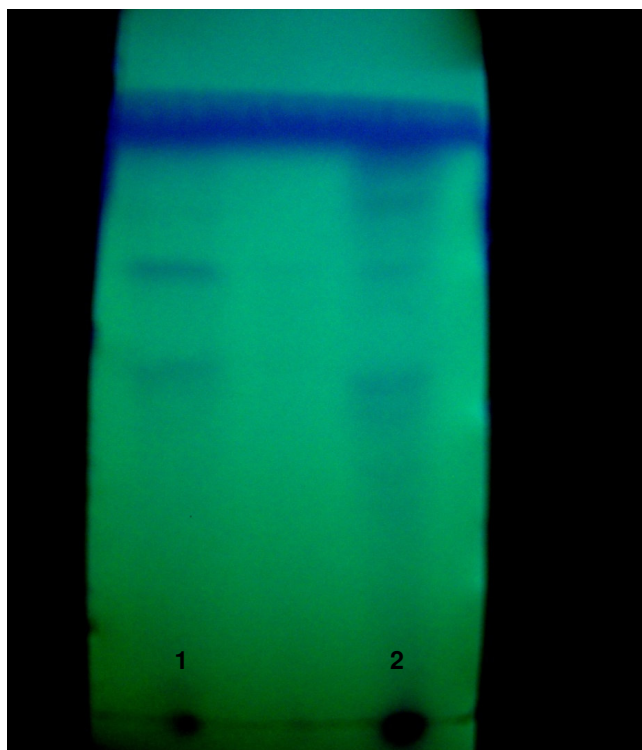


Plate 8: Thin Layer chromatographic analysis showing blue color spots of benzene extract from (1) An-6-shr and (2)An-M-Mag

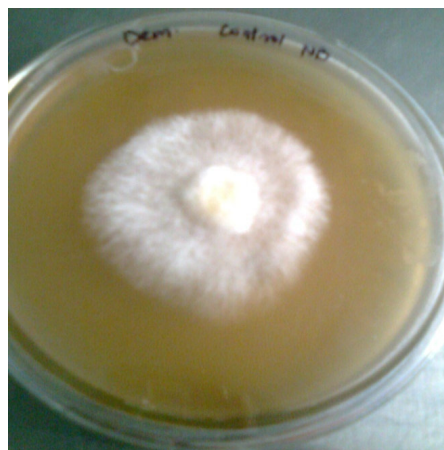


Plate 9: Antifungal activity of *Pseudomonas* strains against *Dematophora* from benzene extract with control

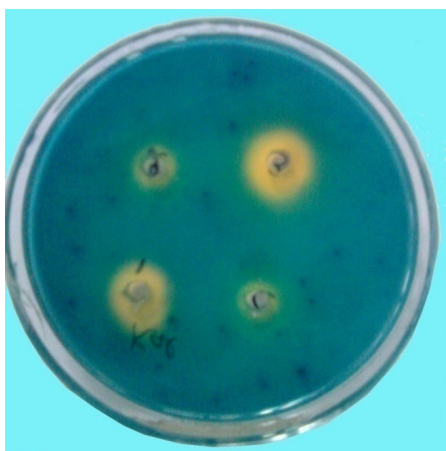


Plate 10: Siderophore activity shown by benzene extract prepared from An-6-shr and An-3-cha

4.13.1 PGP activities of extracted metabolites

The results revealed (Table 13) that 100µl of benzene fraction of crude extract showed siderophore activity on chromeazurol-S agar in the range of 12-16mm diameter of yellow/orange zone and also proteolytic activity on skim milk agar in the range of 12-17mm diameter of clear zone. Benzene extract from any of *Pseudomonas* isolate didn't show phosphate solubilizing and chitinase activity on PVK and chitin agar. None of the chloroform extract from any of the isolate showed any of the PGP activity.

4.13.2 Antifungal activity of extracted metabolites.

All the extracts (chloroform and benzene) were analysed for antifungal activity by well plate assay method on MEA against five indicator test fungal pathogens viz., *Dematophora* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp., *Sclerotium* sp. The Chloroform extract didn't show inhibition against any indicator fungal pathogen. Benzene extract from all the *Pseudomonas* isolated showed antagonistic antifungal activity against one or the other fungal test organisms.

Antifungal activity against:

(a) *Dematophora* sp.

Antifungal activity against plant pathogen *Demathophora* sp. have been shown by three benzene extracts extracted from 72 h old supernatant of isolates An-6-shr, An-3-cha and An-M-Mag in the range of 13.4 to 30.0% inhibition of growth of mycelia in MEA plate after 3 days of incubation at 28°C. The maximum percent growth inhibition was shown by An-6-shr (30.0%I), followed by An-3-cha(20.0%I) & An-M- Mag (13.4%I).

(b) *Fusarium* sp.

Three of benzene extracts from An-6-shr, An-3-cha and An-1-Mag showed antifungal activity against *Fusarium* sp. in the range of 19.4 to 33.3% inhibition. The maximum percent growth inhibition was shown by two extracts

An-6-shr (22.2 %I) An-1-Mag (33.3 %I). The minimum percent growth inhibition was shown by isolate An-3-cha (19.4 %I).

(c) *Phytophthora* sp.

Antifungal activity against plant pathogen *Phytophthora* sp. had been shown by only one benzene extract An-6-shr with percent growth inhibition of 14.7%.

(d) *Pythium* sp.

Out of four, three of benzene extracts from An-6-shr, An-3-cha and An-1-Mag showed antifungal activity against *Pythium* sp. in the range of 18.4 – 24.2% inhibition. The maximum percent growth inhibition was shown by An-3-shr (24.20 %I) followed by An-3-cha (21.2%I) and the minimum was shown by An-1-Mag (18.2 %I).

(e) *Sclerotium* sp :

Out of four extracts from four isolates, three of benzene extracts from An-3-cha, An-M-Mag and An-1-Mag showed antifungal activity against *Sclerotium* sp. (18.4-28.9%I). The maximum percent growth inhibition was shown by An-3-cha (28.9%I) followed by An-1-Mag (26.4 %I). The minimum percent growth inhibition was shown by An-M-Mag (18.4 %I).

The overall results revealed that isolate An-6-shr produced broad spectrum antifungal metabolite at 28°C under shake condition (100rpm) after 72h in PPM medium. Benzene extract prepared from culture supernatant is effective against five fungal pathogens i.e *Dematophora* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp. and *Sclerotium* sp. i.e. in the range of 14.7- 30.0% . Second isolate An-3-cha also produced broad spectrum antifungal metabolite and benzene extract from culture is effective in inhibiting the growth of three important fungal pathogen viz. *Dematophora* sp., *Fusarium* sp. and *Pythium* sp. The inhibiting percentage was in the range of 19.4 to 21.2%I by 100µl. of benzene extract. These two isolates were used in studying the growth promotion of apple

plants in field trials that were conducted at farmer's field in replant site of orchard at Maggota.

Table 21 Evaluation of antifungal activities of extracted antifungal metabolites

<i>Pseudomonas</i> isolates	Extract	Antifungal activity (mm dia. & % inhibition)									
		<i>Dematophora</i> sp.		<i>Fusarium</i> sp.		<i>Phytophthora</i> sp.		<i>Pythium</i> sp.		<i>Sclerotium</i> sp.	
		mm	%I	mm	%I	mm	%I	mm	%I	mm	%I
	Control	30	0.0	36	0.0	34	0.0	33	0.0	38	0.0
An-6-shr	chloroform	-	0	-	0	-	0	-	0	-	0
	benzene	21	30(33.19)	28	22.2(28.08)	29	14.7(16.24)	25	24.2(25.03)	-	0
An-3-cha	chloroform	-	0	-	0	-	0	-	0	-	0
	benzene	24	20(26.52)	29	19.4(25.12)	-	0	26	21.2(23.25)	27	28.9(32.51)
An-M-Mag	benzene	26	13.4(18.34)	-	0	-	0	-	0	31	18.4(24.62)
An-1-Mag	benzene	-	0	24	33.3(35.25)	-	0	27	18.2(20.12)	28	26.4(30.53)
CD _{.05}		1.25	4.17(3.08)	1.25	3.48(2.35)	.726	2.10(1.05)	1.25	3.79(2.45)	1.25	4.96(3.39)

*value in parentheses are transformed values

**Antifungal activity expressed in terms of percent inhibition (% I) of mycelial growth of indicator test pathogen on MEA by well plate assay method after incubation at 28°C for 5 days.

Percent inhibition (%I) = C-T/ T× 100

Where, C: Growth of mycelia in control. T: Growth of mycelia in test.

4.14 Application of fluorescent *Pseudomonas* strains An-6-shr & An-3-cha to apple plants in replant site of orchards at Maggota.

Table 22 Details of replant site and strains of fluorescent *Pseudomonas* species used for field trials

Site of field/ District	Age of Orchard	Strain applied	Concentration of inoculum density (approximate)
Maggota (Jubbal) Shimla District	>25 years	An-6-shr An-3-cha Consortium An-6-shr and An-3- cha (1:1)	Half (H) Full (F) 4x10 ⁸ 8x10 ⁸ 4x10 ⁸ 8x10 ⁸ 4x10 ⁸ 8x10 ⁸

4.14.2 Effect on per cent survival of replanted apple:

Field trial using two best selected strains An-6-shr & An-3-cha on the basis of overall plant growth promoting activities *in vitro* was started in Feb-March 2011 at replant site of the orchard. The survival percentage of apple plants was calculated as the number of plants survived over the number of control plants. Over all 70-90% and 70-80 % survival per cent of replanted plants was

observed in treatments with full and half inoculum' concentrations of individual and consortium inoculums respectively as compare to their respective control (50 %) after nine months of plantation.

Two inoculums concentration of each individual strain (Half & full conc.) and their consortium (i.e. both bacteria in 1:1 ratio) approximate inoculums density 8×10^8 cfu/ml along with control (water) were used. Each concentration of inoculum of individual strain i.e. half conc. (4×10^8 cfu/ml) & full conc. (8×10^8 cfu/ml) and similarly each concentration of consortium i.e. half (4×10^8 cfu/ml) & full (8×10^8 cfu/ml) were used to treat ten apple plants/seedlings (replications) in each set. Liquid formulation of each individual and their consortium were used for application. Before plantation, soil of pits in replant site were treated two times with each formulation. After plantation of treated apple seedlings in replant sites, cyclic treatment of basins of planted seedling was done for 9 months with each formulation. In every field experiment changes in rhizobacterial population i.e. total count of bacteria, fluorescent *Pseudomonas* and fungi was observed. Percent survival of seedlings, plant growth parameters like height and number of nodes. soil parameters like available NPK content of rhizosphere soil, moisture, soil pH and PGP activities in rhizosphere soil of treated plants were also observed before and after treatments.

4.14.3 Effect on plant growth parameter:

4.14.3.1 Plant height

The performance of replanted apple seedlings after cyclic treatments with selected strains of fluorescent *Pseudomonas* sp. (An-6-shr and An-3-cha) in replant site of orchard was much better as compared to control seedlings in term of plant growth parameters i.e. plant height and number of nodes.

Overall on an average there was 27.5 to 120.2% increase in plant height over control seedlings. All the treatments with full inoculum concentration of individual strains and consortium of two strains showed more increasing effect on plant height (98.2 to 120.2%) except in case of strain An-6-shr.



1



2



3



4



5

Plate 11 Effect of *Pseudomonas* strains on apple seedlings after nine months (1) An-6-shr full concentration, (2) An-3-cha full concentration, (3) Untreated control (4) Consortium full concentration (5) An-3-cha Half concentration

The effect of different treatments of fluorescent *Pseudomonas* sp. isolates An-6-shr and An-3-cha on height after 9 months showed that the maximum percent increase in height were recorded with full concentration of An-3-cha (8×10^8 cfu/ml) i.e., 120.2% increase over respective control as compared to half concentration of An-3-cha (107.4% increase) and full concentration of consortia (98.2% increase) followed by half concentration of consortia (89.6%). Two inoculum conc. of An-6-shr individually showed slightly less percent increase in plant height i.e 27.5 to 52.8% as compared to other strains.

4.14.3.2 Number of nodes:

Similarly there was 61.6 to 188.1% more increase in number of nodes over control plants in all the treatments except in one treatment i.e. full concentration of individual strain An-6-shr. Half concentration of all the treatments (individual and consortium) showed 85.3 to 150 percent increase in number of nodes over control. Similarly full concentration of individual strain An-3-cha and consortium showed 61.6 to 188.1% increase over control in number of nodes.

Table 23: Effect of *Pseudomonas* strains on survival and growth of apple in replant site after 3 and 9 months.

Site Maggota	% Survival						% Increase							
	3 months			9months			height of plant				No of nodes/leaves			
	Control	Half	Full	Control	Half	Full	Half	Full	Half	Full	Half	Full	Half	Full
Month s	3 months			9months			3 month		9 months		3months		9months	
An-6-Shr	70	100	100	50	80	70	4.1↓	0.0	52.8	27.5	8.5↓	38.8	85.3	15.27
An-3-Cha	70	70	100	50	70	90	7.4 ↓	4.1↓	107.4	120.2	2.1	7.4 ↓	150.0	188.1
Consortium	70	80	90	50	70	80	12.3	55.3	89.6	98.2	3.1 ↓	50.0	100.4	61.6

4.14.3.3 Effect of cyclic treatment of An-6-shr and An-3-cha on population density of total bacteria, fluorescent *Pseudomonas* sp. and fungal flora in replant sites of orchards at Maggota.

4.13.3.1 Effect on Root colonization and rhizobacterial count

A PGPR that can stimulate plant growth in laboratory will not necessarily have any significant impact on plant in the field unless it is able to persist and

grow in the natural environment. To exert their beneficial effects, bacteria usually must colonize the root surface efficiently.

So the experiment was conducted to the study of the effect of cyclic treatment of best selected strains of fluorescent *Pseudomonas* sp. on total counts of bacteria, fluorescent *Pseudomonas* and fungi in the rhizosphere soil of apple seedlings planted in pretreated soil of replant site pits. All counts were represented in term of cfu/ml of dilution.

The result on the effect of cyclic treatment of best selected strains of fluorescent *Pseudomonas* sp. and their consortium of full (4×10^8 cfu/ml) and half (4×10^8 cfu/ml) inoculums densities were 8×10^8 cfu/ml was studied on the root colonization in terms of effect on total bacterial count, total *Pseudomonas* count and total fungal count in the rhizosphere observed periodically for eight to nine months at an interval of one month starting from March to November. One year old seedlings/plants each of apple were selected for bacterization treatment for field trial experiments to study the effect of best selected fluorescent *Pseudomonas* strains on different plant growth and soil parameters of planted seedlings.

Plants receiving inoculation with liquid formulations of An-6-shr and An-3-cha individual strains and their consortium have significant increase in total bacterial count as well as total fluorescent *Pseudomonas* count (Table 12) as compared to uninoculated controls. Treatment with full concentration of An-6-shr showed the maximum increase in counts of fluorescent *Pseudomonas* from april to October. Overall there was gradual decrease in total bacterial count and increase in total fluorescent *Pseudomonas* count from 9.5×10^5 to 14.5×10^5 cfu/g of soil in all the treatments. Also there was decrease in counts of total fungal flora from 3 to 1.5 cfu/g of soil in all the treatments.

These results indicated towards the possibility of sufficient increase, in number and establishment of fluorescent *Pseudomonas* sp. in rhizosphere (upto 10^6 to 10^8 cfu/g soil) of apple planted in replant problem site area. The results also indicated towards the possibility that the bacterial load of *Pseudomonas* sp.

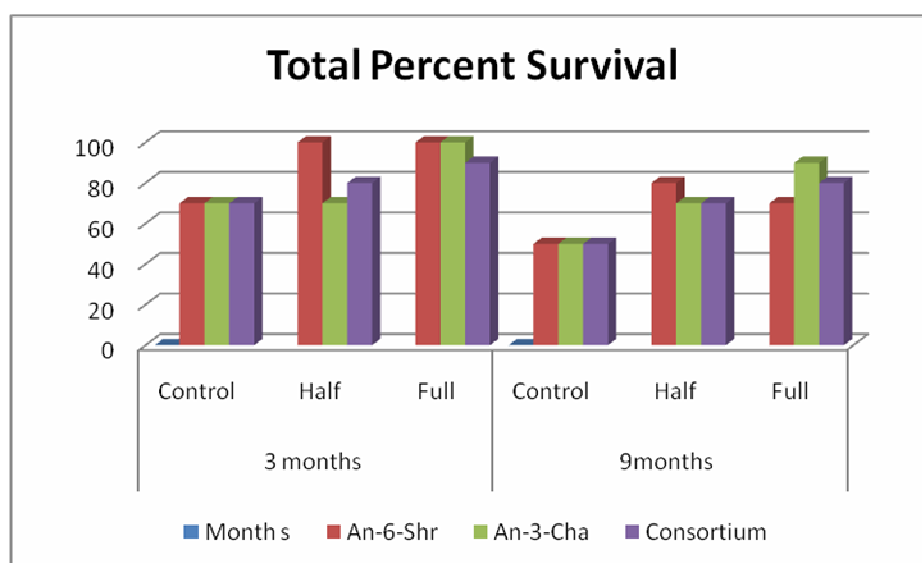


Fig 5 : % survival of plants after nine months in replant area site

inoculums in the rhizosphere might have decrease the deleterious microflora that may be pathogenic flora of fungal and bacterial origin.

Table 24 Effect of cyclic treatment of selected strains of fluorescent *Pseudomonas* strain An-6-shr and An-3-cha on population density of bacteria and fluorescent *Pseudomonas* sp. in rhizosheric soil

Strain	Inoculum Concentration	Total bacterial count cfu/g soil. x 10 ⁵							Total <i>Pseudomonas</i> count cfu/g soil. x10 ⁵						
	Months	April	May	June	July	August	Sept.	October	April	May	June	July	August	Sept.	October
Control		24	25.5	12.5	15.5	13.5	10	8.5	-	-	-	-	-	-	-
An-6-shr	Half	12.5	7	12	9.5	13	15	20	7	2	1.5	9.5	10	11.5	14
	Full	21	19.5	19.5	11	12.5	14.5	19.5	9.5	5	1.5	9	11	13	14.5
An-3-cha	Half	8	15	13.5	12.5	14	18	22	6.2	2.5	4	8.5	9	10	10.5
	Full	6	16.5	8.5	11	13.5	19	23	8.5	5	4	8.8	9.1	12	14
consortia	Half	23	7	9.5	8.5	12	17	20	2.3	5.5	1.5	7.5	8.5	9.8	11.5
	Full	26	10	7	13	15.5	13.5	21.5	1.5	2.5	4	5.5	8	9	13

Table 25: Effect of cyclic treatment on population density of total fungal flora in replant sites of apple at Maggota.

Strain	Total fungal count (cfu/g. x 10 ³)			
	3 months		9 months	
	Half	Full	Half	Full
An-6-shr	3	2.5	1	1.5
An-3-cha	1.5	3	2	1
Consortia	4	3	2.5	1
Control	5		7	

4.14: Effect of *Pseudomonas* strains An-6-shr & An-3-cha and their consortia on PGP potential of rhizospheric soil extract.

4.14.1 Multifarious PGP activities:

Soil extracts of each treatment showed (Table 27) increase in all the PGP activities i.e. P-solubilizing, siderophores, lytic enzyme, HCN and ammonia in all the treatments. PGP activities in rhizosphere soil extract of each treatment was analysed after every month of inoculation. Phosphate solubilizing, siderophores and proteolytic activities expressed in terms of mm. diameter of yellow, pinkish/orange and clear zone produced around the well by 100µl of soil extract on PVK, chrome azurol-S (CAS) and skim milk agar at 28° C after 48h. Overall soil rhizosphere extract of each treatment with individual and consortium strain showed more increase in PGP activities i.e. Phosphate solubilization,

siderophore, proteolytic, HCN and ammonia after 9 months treatments as compared to treatments after 3 months. But after both the period of treatments the overall effects is more as compared to control.

Soil extracts in treatments with full inoculum concentrations of both individual and their consortium strains showed much increase in all PGP activities i.e. phosphate solubilization, siderophore, proteolytic, HCN and ammonia after 9 months. (Table 27).

4.14.2 Antifungal activity

Rhizosphere soil extracts from each treatment of individual and consortium strain were analysed for antifungal activities against four important plant pathogens i.e. *Dematophora* sp., *Fusarium* sp, *Phythium* sp. and *Sclerotium* sp. The results revealed that An-3-cha half concentration showed percent inhibition against all four fungal pathogens (20-36.1%I) followed by An-6-shr half concentration that showed percent inhibition against three fungal pathogens (25-39.4%I), full concentration of consortia showed percent inhibition only against *Dematophora* sp. only (33.3%I) after three months. The results after nine months revealed that the An-6-shr full concentration showed the percent inhibition against all the fungal pathogens in the range of 10.50-47.22%I, followed by An-3-cha half concentration that showed percent inhibition against all fungal pathogens (26.19-44.44%I). The results revealed that the production of antifungal activity increases after nine months against fungal pathogens.

4.15 : Effect of rhizosphere soil of apple plants planted in replant site before and after inoculation

4.15.1 Effect of inoculated *Pseudomonas* strain on soil parameters.

4.15.2 Available N, P and K of rhizosphere soil.

NPK content in the treated rhizosphere soil increased over control. Over all treatments with full concentration of individual strain and consortium were more effective in increasing the NPK content (i.e. percent increase over control) of rhizospheric soil as compared to percent increase in treatments with half concentration of inoculums i.e. 19-23% increase in nitrogen, 40-75% increase in phosphorous and 17-21% increase in potassium.

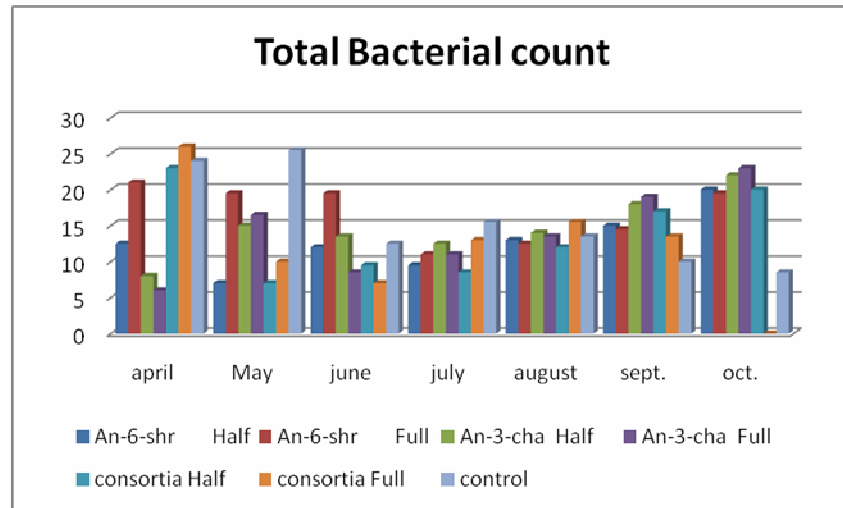


Fig 6 Effect of cyclic treatments of fluorescent *Pseudomonas* strains on the total bacterial population of apple planted in replant area of Maggota

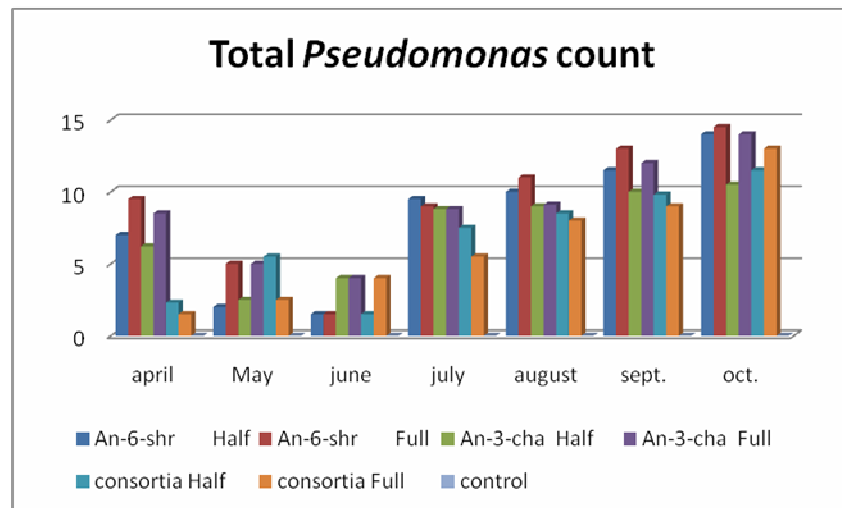


Fig 7 Effect of cyclic treatments of fluorescent *Pseudomonas* strains on the total *Pseudomonas* population of apple planted in replant area of Maggota

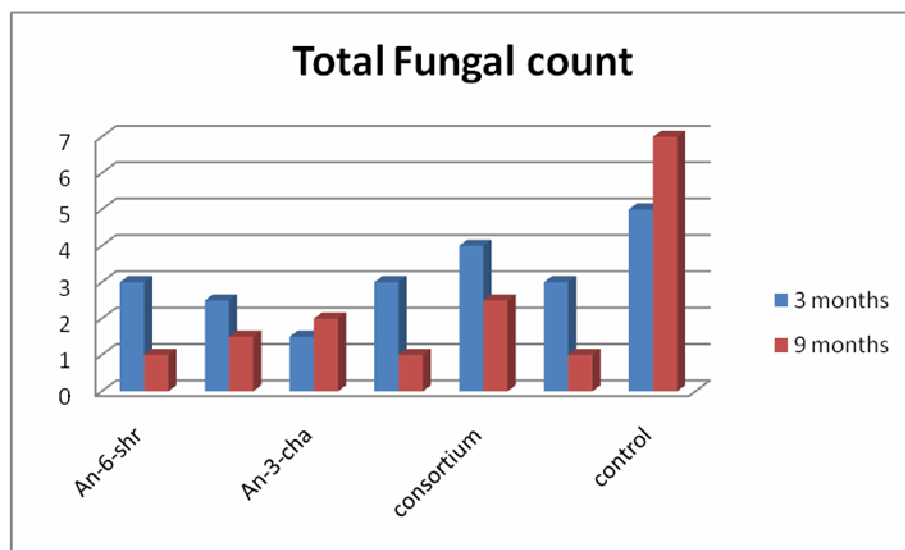


Fig 8: Effect of cyclic treatments of fluorescent *Pseudomonas* strains on the total fungal population of apple planted in replant area of Maggota

Table 26. Effect of cyclic treatment of *Pseudomonas* strains & their consortium on antifungal activity in rhizosheric soil extract after 3 & 9 months

Strain	Antifungal activity (mm dia & % inhibition)															
	AFTER 3 MONTHS															
	Dematophora sp				Fusarium sp.				Pythium sp.				Sclerotium sp.			
	Half		Full		Half		Full		Half		Full		Half		Full	
	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I	mm	%I
Control	36	0.0	36	0.0	30	0.0	30	0.0	32	0.0	32	0.0	38	0.0	38	0.0
An-6-shr	23	36.1(37.28)	-	-	24	20	-	-	21	34.3(36.15)	29	9.3(13.05)	-	-	30	21.05(22.25)
An-3-cha	25	30 (32.15)	23	36.1(38.14)	21	30 (32.05)	26	13.3(15.25)	24	25(27.14)	23	28.1(30.05)	23	39.4(36.50)	-	
consortia	-		24	33.3(36.08)	21	33.3(35.25)	-		-		-		32	15.8(12.80)	-	
control	26	27.7 (26.05)			-				-				31	18.42(20.12)		
CD _{.05}	2.45				1.91				1.64				1.84			

Strain	Antifungal activity (mm dia & % inhibition)															
	AFTER 9 MONTHS															
	Dematophora sp				Fusarium sp.				Pythium sp.				Sclerotium sp.			
	Half		Full		Half		Full		Half		Full		Half		Full	
	mm	%I	m m	%I	m m	%I	m m	%I	mm	%I	m m	%I	mm	%I	mm	%I
Control	36	0.0	36	0.0	30	0.0	30	0.0	32	0.0	32	0.0	38	0.0	38	0.0
An-6-shr	26	27.7(29.17)	19	47.22(45.14)	22	33.33(36.08)	20	39.4(37.08)	26	31.57(28.08)	34	10.5(13.32)	-	-	29	30.95(32.15)
An-3-cha	25	30.55(32.14)	20	44.44(42.05)	24	27.3(25.13)	21	36.4(31.84)	28	26.31(27.19)	-	-	31	26.19(30.01)	30	28.57(30.05)
consortia	22	38.88(35.13)	19	47.22(45.14)	23	30.3(31.12)	19	42.4(39.16)	-		32	15.7(17.90)	32	15.8(17.05)	-	
control	28	22.2(21.16)			26				21.2(22.23)				-			
CD _{.05}	1.34				1.81				1.63				1.54			

Table 27 Effect of cyclic treatment of *Pseudomonas* strains on PGP potential of rhizospheric soil extract

Strain	Months	Phosphate solubilizing			Siderophore			Proteolytic			Ammonia		
		Control	Half	Full	control	Half	Full	control	Half	Full	control	Half	Full
		*mm dia	*mm dia	*mm dia	*mm dia	*mm dia	*mm dia	*mm dia	*mm dia	*mm dia	Color change**		
An-6-shr	3	11	15	16	8	12	15	10	12	15			
	9	16	24	25	11	21	23	12	18	20	-	+++	+++++
An-3-cha	3	12	16	16	12	13	15	13	14	14			
	9	16	20	24	11	20	24	12	19	22	-	+++	++++
Consortium	3	12	15	17	12	13	15	13	13	16			
	9	16	21	25	11	22	23	12	21	23	-	+++	+++

* activities expressed in terms of mm. diameter of yellow , pinkish/orange and clear zone produced around the well by 100ml of soil extract on PVK media, Chrome azurol-S (CAS) agar and skim milk agar at 28° C after 24 h

** - indicates the yellow color; +++ = orange; +++++ = dark orange

4.15.3 pH and moisture content of soil

The pH of soil is determined by a pH meter before and after the treatment. The pH of soil was found to be 6.0 before the treatment and pH of 6.5 was observed after the treatment. The moisture content was 8% before and 7.5 % after experiment.

Table 28 : Effect of strain on *Pseudomonas* strain An-6-shr and An-3-cha on N P K content of rhizosphere soil after nine months of treatment

<i>Pseudomonas</i> strains	% Increase					
	Nitrogen		Phosphorous		Potassium	
	Half	Full	Half	Full	Half	Full
An-6-shr	15	19	30	40	15	17
AN-3-cha	19	21	50	55	17	18
consortium	22	23	60	75	20	21

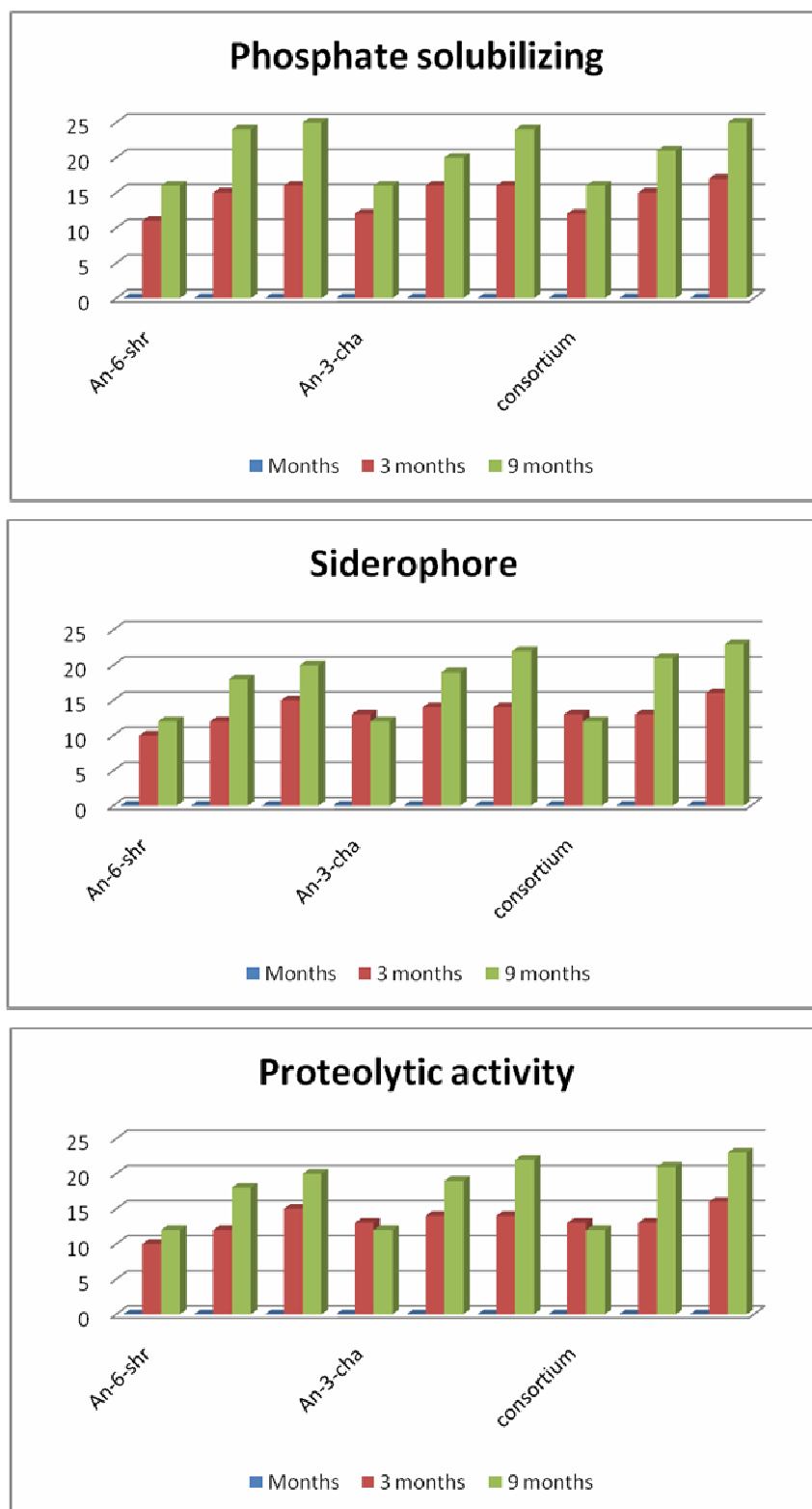


Fig 9: Phosphate solubilizing, Siderophore and proteolytic activity from rhizospheric soil extract after 3 and 9 months of inoculation of *Pseudomonas* strains

Chapter-5

DISCUSSION

Replant disorder or replant disease refers to the problem of re-establishing plants in soil where the same species was previously grown. Plants affected by replant disease may establish poorly, make poor growth and may even die. This is most noticeable when a plant growing in affected soil is compared with one planted at the same time in unaffected or sterilized soil. Roots will grow poorly and fine roots may be rotten. It is caused by complex of fungi, bacteria, nematodes, soil factors such as pH, moisture, stress and insufficient available phosphorus for vigorous growth of young trees. Overcoming replant disease is critical for the successful establishment of high density orchards. The causes of apple replant problem on different sites are highly variable. Not all the soils respond in the same way to the previous replant treatments. Thus a treatment that is beneficial in one orchard may not have any effect in other.

Apple replant disease (ARD) is a complex syndrome that occurs in young apple trees in replanted orchard sites (Mai and Abawi, 1981). Symptoms include death of fine feeder roots, stunted growth above and below ground, and reduced fruit yields. In most sites, biotic factors have been implicated in ARD, with soil borne fungi, bacteria, nematodes, actinobacteria and oomycetes variously cited as causal pathogens in site specific combinations (Mazzola and Gu, 1998).

The plant roots and their immediate environment constitute very dynamic enrichment environment that harbors free living beneficial microorganisms (Kloepper *et al.*, 1989; Glick, 1995). They improve the plant growth through direct or indirect effects on plants by producing plant growth promoting substances. *Bacillus* and *Pseudomonas* sp. have been found to be predominantly in the rhizosphere of various crops (Broadbent *et al.*, 1977). The fluorescent *Pseudomonas* makes up the dominant population in the rhizosphere and possesses several properties that have made them biocontrol and PGPR agents of choice (Johri *et al.*, 1997).

Currently, control of apple replant disease in commercial orchards outside India is accomplished primarily through the use of preplant soil fumigation with chemicals such as methylbromide, metal sodium and chloropicarin. But alternative to methyl bromide control, cultural methods are needed. Moreover this practice discouraged by integrated fruit production programs and is restricted by loss of chemical fumigants (Granatstein and Dauer 2000a).

The PGPRs may have potential scope in the management of biotic stress especially in replant problem sites in old orchards of apple and pear. The microclimatic changes that occur would be conducive to several pests and diseases. Some pathogens and pests will have common hosts resulting in severe crop losses; the efforts should be made to manage these pathogens through microbiological intervention to ensure optimum crop health and productivity. The potential microbial communities which have broad spectrum of growth promoting and disease suppressive activities against major soil borne pathogens are of great relevance.

The development of different strategies using a mixture of PGPR strains especially indigenous fluorescent *Pseudomonas* sp. would be an emerging and important area in crop growth promotion, protection and also in establishment of newly planted trees on old sites of apple and pear. It may reduce the losses caused by replant problem syndrome in economically important fruit crops especially apple.

So this study was carried out to find some alternative by finding out best indigenous plant growth promoting and biocontrol *Pseudomonas* species which may also be efficient P-solubilizer, siderophore, HCN, lytic enzyme and plant growth regulator along with strong biocontrol activity like broad spectrum antifungal metabolite producer. We have studied the total bacterial population and *Pseudomonas* population present in the rhizosphere of apple plants growing in normal orchards and replant problem sites. Different bacterial strains were isolated from the rhizosphere of apple plant grown in orchards of Maggota, (Jubbal) Shimla district area by standard serial dilution method using general i.e.

nutrient agar and specific medium for *Pseudomonas* species i.e. King's B medium (Table 2,3). They were identified up to genus level by using different morphological, biochemical, physiological and molecular (16s rRNA gene) characteristics (Plate 5) according to Bergey's manual of systematic bacteriology. They all were screened out for production of various plant growth promoting and disease suppressing activities using specific media and specific conditions for each activity *in vitro*. and identified the isolates upto genus level and possibly upto species level also. They were also characterized for production of broad spectrum antifungal activities against five important pathogens that are supposed to be involved in replant disease of apple. Two optimum cultural conditions i.e. times of incubation and temperature for high production of antifungal activity were also standardized so that they can be used for mass production of inoculum for field application in old apple orchard that is affected by replant disease. Also tried to isolate and extract broad spectrum antifungal metabolite and evaluate for antifungal activities. The two best fluorescent *Pseudomonas* strains were selected and were applied in replant problem site in the field individually and in the form of consortium. The treatment of rhizospheric soil of replant site pit was done two times at an interval of one day before plantation of new plants by application of two *Pseudomonas* strain An-6-shr and An-3-cha. The cyclic treatment of rhizosphere soil of replanted plants (basin) was also done for 9 months starting from February 2011 to October 2011. Various plant growth and soil parameters of rhizosphere soil, establishment of plants in terms of percent survival were studied. The root colonization by introduced PGP *Pseudomonas* sp. studied by determination of total bacterial count, *Pseudomonas* and fungal count were done to study the influence and establishment of introduced fluorescent *Pseudomonas* sp. in replanted apple rhizosphere.

There is a great scope for increasing the efficiency of the strain, so the present study was undertaken to see whether these plant growth promoting bacteria can effect the establishment of plants in replant problem site of apple orchards.

5.1 ISOLATION AND ENUMERATION OF *Pseudomonas* SP. FROM REPLANT AND NORMAL SITE OF APPLE

Significance difference in total bacterial and fluorescent *Pseudomonas* sp. communities in relation to normal and replant sites of apple was observed (Table 2). Trends in number of total cultured microorganisms were distinct. Total bacterial population was less in replant site as compared to normal site. Densities of *Pseudomonas* sp. was found to be far less or in some cases nil as compared to normal site.

In accordance with the findings of Catska *et al.*, (1982), the densities of *Pseudomonas* in the apple rhizosphere decreasesd over years after replanting. Observed population shift may be related to change in soil moisture, soil temperature, rhizodeposition and root turn over (Yao *et al.*, 2006).It is possible that some specific genus type of plant root may release more rhizodeposits and therefore maintain higher microbial population in the rhizosphere. The microbial population differences in these specific genotype was mirrored the genetic difference between their root stock cultivars as described by different worker (Yao *et al.*, 2006 and Rumberger *et al.*, 2007).

5.2 MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION OF *Pseudomonas* ISOLATES

As evident from the table 3, the given isolated microorganisms from apple rhizosphere soil are gram negative, non spore forming, coccobacillus producing fluorescent pigment and positive for catalase, oxidase with citrate, gelatin hydrolysis and denitrification tests in Huge and Leifson medium they showed only oxidative growth. Indole MR and Voges-proskaur and lactose fermentation test are negative. So according to Bergey's manual of systematic bacteriology and based on the colony morphology, microscopic observation, cultural, biochemical and physiological properties, the isolates were identified as belonging to genus *Pseudomonas* sp.

It may attribute to specific choice of media employed for the isolation *i.e.* nutrient agar and king's B medium. Several others hence supported use of King's

B medium (Kings *et al.*, 1954) for the isolation of fluorescent *Pseudomonas* sp. Enrichment cultures and/or use of selective isolates media formulations have been widely used as techniques for bringing in to pure cultures, the rare or specific forms of microbial diversity in rhizosphere of different crops (Louw, 1970; Thakkar *et al.*, 1993). The two most efficient plant growth promoting genus *i.e.* *Pseudomonas* sp. and *Bacillus* sp. were also in occurrence with the findings (Kapoor *et al.*, 1989; Saxena *et al.*, 2003).

Microorganisms are exposed completely to environmental influences. They must be able to tolerate and react to a wide range of environmental changes. In contrast to pH, ionic composition and water activity, the internal temperature of microorganisms must be equal to that of its environment. As for many chemical reactions, all microbial activity is sensitive to environmental temperature. Microorganisms are known which can grow above 90⁰C or at less than 0⁰C provided that liquid water and nutrients are available but no microorganism will grow at both ends of this range. The growth of most microbes is restricted to a 20 – 40⁰C Span. Each species or a strain has a characteristic minimum, optimum and maximum temperature. At minimum and maximum temperature growth rate occurs at its maximum rate. Temperature optimum is usually only 5-10⁰C below temperature maximum (Forage *et al.*, 1990). The optimal temperature for growth may not be that best suited to product formation especially where the product is predominantly non growth associated as in the case of many secondary metabolites (Woodruff, 1961). Additionally, different strains may have different growth and production optima. So in any mutation or general selection programme independent temperature optimization of each strain for growth and subsequent product formation is often necessary (Dulaney, 1951; Woodruff, 1961).

So, we have studied the effect of varying optimum temperature *i.e.* 4 ⁰C, 28 ⁰C, 37 ⁰C and 41 ⁰C on the growth of *Pseudomonas* isolates. Each microorganism has its own specific minimum, optimum and maximum temperature. This behavior of all the *Pseudomonas* sp. isolates was similar to usual response of mesophilic organisms where metabolic activities get slow down

below and above the optimum temperature. This suggests that organisms are mesophilic in nature. Few of the *Pseudomonas* isolates were found to grow even at temperature ranging from 4⁰C to 41⁰C our result collaborates with Mishra *et al.*, (2005) who reported that *Pseudomonas lurida* grew at temperatures ranging from 4 to 30 ⁰C with a growth optimum at 28⁰C. Also it was reported that for *Pseudomonas lurida*, the optimal growth temperature is 21 ⁰C. Growth can be observed at 4 ⁰C and 41⁰C as well. . Temperature is an important process parameter. It has an influence on microbial physiology and maintenance of an optimal process temperature in industry and is one of the major factors in the economics of the process.

5.3 MOLECULAR IDENTIFICATION

Traditionally, a search for PGPRs involves screening a large number of isolates and identifying a desired phenotypic trait. Once isolates are purified, the main aim is to keep the maximum genetic diversity in the minimum number of isolates, for further biological assays. This goal may be achieved through 16S rRNA-PCR that define differences at the strain level (Lucas *et al.*, 2001).

The *Pseudomonas* RNA homology group shares so many characteristics with othe pseudomonads that strict differentiation is not possible with ordinary phenotypic tests. Therefore, methods based on analysis of the genome have been proposed. The present study was conducted to develop a 16S rRNA gene based PCR protocol for the detection and identification of members of the genus *Pseudomonas*. The *Pseudomonas* specific-PCR primers were subjected to experimental testing on pure cultures. Database searches for 16S rRNA revealed sequences related to *Pseudomonas* that derived from cultures originally identified by others as belonging to different genera. *Pseudomonas* classification has attracted much attention in the recent past and extensive reclassification has been performed. The *Pseudomonas* specific-PCR protocol may offer a rapid diagnostic tool for genus identification. The isolates under our study of 16S rRNA sequencing showed 99% similarity with *Pseudomonas* genus. The isolate An-6-Shr showed 98% homology with *Pseudomonas aeruginosa* NEB 98 with accession number (GU121495.1) and 96% similarity with *Pseudomonas putida*

and *Pseudomonas fluorescens* whereas AN-3-Cha showed 98% homology with *Pseudomonas aeruginosa* strain NBAII AFP-5 16S ribosomal RNA gene, partial sequence acc no [HM439971.1](#). and 96% similarity with *Pseudomonas putida* and *Pseudomonas fluorescens*.

Burke *et al.* (2004) made molecular characterization of functionally important microbes viz., *Bacillus subtilis*, *Pseudomonas fluorescens* and *Streptomyces noursei* from the gut of indigenous silkworm breeds. To confirm isolated microorganism as *Pseudomonas fluorescens* 16S rRNA intervening sequence specific ITSIF (5'AAGTCGTAACAAGGTAG3'); ITS2R (5'GACCATATATAACCCCAAG3') primers were used to get an amplicon size of 500-600 bp. Amplification of the PCR products was confirmed in 1.2 % agarose gel. Sequencing of PCR products was done. The BLAST analysis of the sequences of PCR products for both the forward and reverse primers showed maximum similarity (87-99%) with *Pseudomonas fluorescens* or *Pseudomonas* sp.

Clark and Lanigan (1993) chose a representative strain, P513/18^T from a subset of three phenotypically similar pseudomonads (P513/18^T, P239/01 and P240/09) for 16S rRNA gene sequence comparison. They found *Pseudomonas costantinii* as the closest phylogenetic neighbour with a similarity of 99.9% by binary sequence comparison, followed by *Pseudomonas trivalis* and *Pseudomonas poae*, displaying 99.7 and 99.8% similarity, respectively. Sequences were aligned using the CLUSTAL_X algorithm. Phylogenetic trees, based on 1379 nt were constructed using the neighbour-joining (Nei and Miller, 1990) and maximum likelihood algorithms. *Pseudomonas* sp. found clustering next to strain P513/18^T in the neighbour-joining method also had the same arrangement in the maximum-likelihood algorithm. The separate clustering of novel isolate P513/18^T and the closest phylogenetic neighbour *Pseudomonas costantinii* was supported by a relatively high bootstrap value (75).

5.4 SCREENING OF FLUORESCENT *Pseudomonas* ISOLATES FOR PRODUCTION OF ANTIFUNGAL AND MULTIFARROUS PGP ACTIVITIES

5.4.1 Antifungal activity:

A greater thrust is given for development of biological consortia with multiple modes of action to suppress the fungal, bacterial and nematodal pathogens since there is no spatial segregation of these pathogens in cropping system (Sarma *et al.*, 1994). Sarma and Anandaraj (1998) have suggested consortium approach for disease management in plantation and spice crops. Mutual compatibility of fungal and bacterial antagonist's viz. *Trichoderma harzianum* and fluorescent *Pseudomonas* were studied in order to establish an efficient consortium for management of root rot of black pepper caused by *Phytophthora capsici*. The study revealed that the fungal and bacterial antagonists are compatible Jisha *et al.*, (2002). Application of rhizobacteria resulted in 38.9 per cent enhanced growth of black pepper and 66.6 per cent survival of ginger tillers (Sharma *et al.*, 2000). In successful cropping systems where the economic output per unit area is positive, the underlying microbiological association diversity, their interaction and the overall impact on the growth and productivity need to be understood for practical utility.

A replant problem in apple orchards appears to be related to soil and rhizosphere microflora (Castka *et al.*, 1982). It is a complex disease syndrome and its etiology is still unclear (Yao *et al.*, 2006). In most of the replant sites, abiotic and biotic factors have been implicated in SRD (Merwin *et al.*, 2001). Soil bacteria, plant parasitic nematodes, fungi, actinomycetes, oomycetes especially *Phytophthora* and *Pythium* individually or together are causative agent of the replant disease (Mai and Abawi, 1981; Castka *et al.*, 1982; Merwin *et al.*, 2001; Rumberger *et al.*, 2007). Methyl bromide (MB) has been the fumigant used most widely to control this disease. But alternative to methyl bromide, cultural methods are needed because methyl bromide is phased out due to environmental problems (Yao *et al.*, 2006).

Many soil microorganisms possess multiple beneficial traits of nutrient mobilization, production of plant growth promoting substances (PGP) and biocontrol ability. Such organisms have a greater role in sustaining agricultural production. Plant growth promoting rhizobacteria (PGPR), a group of root associated bacteria, intimately interact with the plant roots and consequently influence plant health and soil fertility. They offer an excellent combination of traits useful in disease control and plant growth promotion.

Several studies have shown that rhizosphere bacterial communities may suppress root infections by soil-borne pathogens. Some bacteria are associated with roots of crop plants and exert beneficial effect on the host and are collectively known as plant growth promoting rhizobacteria (Glick, 1995; Kloepper *et al.*, 1989). Some of these rhizobacteria not only benefit from the nutrients secreted by the plant roots, but also beneficially influence the plant in a direct or indirect way resulting in stimulation of its growth.

Amongst the PGPRs, fluorescent pseudomonads have emerged as the largest and potentially the most promising group of PGPR with their rapid growth, simple nutritional requirements, ability to utilize diverse organic substrates and mobility. Fluorescent pseudomonads produce highly potent broad spectrum antifungal molecules against various phytopathogens, thus acting as effective biocontrol agents. They are well equipped as primary root colonizers. Through several mechanisms *viz.*, production of antibiotics (Gutterson *et al.*, 1988), siderophores (Kloepper *et al.*, 1980), HCN (Defago *et al.*, 1990) and competition for space and nutrients (Elad *and Chet.*, 1987), They inhibit soil borne plant pathogens. They could serve as promising bioinoculants for agricultural system to increase productivity since the action of such bacteria is highly specific, ecofriendly and cost-effective. However, selecting a potential strain with multiple beneficial traits for production of inoculants from natural biodiversity hot spots assumes significance.

PGPR's especially fluorescent *Pseudomonas* sp. can directly promote the growth of plants usually by production of phytohormones and/or by increasing

the available nutrients through production of secondary metabolites like lytic enzymes, phosphate solubilization, and siderophores and/or as biocontrol agents that are able to protect plant from infections by phytopathogenic organisms through production of antibiotics, siderophores, HCN, ammonia and cell wall degrading enzymes (Kloepper *et al.*, 1989; Kloepper, 1993; Glick, 1995). The large scale application of PGPR's to crops as inoculants would be attractive as it would substantially reduce the use of chemical fertilizers, pesticides and methyl bromide etc. which often pollute the environment and reduce the fertility of soil.

Plant growth promoting rhizobacteria can competitively colonize plant roots and stimulate plant growth and/or decrease the incidence of plant diseases. They can be exploited as biological pesticides to decrease the use of chemical pesticides in agriculture. Some *Pseudomonas* sp. especially fluorescent pseudomonads, are particularly suitable to be used as agricultural biocontrol agents because they can produce large amounts of secondary metabolites to protect plants from phytopathogens and stimulate plant growth.

In this light we have screened out many native isolates of fluorescent *Pseudomonas* sp. from apple soil of Rohru area (Maggota, Shimla district) for production of antifungal activity (Table 8) against five important fungal pathogens that are thought to involve in replant disease of apple and other soil borne diseases of apple and further tried to study the effect of three cultural conditions i.e. media, time of incubation and optimum temperature on high production of antifungal activity and mass multiplication, that can be further used for inoculum and consortium development for field application (Table 10-19).

The broad spectrum antifungal metabolites were extracted and evaluated to find out its nature and some other properties (Table 21, Plate 8). It was found that the metabolite is like phenazine type of antibiotic that showed broad spectrum antifungal activities (Table 22, Plate 9). The extracted metabolite also showed siderophore and lytic enzyme (protease) activities. Some broad spectrum antifungal metabolites are known to have siderophore activity and even some siderophores are known to have broad spectrum antifungal activities (Bonsall *et*

al., 1997). But the exact nature and property of extracted metabolite of this study require further detailed investigation.

The most effective mechanism that a PGPR can employ to prevent growth of phytopathogen is the synthesis of antibiotics. Genetically manipulated strains of *Pseudomonas fluorescens* that was over producing the antibiotic *i.e.* Pyoluteroin and 2,4-diacylphorogulcinol was used to protect the cucumber plants against disease caused by *Pythium ultimum* more than the wild strain (Maurhofer *et al.*, 1992; Schnider *et al.*, 1995). Thomshaw and Weller (1998) identified a strain *P. fluorescens* that produced a phenazine antibiotic capable of inhibiting growth of *Gaeumannomyces graminis* var. *tricti*, the casual agent of major root disease of wheat worldwide.

The investigations were carried out on the biocontrol potential of isolated fluorescent *Pseudomonas* sp. These isolates were tested for their in vitro biocontrol potential against five important fungal pathogens *viz.* *Dematophora* sp., *Fusarium* sp., *Phytophthora* sp., *Pythium* sp. and *Sclerotium* sp. that are also supposed to be one of the main factor involved in apple replant disease. Lytic enzyme activity that was shown by the extracted antifungal metabolite with benzene (Table 21) also require further detailed analysis, whether the metabolite have enzyme activity or it is enable to degrade the milk proteins *i.e.* clear area on milk agar plate by some other chemical reaction.

This is in agreement with Pandey *et al.*, (2006) who reported that in petridish assay by the *Pseudomonas* sp. bacterium tested positive for inhibition of the growth of two phytopathogenic fungi. It caused transparent clearing in case of *Alternaria alternate*, and it suppressed the process of conidiation in *Fusarium oxysporium*. The effect of *Pseudomonas putida* in the control of major pathogens such as *Phytophthora parasitica* and *Fusarium* sp. has also been reported (Scher and Baker, 1982). The mechanism of suppression of *Fusarium* wilt of carnation by two fluorescent *Pseudomonas* sp. strains has also been reported by Scher and Baker (1982).

5.5 PHOSPHATE SOLUBILIZATION

Phosphorus is one of the major plant nutrients. It is second only to nitrogen as the most essential to plant growth. Plant obtains phosphorus from the soil in the form of HPO_4^{2-} and H_2PO_4 . However, the major portion of applied phosphate fertilizer gets fixed into unavailable form in the soil resulting in only 10 percent becoming available to plant.

The use of phosphate solubilizing microorganisms as inoculant simultaneously increases the plant crop yield. Strains from the genus *Pseudomonas*, *Bacillus* and *Rhizobium* species are among the most powerful phosphate solubilizers (Rodriguez and Fraga, 1999). Soil microorganisms play an important role in the solubilization of bound phosphates which are either in the form of organic phosphates or inorganic phosphates and make them available to higher plants (Kapoor *et al.*, 1989; Gaur, 1990).

Different forms of insoluble phosphates such as Tricalcium phosphates (TCP), rock phosphate (RP), hydroxyapatite (HAP) and dicalcium phosphate (DCP) have been used to determine the phosphate solubilizing activity of microorganisms in Pikovskaya's medium by Chodat and Gouda, (1961); Kundu *et al.*, (2002). Tricalcium phosphate has been reported to be readily solubilized than rock phosphate (Srivastav *et al.*, 2003). So in the present studies TCP was selected as a substrate for determining the phosphate solubilizing potential of fluorescent *Pseudomonas* sp. isolates. Pikovskaya's medium was selected for screening the phosphate solubilizing potential, as previous studies have shown this medium to be most selective, least susceptible to overgrowth by non phosphate solubilizing bacteria and most reproducible, allowing the growth of a broad diversity of phosphate solubilizing bacteria (Kundu *et al.*, 2002; Srivastav *et al.*, 2003).

In our study, all the indigenous strains of fluorescent *Pseudomonas* isolates from the rhizosphere of apple showed phosphate solubilizing activity both in Pikovskaya's agar plate in the range of 14-26 mm dia. and also solubilize tricalcium phosphate in liquid cultures and make them available as inorganic

phosphorous in the range of 40-75 µg/ml (Table 6). The isolates of fluorescent *Pseudomonas* sp. were found to be more effective in solubilizing TCP *in vitro*. Out of these two strains were also selective for field studies in replant sites.

Phosphorous is an essential nutrient that is required in large quantities for good growth of young replanted plants. Phosphorous deficiency may also be the major cause of poor growth of young trees. Application of efficient PGPR's especially An-6-shr and An-3-cha that are also efficient in phosphate solubilization *in vitro* may be the one reason that have helped replanted apple seedlings to establish and grow well in replant site. (Table 24, Fig 9). PGP activities also showed increase in rhizospheric soil of planted seedling of apple in orchard at Maggota, (Table 27) The influence on the growth and establishment in our studies (Table 24) may also be due to orthophosphorus uptake by young planted plants that may be produced by introduced strains in the form of solubilized inorganic phosphorous.

The results have been shown by other workers that maximum solubilization in liquid medium occurred only after ninth day of incubation, which later decreased with increase in incubation period (Thakkar *et al.*, 1993). Similar observations were also noticed by Gaur., (1990) But in comparison with our studies we have observed that active phosphate solubilization (Table 6) occur in stationary phase of growth, as it is evident from the results that 48 or 72 h. old cell free culture supernatant efficiently solubilizes the tricalcium phosphate in liquid medium. The solubilization of tricalcium phosphate may be probably due to the production of more organic acids that decrease the pH and help in solubilization of insoluble phosphate.

5.6 SIDEROPHORE PRODUCTION

Microbially produced siderophores that are iron chelating compounds can increase and regulate the availability of iron in plants rhizosphere (Neilands, 1982). Now these compounds have come under investigation in plant nutrition along with disease suppression (Reid *et al.*, 1986; O'Sullivan and O Gara, 1992). Kloepper *et al.*, (1980) have clearly demonstrated the role of siderophores in

rhizobacteria mediated antagonism. Siderophores mediated antagonism is implicated in *P. fluorescens* and *P. capsici* antagonistic system (Diby *et al.*, 2001). Many different siderophore of bacteria and fungi have been isolated (Raymond *et al.*, 1984).

Pseudomonas species produced fluorescent yellow water soluble siderophores and these siderophores have been classified as pyoverdins and pseudobactins (O'Sullivan and O'Gara, 1992). The production of these siderophores have been linked to plant disease suppression ability of certain fluorescent *Pseudomonas* species (Loper and Buyer, 1991) and suggested the involvement of these compounds in plant growth promoting and disease suppression by these strains (Kumar and Bezbaruah, 1996).

There are number of reports suggesting the plant species are capable of obtaining iron from microbial siderophores (Park *et al.*, 2005. Cline *et al.*, 1984) demonstrated that the iron from microbial hydroxamate siderophores may become available to plant both in nutrient solution and in soil. Siderophores from fluorescent *Pseudomonas* species have also been implicated in iron uptake by tomato plant (Dass *et al.*, 1986). These reports suggested that some plant species may obtain iron viz., certain microbial siderophores.

In our study almost all the strains of fluorescent *Pseudomonas* sp. showed high production of siderophores (Table 6). The results showed that all the isolates produced siderophores in the range of 12 to 17 mm diameter of pinkish orange zone on chromeazurool-S agar plates (Plate 4) and 78.48-90.80 %SU by reduction of blue color of CAS in liquid assay. These results suggested that siderophores produced by these strains may also have some beneficial role in the growth promotion and disease suppression in the bacterized apple plants planted in replant problem site at Maggota (Table 23) and they also helped the apple plants to establish better in replant site pits. Whether the growth promotion of apple plants in replant site was due to biocontrol by siderophore or due to supply of iron ions as nutrients have to be clearly establish by further extensive experimentation.

5.7 HCN PRODUCTION

In addition of antibiosis mechanism that includes the disease suppressing effect of siderophores and antibiotics, there are a number of different ways in which PGPR's can inhibit phytopathogen (Voisard *et al.*, 1989). Some *Pseudomonas* sp. are able to synthesize HCN to which these *Pseudomonas* sp. are themselves resistant that may be linked to the ability of these strains to inhibit some pathogenic fungi.

Some biocontrol plant growth promoting bacteria produce a wide range of low molecular weight metabolites with antifungal potential. The best known is hydrogen cyanide (HCN) to which the producing bacterium usually a *Pseudomonas*, is resistant. HCN is released as a product of secondary metabolism by several microorganisms and affects sensitive organism by inhibiting the synthesis of ATP-mediated cytochrome oxidase. *Pseudomonas* sp. produce hydrogen cyanide from oxidation of glycine because Fe^{3+} ions and glycine enhance the HCN production by *Pseudomonas* sp. in culture (Bakers and Schippers, 1987).

The ability of the *Pseudomonas* sp. strains to inhibit the growth of *Fusarium in vitro* and produce cyanide has been claimed to be an important in biological control (Defago and Hass, 1990) *Pseudomonas putida* strain BK 8661, which produces siderophores, antibiotics and low levels of hydrogen cyanide, suppressed by the growth of *Septoria tritici* and *Puccinia recondite* .sp. *tritici in vitro* and on wheat leaves.

In this study, almost all the stains of *Pseudomonas* sp. isolates showed production of HCN *in vitro* as evaluated from the color change of pre-dipped picric acid paper strips from yellow to orange brown. The production of HCN by applied PGP strains in the field experiments at replants may also be one of the cause that help in growth promotion of apple seedling (Table 6)

5.8 AMMONIA PRODUCTION

The effect of ammonia excreting strains of *Azotobacter chroococcum* AC₂ was studied on cereals and legumes and they were beneficially influenced by the inoculation of the ammonia excreting strains of *Azotobacter chroococcum* AC₂ (Narula and Gupta, 1987). Stimulation effects of ammonia excreting strains of *Azotobacter* and increase in nitrogen fixation crops might be due to the capacity of particular crop to utilize ammonia (Narula and Gupta, 1987). The enzyme activity in the inoculated soil with ammonia excreting microorganisms was found to be more as compared to uninoculated control.

Ahmad *et al.*, (2006) reported that the plant growth promoting rhizobacteria were found to produce ammonia. Chaiharn *et al.* (2008) also reported the production of ammonia by phosphate solubilizing microorganisms. More than 64% of the isolates produced ammonia.

In our study, all the strain of *Pseudomonas* sp. isolates produce ammonia as observed from the change of color for light yellow to brown (Plate 4). These bacterial strains are also free living in the soil/rhizosphere of apple plant. They all produce many plant growth promoting activities like antifungal, siderophore, phosphate solubilization, phytohormones, HCN, lytic enzymes along with ammonia. The results pointed out the need to study the contribution of these ammonia excreting strains of fluorescent *Pseudomonas* sp. isolates in growth promotion of apple seedlings whether it has any role in reduction of replant problem, yet to be studied.

5.9 LYTIC ENZYMES

Inhibitory effect against various type of microorganisms was due to the lytic enzymes produced by the microorganisms (Kaur *et al.*, 1989; Gupta *et al.*, 1992). Enzyme chitinase and chitinase produced by bacteria, fungi and actinomycetes i.e. *Mucor*, *Trichoderma* and *Pseudomonas* species possess lytic effect that relates to the antagonistic behaviour (Pedraja and Lopez, 1991). Some proteolytic enzymes especially elastase, subtilisin, pronase also possess

bacteriolytic\ cell wall lysing properties against different Gram positive and Gram-negative bacteria (Kaur *et al.*, 1989; Gupta *et al.*, 1992).

These proteases have an important role in decomposing protein substances. In agriculture, these lytic enzyme producing microorganisms may have role as biological control agents for eradication of some fungal and bacterial plant pathogens. Also they may have role in supplying some nutrients to plants as some of these enzymes especially protease/ chitinase have broad substrate specificities i.e. they can degrade and metabolize complex substrate and substances produced as plant root exudates by old trees of apple. They produce some toxin and may other root exudates that are also one of the reasons for replant disease (Politycka and Adamska, 2003). So these PGP strains of fluorescent *Pseudomonas* sp. producing lytic enzymes may also have some role in biocontrol activity or supply small molecular substance as nutrients to young planted trees and may help in their growth promotion and establishment. One of the main functions of the enzymes in soil and in rhizosphere might be their role in the plant nutrition and disease suppression. They transform the organic matter in to mineral constitutes, important for plant growth.

The screening of *Pseudomonas* isolates for protease production on skim milk agar plates showed that maximum number of *Pseudomonas* species were positive for the proteolytic activities, in the range of 11-14mm diameter of clear zone produced on skim milk agar and 46-90µg/ml by liquid assay. (Table 6).

Microbial proteases are an important class of industrial enzymes (Aunstrup, 1980), that are mainly used in detergent, leather, food and feed, photography and pharmaceutical industries. *Pseudomonas* and *Bacillus* species are reported to be prolific producer of proteinases which constitute a major sources of enzymes used in detergents , food , pharmaceuticals. They are often used in the analysis and have role in utilization of waste (Kulp, 1975).

The enzyme activity is merely always higher in rhizosphere soil than in free soil (urease, invertase, catalase, phosphatase, protease and amylase, etc.). The enzymes in the rhizosphere may be produced by microorganisms associated

with plant roots. (Vancura and Jandera, 1986). Another important function of enzymes may be in the formation and regulation of the microbial association in rhizosphere (Jandera, 1982, Lockwood, 1960). The selection and accumulation of some microbial species take place on the roots and in their proximity. The regulation of the synthesis and activity of enzymes may enable the microorganisms to utilize nutrients preferentially from the mixtures of substrates. The substrates most suitable for their growth and thus to gain the superiority under the given conditions.

5.10 PLANT GROWTH HORMONES

The ability of soil microorganisms to synthesize various metabolites, which can affect the soil properties, the growth and chemical composition of plants and their health is considered as one of the most important factors of the soil fertility (Vancura and Jandera, 1986). Plants require a definite levels and ratio of the plant growth hormones to maintain their normal growth and development.

In the present study all the isolates showed production of plant growth regulators in stationary phase of growth in the range i.e. auxins of 9-22µg/ml, gibberellins 34-75 µg/ml and cytokinin in 20-80 µg/ml (Table 7). Two strains that were also used in field trial experiment showed maximum production of all the three regulators. So these two PGPR An-6-shr and An-3-cha when applied in replant site while planting the apple seedlings might have helped to get establish by increasing the root volume especially by production of auxins that become easily available to plants in sick soil. Similarly gibberellins and cytokinins might have helped plants in increasing their growth i.e. height and number of nodes per plant (Table 23). The results revealed that the isolates of *Pseudomonas* sp. isolated from replant sites showed comparatively less production of growth regulators as compared to normal sites.

It is well established that several rhizosphere bacteria especially root colonizing *Pseudomonas* species produce vitamins and growth hormones (auxins and cytokinins), which often lead to certain morphological changes in the plants

(Brown, 1972; Turner and Backmann, 1991). The plants cultivated under sterilized conditions had less gibberellins and auxins than the plant from a non-sterile environment. Treatment of plant root or leaves grown under sterilized conditions with microorganisms producing these substances increase the concentration of the hormones in plant tissue almost to the level encountered in the plant growth under non-sterile conditions (Libbert *et al.*, 1969). The metabolites produced by microorganisms influence the plants beside also the formation of relations among the microorganisms in the rhizosphere association, the availability of minerals nutrients in plant and soil structure.

The auxins type substances were detected by mean of paper chromatography methods (Bukatsch *et al.*, 1956). The production of auxins depend upon the strains and microorganisms and on their age. The maximum IAA was observed in the stationary phase of *Azotobacter* (Brown, 1972) and other species of *Pseudomonas* species and *Bacillus* species.

Many microorganisms producing phytohormones were able to form gibberellins and kinetins, either together with auxins or often alone. *Azotobacter* is one of the microorganisms, which is able to synthesize large amount of biologically active substances. Gibberellic acid (GA₃) and gibberellins like compounds were identified in cultures of various species of *Azotobacter*, *Pseudomonas* and *Bacillus*. Microbial synthesis of gibberellins and other metabolites is an important factor in soil fertility (Katznelson and Kole, 1965). *Azospirillum* spp. are plant growth promoting bacteria whose beneficial effect to be partially due to production of phytohormone including gibberellins (GA₃). The beneficial effect of *Azospirillum* spp. on growth and yield (Okon and Labandera - Gonzales, 1994) or water stress allevation of graminaceous plant of GA production of the bacteria (Kucey *et al.*, 1989).

Karnwal (2009) estimated indole-3-acetic acid production by *Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2 by ELISA as observed by Bano and Musarrat (2003). The concentration of IAA in *Pseudomonas fluorescens* AK1 isolates without tryptophan was 3.1 pmol/ml. A

significant increase in the production of IAA was recorded in the presence of 100, 200 and 500 µg/ml of tryptophan, *i.e.*, 3.8, 5.2 and 6.9 pmol/ml, respectively. Similarly, *P. aeruginosa* AK2 was able to produce IAA without tryptophan in a concentration of 3.3 pmol/ml. A further increase in IAA production (3.9, 4.0 and 4.2 pmol/ml) was observed in the presence of different concentrations of L-tryptophan (100, 200 and 500 µg/ml).

Gibberellins are one of the major groups of growth hormones which play an essential role in the growth and development of plants. They are di-terpenoid acids hydrocarbons produced in good quality by microorganisms. Many microorganisms producing phytohormones were able to form gibberellins and kinetins, either together with auxins or often alone. Gibberellic acid and gibberellins like compounds like compounds were identified in culture of various species of *Azotobacter*, *Pseudomonas* sp. and *Bacilli*. Microbial synthesis of gibberellins and other metabolites is an important factor in soil fertility.

Cytokinins are a group of plant hormone essential for cell division and differentiation in plants. Most occurring cytokinin includes isopentyladenine (IP) and Trans Zeatin (tZ) is derivatives of N₆ prenylated adenine. Plant growth promoting bacteria affect growth of plant by producing metabolites such as plant growth regulators that directly affect the plant growth or by facilitating nutrient uptake by the plant (Glick, 1995). PGPR including *Azotobacter*, *Azospirillum*, *Rhizobium*, *Bacillus* and *Pseudomonas* had been reported to synthesise cytokinins in pure culture (Arshad and Frankenberger, 1993).

5.11 SELECTION OF TWO CULTURAL CONDITIONS FOR HIGH PRODUCTION OF ANTIFUNGAL ACTIVITY AND GROWTH

Understanding which environmental factors are important and how these influences the production of secondary metabolic activities is important. Jisha *et al.*, (2002) found that biological activity and composition of soil microbes are generally affected by many factors including physico-chemical properties of the soil, temperature and vegetation.

Microorganisms require carbon, nitrogen, phosphorus, sulphur, minerals, vitamins and other growth factors. They are sensitive to temperature, oxygen/carbon dioxide in their environment; pH is also a critical factor. Nutrition to support growth is generally not difficult to satisfy since many of the cultures are isolated by growing out on substrate. The challenge is faced to provide the organisms with conditions that allow expression of secondary metabolites and accumulations of unusual metabolites (Demange *et al.*, 1987). There is not such thing as the best culture medium that will allow any microorganisms to express its secondary metabolic capacity and it is impossible to predict what a fresh isolated microorganisms to express its secondary metabolic capacity and it is impossible to predict what a fresh isolated microorganisms will require for metabolite accumulation. A number of media generally employed of expression of secondary metabolism of microorganisms and initial evaluation of media are usually made. Selection of media is complex since the possible variations are so large.

So, the development of media which increase the production of bioactive compounds is very important. Therefore by using this approach chances of finding novel compounds increased and could be worth investigating for microorganism's secondary metabolism. Secondary metabolism becomes; active at later stages of growth phase.

Culture conditions play an important role in cellular growth and also in production of biological activities by microorganisms. As the physiological and nutritional requirement of an organism is genetically predetermined, it is important to provide the appropriate carbon and nitrogen source and also the proper environment for optimal production of activity. So, in our study optimization and standardization of the physical conditions for optimum production of phosphate solubilizing and antifungal activity was done.

5.12 EFFECT OF GROWTH MEDIA

Biosynthesis of bioactive substances is a specific property of some species or even some strains of microorganisms (Issac *et al.*, 1992). This property depends greatly upon conditions of cultivation of microorganisms.

Although a good growth may occur in many media but secondary metabolites may only be produced in a specific medium (Bentley and Keil, 1962). Sometimes a given organisms may produce one metabolite on one medium and a totally different one on another medium (Oxford *et al.*, 1935). Variation in the chemical composition of the medium and its relationship to yield and types of secondary metabolites is well known. Development of medium that produces high yields of desired secondary metabolite is still empirical to a considerable degree.

Different organisms are able to grow in different types of growth media but the growth and biological activity may vary since organisms face different environmental factors affecting the production of biological activity by organisms and provide information for possible parameters affecting biological activity production by organisms *in vitro*. The conducted experiment was focused on the identification of a suitable growth medium for *Pseudomonas* sp. isolates that could induce higher levels of antifungal activity.

In the present study, five standard media, *i.e.* Nutrient Broth, King's B media (KMB), Pigment production media (PPM), Yeast malt (YM), and 1/5 M523 were used for the production of antifungal activity (Rossales *et al.*, 1995). The best medium was found to be King's B medium followed by Yeast Malt media and the minimum production of antifungal activity was observed in pigment production media (PPM) and 1/5 M523 medium at 72h at 25⁰C.

Maximum production of antifungal activity against all the five indicator fungal pathogens by isolates of *Pseudomonas* species was shown after 72h of incubation. Maximum number of isolates produced antifungin PPM medium at 28°C under shake conditions.

Similarly maximum production of antifungal metabolites was observed at 25°C as compared to production at 37°C under shake conditions by all the tested isolates of *Pseudomonas* species from apple. The growth (O.D) of the bacterial isolates was maximum at 25°C after 72h of incubation. So the production conditions for preparations of liquid inoculum and mass multiplication of cells for field trial were fixed as 72h of incubation under shake conditions at 25°C. Generally 28°C temperature is a optimum temperature of growth for almost all the *Pseudomonas* species.

5.13 EXTRACTION AND PRELIMINARY CHARACTERIZATION OF ANTIFUNGAL ACTIVITY

Antibiotic production is an important mechanism by which biocontrol agents, especially PGPR, suppress plant pathogens. However, isolation from natural sources such as roots and soil has been achieved for relatively few antibiotics known to contribute to biological control (Thomashow and Weller, 1998). The basis of antibiosis as a biocontrol mechanism of PGPR has become increasingly better understood over the past few years. A variety of antibiotics have been identified such as 2,4-diacetylphloroglucinol, lipopeptide, phenazine, pyoluteorin, and pyrrolnitrin produced by *Pseudomonads* (Defago *et al.*, 1990; Raaijmakers *et al.*, 1998). Some antibiotics produced by this PGPR group of bacteria are findings new users as experimental pharmaceutical and this group of bacteria may offer an untapped resource for compounds to deal with the alarming ascent of multidrug resistant human pathogenic bacteria. 2,4-diacetylphloroglucinol is an important broad spectrum antibiotic produced by some fluorescent *Pseudomonads* that inhibit a number of phytopathogens. Fluorescent *Pseudomonas* sp. has been studied intensively because of their abundance in the rhizosphere and their abiotics to promote plant growth.either ability stimulating the plant or by suppressing fungal root pathogens (Cook *et al.*, 1993). *Pseudomonads* that produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG or Phl) inhibit a broad spectrum of plant pathogenic fungi and control a variety of root and seedlings diseases (Raaijmakers and Weller, 1998). The population of 2,4-diacetylphloroglucinol producers in the rhizosphere correlated with disease suppressiveness of the soil and in situ antibiotic

production (Raijmakers and Weller, 1998). Diverse 2,4-diacetylphloroglucinol producing *Pseudomonas* sp have been isolated from the rhizosphere of various crop plants and their roles in promoting plant growth and inhibiting root diseases are the subject of ongoing investigations worldwide.

5.13.1 Phenazine

So keeping in view the above studies we here evaluated and characterized the indigenous strains of fluorescent *Pseudomonas* sp. isolated from the rhizosphere of apple growing in normal and replant sites of Shimla district, for antifungal activity along with other direct or indirect plant growth promoting activities viz., siderophores, phosphate solubilization, lytic enzyme. Extracted the antifungal metabolites from 72h old supernatant of the four isolates of *Pseudomonas* species by standard methods that were used for isolation of Phenazine and Pyocyanin (Rosales *et al.*, 1995). Evaluated the extracted antifungal metabolites for preliminary characteristics i.e., antifungal activity against five major indicator test fungi viz; *Fusarium* sp, *Dematophora* sp, *Pythium* sp., *Phytophthora*. (Table 32). These are major soil borne pathogens of apple. Also analysed extracted metabolites for siderophore activity, phosphate solubilizing, proteolytic and chitinase activity by well plate method (Table 31). These molecules i.e., siderophores are also supposed to be major metabolites produced by PGPR that also inhibit the growth of phytopathogens. Analysis of antifungal metabolites by thin layer chromatography and UV-visible spectral properties was also done that give an idea whether extracted antifungal metabolites related to Phenazine group of antibiotics. Our studies corroborate with earlier findings by other workers (Rosales *et al.*, 1995). It is concluded that four strains produce antifungal metabolites Phenazine and pyocyanin, but only phenazine showed inhibitory activity against plant pathogenic fungi. Presence of pyocyanin can be directly observed by the production of blue color and phenazine produce yellow green color. The extracted antifungal metabolites from different isolates of *Pseudomonas* sp. gave Rf value between the range of 0.49 – 0.53 and also showed maximum absorption at wavelength i.e. at 254nm (Plate 8). This study also corroborate with the other studies (Rosales *et al.*, 1995). These

result gave an idea that extracted antifungal metabolites may be related to the group of antibiotic i.e., Phenazine. Further exact characterization whether the extracted metabolites belong to Phenazine or some other group of antibiotic need more study. Further well characterstization for other characteristics of purified metabolites produced by our isolates from apple required more detailed studies. Whether this specific antifungal antibiotic (purified) have any role in biocontrol potential of specific strain and hence in reduction of replant problem of apple require further detailed studies.

5.14 FIELD APPLICATION

5.14.1 Effect of cyclic treatment of best selected PGP strains of fluorescent *Pseudomonas* sp. An-6-shr and An-3-cha on growth of apple plants in replant site.

5.14.1.1 Root colonization and rhizobacterial count

A PGPR that can stimulate plant growth in laboratory will not necessarily have any significant impact on plant in the field unless it is able to persist and grow in the natural environment (Glick, 1995). So the experiment was conducted to study the effect of cyclic treatment of best selected fluorescent *Pseudomonas* sp. An-6-shr and An-3-cha on total bacterial and total *Pseudomonas* count in the rhizosphere soil of apple plants planted in pretreated/bacterized soil of replant problem site pit.

It is generally assumed that root colonization by introduced bacteria is essential for biocontrol of root pathogen and that by increasing the population of introduced bacteria on the root should enhance disease control (Suslow, 1982).

The result on the effect of cyclic treatment of best selected strain of fluorescent *Pseudomonas* sp. An-6-shr and An-3-cha individually as well as their consortium (1:1) each of inoculum density approximately 8×10^8 cfu/ml studied on the root colonization in terms of effect on total bacterial count and on total fluorescent *Pseudomonas* count in the rhizosphere that was observed periodically for 9 months starting from February 2011 to October 2011. Two inoculum

concentrations of each strain and their consortium i.e. full concentration (8×10^8 cfu/ml) and half concentration (4×10^8 cfu/ml) were used in the form of liquid formulation.

After each cyclic application of *Pseudomonas* sp. An-6-shr and An-3-cha there was gradual increase in total *Pseudomonas* count in the bacterized rhizosphere (Table 24) plants of apple. The *Pseudomonas* strains seem to get established upto appropriate concentration in the rhizosphere of treated apple plants. Over all total *Pseudomonas* count increased in all the treatment after 3 and 9 months of treatment (Table 24, Fig 7) as compared to control treatments. The results also indicated towards the possibility of decrease in the total bacterial count in the soil (Table 24, Fig7). Similarly, decrease in fungal count was observed indicating towards the possibility of reduction of pathogenic bacterial and fungal flora might be due to the biocontrol ability of the introduced strains i.e. An-6-shr and An-3-cha.

These results indicated toward the possibility of sufficient increase in number and establishment of fluorescent *Pseudomonas* sp. An-6-shr and An-3-cha in rhizosphere of apple plants planted in replant site.

Our studies are in accordance with some studies in which they have tried to assess the effect of bacterial population size on pathogen population and of disease severity on root cell and growth promotion of apple plants by specific strains of bacteria and of potato plants (Kloepper and Schroth, 1978).

5.14.1.2 Percent Survival, Plant growth promotion in terms of height and number of nodes

With increasing awareness of environment hazards, biobased technologies for sustainable agriculture and biopesticides can provide long lasting effective solutions. The use of PGPR as biofertilizers is one of most promising biotechnologies to improve primary production with low inputs in fertilizer through any of the mechanism possible; biocontrol nutrient mobilization, phytohormones production and nitrogen fixation (Glick, 1995). Interaction between bacteria and roots of plants has been beneficial/harmful or neutral and

the delicate balance is a consequence of both soil and plant type (Latour *et al.*, 1996). The free living bacteria that provide benefit to plant hence been termed as PGPR (Kloepper *et al.*, 1989).

In this study the indigenous strains of fluorescent *Pseudomonas* were screened out for production of multifarious PGP activities affecting directly or indirectly in the growth promotion of plant viz. antifungal, siderophores, ammonia, phosphate solubilizing and proteolytic activities. The results of experimental trial in replant site of the orchard at Maggota (Rohru), the most prone area of Shimla District indicated that the best selected *Pseudomonas* strains were able to increase the growth and establishment of replant apple plants. This might be due to the production potential of these PGP strains for multifarious activities, solubilize phosphate, produce siderophore, proteolytic activities, PGR's, HCN ammonia, as there was maximum percent increase in height of plant, number of nodes and survival over control (Table 23) and antifungal activities. These experiments were tried in replant site first time in India with PGPR, especially fluorescent *Pseudomonas* sp. for the management of replant problem of apple.

Out of 7 strains of *Pseudomonas* sp. we have selected two best strains viz., An-6-shr and An-3-cha depending upon the production of maximum plant growth promoting activities *in vitro*. Best selected strains An-6-shr and An-3-cha were tried in field to study the effect on plant growth parameters like percent survival, plant height, number of leaves/nodes, and soil parameters like moisture, pH and NPK of rhizosphere soil of apple plants planted in the replant problem site of orchards in Magauta (Distt. Shimla)

Seventy plants of apple were selected for bacterization treatment for field trial experiments to study the effect on different growth parameters of planted apple plants. The performance of apple replanted plants after cyclic treatment with fluorescent *Pseudomonas* sp. An-6-shr and An-3-cha in replanted problem site field was much better as compared to control plants in term of all growth parameters viz. percent survival, plant height, number of leaves/nodes. All the

treatment with especially full concentration of individually as well as consortium were found be superior over their respective control In terms of percent survival , growth and number of nodes (Table 23). The study was also supported by the experiment that was performed in *vitro* by evaluating the rhizosphere soil extracts of treated plant for multifarious PGP and antifungal activities (Table 26, 27) and indicated towards that the increasing effect of introduced PGP strains on growth promotion and percent survival might be due to production potential of these strains in the field. The proper establishment and better effect of the PGPR's in the field may also be due to PGP use of these indigenous strains that didn't get much competence from other existing micro flora and also due to their production potential for broad spectrum antifungal activities. This intrinsic property of strains have made them more compatible and over powered by reducing and killing the pathogenic as well as other deleterious flora.

This study was supported by the finding of many workers like Dey (2004) have studied the growth promotion and yield enhancement of peanut by application of plant growth rhizobacteria. Plant growth promoting *Pseudomonas fluorescens* possess the IAA and siderophores production, ammonification, phosphate solubilizing attributes and also inhibited *Asperigillus niger* and *A. flavus* in *vitro*.

Srivastav *et al.*, (1999) who studied colonization of wheat root by fluorescent *Pseudomonas* or wheat root by fluorescent *Pseudomonas* and find that root and shoot length of wheat plant was increased by fluorescent *Pseudomonas* after 30 days growth in non sterilized soil. After 120 days, the difference between root length between untreated control and treated plant was statistically non significant.

5.14.1.3 Available NPK in rhizosphere soil of treated apple plants

Nitrogen (N) is one of the major nutrients required for the nutrition of plants. Total nitrogen present is nearly 90-99% of the organic forms and 1-5% in the inorganic form as ammonium and nitrate ion. Normally a plant contain N in the range of 0.2-4% of dried plant tissue nitrogen found in the horizon of the soil,

mostly in organic material. In the soil solution organic N is gradually transformed into ammonia (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) by microbial processes. Organic N is in itself, of very little use to plants as it cannot be absorbed as such. It is therefore necessary to estimate the difference from of available N (NO_3^- N and NO_2^- N together and hardly exceed 1 percent of the total N in normal soil) it also represent the activities of plant growth promoting rhizobacteria and hence increase the soil fertility due to microbiological actions of beneficial microorganism. The available N in soil refers to a fraction of the total-N which is converted into forms accessible to the plant. The sum total of ammonia and nitrate is smaller than the total-N which becomes available to plants. The difference being attributed to the mineralization of the organic-N during crop growth cycle.

The total K content of micro flora varies from 0.5 to 2.5 percent the total K is distributed in mineral form fixed, non-exchangeable and temporarily retrograded K (1-10%) and exchangeable and water soluble K. The K concentration in the soil solution is quite reduced on an average of 5.20 mg/l. Both water soluble and exchangeable K are most accessible to the plant. Available K can be separated into that, immediately available, which is the water soluble and exchangeable, and that potentially available or fixed. The neutral normal ammonium acetate extract contains both water soluble and exchangeable K so K extracted by this method is equated as available k.

The results on available N,P,K content of rhizospheric soil the of bacterized apple plants planted in replant problem pit (Table 28) indicated towards the possibility that percent increase in available N,P,K content might be due to effects of PGP strains. The *Pseudomonas* species An-6-shr and An-3-cha have showed positive influential effect on the solubilization of complex substrate present in soil rhizosphere.

Chapter-6

SUMMARY AND CONCLUSION

The present study on antifungal activity produced by native strains of *Pseudomonas* species aims at the isolation and selection of fluorescent *Pseudomonas* sp. producing broad spectrum antifungal activity along with other direct or indirect plant growth promoting activities so as to select superior strain that can be used as bioinoculant and biopesticides that may help to manage replant problem of apple at Maggota. The investigation also include production and extraction of antifungal metabolites from these isolates that would also help to select superior strain along with standard as marker for identification of bacterial strains in vitro and in situ.

The fluorescent *Pseudomonas* sp. were screened and characterized for the production of phosphate solubilizing, antifungal, siderophores, HCN and ammonium production so as to select the best plant growth promoting strains for field trials and management of replant problem of apple. Antifungal metabolite extracted from *Pseudomonas* isolates and evaluated for antifungal and other PGP activities and analyzed by TLC for Rf and color of spot.

The salient features of the results are gives below:

- Maximum counts of *Pseudomonas* sp. present in soil samples collected from normal rhizosphere sites of both apple plants as compared to counts present in rhizosphere of replant sites.
- On the basis of morphological, physiological and biochemical characterization, most of the isolates belong to *Pseudomonas fluorescens* and other belongs to *Pseudomonas aeruginosa*.
- Maximum numbers of strains were found resistant for HgCl₂, crystal violet, Kanamycin and streptomycin. No zone production was seen against naphthalene, penicillin and bavistin.

- Maximum *Pseudomonas* sp. isolates showed antifungal activity against *Dematophora* sp., *Phytophthora* sp., *Fusarium* sp., *Pythium* sp. and *Sclerotium* sp. The two isolates viz., An-6-shr and An-3-cha showed antifungal activity against all the five indicator fungi.
- All the isolates of *Pseudomonas* sp. have also been characterized for the production of different plant growth promoting activities *in vitro* viz., Phosphate solubilizing siderophores, HCN, ammonia and protease activity. The two isolates viz., An-6-shr and An-cha were found to be the best in production of plant growth promoting activities.
- The maximum production of antifungal activity was in An-3-cha (52.6 %) and An-6-shr (50.6%) on Nutrient broth and Yeast Malt. The antifungal activity by maximum number of *Pseudomonas* strains was produced on Kings B media and Yeast Malt.
- Thin layer chromatographic analysis of extracted antifungal metabolite on silica gel-G from all fluorescent *Pseudomonas* isolates showed Rf value in the range of 0.49 to 0.53 and development of light green spot after spraying with DSA. The extracted antifungal metabolites in benzene layer showed siderophore and proteolytic activity.
- Benzene layer extracted metabolites showed antifungal metabolites against *Dematophora* sp., *Fusarium* sp., *Phytophthora* sp. and *Pythium* sp. According to literature it was concluded that all the extracted antifungal metabolites belongs to group of broad spectrum antibiotic phenazine which is effective in controlling the soil borne root diseases of plants.
- 16 S rRNA gene analysis was done using specific primers for fluorescent *Pseudomonas* sp *sensu stricto*. or type-1 group. This group compare *P.aeruginosa*, *P.putida* & *P.fluorescens* potential PGPR bacteria.
- The two best selected fluorescent *Pseudomonas* sp. isolates viz., An-6-shr and An-3-cha have shown increase in various plant and soil parameters after the cyclic treatment of one year in replant site pits of apple.
- Results on establishment and survival of apple plant in replant sites and plant growth and other parameters were observed after 9 months

- 70-90% and 70-80 % survival per cent of replanted apple seedlings/plants was observed in treatments with full and half inoculum' concentrations respectively as compare to their respective controls (50%).
- These results indicated towards the possibility of sufficient increase, in number and establishment of fluorescent *Pseudomonas* sp. in rhizosphere (up to $\times 10^6$ to $\times 10^8$ cfu/g soil) of apple seedlings planted in replant problem site area. The results also indicated towards the possibility that the bacterial load of *Pseudomonas* sp. inoculums in the rhizosphere might have decrease the deleterious micro flora that may be pathogenic flora of fungal and bacterial origin.
- The performance of apple replanted seedlings/plants after cyclic treatment with fluorescent *Pseudomonas* in replanted problem site of the field at different locations was much better as compared to control seedlings (treated with water) in term of all growth parameters viz. plant height, plant girth, and number of nodes/leaves etc.
- Increase in all the PGP activities i.e. P-solubilizing, siderophores, lytic enzyme, HCN and ammonia was observed in rhizosphere soil extracts in all the field experiments at different locations. Antifungal activities against four important plant pathogens i.e. *Dematophora*, *Fusarium*, *Pythium* and *Sclerotium* species was found to be increased from 9.2 to 47.2% inhibition of growth of mycelia *in vitro*.
- The pH of soil was found to be 6 before the treatment and pH of 6.5 was observed after the treatment.

It is concluded that all the maximum number of strains has shown antifungal activity. The two best selected strains viz., An-6-shr and An-3-cha has shown maximum plant growth promoting activities and they have also shown increase in various plant and soil parameters after 8 months of cyclic treatment. So these strains can be further used for solving the replant problem to some extent. There is a great scope for increasing the efficiency of the strain so the present study was undertaken to see whether these plant growth promoting rhizobacteria can affect the establishment of apple plants in the replant problem site.

Chapter-7

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DR. Y. S. PARMAR UNIVERSITY OF HORTICULTURE AND FORESTRY
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DEPARTMENT OF BASIC SCIENCE

Title of Thesis : “Studies on the role of antifungal metabolites producing native strains of *Pseudomonas* species to overcome replant problem of apple.”

Name of Student : Kalpana Mehta

Admission Number : F-2009-04-M

Name of Major Advisor : Dr. (Mrs.) Mohinder Kaur

Major field : Microbiology

Minor field(s) : i) Biochemistry
ii) Forest product

Year of award of Degree : 2012

Number of pages in Thesis : 158+II

Number of words in Abstract : 353

ABSTRACT

Under the present investigation the aim was to isolate, identify and select native plant beneficial fluorescent *Pseudomonas* sp. producing antifungal activity from apple growing in normal and replant sites of Shimla district. They all were screened out for the production of other direct and indirect plant growth promoting activities viz; siderophores, HCN, Ammonia, P-solubilizing, lytic enzymes, in vitro and plant growth regulators viz; auxins, gibberellins, and cytokinins. Molecular technique has been standardized that would be used as a tool to identify, compare and characterize different plant root colonizing plant beneficial *Pseudomonas* sp. in vitro and in situ. This would be helpful in selecting strain of fluorescent *Pseudomonas* isolates for inoculants culture analysis, rapid differentiation & identification of species, and detection of strain relatedness. For **16S rRNA gene analysis** specific primers were used so to find their taxonomic affiliation to fluorescent *Pseudomonas* group (sensu stricto) sub group of gamma proteobacteria and to three most potential PGPR bacteria ie *P.aeruginosa*, *P.putida* & *P.fluorescens*. two strains An-6 shr and an-3-cha that were used in field trials showed 96-98% similarity to *P.aeruginosa*, *P.putida* and *P.fluorescens* after performing NCBI BLAST with their 16s rRNA gene sequences.

Five different media NB, PPM, 1/5M523, YM and King's B medium for growth and high production of antifungal activity against five different fungal pathogens. Also time of incubation and temperature of incubation were standardized for mass multiplication of inoculums for field trials. The antifungal metabolites were extracted with benzene and chloroform and were tentatively identified on thin layer chromatography (TLC) at R_f 0.5, 0.5, 0.49 and 0.51. Antifungal activities of *Pseudomonas* sp. help in suppression of plant pathogenic fungus in soil and these may be the reasons of better establishment of apple seedling treated with fluorescent *Pseudomonas* strain and results in increase in *Pseudomonas* count and decreased total rhizobial count. Therefore these strains can be exploited for the management of replant problem of apple. These selected genotypically and phenotypically characterized strains of *Pseudomonas* sp. which were found to be potent and can have great importance in the field of agriculture, especially in the treatment of apple plants in replant problem area.

Signature of Major Advisor

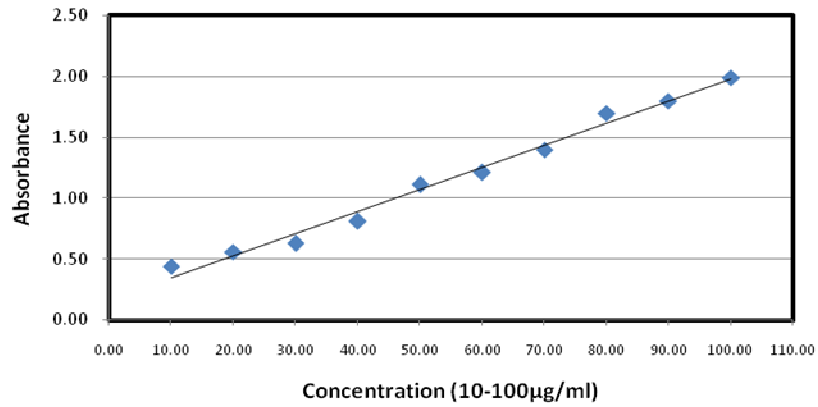
Countersigned

Signature of the Student

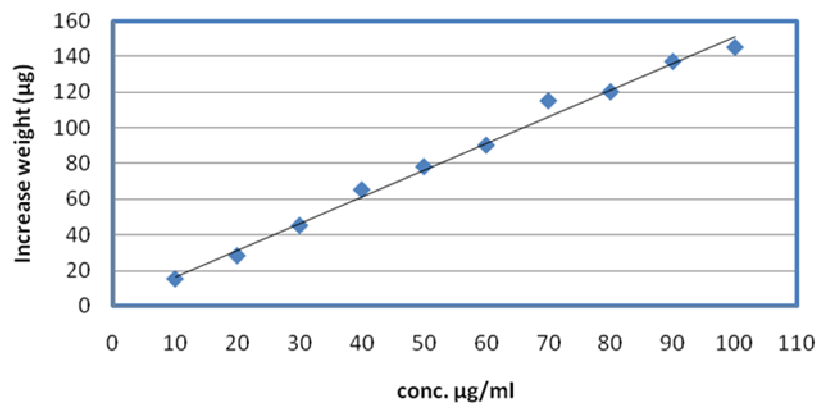
Professor and Head
Department of Basic Sciences
Dr. YSP, UHF, Nauni, Solan (H.P.)

APPENDIX-I

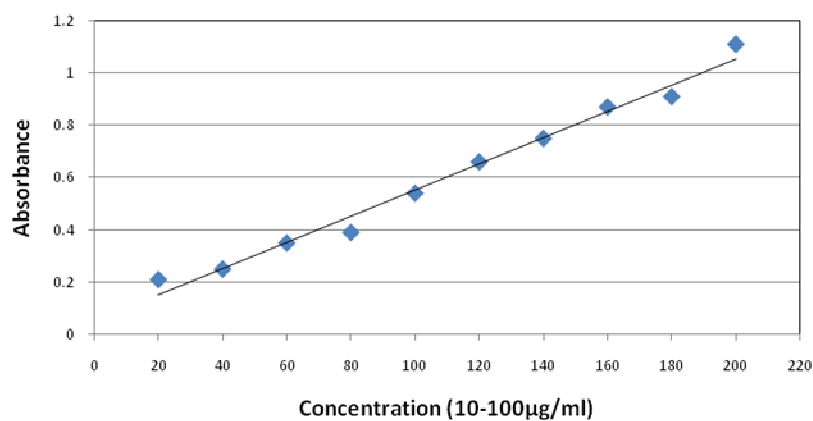
Standard curve for Gibberellins



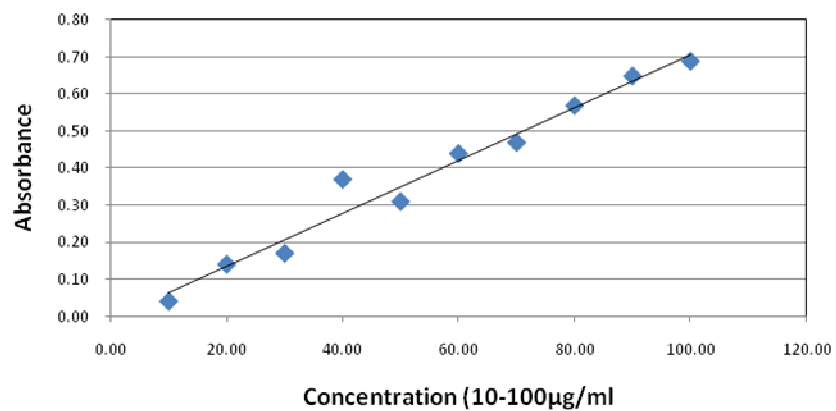
Standard curve for cytokinins



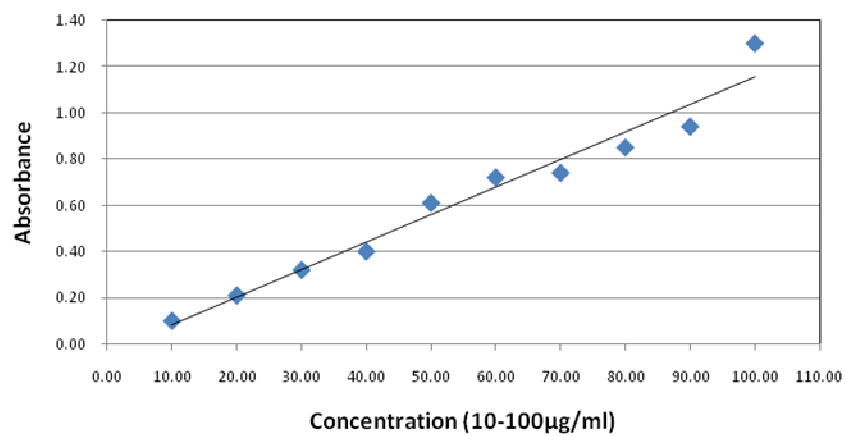
Standard curve of Auxin



Standard curve for Phosphate solubilization



Standard curve for Proteolytic assay



APPENDIX-II

Anova Table . Growth Inhibition by HgCl₂

Source of variation	Df	MSS	F
Treatment	6	241.14	316.50
Error	42	0.76	
Total	62		

Anova Table Growth Inhibition by crystal violet

Source of variation	Df	MSS	F
Treatment	6	420.14	802.09
Error	42	0.52	
Total	62		

Anova Table Growth Inhibition by Kanamycin

Source of variation	Df	MSS	F
Treatment	6	1264.2	1659.31
Error	42	0.76	
Total	62		

Anova Table Growth Inhibition by Streptomycin

Source of variation	Df	MSS	F
Treatment	6	589.81	952.77
Error	42	0.61	
Total	62		

Anova Table Phosphate solubilizing plate assay

Source of variation	Df	MSS	F
Treatment	6	56.71	30.54
Error	14	1.85	
Total	20		

Anova Table Phosphate solubilizing Quantitative assay

Source of variation	Df	MSS	F
Treatment	6	821.10	387.31
Error	14	2.12	
Total	20		

Anova Table Siderophore plate assay

Source of variation	Df	MSS	F
Treatment	6	20.85	20.85
Error	14	1.00	
Total	20		

Anova Table Siderophore Quantitative assay

Source of variation	Df	MSS	F
Treatment	6	33.86	44.60
Error	14	0.59	
Total	20		

Anova Table Proteolytic plate assay

Source of variation	Df	MSS	F
Treatment	6	4.42	3.10
Error	14	1.42	
Total	20		

Anova Table Proteolytic Quantitative assay

Source of variation	Df	MSS	F
Treatment	6	1151	366.50
Error	14	3.14	
Total	20		

Anova Table Auxin Production

Source of variation	Df	MSS	F
Treatment	6	82.42	44.38
Error	14	1.85	
Total	20		

Anova Table Gibberellin production

Source of variation	Df	MSS	F
Treatment	6	79843	5127.5
Error	14	15.57	
Total	20		

Anova Table Cytokinin production

Source of variation	Df	MSS	F
Treatment	6	1942.9	715.7
Error	14	2.71	
Total	20		

Anova Table Antifungal activity against *Dematophora* sp

Source of variation	Df	MSS	F
Treatment	6	1161.2	181.86
Error	14	6.3	
Total	20		

Anova Table Antifungal activity against *Phytophthora* sp

Source of variation	Df	MSS	F
Treatment	19	1093.9	1203.52
Error	40	0.908	
Total	60		

Anova Table Antifungal activity against *Fusarium* sp

Source of variation	Df	MSS	F
Treatment	6	1376.5	198.8
Error	14	6.92	
Total	20		

Anova Table Antifungal activity against *Sclerotium* sp

Source of variation	Df	MSS	F
Treatment	6	1303.8	1149.14
Error	14	1.13	
Total	20		

Anova Table Antifungal activity against *Pythium* sp

Source of variation	Df	MSS	F
Treatment	6	13.76	204.20
Error	14	0.067	
Total	20		

Anova Table Effect of antifungal metabolite on siderophore production plate assay

Source of variation	Df	MSS	F
Treatment	5	170	255.15
Error	12	0.66	
Total	17		

Anova Table Effect of antifungal metabolite on proteolytic activity

Source of variation	Df	MSS	F
Treatment	5	165.20	247.80
Error	12	0.66	
Total	17		

Anova Table Antifungal activity from metabolite against *Dematophora* sp.

Source of variation	Df	MSS	F
Treatment	5	699.24	233.27
Error	12	2.99	
Total	17		

Anova Table Antifungal activity from metabolite against *Fusarium* sp.

Source of variation	Df	MSS	F
Treatment	5	828.00	472.42
Error	12	1.75	
Total	17		

Anova Table Antifungal activity from metabolite against *Phytophthora* sp.

Source of variation	Df	MSS	F
Treatment	5	243.01	247.96
Error	12	.98	
Total	17		

Anova Table Antifungal activity from metabolite against *Pythium* sp.

Source of variation	Df	MSS	F
Treatment	5	12.43	238.89
Error	12	0.052	
Total	17		

Anova Table Antifungal activity from metabolite against *Sclerotium* sp.

Source of variation	Df	MSS	F
Treatment	5	820.33	224.75
Error	12	3.64	
Total	17		

Anova Table Effect of time of incubation on antifungal activity against *Dematophora* after 48h.

Source of variation	Df	MSS	F
Treatment	6	3617.8	1945.46
Error	70	1.86	
Total	104		

Anova Table .. Effect of time of incubation on antifungal activity against *Fusarium* after 48h.

Source of variation	Df	MSS	F
Treatment	6	2904.3	697.99
Error	70	4.16	
Total	104		

Anova Table .. Effect of time of incubation on antifungal activity against *Phytophthora* after 48h.

Source of variation	Df	MSS	F
Treatment	6	1877.9	500.91
Error	70	3.74	
Total	104		

Anova Table Effect of time of incubation on antifungal activity against *Pythium* after 48h.

Source of variation	Df	MSS	F
Treatment	6	2874.4	1805.15
Error	70	1.59	
Total	104		

Anova Table Effect of time of incubation on antifungal activity against *Sclerotium* after 48h.

Source of variation	Df	MSS	F
Treatment	6	3056.6	1405.73
Error	70	2.17	
Total	104		

Anova Table Effect of time of incubation on antifungal activity against *Dematophora* after 72h.

Source of variation	Df	MSS	F
Treatment	6	3523.0	1818.39
Error	70	1.93	
Total	104		

Anova Table Effect of time of incubation on antifungal activity against *Fusarium* after 72h.

Source of variation	Df	MSS	F
Treatment	6	2773.2	2177.28
Error	70	1.27	
Total	104		

Anova Table Effect of time of incubation on antifungal activity against *Phytophthora* after 72h.

Source of variation	Df	MSS	F
Treatment	6	2464.5	1669.89
Error	70	1.47	
Total	104		

Anova Table Effect of time of incubation on antifungal activity against *Pythium* after 72h

Source of variation	Df	MSS	F
Treatment	6	3411.2	2403.89
Error	70	1.41	
Total	104		

Anova Table ... Effect of time of incubation on antifungal activity against *Sclerotium* after 72h.

Source of variation	Df	MSS	F
Treatment	6	2769.4	1550.16
Error	70	1.78	
Total	104		

Anova Table .. Effect of temperature of incubation on antifungal activity against *Dematophora* at 25°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	3541.4	1598.01
Error	70	2.21	
Total	104		

Anova Table .. Effect of temperature of incubation on antifungal activity against *Fusarium* at 25°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	2764.8	1781.13
Error	70	1.55	
Total	104		

Anova Table Effect of temperature of incubation on antifungal activity against *Phytophthora* at 25°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	3126.2	1737.69
Error	70	1.79	
Total	104		

Anova Table .. Effect of temperature of incubation on antifungal activity against *Pythium* at 25°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	3195.3	2631.20
Error	70	1.21	
Total	104		

Anova Table .. Effect of temperature of incubation on antifungal activity against *Sclerotium* at 25°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	2716.0	1302.02
Error	70	2.08	
Total	104		

Anova Table .. Effect of temperature of incubation on antifungal activity against *Dematophora* at 37°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	3625.7	1815.82
Error	70	1.99	
Total	104		

Anova Table Effect of temperature of incubation on antifungal activity against *Fusarium* at 37°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	2425.1	1242.91
Error	70	1.95	
Total	104		

Anova Table .. Effect of temperature of incubation on antifungal activity against *Phytophthora* at 37°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	2097.6	892.31
Error	70	2.35	
Total	104		

Anova Table .. Effect of temperature of incubation on antifungal activity against *Pythium* at 37°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	2985.9	1963.01
Error	70	1.52	
Total	104		

Anova Table Effect of temperature of incubation on antifungal activity against *Sclerotium* at 37°C after 72h.

Source of variation	Df	MSS	F
Treatment	6	2503.9	1095.59
Error	70	2.28	
Total	104		

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10+2	Second (53%)	Himachal Pradesh Board of School Education, Dharamshala, H.P.	2004
B.Sc. Biotechnology	First(79%)	Punjab Technical University, Jalandhar.	2009

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Government/Univ./SAARC

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any other financial assistance
received during study period

(Kalpana Mehta)