PRELIMINARY CHARACTERIZATION OF FLUORESCENT *PSEUDOMONAS* DIVERSITY PRODUCING PLANT GROWTH REGULATORS IN NORMAL AND REPLANT SITE OF APPLE AND PEAR

Thesis

by

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CERTIFICATE-I

This is to certify that the thesis entitled, **"Preliminary characterization of fluorescent *Pseudomonas* diversity producing plant growth regulators in normal and replant site of apple and pear "** submitted in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE** in **Basic Sciences (Microbiology)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) is a record of bonafide research work carried out by **Miss. Ritika Kapoor (F-2007-17-M)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

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

Place: Nauni-Solan
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CERTIFICATE-II

This is to certify that the thesis entitled, **“Preliminary characterization of fluorescent *Pseudomonas* diversity producing plant growth regulators in normal and replant site of apple and pear”** submitted by **Miss Ritika Kapoor (F-2007-17-M)** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE** in **Basic Sciences (Microbiology)** has been approved by the Student’s Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"Preliminary characterization of fluorescent *Pseudomonas* diversity producing plant growth regulators in normal and replant site of apple and pear"** submitted to Dr Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan (HP) by **Miss. Ritika Kapoor (F-2007-17-M)** in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE** in **Basic Sciences (Microbiology).**

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ABBREVIATIONS USED

%	per cent
IAA	indole-3-acetic acid
GA_3	gibberellic acid
^{0}C	degree celcius
cm	centimeter
et al.	and others
Fig.	figure
g	gram
U. V	ultra violet
nm	nano meter
O.D	optical density
Kg	kilogram
PGPR	plant growth promoting rhizobacteria
PGRs	plant growth regulators
ACC	amino cyclopropane-1-carboxylic acid
TLC	thin layer chromatography
Rf	resolution front
g/l	grams per liter
h	hour
M	molar
min	minute
mg	milli gram
mg/l	milligram per liter
mM	mill molar
mm	Millimeter
N	normal
rpm	revolutions per minute
µl	microlitre
µg	microgram
µg/ml	microgram per milli liter
v/v/v	volume by volume by volume
w/v	weight by volume
PSB	Phosphate solublizing bacteria

Chapter-1

INTRODUCTION

The replant problem has become very serious problem in horticultural crops and it is distributed worldwide commonly encountered in establishing new orchards on old sites (Mai and Abawi, 1981). Poor growth of newly planted trees (pear and apple) is common in replanted orchards. The disease is a complex syndrome that affects young trees in replant sites and reduces growth survival and yield of replanted trees. The possible solution to the problem is to introduce appropriate novel and indigenous important plant growth promoting bioagents so as to have better and effective root colonization and to inoculate planting material to soil.

Pseudomonas species are diverse and versatile group of indigenous microflora of almost all horticulture and forestry crops. They directly enhance the plant growth by increasing the available nutrients through variety of mechanisms like production of siderophores that chelate iron and make it available to the plant roots, and as biocontrol agents that are able to protect plant from infections by phytopathogenic organisms through production of antibiotics, siderophores, HCN, ammonia, cell wall degrading enzymes, solubilization of minerals such as phosphorus and synthesis of plant growth regulators (Glick, 1995). PGPR may have one or more combination of these mechanisms in the rhizosphere and promote the growth and protect the roots of plants and may also help them to establish under harsh and stress conditions. Fluorescent *Pseudomonas* species have emerged as largest and potentially most promising group of plant growth promoting rhizobacteria. Their potential to synthesize different secondary metabolites with diverse and multiform activities is an important function of soil fertility and sustainability of crops. Their potential to synthesize different secondary metabolites with diverse biological activities is the important function of soil fertility and sustainability of crops. They affect the soil properties, chemical composition of plant and their health.

The large scale application of PGPR's to crops especially apple and pear in replant site as inoculants would be attractive as it would substantially reduce the use of chemical fertilizers under these harsh and stress conditions and also promote to improve their growth. Hence they may help to solve the replant problem of apple and pear.

Plant growth regulators are the organic substances that influence physiological processes of plants at very low concentrations when produced endogenously by plants, they are referred to as phytohormones (plant hormones), where as the term plant growth regulators (PGRs) include many synthetic and naturally occurring compounds. Nickell (1982) defined this term i.e. plant growth regulators as " either natural or synthetic compounds including microbial plant growth regulators that are applied directly to a target plant to alter its life processes or its structure to improve quality, increase yields or facilitate harvesting". The production of plant growth regulators induces additional root hair and lateral root formation (Tien *et al.*1979). Thereby enhancing the plant's ability to take up nutrients from soil and increasing the yield. Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrient from the environment, thus enhancing the chance of survival.

Most root promoting bacteria synthesize indole acetic acid (IAA) and their effects on plant mimic that of exogenous indole acetic acid (Alvares *et al.,* 1989; Meuwley and Pilet, 1981). Microorganisms inhabiting rhizosphere of various plants are likely to synthesize and release auxins as secondary metabolites. Improved plant growth via plant associated bacteria has been reported in many plants microbes interactions. Bacteria that inhabit the rhizosphere may influence plant growth directly by contributing to host plants endogenous pool of plant growth regulators; indole acetic acid (Lee *et al*., 2004); via phosphate solubilization activity, by enhancing bacterial root and production of siderophores. Microbial synthesis of plant growth regulators is an important factor of soil fertility (Kampert and Sterezyk, 1975). Gibberellins, cytokinins and indole acetic acid are secondary metabolites which are important biotechnology and economically products produced commercially from fungi for agricultural and horticultural industry (Decan, 1984).

So it is important to study and understand some of plant growth promoting traits, the production and characterization of bacterial plant growth regulators from the plant growth promoting indigenous rhizobacteria. So that they can be characterized, developed and mass multiplied as potent PGPR indigenous strains of fluorescent *Pseudomonas* sp. producing plant growth regulators for management of replant problem in Mandi district of Himanchal Pradesh.

Hence the study is proposed with the following objectives:

1. Isolation, enumeration, identification and characterization of fluorescent *Pseudomonas* producing plant growth regulators (auxins, gibberellins, cytokinins).
2. Preliminary characterization of plant growth regulators.

CHAPTER-II

REVIEW OF LITERATURE

The rhizosphere of plant is an important soil ecological environment for plants and microbial interactions. The vast variety of beneficial microorganisms' interactions can be useful, harmful or neutral (Beringer, 1990). Not much work has been carried out on rhizosphere of pear and apple for the influence and characterization of plant growth promoting and disease suppressing metabolites produced by plant growth promoting rhizobacteria especially *Pseudomonas* sp. and also for replant problem.

Decrease in growth was found after planting apple rootstocks which is referred to as "Specific Replant Disease" (SRD) (Hoestra, 1968). Besides the SRD on apple fruit trees, however a "Specific pear replant disease" has also been found (Benson, 1973). Number of reports suggesting that the soil-borne pathogens: fungi, actinomycetes and saprophytic phytotoxic microorganisms are the main biological cause of SRD (Hein, 1974; Castka, 1993).

One of the factors giving rise to soil sickness in apple orchards is the rhizosphere microflora (Catska *et al*., 1982). The composition of the microbial population in the rhizosphere changes with increasing age of apple trees. The presence of fluorescent *Pseudomonas* in the rhizosphere of old apple was rare, but the planting of apple seedlings into sick soil induced their proliferation. Apple replant disease is complex syndromes that affect young trees in replanted orchard site causing necrotic lesions on feeder roots, stunted tree growth and reduced cumulative growth (Yao *et al*., 2006 and Rumberger *et al*., 2007). This is a complex disease and is often encountered in establishing new orchards on old sites. Methyl bromide (MB) has been the fumigant used most widely to control Apple Replant Disease (ARD). However soil fumigation suppressed ARD in only 50% of all replanted orchards. Soil bacteria, plant parasitic nematodes, fungi, actinomycetes and oomycetes have all been the causative agent of ARD. So, *Pseudomonas* species is the alternative source in the suppression of ARD (Mazzola, 2007).

Among some beneficial rhizosphere microorganisms the fluorescent *Pseudomonas* are mainly known to stimulate the growth of several annual crops (Kloepper *et al*., 1980) or perennial trees. Symptoms of the SRD have shown to decrease by plant-growth promoting (PGPR) *fluorescent- putida* type *Pseudomonas* rhizobacteria inoculated in several ways.

2.1 ISOLATION AND CHARACTERIZATION OF RHIZOSPHERE BACTERIA

The microorganisms from the rhizosphere can be isolated by media enrichment technique in which the specific media is chosen and supplied with some specific nutrients that are required for particular types of microbes. There are various beneficial microorganisms in rhizosphere of plant (Beringer, 1990). Beneficial effects may be due to the production of secondary metabolites such as antibiotics (Howell and Stipanovic , 1980). Hydrogen cyanide (Kunz and Nagappan, 1989), production of iron-chelating siderophores (Kumar and Dube, 1993), phosphate solubilization (Glick, 1995; Dave and Patel, 1999) and ammonia production (Lata & Saxena 2003). The production of secondary metabolites has been generally studied in submerged cultures. Bu' Lock (1961) observed under these conditions viz., growth phase and production phase. It has been observed that at the start of fermentation, all the nutrients were present in excess. After the depletion of any one of the nutrients, growth rate diminishes. During this stage of limited growth metabolisms of microorganisms became imbalanced (Buckland *et al*., 1975). Secondary metabolism is restricted to plant and microorganisms and is species or strain specific.

The secondary metabolites produced by bacteria and fungi have been classified into nine families and sub-families, on the basis of their chemical structure. These compounds are reported to vary in size from small amino acid derivative to much larger monocyclic compounds. Two of these, the macrolides and cyclic peptides are unique and exhibit biological activities. Berdy (1974), Omura and Nakagawa (1984) noted that macrolides possessed antiparasitic, antibacterial and antifungal activity. Secondary metabolites are now being used for applications other than as antibacterial, antifungal and antitumor agents. These applications included are against parasites and insects as well as for animal and plant growth stimulation, immuno-suppression and other pharmacological activities. Further application are possible in various areas of pharmacology and agriculture, industrial, biological and medicinal purposes with wide range of biological activities i.e. antibacterial, antifungal, antiviral, proteolytic and insecticidal etc. found in the molecules. The rhizosphere plant involves colonization by a variety of naturalistic or parasitic relations with the plant depending upon the type of microorganisms, soil nutrients status, plant defense system and soil environment (Srivastava *et al*., 1999).

Microorganisms that grow in the rhizosphere are ideal for use as biocontrol agents since the rhizosphere provide the front line defense for roots against attack by pathogen (Weller, 1988). *Pseudomonas* species are receiving much attention as biocontrol agents.

Van and Schippers (1989) has shown that *Fusarium* wilt of stem was significantly reduced by root bacterization with *Pseudomonas* sp. strain SC 5417. Kloepper *et al*. (1980) has reported that wilt conductive soil was rendered temporarily suppressive to *Fusarium* wilt to flax when a strain of *Pseudomonas* sp. or its purified siderophore was added. Similarly, Scher and Baker (1982) found that strain of *Pseudomonas putida* suppressed the incidence of *Fusarium* wilt of flax, cucumber and radish when incorporated into wild conductive soil.

2.2 PRODUCTION OF PLANT GROWTH REGULATORS BY PGPR's

AUXINS

Plant growth promoting rhizobacteria (PGPR) are considered to promote plant growth directly or indirectly. *Pseudomonas* bacteria, specially *P. fluorescens* and *P. putida* are the most important kinds of PGPR (Khakipour *et al*., 2008). Production of auxin is one of the main reasons to promote yield because of inoculation with this bacteria. In this research fifty strains of fluorescent *Pseudomonas* belonging to microbial bank of Soil and Water Research Institute, isolated from Iran soils, selected and evaluated for secretion of auxin compounds. In HPLC device, 72% of the strains exuded at least one type of indole auxin composites. The amount of exuded IAA by *P. fluorescens* strains was varied from zero to 31.6 mg/l while it was producing from zero to 24.08 mg/l in *P.putida*. The amount of exuded IAM by *P. fluorescens* and *P. putida* was between 0-16.2 and 0-17.2 mg/l, respectively. These strains also exuded 0-7.2 mg/l ILA for P. fluorescens and 0-10 mg/l for *P.putida*. Neither of experimented strains exuded the IBA. The results showed that 65% of the studied *P. fluorescens* used indole acetamide (IAM) pathway to synthesize IAA and 35% used the IAM and IPyA path, while 48% of the *P. putida* through IAM, 41% through both paths and 7% used the only IPyA path towards IAA synthesize. But 78% of the strains studied in spectrophotometery exuded auxins which their amounts were producing between 0-7.09 mg/l for *P. fluorescens* and 0-4.40 mg/l for *P. putida* strains.

Buvana (2002) reported that inoculation of rice CV ADT 36, CO 47, IR 50 and maize CV C01 with *Azorhizobium* in combination with cell wall degrading enzyme, growth regulators, 2,4-D and flavonoid naringenin induce nodule like structure and increased shoot growth, number of lateral rootlets and nitrogenase activity. Further 2, 4-D induced the lateral root formation and also acted as stimulator for rhizobial colonization

According to Dilfuza (2007) the growth promotion of winter wheat after inoculation showed that most of bacterial strains were responsible for increasing only the root dry weights. There were no significant effects on shoot growth. All bacterial strains produce IAA regardless of their origin, rhizosphere and phyllosphere. The highest concentraton of IAA (2.0 µg/ml to 2.70 IAA ml^{-1} filterate) were produced by bacterial strains *Pseudomonas fluorescence* isolated from rhizosphere of peas.

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 IAA 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 were on average 35 to 50 percent longer than the roots from seeds treated with the indole-3-acetic acid deficient mutant and roots from uninoculated seeds.

From a total of 30 fluorescent *Pseudomonas* isolates (15 *P. fluorescens* and 15 *P. aeruginosa*) were obtained from different plant rhizosphere and were characterized on the basis of biochemical tests and plant growth-promoting activities. *Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2 showed the best plant growth-promoting activity. These isolates were tested for their ability to produce indole acetic acid in pure culture in the absence and presence of L-tryptophan at 50, 100, 200 and 500 µg/ml. For both strains, indole production increased with increases in tryptophan concentration (0.5, 1.2, 4.3 and 9.3 µg/ml; and 0.2, 0.7,3.8, and 8.3 µg/ml, respectively). *P. aeruginosa* AK2 was less effective in production of indole acetic acid than P. fluorescens AK1. Inoculation of rice seeds with *P. fluorescens* AK1 and *P. aeruginosa* AK2 showed a good level (2.30 pmol/ml and 2.1 pmol/ml) of indole acetic acid compared to uninoculated seeds (1.6 pmol/ml) (Karnwal, 2009).

Barea and Agilar (1982) reported that *Glomus mosseae ,* a representative species of Endogonaceae (Phycomycetes) able to form vescicular-arbuscular mycorrhiza, was investigated for phytohormones production. Spores of *G.mosseae* were axenically germinated in water and the resultant mycelium growth was assayed by standard procedures for extracting plant hormones from microbial cultures. Paper partition chromatography and specific bioassays were used to separate and identify plant growth-regulating substances, one with R_f corresponding in position to authentic gibberellic acid, and four substances with the properties of cytokinins.

Farah Ahmad *et al*. (2005) observed that total of 21 bacterial isolates (*Azotobacter* sp., 10 and fluorescent *Pseudomonas* sp., 11) were isolated from different rhizospheric soils in the vicinity of Aligarh city and characterized as per standard methods. These isolates were further tested for the production of indole acetic acid (IAA) in a medium with 0, 1, 2 and 5 mg/ml of tryptophan. A low amount (2.68-10.80 mg/ml) of IAA production was recorded by *Azotobacter* strains without tryptophan addition. Seven *Azotobacter* isolates showed high level (7.3 to 32.8 mg/ml) production of IAA at 5 mg/ml of tryptophan while at 1 and 2 mg/ml the production was in the range of 1.47 to 11.88 and 5.99 to 24.8 mg/ml, respectively. Production of IAA in fluorescent *Pseudomonas* isolates increased with an increase in tryptophan concentration from 1 to 5 mg/ml in the majority of isolates. In the presence of 5mg/ ml of tryptophan, 5 isolates of *Pseudomonas* produced high levels (41.0 to 53.2 mg/ml) of IAA while 6 other isolates produced IAA in the range of 23.4 to 36.2 mg/ml. Production of IAA was further confirmed by extraction of crude IAA from 3 isolates of *Azotobacter* (Azs1, Azs6 and Azs9) and three isolates of *Pseudomonas* (Ps1, Ps4 and Ps7) and subsequent TLC analysis. A specific spot from the extracted IAA preparation was found corresponding with the standard spot of IAA with same Rf value. *Pseudomonas* isolates (Ps1, Ps4 and Ps7) showed inhibitory effects on the growth of root elongation of *Sesbania aculeata* and *Vigna radiata* at all concentrations of tryptophan compared to the control. However, the isolates of *Azotobacter* (Azs1, Azs6 and Azs9) demonstrated stimulatory effects on both plants. Increasing the concentration of tryptophan from 1 mg/ml to 5 mg/ml resulted in decreased growth in both *S. aculeata* and *V. radiata*. On a comparative basis isolate Azs9 was most promising in promoting plant growth. On the other hand, high concentration of exogenous tryptophan could exhibit toxic effects on plant growth.

Bhatia *et al.* (2005) studied the isolates of fluorescent *Pseudomonas* that were isolated from the rhizosphere of sunflower, potato, maize and groundnut. All the isolates produced fluorescent pigment in succinate broth and displayed siderophores production, production of hydrogen cyanide (HCN) and indole acetic acid (IAA) and root colonization by all the isolates resulting in plant growth promotion.

The ability to produce the plant hormone IAA is wide spread among fungi and bacteria (Hutzinger, 1970). Micro-organisms, which commonly inhibit aerial or subterranean surfaces of plants, have been shown to be capable of indole acetic acid synthesis (Wichner and Libbert, 1968). Such micro-organisms include rhizobia (Johri et al., 1997), mycorrhizal fungi (James et al., 1985). *Pseudomonas* (Loper and Buyer, 1991), *Azospirillum brasilense* (Tien et al., 1979) and *Azotobacter paspali* (Barea and Brown, 1974).

Ansar *et al*. (2009) reported that the inability to induce adventitious roots is often a limiting factor in conventional cuttings and tissue culture. In this study, several criteria were taken into consideration in determining the best rooting treatment in olive cultivar Moraiolo. Among the indolebutyric acid (IBA) and naphthaleneacetic acid (NAA) hormones, tested for maximum percentage of rooted shoots, root number, root length and quality of roots, IBA at 1.5 mg l-1 concentration proved to be the best one for rooting of Moraiolo cultivar of olive producing maximum root initiation in 86.67% shoots, 5.03 number of roots per rooted explant and 4.95 cm root length. The roots produced on IBA were longer with better quality shoots whereas NAA produced poor response with necrotic leaves and leaf abscission.

Sridevi and Millaiah (2007) observed that indole-acetic acid production by *Rhizobium* isolates started after 24h and reached to maximum after 72h when bacteria reached stationary phase of growth, then decreased slowly. The pattern of growth was similar in all these isolates but they differed only in IAA production.The decrease in IAA production after 72h might be due to release of IAA oxidase and peroxidase. The isolates preferred L-tryptophan for maximum IAA production. The effect of different concentration of L-tryptophan revealed maximum growth and IAA production were observed at 3 µg/ml.

It is clear that *Rhizobium* isolates differ significantly in auxin production. the ability of *Rhizobium* isolates to produce IAA in tryptophan supplemented medium suggested the possibility that symbiont was responsible for higher IAA content of root nodule.

Claude (1996) attempted to improve the accuracy of Avena coleoptile straight growth test for auxin. The modifications introduced resulted in a substantial improvement of commonly used coleoptile elongation test. The proposed test retains the simplicity of physical requirement and of manipulation characteristic of all elongation tests. The main feature of proposed test is that it permits the measurement of elongation of coleoptile's segments as being directly proportional to the concentration rather than to logarithmic concentration of IAA

Khalid *et al*. (2004) reported that a large number of bacteria were isolated from the rhizosphere soil of wheat plants grown at different sites. Thirty isolates showing prolific growth on agar medium were selected and evaluated for their potential to produce auxins *in vitro*. Colorimetric analysis showed variable amount of auxins (ranging from 1.1 to 12.1 mg/ml) produced by the rhizobacteria in vitro and amendment of the culture media with 1-tryptophan (1-TRP), further stimulated auxins biosynthesis (ranging from 1.8 to 24.8 mg/ml). HPLC analysis confirmed the presence of indole acetic acid and indole acetamide (IAM) as major auxins in culture filtrates of these rhizobacteria. A series of laboratory experiments conducted on two cultivars. Of wheat under genobiotic (axenic) conditions demonstrated increase in root elongation (17.3%), root dry weight (up to 13.5%), and shoot dry weight (36.3%) of inoculated wheat seedlings. Linear positive correlation (r=0.99) between in vitro auxins production and increase in growth parameters of inoculated seeds was found. Based upon auxins biosynthesis and growth promoting activity, four isolates were selected and designated as plant growth promoting rhizobacteria (PGPR). Auxins biosynthesis in sterilized verses non sterilized soil inoculated with selected PGPR was also monitored that revealed superiority of the selected PGPR over indigenous micro flora. Peat based seed inoculation with selected PGPR isolates exhibited stimulatory effects on grain yields of tested wheat cultivars. In pot (up to 14.7% increase over control) and field experiments (up to 27.5 % increase over control); however, the response varied with cultivars. and PGPR strains.

Production of indole acetic acid under different cultural conditions by *Rhodopseudomonas palustris, Rhodobacter sphaeroides, Rhodocyclus gelatinosus* and *Rhodocyclus tenicis* (Munjam *et al.*2002). Indole acetic acid production increased in the presence of tryptophan. L-glutamic acid was best nitrogen source for production by *Rhodocyclus gelatinous, Rhodobacter sphaeroides* responded almost uniformly to different nitrogen sources. Illumination conditions favoured the indole acetic acid production in *Rhodobacter sphaeroaides* while in *Rhodocyclus* preferred malate as electron donor followed by succinate, while *Rhodobacter* preferred formate followed by malate for indole acetic acid production.

The *Rhizobium* species isolated from healthy and mature root nodules of leguminous trees *Dalbergia lenceolaria* preferred mannitol and KNO_3 for the growth as carbon and nitrogen source (Ghosh and Basu, 2001). The bacterium produced a high amount (22.3µg/ml) of IAA and L-tryptophan supplemented in basal medium. Growth of indole acetic acid and production was maximum when it reached the stationary phase of growth. The cultural requirements were optimized for maximum growth and indole acetic acid production by the *Rhizobium* species was increased by 270.8 percent over control when the medium was supplemented with mannitol (1 percent W/V), sodium dodecyl sulphate (SDS) (1mg/ml), L-asparaginine (0.02 percent W/V) and biotin (1µg/ml) in addition to L-tryptophan (2.5)mg/ml).

Maria *et al*. (2000) investigated that ethylacetate extracts from superimposed liquid concentrated cell cultures of *Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas putida, Serratia sp.* and *Klebsiella pneumoniae starins* obtained from the rhizosphere of rice cultivated in Tolima region, Colombia S. A., have shown to be producers of extracellular indole-3-acetic acid at concentrations from 3.5mg/ml. *Azotobactervinelandii*, and *Klebsiella pneumoniae* yielded the highest concentrations. *Pseudomonas* sp. was found in vitro to antagonize the *Phytophyhora infestans,* mainly by the production of antibiotics were additional activities observed.

Lee *et al*. (2004) reported that *Gluconacetobacter diazotrophicus* is an endophyte of sugarcane frequently found in plants grown in agriculture area where nitrogen fertilizer input is low. Results from this laboratory, using mutant strains of *G. diazotrophicus* unable to fix nitrogen, suggested that there are two beneficial effects of *G. diazotrophicus* on sugarcane growth : one dependent and one independent on nitrogen fixation. A plant growth promoting substance, such as indole-3- acetic acid, known to be produced by *G. diazotrophicus*, could be a nitrogen fixation independent factor.

Maor *et al.* (2004) observed that plant pathogenic fungus *Colletotrichum gloeosporioides* f. sp. *Aeschynomene* utilizes external tryptophan to produce indole-3-acetic acid through the intermediate indole-3-acetamide (IAM). They studied the effects of tryptophan, IAA, IAM on IAA biosynthesis in fungal axenic cultures and on planta IAA production by fungus. IAA biosynthesis was strictly dependent on external tryptophan and was enhanced by tryptophan and IAM. The fungus produced IAM and IAA in planta during the biotrophic and necrotrophic phases of infection. The amounts of IAA produced per fungal biomass were highest during the biotrophic phase. IAA production by this plant pathogen might be important during early stages of plant colonization.

Quantum chemical methods AM1 and PM3 and chromatographic methods were used by Zakharova *et al*. (1999) to qualitatively characterize pathways of bacterial production of indole-3-acetic acid. The standard free energy changes for the synthesis of tryptophan (Trp) from chorismic acid via anthranilic acid and indole were calculated, as were those for several possible pathways for the synthesis of IAA from Trp, namely via indole-3-acetamide (IAM), indole-3-pyruvic acid (IPyA) and indole-3-acetonitrile (IAN). The $\Delta G^{0'}_{sum}$ for Trp synthesis from chorismic acid was -402 (-434) kJ mol^{-1} (values in parantheses were calculated by PM#). The $\Delta g^{0'}_{sum}$ for IAA synthesis from Trp were -565(-548) kJ mol^{-1} for the IAN pathway, -481(-506) kJ mol^{-1} for the IAM pathway, and -289(-306) Kj mol^{-1} for IPyA pathway. By HPLC analysis, the possibility was assessed that indole, anthanilic acid and Trp might be utilized as precursors for IAA synthesis by *Azospirillum brasilense* strain Sp. 245. The results indicate that there is a high motive force for Trp synthesis from chorismic acid and for IAA synthesis from Trp, and make it unlikely that anthranilic acid and indole act as the precursors to IAA in a Trp-independent pathway.

Pandey *et al*., (2006) studied the interactions and the importance of a unique relationship in a plant growth promoting consortium comprising two species, *Burkholderia* sp. MSSP and *Sinorhizobium meloti* PP3. They are rhizospheric isolates with abilities to produce indole-3-acetic acid and solubilize inorganic phosphate. The organisms were grown as monospecies or mixed –species culture and studied for growth profile, IAA production and phosphate solubilization. *Burkholderia* sp. MSSP was marked with green fluorescent protein reporter gene to monitor growth in mixed- species culture. The growth rate of PP3 increased in mixed species culture, while that of MSSP remained unaffected. IAA production increased about 50% in mixed – species culture, compared to maximum IAA released in individual trials. The amount of phosphate solubilized was not affected. The two strains were tested on *Cajanus cajan* for their plant growth promoting activities in sterile soil. Inoculation of either MSPP or PP3 resulted in significant increase in seedling length and weight. In accordance with the findings of *in vitro* experiments, exceptional increase in seedling growth grown was recorded in mixed species, co-inoculated consortium.

Bandara *et al.* (2006) reported that the endophytic microbes promote plant growth and enhanced resistance to various pathogens. They further showed that the endophytes isolated from *Oryza sativa* used as the test plant produced two types of interactions; biofilms (bacteria attached to mycelia) and mixed cultures with no such attachments. Production of indole acetic acid like substances (IAAS) of biofilms was higher than that of mixed cultures, fungi or bacteria. Bacteria and fungi produced higher quantities of indole acetic acid like substances (IAAS) than mixed cultures. In mixed cultures, the potential of IAAS and pH of the biofilms, indicating that IAAS was the main contributer to the acidity. However such relationships were not observed in mixed cultures. Microbial acid production was important for suppressing plant pathogens. Thus the biofilm formation in endophytic environment seems to be very important for healthy plant growth. However, it is unlikely that an interaction among endophytes takes place naturally in the endophytic environment, due to physical barriers of plant tissues. Further, critical cell density dependent quorum sensing leads to biofilm formation may not occur in the endophytic environment as there is a limited space. As such in vitro production and application of beneficial biofilmed inocula of endophytes are important for improved plant production in any-ecosystem. The conventional practice of plant inoculation with monocultures or mixed cultures of effective microbes may not give the highest microbial effect, which only can be achieved by biofilm formation.

It was found that the production depends on the strain of the microorganisms and on their age. The maximum indole acetic acid production was observed in the stationary phase and the indole acetic acid was transformed into indole-3-carbonic acid during further aging of the culture. The formation of indole acetic acid and other auxins has been proved also on *Pseudomonas putida* and *Pseudomonas fluorescens* by means of high performance liquid chromatography and mass spectrometry methods. Indole-3-acetaldehyde (IAH) has been determined in *Pseudominas* culture in amounts of 100 to 200 per gram of bacterial dry mass. It seems likely, that indole acetic acid production is not so high under natural soil conditions. With regard to the value reported for maize for pellet corresponding to 0.56µg indole acetic acid per gram of root dry mass at a distance of 1 to 4 mm form the root tip, it is possible to assume that indole acetic acid IAA produced by the bacteria may influence its level in plant roots (Vancura and Macura, 1960).

Bacterial isolates (*Azotobacter* and fluorescent *Pseudomonas* sp. were isolated from different rhizospheric soils. These isolates were tested for the production of indole acetic acid in a medium with 0, 1, 2 and 5 mg/ml. A low (2.68-10.80mg/ml) amount of indole acetic acid production was recorded by *Azotobacter* strains without tryptophan addition. *Azotobacter* isolates showed high level (7.3 to 32.8 mg/ml) of indole acetic acid production at 5mg/ml of tryptophan while at 1 and 2 mg/ml, the production was in the range of 1.47 to 11.88 and 5.99 to 24.8 mg/ml, respectively. Production of indole acetic acid in fluorescent *Pseudomonas* isolates increased with an increase in tryptophan concentration from 1 to 5mg/ml in the majority of isolates. Production of indole acetic acid from three isolates of *Azotobacter* and three isolates of *Pseudomonas* and subsequent TLC analysis. A specific spot from the extracted indole acetic acid preparation was corresponding with the standard spot of IAA with same Rf value (Ahmad *et al*. 2005).

Further bacterial associated with the roots of greenhouse tropical orchids were shown to produce indole acetic acid and to excrete it into the culture medium (Tsavkelova *et al.* 2005). The presence and activity of indole acetic acid were demonstrated colorimetrically and by thin layer chromatography and by bioassays. The associated bacteria varied in their ability to excrete indole compounds (1-28µg/ml nutrient broth). Addition of tryptophan to the growth medium enhanced the phytohormones production. Addition of 200µg/ml tryptophan, the bacteria *Sphingomonas sp. Mycobacterium sp., Bacillus sp.* and *Rhizobium sp.* produces indole acetic acid. *Cellulomonas, Rhodococcus sp.* and *Micrococcus luteus* produce indole acetic acid. Auxins production depends upon the cultivation conditions and the growth phase of bacterial cultures.

Lindow *et al.* (1998) observed that the relatively high percentage of epiphytic bacteria on pear leaf and fruit surfaces had the ability to produce indole-3-acetic acid in culture media supplemented with tryptophan. While over 50 percent of the strains exhibited high acetic acid production as evidenced by both colorimetric and high performance liquid chromatography analysis of culture supernatants. A majority of strains that produced high amount of indole acetic acid were identified in *Erwinia herbicola*, while some strains of *Pseudomonas syringae*, *Pseudomonas viridiflava*, *Pseudomonas fluorescens, Pseudomonas putida* and *Rahnella aquaticus* that produced high amounts of indole acetic acid also were found on pear. Fruit russeting was significantly increased in 39 out of 46 trials over an 8 period in which indole acetic acid producing bacteria were applied to trees as compared with control trees. A linear relationship was observed between fruit russet severity and the logarithm of the population size of different indole acetic acid producing bacteria on trees in the 30 days after inoculation, when normalized for the amount of indole acetic acid produced by each strain in the culture. On average, the severity of fruit russet was only about 77 per cent that on control trees when trees were treated at the time of bloom with *Pseudomonas fluorescens* strains which does not produce indole acetic acid.

VasanthaKumar *et al.* (2004) reported that bacteria isolated from galls that were identified as a member of family *Enterobacteriaceae* caused galls on 50 to 100 percent of micropropagated cranberry plants that were inoculated. Four of fifteen isolates identified as *Pseudomonas* sp. caused galls on 10 to 83 percent of plants inoculated. Twelve of fifteen isolates identified as either *Agrobacterium* sp. or *Rhizobium* sp. caused galls on 10 to 50 percent of plants inoculate, but the galls were smaller than those caused by members of the family Enterobacteriaceae or *Pseudomonas sp*. There was a positive correlation between the ability of bacteria to produce indole acetic acid in vitro and cause galls. Bacteria were isolated from plant and soil samples collected from beds where stem gall had been observed in the past 2 years and beds where stem gall had never been observed. Indole acetic acid producing bacteria were common in all samples. The result of this study support that indole acetic acid producing bacteria cause cranberry stem gall and suggest that rather than one bacterial sp. being the cause, multiple strains of bacteria that produce indole acetic acid may be responsible for gall formation. *Erwinia herbicola* produces large quantities of indole acetic acid in culture media supplemented with L-tryptophan.

Lata *et al*. (2006) on the basis of TLC and HPLC analysis found that *Pseudomonas stutzeri* strain produce plant hormone auxin (indole-3-acetic acid). The yield of IAA with and without tryptophan were 188 ± 25 and 134 ± 10 µg/ml respectively. The IAA was clearly identified on TLC in the samples extracted from culture *Pseudomonas stutzeri* with and without added to culture media.

GIBBERELLINS

Sang Mo Kang et al. (2009), reported that plant growth-promoting rhizobacteria with gibberellins (GA)-producing potential were isolated from soil and screened for plant growth promotion. A new strain, *Acinetobacter calcoaceticus* SE370, produced extracellular GA and also had phosphate solubilising potential. It produced 10 different gibberellins, including the bioactive GA_1, GA_3 and GA_4 which were, 0.45, 6.2 and 2.8 ng/ 100 ml respectively. The isolate solubilised tricalcium phosphate and lowered pH of the medium during the process. Culture filtrates of the organism after growth on broth promoted growth of cucumber, Chinese cabbage and crown daisy.

Cheruth *et al*. (2009) used different growth regulators and retardants were used to analyze their effect on photosynthetic characteristics of *Catharanthus roseus*. The plant growth retardant used was paclobutrazol. The synthetic growth regulator used was gibberellic acid. The exogenously applied non traditional growth regulator was an elicitor *Pseudomonas fluorescence*. It was concluded that these growth retardant and growth regulator altered the photosynthetic characteristics.

Fusarium strains were screened to select the potential strains for the production of gibberellins in culture media in flasks (Srivastava *et al.* 2003). Quantitative estimation of gibberellins from different strains of *Fusarium* was done spectrophotometrically which showed the presence of variable amount of gibberellins. Range of gibberellins production varied between 0.66 to 600 mg g-1 dry weights of the mycelium. Most of the strains produced 50-180 mg gibberellins on mycelium dry weight basis. Thin layer chromatography and high performance liquid chromatographic separation confirmed the presence of GA_3, $GA_{4,}$ $GA_{7.}$

Bottini *et al.* (1989) observed that gibberellic acid production in vitro by *Azospirillum sp.* and effects of *Azospirillum sp.* on infected plants were studied GA_1 and GA_3 were characterized by capillary gas chromatography, mass spectrometry from antibiotic cultures of *Azospirillum lipoferum*. In addition , the bacteria have also been seen to metabolize exogenous gibberellic acid GA_5 .

Joo *et al.* (2001) investigated the growth promotion of red pepper plug seedlings by *Bacillus cereus, Bacillus marcroides* CJ-29 and *Bacillus pumilis* CJ-69 isolated from the rhizosphere. Gibberellins a well known plant growth promoting hormone, were detected in the culture broth of rhizobacteria. Among the GA_s the contents of the $GA_{1,}$ GA_2, $GA_{3,}GA_4$ and GA_7were comparatively physiologically active than that of others, suggesting that the growth promoting effect was obtained from the GA_s.

Perrig *et al.* (2007) evaluated phytohormone in two strains of *Azospirillum* used for inoculant formulation in Argentina. GA_3 production was significantly higher in Cd (0.66 µg/ml) than in Az 39 (0.30 µg/ml). Inoculation assays have demonstrated variability in growth promotion and yield increases. Results of study shown that two strains possess the capacity to produce and release plant growth promoting substances such as IAA, GA_3.

Vikram *et al*. (2007) indicated that phosphate solubilising bacteria isolated from crops grown in vertisols were tested for production of plant growth promoting substances. All the thirty isolates of PSB were able to produce IAA and GA. The maximum amount of GA was produced by strain PSBV-5 (9.80 μg/25 ml broth) and minimum amount of GA was produced by PSBV-30, PSBV-20 (both 0.60 μg/25 ml broth).

Manero *et al.* (2001) observed that the plant growth promoting rhizobacteria (PGPR), *Bacillus pumulis and Bacillus lichniformis,* isolated from the rhizosphere of the alder have a strong growth-promoting activity. Bioassays data showed that the dwarf phenotype induced in the alder seedlings by paclobutrazol was effectively reversed by the application of extract from media incubated with both bacteria and also by exogenous GA_3. Full scan gas chromatography- mass chromatography analyze o the extracts of these media showed the presence of GA_1, GA_3, GA_4, GA_{20}, in addition to the isomer 3-epi-GA_1 and iso GA_3 isotope dilution analysis indicated that epi GA_1 was an artifact, likewise iso GA_3 is also probably an artifact spontaneously formed during extraction. In both cuture media, GA_1 was present in higher concentration (130-150 mg/ml) than GA_3 (50-60 mg/ml), GA_4 (8-12 mg/ml) and GA_{20} 2-3mg/ml). The data indicated that culture of both bacteria accumulate bioactive C19-gibberellins active in host plant. The evidences suggested that the growth promotion by rhizobacteria could be mediated by bacterial GA_s.

I*n vitro* biocontrol ability of *Triciderma harzianum* on the phytopathogen *Alternaria alternate* improved in presence of growth regulator gibberellic acid or indole acetic acid. These plant hormones decreased the secretion of endopolygalacturonase of *A. Alternaria* by approximately 20% did not modify endochitinase secretion of *T.* harzianum and did not alter germination of conidia or mycelia growth of any of these fungi (Angelona Roco , 2001).

The optimal cultural parameters for gibberellic acid 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. (Karakoc and Aksoz, 2006). The highest level of gibberellic acid (250.06 mg/l) production was obtained in nutrient broth when the bacterial culture was incubated at 30^{o}C for 72h at pH7 on a rotatary shaker and in dark conditions.

Rhizosphere and rhizoplane of fababean, melochia, sesame and soyabean plants are inhabited with fungi mostly *Aspergillus flavus, Aspergillus niger, Fusarium oxysporium, Penicillum fungiculosum* and *Rhizopus stolonifer (*Hasan, 2002). All fungal species produced gibberellins but *Fusarium oxysporium* was found to produce both GA_3 was 10 days in mycelium and 15 days in filtrates at 28^{o}C. The contents of GA_3 and indole acetic acid were significantly increased at 0.5 and 1 percent NaCL after 5 days, but they were lowered at 4 percent NaCL.

CYTOKININS

Ghazala Nasim *et al.* (2006) studied effect of different concentrations of cytokinins on growth of four species of soil fungi namely, *Aspergillus oryzae, A. terreus Thom, A. niger van Tieghem and Alternaria alternate* (Fr.) Keissler. The hormone was applied singly in various concentrations. Increased growth rate and biomass production revealed significant values when treated with dilute solutions of cytokinins at 15, 30 and 45 mgL-1. For all test fungi fresh weights and dry weight values dropped significantly when treated with 60 mgL-1 concentration of the hormone solution. The data on fresh and dry biomass revealed that the highest biomass increase was obtained for *A. alternata*. Fresh biomass of *A. alternata* showed 39.9% increase when treated with 45 mgL-1 concentration of hormone solution in comparison to control, whereas an increase of 43.75% was obtained in the case of dry weight. At 60 mgL-1, a significant fresh biomass suppression of 17.9% and 17.64% was observed for *A. niger* and *A. oryzae*, respectively. The highest loss for dry biomass was noticed in *A. alternata* (18.75%).

Taller *et al.* (1989) observed that the *Azotobacter vinelandii* was grown to stationary phase in defined medium. The cell free culture medium was analyzed for cytokinins content by XAD-2 and Sephadex LH-20 chromatography, thin layer chromatography, tobacco callus bioassay, and enzyme immunoassay. In addition to being found in *Azotobacter sp*., cytokinins have been also found in culture medium of *Arthrobacter sp.* and *Bradyrhizobium* (Philip and Torrey, 1972). Three cytokinins active fractions were detected and tentatively identified as trans-zeatin, isopentyladenosine and isopentyladenine. The total cytokinins activity was equivalent to 0.75 µg of kinetin per liter.

Cytokinins like substances synthesized by planktonic bacteria isolated from littoral and pelagial zone of lake in spring and summer were carried out by Donderski and Gluchowska (2000). They observed that 62.5 percent bacteria isolated in summer and 12.5 percent of bacteria isolated in spring were able to produce cytokinins like substance. Among synthesized substances of cytokinins they found isopentyladenine, zeatin and zeatin riboside. The amounts of cytokinins like substances produced converted into 1 g dry mass of bacteria were as follows: 9.97-21.59 µg for isopentyladenine; 3.08-35.08 µg for zeatin and 0.35-18.69 µg for zeatin riboside. Various taxonomic groups of bacteria were capable of synthesizing of these compounds, such as *Vibrio, Bacillus, Aeromonas, Achromobacter* genera and *Enterobacteraceae* family. Among the analyzed bacteria it was only the strain of *Achromobacter sp*. that produced two compounds at the same time that is zeatin and zeatin riboside.

It is now recognized that some plant growth promoting rhizobacteria may promote plant growth by secreting plant growth hormones (Lifshitz *et al*., 1987). Plant growth hormone cytokinins can be produced by many bacterial generas and its occurrence was observed in many terrestrial soil, rhizosphere and mycorrhizosphere of pine (Kampert *et al*., 1975) which suggested that bacterial cytokinins may affect the growth and developments of plants. Most of bacterial isolates with cytokinins productivity were *Flavobacterium*, *Acinetobacter*, *Bacillus*, *Arthrobacter*, *Aerobacter*, *Chromobacterium* and *Pseudomonas* sp. (Maruyama, 1986).

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. A bright red pigment prodigiosin characteristics of *Serratia marcescens* similar pigment was isolated from the many prokaryotes including *Pseudomonas magnesiorubra* and *Vibrio psychroerythrus* (D' Aoust and Gerber 1974) which have common tripyrrole structure referred as prodigeosene and differ from one organism to another in the nature of substituents attached to the nucleus.

Arkhipova *et al.* (2005) observed that the hormone production by microorganisms selected as antagonists of pathogenic fungi and the effect of their introduction into soil on hormone content and growth of lettuce plant were studied. Hormones in bacterial cultural media and plant extracts were immunopurified and assayed using specific antibodies, indole-3-acetic acid, abscisic acid (ABA) and cytokinins zeatin riboside (ZR), dihydrozeatinriboside and isopentyladenosine. Zeatin riboside was shown to be main cytokinins present in the bacterial cultural media as a complex with high molecular component. Inoculation of lettuce plants with bacteria increased the cytokinins contents in both shoots and roots.

Cytokinins were detected in culture filterates of *Azotobacter chroococcum*, *Azotobacter beiferinckii*, *Azotobacter vinelandii*, *Pseudomonas fluorescens* and *Pseudomonas putida* (Nieto and Frankenberger, 1989). The most prolific cytokinins producer was *Aztobacter chroococcum*. Several purine ring constituents and isoprenoid compounds were tested for their suitability as physiological precursors to cytokinins biosynthesis. The isolation and quantification of zeatin riboside, dihydrozeatinriboside, t-zeatin and c-zeatin, isopentyladenine and its riboside were monitored through high performance liquid chromatography (HPLC), U.V. spectrometry and bioassay. The stimulation of *Azotobacter chroccoccum* to produce cytokinins by the addition of adenine and isopentyl alcohol was monitored during the growth of the bacterium and tested under various environmental conditions in liquid media.

Gonzalez-Lopez *et al.* (1986) assessed plant growth regulator production by *Azotobacter Vinelandii* in four media and found up to fourfold more cytokinins production in dialyzed soil medium (1.78-4.4 µg kinetin equivalents per milliliter) compared with other media. This suggested that cytokinins production in soil may be considerably greater than that observed in culture media.

Salamone *et al.* (2001) proposed the mechanisms by which rhizobacteria enhance plant growth through the production of plant growth regulators. Five plant growth promoting bacteria rhizobacterial (PGPR) strains produced the cytokinins dihydrozeatin riboside (DHZR). In pure culture cytokinins production by *Pseudomonas fluorescens,* rifampcin resistant mutant and two pho A-derived mutants with reduced capacity to synthesize cytokinins was further characterized in pure culture using immunoassays and thin layer chromatography (TLC). *Pseudomonas fluorescens* produced higher amounts of three cytokinins that is isopentyladenosine (IPA), trans-zeatin ribose (ZR) and dihydrozeatin riboside (DHZR) during stationary phase. Isopentyladenosinewas the major adenosine produced, but the proportion of zeatin riboside and dihydrozeatin riboside (DHZR) accumulated by the two mutant strains increased with the time. Addition of 10^{-5} M adenine increased the cytokinins production in 96 and 168 hour culture of the strains *Pseudomonas fluorescens.*

Agrobacterium tumefaciens infects plants and induces the formation of the tumors called “crown gall” by integrating the transferred DNA (T-DNA) region of the Ti-plasmid into the plant nuclear genome. Tumours are formed because of the T-DNA encodes the enzymes that modify the synthesis of two plant growth hormones, auxins and cytokinins. The cytokinins synthesis enzyme Tmr which is encoded by the Agrobacterium T-DNA region is targeted to and functions in plastids of infected plant cells, despite having no typical plastid-targeting sequence. Evidences provided that Tmr is an adenosine phosphate-isopentenyltransferase that creates a new cytokinins biosynthesis bypass by using 1-hydroxy -2-methyl-2-butenyl 4-diphosphate as a substance. Unlike in the conventional cytokinins biosynthesis pathways in the plants, trans-zeatin type cytokinins re produced directly without the requirement for P450 monooxygenase-mediated hydroxylation. These results demonstrate that *Agrobacterium tumefaciens* modifies cytokinins biosynthesis by sending a key enzyme into plastids of the plant to promote tumorigenesis (Sakakibara *et al.* 2006).

2.3 ANTIFUNGAL ACTIVITY

Tari *et al.* (1988) isolated a soil bacterium *Pseudomonas corrugates* from nine temperate site of Indian Himalayan Region (IHR) and examined its antagonistic activities against two phytopathogenic fungi, *Alternaria alternate* and *Fusrium oxysporum.* Although the bacterium did not show inhibition zones due to production of diffusible antifungal metabolites, reduction in growth between 58% and 49% in both test fungi, *Alternaria alternate* and *Fusrium oxysporum* was observed due to production of antifungal metabolites. The antagonism was observed to be affected by growth medium, pH and temperature.

Singh and Sinha (2005) studied influence of time of application of *Pseudomonas fluorescens* in suppressing sheath blight of rice. This investigation was undertaken to list the affectivity of potential strains of *Pseudomonas fluorescens* against sheath blight of rice under field conditions, applied as three time sequences. Foliar sprays with Pfr-1, seven days before pathogen inoculation resulted in maximum reduction in sheath blight severity (59.6-64.4) and incidence (36.7-40.4) and increased grain yield (30.6-32.3) and 1000-grain weight (27.2-29.5).

Bhatia *et al*. (2005) studied enhancement of plant growth and suppression of collar rot of sunflower caused by *Sclerotium rolfsii* through fluorescent *Pseudomonas*. Ten isolates of fluorescent pseudomonad were isolated from rhizosphere of sunflower, potato, maize and groundnut. All the isolates produced fluorescent pigment in succinate broth and displayed siderophores production. Production of hydrogen cyanide (HCN) and indole acetic acid (IAA) by all the isolates was recorded besides phosphate solubilization. Bacterization of sunflower seeds, with fluorescent *Pseudomonas* PS-I and PS-II resulted in increased seed germination, root length; shoot weight, fresh and dry weight of roots and shoots and yield of sunflower. Seed bacterization with strain of *Pseudomonas* sp. PS-I and PS-II reduced incidence of collar rot by 69.8 per cent and 56.9 per cent, respectively, in *Scerotium rolfsii* infested soil, making the organism potential biocontrol agent against collar rot of the sunflower.

2.4 SIDEROPHORES

Lankford (1973) coined the term siderophore to describe low molecular weight molecules that bind ferric iron with an extremely high affinity. Siderophores was derived from Greek term meaning iron carrier (Ishimaru, 1993). The molecular weight of siderophores range from approximately 600 to 1500 Daltons and siderophores must be actively transported. Once actively transported into the periplasm, the iron siderophore complex is bound to a periplasm binding protein (Braun, 2002).

The *Pseudomonas* is known to produce siderophores of the hydroxamate class. Depending on the source, these have been named as ferribactin, pyoverdin and pseudobactin. Further none of these have yet been completely characterized in a chemical sense (Neilands, 1981).

Neilands and Leong (1986) suggested that there are at least three ways by which siderophores could affect plant life. In the first the chelates act in the soil to solubilize and transported Fe (III), a very important mineral in plant nutrition. A second possible effect of siderophores may be via the facilitation of plant disease. In a survey they detected that gram-negative bacterial phytopathogens including species of *Agrobacterium, Erwinia* and *Pseudomonas* showed the presence of siderophores or siderophores like compounds. Siderophores are by now finely established as one very important determinant of virulence for infections of animals and plant. The third mechanism is a type of bio-control in which certain microbial species, such as fluorescent *Pseudomonas* discourage the growth or metabolic activities of competing micro-organisms. Antibiosis, hormone effect and lytic activity may work in combination with siderophores to afford a micro-environment at the root surface that favors plant growth. Despite the considerable structural variation found among siderophores they all form six coordinated octahedral complexes with Fe (III). The two methods most commonly used to identify compound as a siderophore are bioassays and chemical detection. Among these chrome azurol-S (CAS) assay method is commonly used (Schwyn and Neilands, 1987). This assay was independent of the structure of siderophore and thus was very useful in screening the organisms for siderophore production. The assay is based on the observation that when a strong chelator removes iron from a CAS dye/iron III or detergent complex, the dye colour turns from blue to orange or yellow. Mainly siderophores fall into two categories viz. catechol-phenolates and hydroxamate type. As most of the ferric complexes are colored, simple addition of ferric chloride or ferric perchlorate to the spent medium may serve to detect the presence of hydroxamate or catechols. Hydroxamates are orange colored with maximum absorbance at 425-450 nm and catechols are wine colored with maximum absorbance at 495 nm. The *Pseudomonas* is known to produce siderophores of the hydroxamate class. Depending on the source, these have been named as ferribactin, pyoverdin and pseudobactin. Further none of these have yet been completely characterized in a chemical sense (Neilands, 1981).

2.5 PHOSPHATE SOLUBILIZATION

Compared with the other major nutrients, phosphorus is by far the least mobile and available to plants in most soil conditions. Although phosphorus is abundant in soils in both organic and inorganic forms, it is frequently a major or even the prime limiting factor for plant growth. The bioavailability of soil inorganic phosphorus in the rhizosphere varies considerably with plant species, nutritional status of soil and ambient soil conditions (Khan *et al.,* 2006).

The phosphate solubilizing bacteria isolated from the rhizosphere of plants, green manuring crops serve twin purpose of solubilizing the cheaper insoluble phosphates and improving the overall soil health. Among the macronutrients, the role of phosphorus in increasing the yield and some times in improving the quality of crops is well known. Low recovery of phosphorus (10-30%) from applied fertilizer due to fixation characteristics in soil coupled with recent steep hike in prices of phosphatic fertilizers have further complicated the problems of its use by the farmers (Dubey, 2000). It is in this context that there is a need to have a comprehensive approach to application for sustainable crop production to enhance its use efficiency.

The soil environment surrounding plants roots, known as rhizosphere is the zone of intense microbial activity (Gaur, 1990) and is also the zone of soil from which plants derive most of the nutrients. The number of organisms in the rhizosphere may be as much as 100 times greater than elsewhere in the soil (Brandy, 2001). Many bacterial, fungal, yeast and actinomycetes species capable of solubilizing sparingly soluble P in pure culture have been isolated from soil and rhizosphere samples (Kucey *et al.,* 1989).

Swaby and Sperber (1958) reported that the population of phosphate dissolving microorganisms is more in the rhizosphere (20-40% of the total population) as compared to non-rhizosphere (10-15 % of total population). It has been found that rhizosphere of legumes support greater number of P-solubilizing microorganisms than do non-legumes (Sobieszczanski, 1961) and that some legumes support more P-solubilizing organisms than other (Paul and Sundara Rao, 1971).

The element phosphorus enjoys an important position in the nutrition and life cycle of plants and animals. Pierre (1993) referred to it as the “Master Key” element in crop production and is known to be involved in a plethora of functions in the plant growth, development and metabolism. It is a component of some important cellular constituents including nucleic acids, phospholipids, coenzymes, phosphorylated sugars, nucleotides and phytin. It is involved in energy transformation in plant and other organisms and is an important component of genetic material. It is associated with several vital functions and is responsible for several characteristics of plant growth such as utilization of sugars and starch, photosynthesis, nucleus formation and cell division, fat and albumin formation, cell organization and the transfer of hereditary characters (Arnon, 1956).

Both organic and inorganic forms of phosphorus occur in soil. The organic forms of phosphorus are the compounds of phytins, phospholipids, nucleic acids and inositol phosphates while the inorganic forms are the compounds of calcium, iron, aluminum and fluorine (Brandy, 2001).

2.6 HYDROGEN CYANIDE AND AMMONIA

Hydrocyanic acid production was observed in a variety of *Pseudomonas aeruginosa* by means of the picrate paper method and verified by the prussian-blue test. The yield of HCN at 26°C was about three times higher than that at 37°C. The maximum HCN concentration in the cultures was reached after 24 hours. Experiments with synthetic media showed that HCN could be formed with glycine as the only nitrogen source. Cultures with glutamic acid and asparagines did not yield amounts accessible for accurate determinations. Experiments in synthetic glycine media with addition of small amounts of nutrient broth or yeast extract suggest that these substances contain factors which accelerate the HCN production (Lorck, 1948).

Among the factors involved in plant microbe interactions as well as in microbe-microbe interactions, in the rhizosphere siderophores and hydrocyanic acid (HCN) have received special attention. HCN is released as a product of secondary metabolite by several micro-organisms and affects sensitive organisms by inhibiting the synthesis of ATP-mediated cytochrome oxidase (Knowles, 1976).

Therefore, depending on the target organisms HCN producing micro-organisms are regarded as harmful when they impair plant health and beneficial when they suppress unwanted components of the microbial community (Schippers *et al.,* 1987).

Pseudomonas fluorescens NCIB 11764 was capable of utilizing cyanate (OCN^-) as a sole nitrogen source for growth. Crude cell extracts from cells grown on cyanate, but not on ammonium sulfate, were induced for an enzyme catalyzing cyanate conversion to ammonia. Enzymatic activity was shown to be bicarbonate dependent and specific for cyanate as a substrate and even ammonia as substrate (Kunz and Naggapan*.,* 1989).

Control of *Pythium* damping-off sugar beet by seed treatment with crop straw powders and a biological control agent was studied by Bardin *et al.* (2004). Seed treatment with non-sterilized powered strain from crop was tested for the control of *Pythium* damping-off sugar beet. Wheat straw powder coated on sugar beet seeds increased the incidence of *Pythium* damping-off but this effect was reversed by the co-inoculation of wheat straw with the bio-control agent *Pseudomonas fluorescens* 708. Coating sugar beet seeds with *P. fluorescens* 708 and flax or pea straw also increased the efficiency of bacterial strain for the control of *Pythium* damping-off. Fermentation of pea straw led to the accumulation of volatile ammonia, which was produced by the reduction of the large amount of nitrate stored in the straw. Reduction of nitrate and therefore, the release of volatile ammonia did not occur in sterilized pea straw. However, fermenting sterile pea straw with bacteria from different genera restored nitrate reduction and release of volatile ammonia, suggesting that microorganisms associated with pea straw are responsible for the conversion of nitrate into volatile ammonia which in tern control *Pythium* damping-off disease in sugar beet.

2.7 LYTIC ENZYMES

Enzymes are the organic catalysts produced generally by micro-organisms, differing from other catalysts and constitute the tools which determine the course of the multitude of life processes (Buchanan and Buchanan, 1958). Various kinds of enzymes are produced by micro-organisms. Lytic enzymes induced by *Pseudomonas fluorescens* and other bio control organisms mediate defense against the anthracnose pathogen in mango leading to improved yield attributes (Viveknathan *et al.,* 2004).

An enzyme chitinase and chitobiase produced by some bacteria and fungi like *Mucor, Trichoderma* and *Pseudomonas* species possessed lytic effect which was related to antagonistic behaviour (Chet 1984; Gooday, 1986). Some proteolytic enzymes especially elastase, subtilisin were also reported to possess bacteriolytic properties against different gram negative and gram positive bacteria (Burke and Pattee, 1967; Kaur *et al.,* 1988). An extra cellular enzyme proteinase was isolated form *Pseudomonas fluorescens* extract possessing anti microbial activity and it was discovered that calcium is required for the structural integrity of the proteinase as well as for activity (Mc Keller and Cholltte, 1986).

Many researchers have successfully isolated a wide array of organisms from a variety of environments that produce many active and stable enzymes including proteases (Durham *et al.,* 1987), amylase (Horikoshi, 1971), lipases. Enzymes that are stable and active at temperature extremes are very much in demand for various industrial processes (Steele and Stowars, 1991). Development of new solidifying agents such as *Pseudomonas* sp. produced gellan gum, Gelrite, facilitated the isolation of thermophilic organisms.

A soil isolate, identified as *Pseudomonas* species produced an extra cellular protease enzyme of 14.4 K Da molecular weight. The kinetic properties of the purified fraction of the bacterial protease were studied experimentally and the rate of casein hydrolysis was predicted by a model based on artificial neutral network. The various kinetic factors studied were incubation time, initial enzyme concentration, initial substrate concentration, pH and temperature. The prediction error in stimulating hydrolysis was less than one per cent (Dutta *et al.,* 2005).

Morihara (1964) observed that some strains of *P. aeruginosa* produce elastase but other do not. Elastase positive strains can produce other proteinases in either synthetic or natural medium in clear contrast to the elastase negative strains which can produce proteinases only in synthetic medium containing Ca ions. The extra cellular lipase was purified from *Pseudomonas aeruginosa* EFZ which exhibited both lipase as well as little of esterase activity (Gilbert *et al.,* 1991).

Chapter-III

MATERIAL AND METHODS

The present investigation entitled, **“Preliminary characterization of fluorescent *Pseudomonas* diversity producing plant growth regulators in replant problem and normal site of pear and apple”** was carried out in the Department of Basic Science, Dr Y.S. Parmar University of Horticulture & Forestry, Nauni- Solan, Himachal Pradesh. The materials used and methodology adopted is described in this chapter.

3.1 ISOLATION OF FLOURESCENT *PSEUDOMONAS* SPECIES FROM RHIZOSPHERE OF APPLE & PEAR

3.1.1 Collection of soil sample

Soil samples along with pieces of roots were collected from the rhizosphere soil of pear and apple orchards in Sanyardi and Nagwain (Mandi district). Four samples were taken around few to 10 cm apart from root and 30 -45 cm deep from five different plants and mixed to make it one composite samples. Similarly composite samples were collected from five different normal and three different replant sites of apple in Mandi fields.

3.1.2 Isolation and identification

Ten grams of rhizosphere soil from pear and apple orchard (normal and replant area) were shaken vigorously in 90 ml sterile water blank in 250 ml Erlenmeyer flask for 30 minutes. Dilutions were made using 9 ml sterile water blanks to give ten fold dilution series from 10^{-1} to 10^{-5} for each soil sample. 0.1 ml of each diluted sample was added to each Petri plate and about 15-20 ml of molten and sterilized media was added to each Petri plate and mixed gently in all directions. The media employed for the isolation of

Pseudomonas sp. were nutrient agar and King's media B. Plates were incubated for 24-48 h. at 28 ± 2^{0}C for *Pseudomonas* sp.

The most predominant *Pseudomonas* sp. isolates showing greenish yellowish fluorescent pigment were assumed to be fluorescent colonies were streaked on different media along with king's medium for purification and further observation of colony morphological features and also pigment production on other medium ie, Pikovskaya's, King's, Succinate media, nutrient agar media etc. Then identified on the basis of morphological and biochemical tests as per their genera lay down in Bergey's manual of systematic bacteriology and were confirmed from the Department of Basic Sciences, Dr Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan (H.P). The selected strains were maintained on the nutrient agar slants at 4^{0}C and were sub-cultured periodically on the same media at 28 ± 2^{0}C. They all were maintained and preserved in 20% glycerol at -20 ^{0}C. all the experiments were conducted after raising fresh cultures. The best selected strains showing maximum production of all the plant growth promoting activities sent to microbiological type culture collection (MTCC) and gene bank IM Tech, Chandigarh for ribotyping and identification.

3.1.3 Chemicals and reagents:

Various reagents and chemicals used in the present studies were procured form B.D.H, C.D.H, Hi-media, Thomas-baker and nice chemicals. Most of chemicals used were of analytical grade.

3.1.4 Composition of media used in this study:

a) Nutrient agar medium:

Composition per liter

Peptone : 5.0 g
Beef extract : 3.0 g
NaCl : 8.0 g
Agar : 20.0 g
pH : 7.0

b) Succinate medium:

Composition per liter

K_2HPO_4 : 6.0 g
KH_2PO_4 : 3.0 g
$(NH_4)_2SO_4$: 1.0 g
$MgSO_4$: 0.2 g
Succinic acid : 0.2 g
pH : 7.0

c) Pikovskaya's medium

Composition per liter

Yeast extract : 0.5 g
Glucose : 10.0 g
$Ca_3 (PO_4)$: 5.0 g
KCl : 0.2 g
$MgSO_4.7H_2O$: 0.1 g
$FeSO_4$: Trace (0.002%)
$MnSO_4$: Trace (0.002%)
$(NH_4)_2SO_4$: 0.5 g
Bromocresol purple : 0.1 g
Agar : 20.0 g

d) King's medium B:

Composition per liter

Protease peptone : 20.0 g
K_2HPO_4 (anhydrous) : 1.5 g
$MgSO_4.7H_2O$: 1.5 g
Glycerol : 15.0 ml
pH : 7.2

e) Potato dextrose agar

Composition per liter

Potato : 200.0 g
Dextrose : 20.0 g
Agar : 20.0 g

f) Skim milk agar

Composition per liter

Nutrient agar : 1 liter

Prepare separately 5 per cent of skim milk and after autoclaving both were mixed.

g) Peptone water broth

Composition per liter

Peptone : 10.0 g
NaCl : 5.0 g
pH : 7.2 ± 0.2

h) CAS-HDTMA agar

Chrome azurol-S : 60.5 mg
Water : 500 ml
Agar : 20 g

Add 100 ml 1 mM $FeCl_3$. $6H_2O$ in 10 mM HCl. Add slowly to HDTMA solution (72.9 mg HDTMA in 40 ml H_2O).

J) Fermentation broth:

Peptone : 10.0g
Dextrose : 5.0g
Sodium chloride : 15.0g
Phenol red : 0.018g
pH : 7.3

K) Malt extract agar

Composition per liter

Malt extract : 20.0 g

Agar : 20.0 g

L) Semi solid nitrate medium

Composition per liter

Beef extract : 3.0 g

Peptone : 5.0 g

Potassium nitrate : 1.0 g

Agar : 1.0 g

M) Semi solid non nitrate medium

Composition per liter

Beef extract : 3.0 g

Peptone : 5.0 g

Agar : 1.0 g

3.1.5 Biochemical Tests:

3.1.5.1 Sugar fermentation metabolism

Fermentation metabolism was used for testing the presence of fermentation metabolism in *Pseudomonas* isolates. Fermentation broth was taken into fermentation tubes and was inoculated with Pseudomonas bacterium and one uninoculated tube of fermentation broth was kept as a comparative control. All inoculated and uninoculated tubes were incubated at 28^0C for 24-48h. Change in colour and appearance of bubbles showed the presence of fermentation metabolism.

3.1.5.2 Oxidase Test:

Soak small piece of filter paper in 1% aqueous tetra methyl- p- phenylenediamine dihydrochloride or oxalate. Some filter paper give blue colour & these must not be used. `Scrap some of fresh young culture with a clean platinum wire or a glass rod and rub on filter paper. A blue colour with in ten seconds is positive oxidase test. No change in colour is negative test. Old cultures are unreliable.

3.1.5.3 Catalase test:

On a clean glass slide take a loopful of growth emulsified with a loopful of hydrogen peroxide. Immediately presence of effervescence /air bubbles indicate positive results.

3.1.5.4 Gelatin liquification test:

Nutrient gelatin deep 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 30^0C for 4-7 days. After incubation, the tubes were placed in refrigerator at 4^0C for 15 minutes. Deep gelatin tubes that remain liquefied produced gelatin and showed positive test for gelatin hydrolysis and those tube that remain solid demonstrated negative reaction for gelatin hydrolysis.

3.1.5.5 Growth at 4^0C, 25 ^{0}C and 41^0C

0.5 ml of overnight culture was inoculated in test tubes containing 10ml nutrient broth. The tubes were incubated at 4^0C and 40^0C. the growth was observed at absorption at 540nm.

3.1.5.6 Denitrification test

Nitrate agar containing potassium nitrate(1%) was used to inoculate with *Pseudomonas* culture in semisolid nitrate medium. Inoculated tube kept in iced water for 10-15 minutes or until medium is completely chilled cold and semisolid medium remained firm so that overlay agar will stay on top. Aseptically poured entire contents of one agar overlay tube into each inoculated tube of chilled medium. Set each tube in agar until overlay agar has solidified. Incubated all inoculated and uninoculated tubes at 30^0C for 48h. Inoculated one tube of semisolid non nitrate media. Place inoculated tube in ice bath for 10-15 minutes or until medium is completely chilled cold make semisolid medium firmer so that overlay agar will stay on top. Aseptically poured entire contents of one agar overlay tube into each inoculated tube of chilled medium. This should be poured so that it gently runs down the slide of tube and slowly layers over top of nitrate medium. Set each tube in agar until overlay agar has solidified. Incubated all inoculated and inoculated tubes at 30^{0}C for 48h.

3.2 CHARACTERIZATION OF BACTERIAL ISOLATES FOR PLANT GROWTH PROMOTING AND DISEASE SUPPRESSING ACTIVITIES

3.2.1 Indicator test fungi

Plant pathogenic indicator test fungi viz., *Fusarium oxysporum*, *Pythium* sp., *Alterneria* sp., *Sclerotium* sp. and *Rhizoctonia solani* were procured from the Department of Plant Pathology (MPP) and from the Department of Basic Sciences of Dr Y S Parmar University of Horticulture & Forestry Nauni-Solan (HP). These fungal cultures were maintained on malt extract agar (MEA) at 4^{0}C and sub-cultured periodically at 28 $\pm$ 2^{0}C on same medium at 28 $\pm$ 2^{0}C.

3.2.2 Preparation of inoculum :

Inoculum of indicator test fungi was prepared by placing a bit of test culture in the centre of prepoured malt extract agar (MEA) plates and incubating the plates at 28 $\pm$ 2^{0}C for 72 h.

3.2.3 Preparation of culture supernatant

0.5 ml of 24 h. old inoculum of all bacterial strains i.e. *Pseudomonas* sp. strains isolated from the rhizosphere of pear and apple orchards were inoculated in 100 ml nutrient broth in Erlenmeyer flasks. Flasks incubated at 28^{0}C for *Pseudomonas* sp. respectively for 72 h. under shake condition (90 rpm) on rotary shaker. Cell free culture supernatants prepared by centrifugation at 10,000 rpm at 4^{0}C for 20 min and preserved in the form of small aliquots (5 ml) in tubes in deep fridge or at 4^{0}C.

3.3 SCREENING AND PRODUCTION OF PLANT GROWTH PROMOTING ACTIVITIES BY *PSEUDOMONAS* SP.

3.3.1 Plant growth regulators

Pseudomonas sp. isolated from the rhizosphere soil of pear and apple orchards were screened out for the production of plant growth regulators viz., auxins, gibberellins and cytokinins (Mahadevan and Sridhar, 1986).

3.3.1.2 Production and estimation

For production of auxins, gibberellins and cytokinins, test organisms were grown in nutrient broth for 72 h. at 28 ± 2^{0}C for *Pseudomonas* under shake conditions. Supernatant was prepared by centrifugation of cultures at 10,000 rpm for 20 minutes and was stored in deep fridge or at 4^{0}C.

3.3.1.3 Auxins

Quantitative measurement of auxins was done by colorimetric method (Gorden and Paleg, 1957) with slight modifications. 2 to 3 drops of orthophosphoric acid was added to 2 ml supernatant and 4 ml of salper reagent (1 ml of 0.5 M $FeCI_3$ in 50 ml of 30 % $HCIO_4$: prepared fresh). This mixture was incubated for 60 minutes in dark. Absorbance was measured at 535 nm. Concentration of auxins was estimated by preparing calibration curve using indole acetic acid (IAA) as standard (10-100 µg/ml).

3.3.1.4 Gibberellins

The gibberellins were estimated calorimetrically 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 HCI was added and mixture was incubated at 20^{0}C for 75 min. For blank 5 ml of 5 per cent HCI was used. Absorbance was read at 254 nm concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA_3,Hi-media) as standard (100-1000 µg/ml).

3.3.1.5 Cytokinins

Radish cotyledons expansion bioassay test was employed (Letham, 1971) for assay of cytokinins like substances the radish seeds (*Raphanus sativus* L. cultivars Japanese white) were germinated in total darkness for 48 h. at 28^0C. After removing the seed coat, smaller cotyledons were transferred to sterilized Petri dishes containing the test solution on filter paper strips. 12 cotyledons were placed in each Petri dish and were included at 25^0C under fluorescent light for 3 days. Then cotyledon on filter paper strips in Petri dish 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 (100-1000 µg/ml).

3.3.2 Antifungal activity

Antifungal activity of each test strain of *Pseudomonas* sp. was checked by standard well/bit plate assay method (Vincent, 1947 Fleming *et al.,* 1975). 72 h. old culture bit of indicator fungi were placed on the one side of prepoured malt extract agar (MEA) plates with the help of sterile well cutter and inoculating needle. On the other side of plates, well was cut with the help of sterile cork borer, 100 µl of 72 h. old cell free culture supernatant of each test bacterial strain isolated from the rhizosphere soil of pear and apple orchards was added to each well. Plates were incubated at 28 ± 2^0C for 3 - 5 days. Antifungal activity expressed in terms of percent inhibition of fungal growth and calculate from equation:

$$\text{Percent inhibition} = \frac{C-T}{C} \times 100$$

T = Growth of mycelia in treatment
C = Growth of mycelia in control

3.3.3 Siderophores

3.3.3.1 Plate assay

Siderophores production was detected by chrome azurol -S (CAS) plate assay method (Schwyn and Neilands, 1987). 72 h old culture bit/well of each test bacteria i.e. *Pseudomonas* sp. was placed on prepoured chrome azurol-S agar (CAS) plates. Plates were incubated at 28^{0}C for *Pseudomonas* sp. for 24 h. Production of siderophore was expressed in terms of mm diameter of pinkish/orange halo produced around the bit at 28^{0}C in 24 h.

3.3.3.2Liquid assay

For quantitative estimation of siderophores, chrome azurol-S (CAS) liquid assay method will used. Seventy two hour old cell free culture supernatant of each test bacterial strains grown in 100 ml iron free Nutrient medium on rotary shaker was taken by centrifugation at 10,000 rpm at 4^{0}C for 20 minutes. 0.5 ml of cell free supernatant was mixed with 0.5 ml CAS assay solution (1.5 ml of 1 mM $FeCI_3$, $6H_2O$ in 10 mM HCl + 7.5 ml of 2 mM CAS stock solution dissolved in 50 ml of 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).

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

Ar = Absorbance of reference at 630 nm

As = Absorbance of supernatant at 630 nm

3.3.4 Phosphate solubilization
3.3.4.1Production

For production of phosphate solubilizing activity, test organisms were grown in Pikovskaya's broth for 72 h. at 28 ± 2^0C for *Pseudomonas* sp. under shake conditions. Supernatant was prepared by centrifugation of cultures at 10,000 rpm for 20 minutes and was stored in deep fridge at 4^0C.

3.3.4.2 Plate assay

For estimation of phosphate solubilizing capacity of test bacteria (Pikovskaya's 1948) Pikovskaya agar plates with known amount of inert phosphorus ($Ca_3(PO_4)_2$) source was prepared. All the strains were screened out for phosphate solubilizing activity by growing the cultures in nutrient agar plates at 28 ± 2^0C for *Pseudomonas* sp. for 72h. Phosphate solubilization expressed in terms of mm diameter of yellow colored zone produced around well at 28^0C for *Pseudomonas* sp. The phosphate solubilizing efficiency is calculated from equation:

$$\text{Phosphate solubilising efficiency} = \frac{Z-C}{C}\times 100$$

Z = Diameter of zone (mm diameter)
C = Diameter of well (mm diameter)

3.3.4.3 Quantitative assay

Quantitative estimation of phosphorus solubilizing activity was done by spectrophotometric method by using Pikovskaya's broth with known amount of inert phosphorus source (tri-calcium phosphate). 0.5 ml of 18h old inoculum of test bacterial strain was inoculated in 100 ml of Pikovskaya's broth and was incubated at 28 ± 2^oC for *Pseudomonas* species for 72h under shake conditions (90 rpm). Supernatants were collected after centrifugation at 10,000 rpm for 20 minutes at 4^0C. To 1.0 ml of supernatant, 5 ml of ammonium molybdate reagent (15 g of ammonium molybdate in 400 ml of distilled water, 342 ml of 12 N conc. HCl was added and cooled and final volume was made to one liter with distilled water) was added along with shaking. 1 ml of working solution of chlorostannous acid (40 %) (I.e. 0.5 ml of stock solution was added to 65.5 ml distilled water to make final volume 66 ml) was added. Stock solution was made by dissolving 10 g of chlorostannous acid in 25 ml concentrated HCl. Total volume of reaction mixture was immediately made 25 ml. Absorbance was measured at 660 nm using red filter. Corresponding amount of soluble phosphorus was calculated from standard curve of KH_2PO_4 (10-100µg/ml).

3.3.5 HCN Production

Bacterial isolates were screened out for the production of hydrogen cyanide (HCN). Bacterial cultures were streaked on prepoured plates of King's medium B amended with 1.4 g/l glycine (Bakker and Schippers, 1987). Whatman No.1 filter paper strips were soaked in 0.5 per cent picric acid in 2 per cent sodium carbonate and were placed in the lid of each petriplates. Petriplates were sealed with parafilm and were incubated at 28^0C for 1-4 days. Uninoculated control was kept for comparison of results. Plates observed for change of color of filter paper from yellow (-) to dark brown (+++) to orange brown (+++++).

3.3.6 Ammonia Production

Ammonia production was checked according to Lata and Saxena (2003). *Pseudomonas* sp. strains were grown in peptone water (5 ml) in tubes. Tubes were incubated at 28^0C for *Pseudomonas* sp. for 4 days. After 4 days, 1ml of Neissler's reagent was added to each tube. Presence of faint yellow colour (+) indicated small amount of ammonia and deep yellow (++) to brown color (+++++) indicated large amount of ammonia production.

3.3.7 Proteolytic activity

3.3.7.1 Plate assay method

All *Pseudomonas* sp. strains were screened out for proteolytic activity by well plate assay method on skim milk agar plates. 100 µl of 72 h old all free culture supernatant of each bacterial strain was added to each well already cutted on skim milk agar plate with the help of sterile cork borer. Plates were incubated at 37^{0}C for 24-48 h and observed for proteolysis i.e. clear zone (mm dia) produced around the well (7 mm).

3.3.7.2 Quantitative assay

The proteinase assay was based on casein digestion and employed in five minute assay time period. The casein substrate was 2% solution in 0.05M Tris buffer. One milliliter of substrate solution was incubated at 37 ^{0}C with 1ml of enzyme dilution in same Tris buffer for 5 minutes. The mixture was then precipitated with 3 ml of 5%TCA. The control consisted of same components. The precipitate was removed by filtration through Whatman no.2 paper.

The filtrate was assayed for relative concentration change in TCA soluble protein components. Enzyme activity was found to be linear with in 5 minutes assay period and was also proportional to enzyme concentration. The unit of activity was defined as hydrolysis of one equivalent milligram of protein per milliliter of enzyme per minute under conditions specified in assay.

3.4 Effect of different media on the production of plant growth regulators by *Pseudomonas* sp. at different incubation period

Effect of media on the production of plant growth regulators i.e. auxins, gibberellins and cytokinins was studied by growing (four *Pseudomonas* sp. PN-4-SAN, PN-10-SAN AN-2-Nag, AN-4-Nag) the test organisms were grown in five different types of media ; succinate media, king's media, nutrient media, peptone water and trypticase soyabroth. 0.5ml of inoculum of overnight culture (18 h old) of test organism was used to inoculate 100 ml of media in 250 ml of Erlenmeyer flask. Flasks were incubated at 28 ^{0}C for 0, 4, 8, 24, 48, 72 h under shaken conditions (90rpm). Supernatant were harvested by centrifugation at 10,000 rpm for 30 minute at 4^{0}C and were used for estimation of auxins, gibberellins and cytokinins. The best media was selected for production of each plant growth regulator and was used for further studies. The experiment was conducted in triplicates and average mean value was presented.

3.5 Extraction and separation of plant growth regulators

All the three plant growth regulators i.e. auxins, gibberellins and cytokinins were extracted and separated from supernatant by chromatographic methods that are thin layer chromatography (TLC). (Mahadevan and Sridhar, 1986). *Pseudomonas* strains will be grown in their best respective medium at 28^0C for 72 hrs under shake conditions. Supernatants will be harvested by centrifugation at 10,000rpm for 30 min.

3.5.1 Extraction of auxins:

Supernatant (100 ml)

↓

Acidified with 1N HCl to pH 3.0

↓

Extracted with 100 ml of di-ethyl ether (3X) in separatory funnel

↓

Kept at 4^0C for 1 hour

↓

Pooled all the ether extract and reduced the volume to 25 ml on a rotary evaporator at 40^0C

↓

Partitioned the ether fraction with 25ml of sodium bicarbonate 5 % (3X)

↓

Acidified the bicarbonate fraction to pH 3.0 with 6 N HCl

↓

Extracted with 50 ml of di-ethyl ether (3X)

↓

Evaporated and concentrated the ether fractions on a rotary evaporator at 40^0C

↓

Dissolved the residue in 2 ml of methanol

↓

Used this fraction for TLC and bioassay

Separation of auxins

Spotted 100 µl of the methanol extract on thin layer plate spreader with silica gel G and developing were done in solvent such as isopropanol-water (30:20. v/v) for 12-14 hours. Plates were sprayed with Salper reagent.

3.5.2 Extraction of gibberellins

100 ml of supernatant mixed with 250 ml of saturated NaHCO3 solution in separatory funnel

↓

Extracted with 300ml ethyl acetate (2X)

↓

Aqueous layer Acidified to pH 2.5 with 5 N HCl

↓

Equal volume of ethyl acetate added and shaked vigorously for 5 minutes

↓

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

↓

All ethyl acetate fractions pooled and dried over Na_2SO_4

↓

Evaporated the ethyl acetate extract on a rotary evaporator at 40^0C

↓

Residue dissolved in 2 ml of ethanol for thin layer chromatography and in 2 ml of water with Tween 20 for bioassay

Separation of gibberellins

Gibberellins were separated by thin layer chromatography using silica gel-G plates were developed in solvent isopropanol; ammonium hydroxide; water (10:1:1 v/v/v) and silica gel-G plates were heated at 120^{0}C for 10 minutes in oven and sprayed with water: concentrated sulphuric acid (30: 70 v/v)

3.5.3 Extraction of cytokinins

Supernatant

↓

Acidified to pH 3.0 with 0.1 N HCl

↓

Extracted with di-ethyl ether (3X) using separatory funnel

↓ ↘ Organic phase discarded

pH of aqueous phase adjusted to 7.8 with NH_4OH (0.1 N)

↓

Extracted with equal volume of n- butanol (3X)

↓

Fractions combined and evaporated under reduced pressure at 40^{0}C using rotary evaporator

↓

Residue dissolved in 0.1 N HCl (10 ml)

↓

Dowex -50 column chromatography

↓

Eluted with the 0.1 N NH_4OH (200 ml)

↓

Evaporated the elutate to dryness

↓

Residue dissolved the in minimum quantity of distilled water and used for bioassays.

Separation of Cytokinins

Cytokinins were separated by thin layer chromatography using silica gel-G plates with solvent n-butanol: ammonium hydroxide (I N): water (7:1:2). The plates were examined under U.V light (254 nm) and observed for the fluorescent spots.

3.6 EVALUATION OF BACTERIAL PLANT GROWTH REGULATORS BY DIFFERENT BIOASSAYS

3.6.1 Auxins:

Avena coleoptile straight test:

Coleoptiles (0.1 cm) length of 3 days old seedlings will be floated in Petri dish containing 1ml solution of test extracted solution, 1ml standard (IAA:10µg or 100µg) and 1ml water (blank) and will be incubated at 28^0C for 48h in dark. Length of section will be measured before and after the experiment.

Observations: Change in length of segment

3.6.2 Gibberellins:

α- amylase release test:

Rice seeds

↓

Cutted apical halves without embryo

↓

Sterilized with 1% sodium hypochloride solution for 10-15 minutes

↓

Rinsed with sterilized water (3X)

↓

Weighed in group of five

↓

Added 1ml of sterilized distilled water (blank), test solution (supernatant or extracted sample and standard solution 10-100 µg

↓

Incubate 30^0C for 4 days in dark

↓

Discard the test solution

Endosperm rinsed with small volume of sterilized distilled water

↓

Grinded in 5ml of 0.2% of $CaCl_2$ (200 ml)

↓

Mixture was centrifuged at 2000rpm for 15min

↓

1ml of filtrate add 4ml of acetate buffer containing 0.25% starch solution will be added and will be incubated at 40^0C for 30 min.

↓

5ml of 0.03N iodine solution added to reaction mixture

↓

Absorbance measured at 700nm

↓

Zero hour control was maintained i.e. 5 ml of acetic acid will be added to first step.

Observations: OD at 700nm using red filter.

α- amylase activity: %unit = $\frac{C-E}{C}\times 100$

C = OD at initial starch solution

E = OD at the end of the reaction

3.6.3 Cytokinins

Radish cotyledons expansion test:

The radish seeds, smaller cotyledons were transferred to sterilized Petri dishes containing the test solutions (7ml) or water (blank) on filter paper strips. 12 cotyledons were placed in each Petri dish and were incubated at 25^0C under fluorescent light for 3 days. The cotyledons were filtered and weighted. The bioassay doses response curve drawn (initial weight – final weight) was expressed as increase in weight against concentration of cytokinins present in the extract will calculate by preparing standard doses responses curve by using kinetin as standard.

Observations: Change in Weight of cotyledons.

STATISTICAL ANALYSIS: The data obtained will be subjected to analysis of variance technique using Completely Randomized Design (CRD).

Chapter-IV

EXPERIMENTAL RESULTS

To determine the status of total bacterial and *Pseudomonas* sp. total of fifteen composite rhizosphere soil samples along with portion of roots from apple and pear were collected from two sites i.e., Nagwain and Sanyardi of normal and replant area of Mandi district.

4.1 ISOLATION, ENUMERATION AND IDENTIFICATION

Total bacterial and *Pseudomonas* sp. were isolated and enumerated from the composite soil sample of apple and pear by standard pour plate and serial dilution method using nutrient agar and King's B medium. Total bacterial count from both normal and replant site were recorded after 48 h of incubation that are presented in terms of log of colony forming unit (cfu) per gram of soil using different dilution range (10^{-2} to 10^{-4}).

Table 1. Survey and collection of rhizosphere soil samples from apple and pear in normal and replant sites of Mandi district viz Nagwain and Sanyardi.

Plant	Site name	Type of site	Total no of #composite soil sample	Total number of isolates	Symbolic designation to isolates
		****Normal/*Replant**			
Apple	Nagwain	5	5	10	AN-Nag
	Nagwain	3	3	3	AR-Nag
Pear	Sanyardi	4	4	14	PN-San
	Sanyardi	1	3	3	PR-San

* Replant site (R) is the site which often encountered in establishing new orchards on old sites and poor growth of newly planted trees is common.

** Normal site (N) where the apple and pear plants are growing in normal orchards

Composite soil samples made by mixing the soil sample that were collected randomly from five different plant rhizosphere in the same site.

The results (Tables 2) showed that there was not much difference in total population of bacteria in soil samples collected from normal site of apple and pear as compared to total bacterial population present in rhizospheric soil from replant site of apple and pear.

There were too many total bacterial and *Pseudomonas* to count in 10^{-1} dilution in normal and replant site of apple and pear. Total viable count of bacteria ranged from 4.97 to 6.3 log cfu / g soil in both normal and replant site of rhizosphere of apple and pear and total viable count of *Pseudomonas* ranged from 0 to 5.4.

But there was significant difference found in total population of *Pseudomonas* in rhizospheric soil from normal site of apple and pear as compared to population present in replant site. The population level of Pseudomonas was much higher in normal area rhizospheric soil as compare to replant area rhizospheric soil. The results are also well documented and clearly observable in bar diagrams (Fig. 1 – 4).

Maximum isolates showed typical colony morphological features as that of *Pseudomonas* sp. The maximum isolates were found to be aerobic, fluorescent pigment (greenish yellow) producer, Gram negative, small rods and non spore producing bacteria (Table 3). Maximum strains were found to be positive for catalase, oxidase, gelatin liquification, denitrification and fermentation metabolism tests and negative for utilization of lactose and sucrose. Maximum strains were positive for oxidative fermentation of glucose without any gas formation. Observations recorded in growth characteristics of the isolates at different optimum temperature i.e., 4^0C, 25 ^{0}C and 41^0C to differentiate between three major different *Pseudomonas* species (Table 4a, b).

Based on colony morphology, microscopic observation, biochemical and physiological properties the maximum isolates were identified as fluorescent *Pseudomonas* sp.

Table 2. **Status of total bacterial and total fluorescent *Pseudomonas* viable density present in normal and replant site of apple**

Plant	**Type of site/ Name of site**	**No. of samples**	**Population status (viable count)**					
			Total bacterial count (log cfu/g soil)			**Total *Pseudomonas* count (log cfu/g soil)**		
			10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}
Apple	Normal/Nagwain	1	5.1	5.7	6.1	4.4	5	5.4
		2	5.1	5.8	6.3	4.4	5	5
		3	5.1	5.8	6.1	4.5	4.9	5.3
		4	5.1	5.7	6	4.6	5	5
		5	5	5.8	6	4.7	5.2	5.3
	Replant/Nagwain	1	5.1	5.7	6.1	4.6	5.1	5
		2	5	5.6	6.2	4.1	4.6	0
		3	5.1	5.5	6.2	3.8	4.3	0
Pear	Normal/Sanyardi	1	5.11	5.43	6	4.4	5	5
		2	5.1	5.7	6.3	4.6	4.9	5.3
		3	5	5.47	6.1	4.6	5	5
		4	5.16	5.79	6.2	4.3	5	5.3
	Replant/Sanyardi	1	5.17	5.67	6.3	4.6	5	5
		2	5.13	5.7	6.2	3.9	4.3	0
		3	4.97	5.65	6.3	3.8	4.6	0

There are too many counts in 10^{-1} dilution in normal and replant sites of apple and pear

4.1.3 Morphological, physiological and biochemical characteristics of fluorescent *Pseudomonas* isolates from rhizospheric soil of apple and pear

Table 3. Morphological characterization of 72 h old fluorescent *Pseudomonas* sp.

Plant	*Pseudomonas* Isolate	Colony morphology				Gram reaction	Spore staining	Pigmentation/ fluorescence	Levan/ slime production
		Shape	Elevation	Edge	Opacity				
Apple	AN-1-NAG	Coccobacillus	Raised	Entire	Transparent	-	-	Grayish	Mucoid
	AN-2-NAG	Coccobacillus	Raised	Entire	Translucent	-	-	Grayish	Mucoid
	AN-3-NAG	Rods	Flat	Entire	Translucent	-	-	Dark brown	-
	AN-4-NAG	Coccobacillus	Raised	Entire	Translucent	-	-	Brown	Mucoid
	AN-5-NAG	Coccobacillus	Flat	Entire	Translucent	-	-	Grayish	-
	AN-6-NAG	Coccobacillus	Flat	Entire	Translucent	-	-	Grayish	-
	AN-7-NAG	Coccobacillus	Flat	Entire	Transparent	-	-	Greenish	-
	AN-8-NAG	Irregular	Flat	Entire	Translucent	-	-	Yellow green	Mucoid
	AN-9-NAG	Irregular	Raised	Entire	Translucent	-	-	Grayish	Mucoid
	AN-10-NAG	Coccobacillus	Flat	Entire	Translucent	-	-	Greenish	Mucoid
	AR-1-NAG	Coccobacillus	Raised	Entire	Translucent	-	-	Yellow green	-
	AR-2-NAG	Rods	Flat	Entire	Translucent	-	-	Grayish	-
	AR-3-NAG	Rods	Flat	Entire	Transparent	-	-	Greenish	-
Pear	PN-1-SAN	Rods	Flat	Entire	Transparent	-	-	Yellow green	-
	PN-2-SAN	Rods	Flat	Entire	Translucent	-	-	Grayish	Mucoid
	PN-3-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Brown	Mucoid
	PN-4-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Greenish	Mucoid
	PN-5-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Greenish	Mucoid
	PN-6-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Greenish	-
	PN-7-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Yellow green	-
	PN-8-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Greenish	Mucoid
	PN-9-SAN	Irregular	Raised	Entire	Translucent	-	-	Greenish	-
	PN-10-SAN	Rods	Raised	Entire	Translucent	-	-	Greenish	-
	PN-11-SAN	Irregular	Raised	Entire	Translucent	-	-	Yellow green	Mucoid
	PN-12-SAN	Rods	Flat	Entire	Translucent	-	-	Grayish	-
	PN-13-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Pinkish	-
	PN-14-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Grayish	Mucoid
	PR-1-SAN	Coccobacillus	Flat	Entire	Translucent	-	-	Grayish	-
	PR-2-SAN	Coccobacillus	Raised	Entire	Translucent	-	-	Grayish	-
	PR-3-SAN	Rods	Raised	Entire	Translucent	-	-	Greenish	-

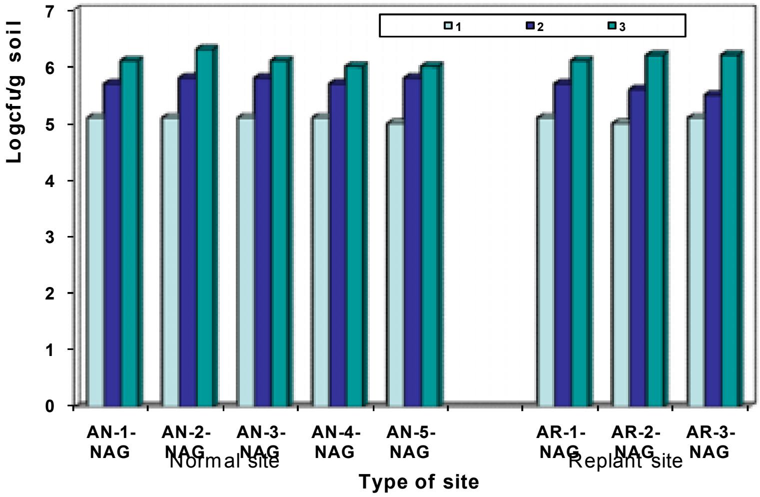

Fig. 1. **Bar diagram showing total bacterial viable density (log cfu/ g of soil) present in normal and replant site of apple in Nagwain (Mandi district).**

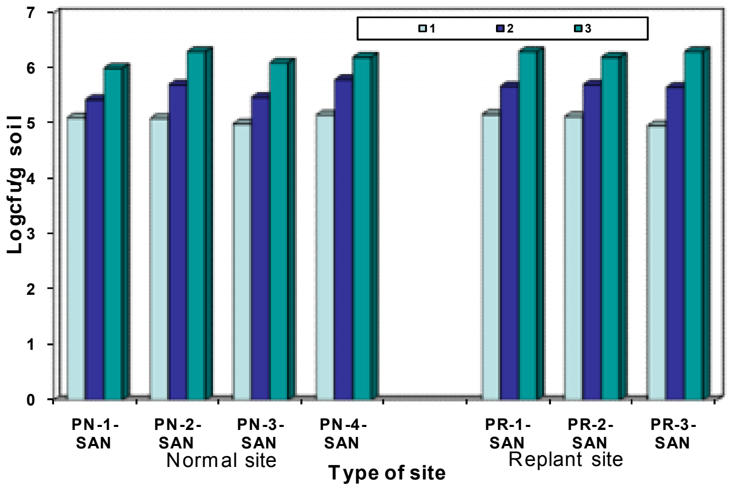

Fig. 2. **Bar diagram showing total bacterial viable density (log cfu/ g of soil) present in normal and replant site of pear in Sanyardi (Mandi district).**

Log cfu/g soil

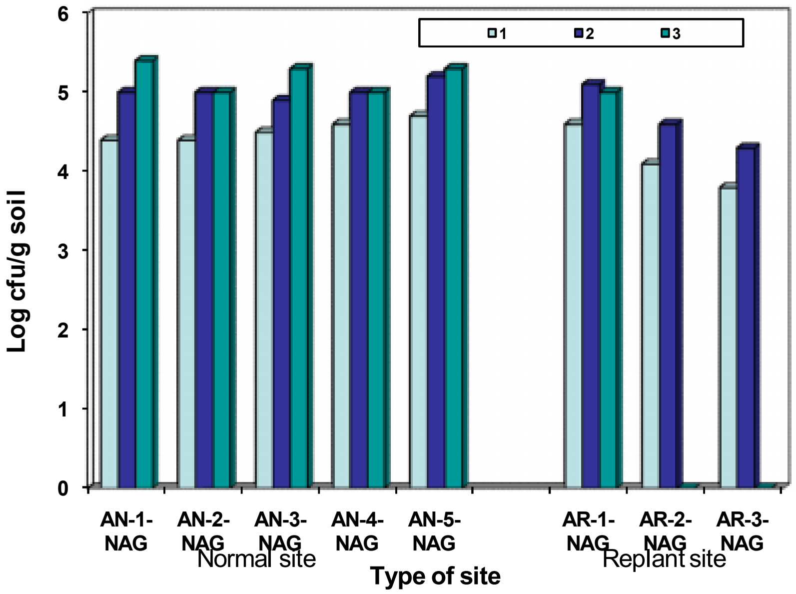

Fig. 3. Bar diagram showing total *Pseudomonas* viable density (log cfu/ g of soil) present in normal and replant site of apple in Nagwain (Mandi district).

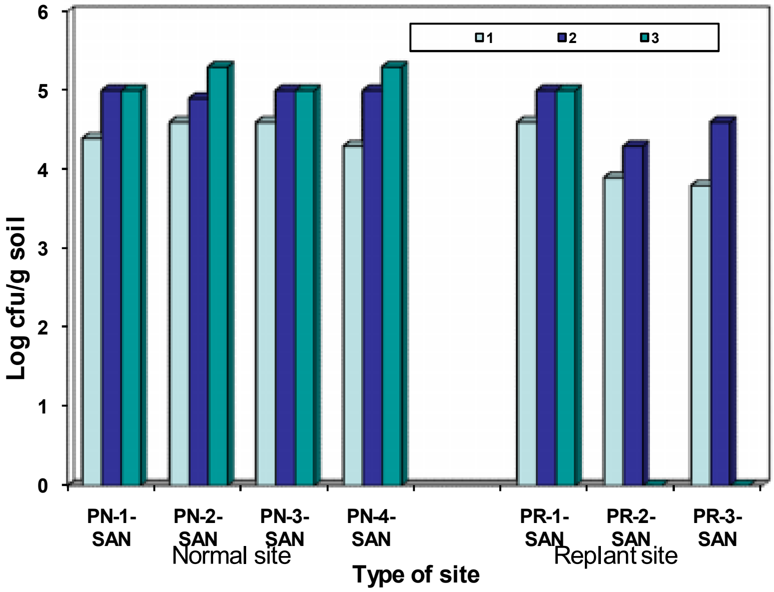

Fig. 4. Bar diagram showing total *Pseudomonas* viable density (log cfu/ g of soil) present in normal and replant site of pear in Sanyardi (Mandi district).

Table 4(a) Physiological and biochemical characterization of 72h old fluorescent *Pseudomonas* isolated from rhizosphere of apple and pear

Plant	*Pseudomonas* Isolate	Catalase test	Oxidase test	Growth at temperatures			Gelatin liquification	Metabolism of glucose	Denitrification test
				4°C	25°C	41°C			
Apple	AN-1-NAG	+	+	-	+	+	+	+	+
	AN-2-NAG	+	+	+	+	-	+	+	+
	AN-3-NAG	+	+	-	+	+	+	+	+
	AN-4-NAG	+	+	+	+	-	+	+	+
	AN-5-NAG	+	+	+	+	-	+	+	+
	AN-6-NAG	+	+	-	+	+	+	+	+
	AN-7-NAG	+	+	-	+	+	+	+	+
	AN-8-NAG	+	+	+	+	-	+	+	+
	AN-9-NAG	+	+	+	+	-	+	+	+
	AN-10NAG	+	+	-	+	+	+	+	+
	AR-1-NAG	+	+	-	+	-	+	+	-
	AR-2-NAG	+	+	-	+	+	+	+	+
	AR-3-NAG	+	+	-	+	-	+	+	-
Pear	PN-1-SAN	+	+	-	+	+	+	+	+
	PN-2-SAN	+	+	-	+	+	+	+	+
	PN-3-SAN	+	+	+	+	-	+	+	+
	PN-4-SAN	+	+	+	+	-	+	+	+
	PN-5-SAN	+	+	-	+	+	+	+	+
	PN-6-SAN	+	+	+	+	-	+	+	+
	PN-7-SAN	+	+	+	+	-	+	+	+
	PN-8-SAN	+	+	-	+	+	+	+	+
	PN-9-SAN	+	+	-	+	+	+	+	+
	PN-10-SAN	+	+	+	+	-	+	+	+
	PN-11-SAN	+	+	+	+	-	+	+	+
	PN-12-SAN	+	+	-	+	-	+	+	-
	PN-13-SAN	+	+	-	+	+	+	+	+
	PN-14-SAN	+	+	-	+	+	+	+	+
	PR-1-SAN	+	+	-	+	+	+	+	+
	PR-2-SAN	+	+	-	+	-	+	+	-
	PR-3-SAN	+	+	-	+	-	+	+	-

-	Indicates no activity
+	Indicates activity

Table 4(b) Physiological and biochemical characterization of 72h old fluorescent *Pseudomonas* isolated from rhizosphere of apple and pear

Plant	*Pseudomonas* Isolate	Tween 80 hydrolysis	Poly β hydroxy butyrate	Lecithinte test	Growth on different organic substrates				
					Glucose	Lactose	Sucrose	Succinic acid	Glycerol
Apple	AN-1-NAG	+	-	-	+	-	-	+	+
	AN-2-NAG	+	-	+	+	-	-	+	+
	AN-3-NAG	+	-	-	+	-	-	+	+
	AN-4-NAG	+	-	+	+	-	-	+	+
	AN-5-NAG	-	-	+	+	-	-	+	+
	AN-6-NAG	+	-	-	+	-	-	+	+
	AN-7-NAG	+	-	-	+	-	-	+	+
	AN-8-NAG	-	-	+	+	-	-	+	+
	AN-9-NAG	+	-	+	+	-	-	+	+
	AN-10-NAG	+	-	-	+	-	-	+	+
	AR-1-NAG	+	-	-	+	-	-	+	+
	AR-2-NAG	+	-	-	+	-	-	+	+
	AR-3-NAG	+	-	-	+	-	-	+	+
Pear	PN-1-SAN	+	-	-	+	-	-	+	+
	PN-2-SAN	+	-	-	+	-	-	+	+
	PN-3-SAN	+	-	+	+	-	-	+	+
	PN-4-SAN	+	-	+	+	-	-	+	+
	PN-5-SAN	-	-	-	+	-	-	+	+
	PN-6-SAN	+	-	+	+	-	-	+	+
	PN-7-SAN	+	-	+	+	-	-	+	+
	PN-8-SAN	+	-	-	+	-	-	+	+
	PN-9-SAN	+	-	-	+	-	-	+	+
	PN-10-SAN	+	-	+	+	-	-	+	+
	PN-11-SAN	+	-	+	+	-	-	+	+
	PN-12-SAN	+	-	-	+	-	-	+	+
	PN-13-SAN	+	-	-	+	-	-	+	+
	PN-14-SAN	+	-	-	+	-	-	+	+
	PR-1-SAN	+	-	-	+	-	-	+	+
	PR-2-SAN	+	-	-	+	-	-	+	+
	PR-3-SAN	+	-	-	+	-	-	+	+

-	Indicates no activity
+	Indicates activity

BIOLOGICAL ACTIVITIES

4.2 SCREENING OF FLUORESCENT *PSEUDOMONAS* SP. ISOLATES FOR THE PRODUCTION OF DIFFERENT PLANT GROWTH PROMOTING ACTIVITIES

4.2.1 Production of pant growth regulators viz. auxins, gibberellins and cytokinins by isolates from:

All the isolates of *Pseudomonas* species isolated from rhizosphere soil of apple and pear were screened out for the production of plant growth regulators viz., auxins, gibberellins and cytokinins by their specific spectrophotometeric and bioassay method by using 72 h old culture supernatants. The result are presented in Table 5.

Almost all the isolates of fluorescent *Pseudomonas* sp. produced plant growth regulators. The production of each plant growth regulator varies from species to species among the organism that may be strain specific. Each growth regulator produced under standard cultural condition i.e., 28^{o}C for 72 hr under shaken condition as estimated from their respective standard curves produced and was expressed in terms of concentration (μg/ml).

4.2.1.1 Auxins

The maximum auxins production was shown by *Pseudomonas* sp. by four isolates from apple and seven isolates from pear of fluorescent *Pseudomonas* sp. in the range of 15 μg/ml to 30μg/ml. All isolates differed statistically and significantly from each others in terms of production of auxins. Some strains i.e., six from apple and eight from pear showed less production of auxins i.e., in the range 9 μg/ml to 10 μg/ml. Two strains of fluorescent *Pseudomonas* sp. isolated from apple showed more less concentration i.e., 5 μg/ml and two strains one each from apple and pear showed very less concentration of auxins i.e., 1 μg/ml.

4.2.2 Gibberellins

Out of thirty isolates of *Pseudomonas* species seventeen isolates i.e, eight from apple and nine from pear produced gibberellins in the range of 40-

Table5. Screening of fluorescent *Pseudomonas* for the production of plant growth regulators i.e. auxins, gibberellins and cytokinins

Plant	Bacterial isolates	Plant growth regulators		
		Auxins *	Gibberellins **	Cytokinins ***
		conc. (µg/ml)	conc. (µg/ml)	conc. (µg/ml)
Apple	AN-1-NAG	22.5	50	100
	AN-2-NAG	30	60	100
	AN-3-NAG	17.5	60	80
	AN-4-NAG	10	25	100
	AN-5-NAG	1	55	80
	AN-6-NAG	7	35	80
	AN-7-NAG	7	20	30
	AN-8-NAG	15	25	80
	AN-9-NAG	8.5	50	80
	AN-10-NAG	5	55	100
	AR-1-NAG	5	48	150
	AR-2-NAG	7.5	25	70
	AR-3-NAG	7	50	30
C.D.		**1.43**	**1.63**	**1.63**
Pear	PN-1-SAN	15	50	100
	PN-2-SAN	30	55	150
	PN-3-SAN	9	54	80
	PN-4-SAN	17.5	21	150
	PN-5-SAN	7.5	50	80
	PN-6-SAN	7	45	100
	PN-7-SAN	10	48	80
	PN-8-SAN	17	21	150
	PN-9-SAN	15	50	80
	PN-10-SAN	30	55	80
	PN-11-SAN	7.5	48	30
	PN-12-SAN	10	25	80
	PN-13-SAN	17.5	22	80
	PN-14-SAN	8.5	21	30
	PR-1-SAN	1	19	80
	PR-2-SAN	7	21	70
	PR-3-SAN	7	19	70
C.D.		**1.40**	**1.63**	**1.63**

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 the standard curve of indole acetic acid (IAA) (10-100 µg/ml), of gibberellic acid (GA_3) (100-1000 µg/ml) and of kinetin (100-1000 µg/ml).

- Each value is mean of triplicate.

to 60 μg/ml. while rest of thirteen strains produced gibberellins in the range of 19 μg/ ml to 25 μg/ml in this level of production five strains are that of apple and eight that of pear. All isolates differed statistically and significantly from each others in terms of production of gibberellins.

4.2.3 Cytokinins

All the thirty isolates of fluorescent *Pseudomonas sp***.** produced cytokinins as calibrated from the change in weight of radish cotyledon when treated with 72 hr supernatants. The production pattern for cytokinins by all the isolates of fluorescent *Pseudomonas* sp. isolated from rhizospheric soil of apple and pear is as follow

34.4% (i.e., ten isolates out of total twenty nine isolates both from rhizospheric soil of apple and pear produced cytokinins in the high range i.e., 100 μg/ml to 150 μg/ml. Similarly 44.3% isolates (i.e., twelve isolates) produced this regulators of upto 80 μg/ml concentration and only 10.3% (i.e., three isolates) produced 70 μg/ml. only four isolates i.e., 13.7% produced less amount of cytokinins i.e., 30 μg/ml as compared to other isolates.

4.2.1 Production of antifungal activity by isolates from apple and pear against:

Isolates of *Pseudomonas* species were screened out for the production of antifungal activity by well plate assay method against six indicator test fungi viz. *Fusarium oxysporum, Alternaria* sp*.*, *Pythium* sp., *Rhizoctonia solani* and *Sclerotium* sp. The results (Table 6) showed that maximum per cent inhibition was found against *Pythium* sp. by both *Pseudomonas.*

4.2.1.1 *Fusarium oxysporum*

Maximum strains of *Pseudomonas* species isolated from rhizosphere soil of apple and pear showed antifungal activity against *Fusarium oxysporum*. Maximum per cent growth inhibition has been shown by four *Pseudomonas* strain PN-4-SAN, PN-8-SAN and PN-10-SAN (52%) whereas maximum replant isolates showed no activity (Table 6, Plate 1). It was observed that there was statistically significant difference between all isolated bacterial strains.

4.2.1.2 *Alternaria* sp.

Most of *Pseudomonas* species isolated from rhizosphere soil of apple and pear showed antifungal activity against *Alternaria* sp. Maximum per cent growth inhibition has been shown by *Pseudomonas* strain AN-6-NAG and PN-5-SAN (52%) followed by four isolates AN-3-NAG, AN-5-NAG, AN-7-SAN, PN-9-SAN and PN-10-SAN. It was observed that there was statistically significant difference between all isolated bacterial strains. Where as replant isolates showed no or weak activity (Table6, Pate2). There was statically significant difference between all strains isolated from rhizosphere soil of pear orchards.

4.2.1.3 *Pythium* sp.

Antifungal activity by *Pseudomonas* strains against plant pathogen *Pythium* sp. has been shown by all strains isolated from normal site. *Pseudomonas* strain showed maximum per cent growth inhibition AN-9-SAN, PN-2-SAN and PN-11-SAN (52 %) followed by AN-3-SAN, AN-5-SAN (46.66 %) while AR-2-NAG, AR-3-NAG isolates showed no activity against this plant pathogen (Table 6, Plate3). It was observed that all bacterial isolates differed significantly from each other.

4.2.1.3 *Rhizoctonia solani*, *Dematophora* and *Sclerotium* sp.

All the strains of *Pseudomonas* isolated from the rhizospheric soil of normal and replant site of apple and pear did not show antifungal activity against any of these two plant pathogen i.e. *Rhizoctonia solani, Dematophora sp.* and *Sclerotium* sp.

Table6. In vitro production of antifungal activity by fluorescent *Pseudomonas* sp. isolated from rhizospheric soil of apple and pear in normal and replant sites against different indicator test fungi.

Plant	**Bacterial isolates**	**Antifungal activity indicator test fungi(% inhibition)***		
		Fusarium oxysporum	***Alternaria* sp.**	***Pythium* sp.**
Apple	AN-1-NAG	22.60 (28.38)	33.30 (35.24)	40.00 (39.23)
	AN-2-NAG	40.00 (39.23)	45.30 (42.30)	22.60 (28.38)
	AN-3-NAG	32.00 (34.4)	48.80 (44.31)	46.66 (43.08)
	AN-4-NAG	40.00 (39.23)	00.00 (00.00)	40.00 (39.23)
	AN-5-NAG	46.66 (43.08)	48.80 (44.31)	46.66 (43.08)
	AN-6-NAG	00.00 (00.00)	52.20 (46.26)	21.30 (27.49)
	AN-7-NAG	00.00 (00.00)	48.80 (44.31)	36.00 (36.87)
	AN-8-NAG	22.66 (28.42)	50.00 (45)	34.60 (35.67)
	AN-9-NAG	00.00 (00.00)	00.00 (00.00)	52.00 (46.15)
	AN-10NAG	00.00 (00.00)	47.70 (43.68)	36.60 (36.87)
	AR-1-NAG	00.00 (00.00)	33.30 (33.30)	52.00 (46.15)
	AR-2-NAG	22.60 (28.38)	00.00 (00.00)	00.00 (00.00)
	AR-3-NAG	22.60 (28.38)	33.30 (35.24)	00.00 (00.00)
C.D.		**1.13 (0.08)**	**0.73 (0.12)**	**1.19 (0.39)**
Pear	PN-1-SAN	46.66 (43.08)	45.30 (42.30)	28.00 (31.95)
	PN-2-SAN	44.00 (41.56)	45.30 (42.30)	52.00 (46.15)
	PN-3-SAN	37.30 (37.64)	50.00 (45)	34.60 (36.03)
	PN-4-SAN	52.00 (46.15)	50.00 (45)	17.33 (24.60)
	PN-5-SAN	37.30 (37.64)	52.20 (46.26)	17.33 (24.60)
	PN-6-SAN	34.66 (36.07)	36.60 (37.23)	28.00 (31.95)
	PN-7-SAN	25.33 (30.22)	00.00 900.00)	22.60 (28.38)
	PN-8-SAN	52.00 (46.15)	36.60 (37.23)	32.00 (34.40)
	PN-9-SAN	00.00 (00.00)	48.80 (44.31)	44.00 (41.56)
	PN-10-SAN	52.00 (46.15)	48.80 (44.31)	44.00 (41.56)
	PN-11-SAN	42.66 (40.78)	45.30 (42.30)	52.00 (46.15)
	PN-12-SAN	48.00 (43.85)	50.00 (45)	44.00 (41.56)
	PN-13-SAN	00.00 (00.00)	00.00 (00.00)	25.33 (30.22)
	PN-14-SAN	00.00 (00.00)	42.20 (40.57)	20.00 (26.57)
	PR-1-SAN	00.00 (00.00)	33.30 (35.24)	17.33 (24.60)
	PR-2-SAN	00.00 (00.00)	00.00 (00.00)	20.00 (26.57)
	PR-3-SAN	00.00 (00.00)	00.00 (00.00)	17.33 (24.60)
C.D.		**0.98 (0.48)**	**1.70 (0.56)**	**1.31 (0.27)**

Values in parenthesis are arc sin transformed

All the isolates did not show antifungal activity against *Rhizoctinia Dematophora* and *Sclerotiumsp*

* Antifungal activity expressed in terms of mm diameter of growth inhibition of mycelium or percent growth inhibition against different fungal pathogens as

$$\times\ \frac{C-T}{C}\ 100 = \%\ \text{inhibition}$$

C: Growth of mycelium in control

T: Growth of mycelium in treatment

4.2.2 Siderophores:

Strains of *Pseudomonas* species were screened out for the production of siderophore on Chrome azurol-S (CAS) plate and also evaluated by quantitative liquid assay method using 72 hours old culture supernatants. The results (Table7, Plate 5) were obtained in the form of pinkish/orange colored zone (mm diameter) produced around the well (7 mm) on Chrome azurol-S (CAS) agar plate at 28^{o}C for 24 h whereas in liquid assay method, results were expressed in the form of reduction in blue color as compared to reference and expressed as percent S.U. (siderophore units)

The siderophore production varied among all the *Pseudomonas* and strains. The maximum siderophore production in terms of orange zone was shown by for isolates of from apple and four isolates from pear in the range of 28mm to 32mm diameter of yellow/ orange zone on CAS plate at 28 ± 2^{0}C for 24 hours followed by thirteen isolates i.e., 44.8% that showed siderophore production in the range of 21-27mm diameter while rest of 27.5% isolates i.e., eight showed activity in the range of 18-20 mm diameter. PR-2-SAN was statistically at par with AR-2-NAG, AR-3-NAG, PN-5-SAN and PN-14-SAN while other strains were statistically different from each other.

All bacterial isolates were also quantified for production of siderophores by quantitative Chrome azurol-S (CAS) liquid assay method. The results (Table65) showed that maximum siderophore production in terms of percent siderophore units (% SU) was shown by produced by *Pseudomonas* strain PN-6-SAN (60 % SU) followed by three isolates for pear and one isolate for apple that showed siderophore production in the range of 52-56% S.U. 50.6% of the isolates i.e., seven from apple and ten from pear showed production between 41-47% S.U. rest of the isolates i.e., 27.5% i.e., five from apple and three from pear showed production between 29-39% S.U. Results revealed that there was significant difference between all isolates.

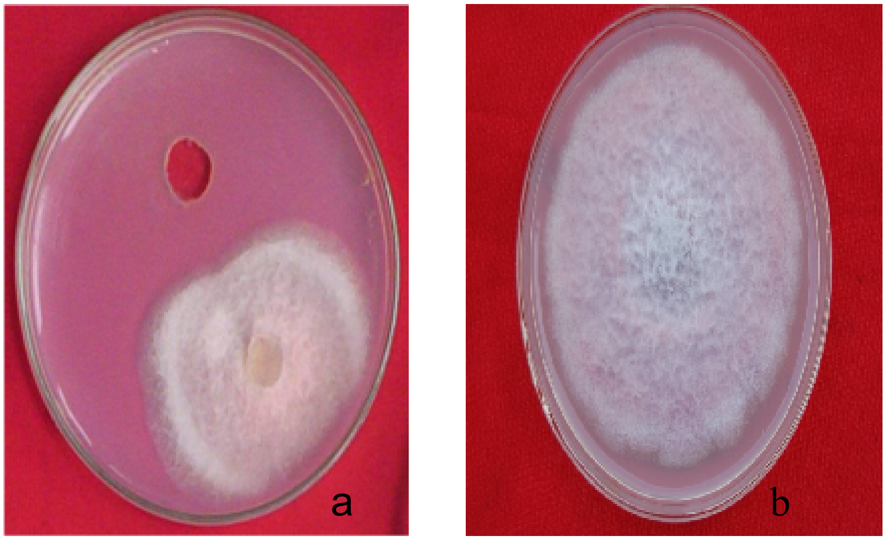

Plate 1: **Antifungal activity of *Pseudomonas* strains viz. PN-10-SAN (a), control (b) against *Fusarium oxysporum* at $28^{o}C$ for four days**

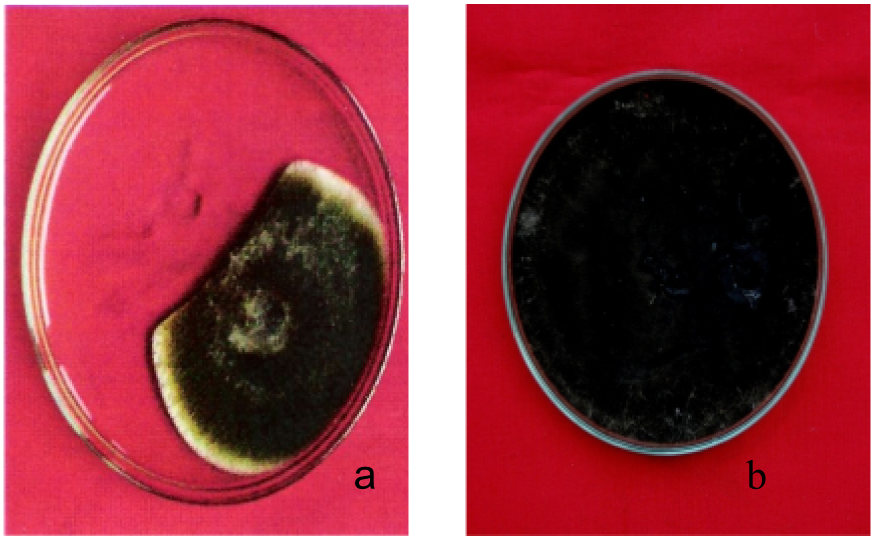

Plate 2: **Antifungal activity of *Pseudomonas* strains viz. PN-4-SAN (a) control (b) against *Alternaria sp.* at $28^{0}C$ for six days**

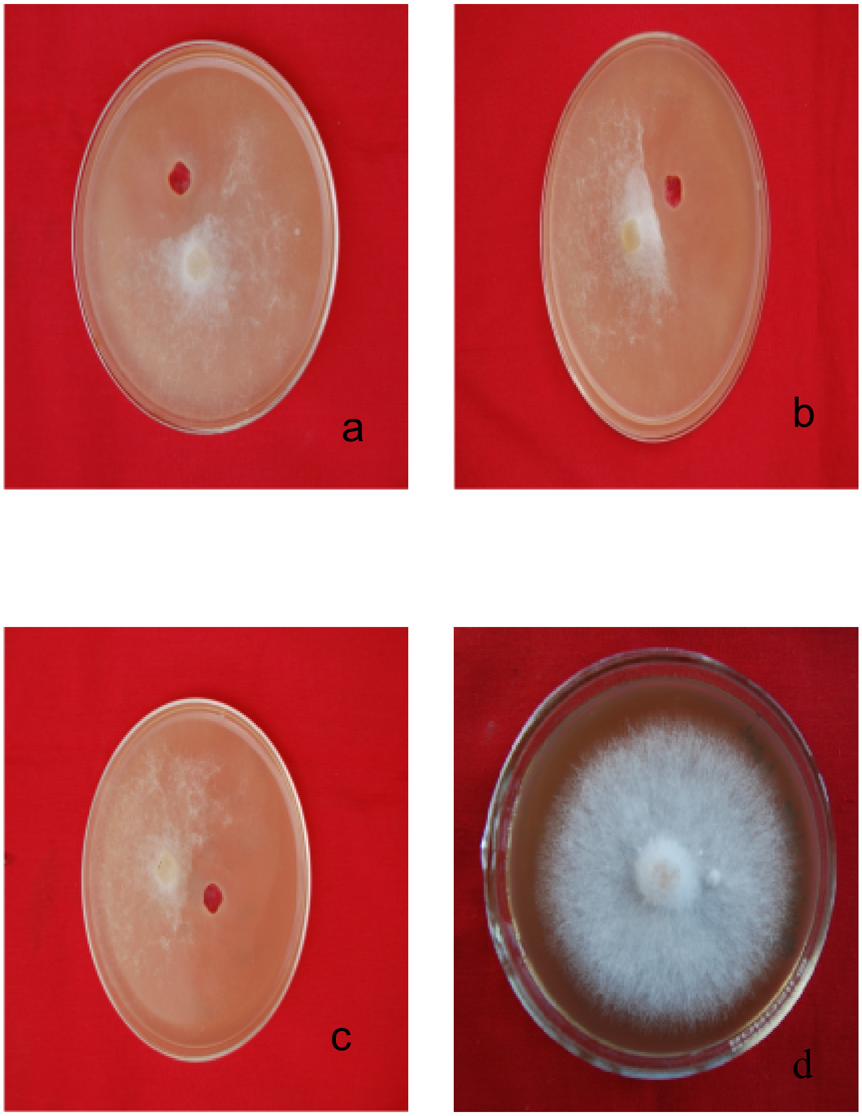

Plate 3: Antifungal activity of *Pseudomonas* strains viz. AN-10-NAG (a), PN-2-SAN (b), PN-11-SAN (c), control (d) against Pythium at 28^{0}C after four days

Table7. Siderophore production by fluorescent *Pseudomonas* sp. isolated from the rhizosphere soil of apple and pear in normal and re

Plant	**Bacterial isolates**	**Siderophore activity Assay method**	
		Plate	**% siderophore unit** (% SU)**
		Pinkish/Orange Zone (mm dia)*	
Apple	AN-1-NAG	28	41 (39.8)
	AN-2-NAG	28	41 (39.8)
	AN-3-NAG	20	47 (43.28)
	AN-4-NAG	21	41 (39.8)
	AN-5-NAG	27	45 (42.13)
	AN-6-NAG	28	39 (38.65)
	AN-7-NAG	29	33 (35.06)
	AN-8-NAG	21	41 (39.8)
	AN-9-NAG	23	41 (39.8)
	AN-10-NAG	25	52 (46.15)
	AR-1-NAG	26	29 (32.58)
	AR-2-NAG	19	33 (35.06)
	AR-3-NAG	19	39 (38.65)
C.D.		**1.61**	**1.66 (0.60)**
Pear	PN-1-SAN	26	41 (39.8)
	PN-2-SAN	27	52 (46.15)
	PN-3-SAN	24	43 (40.98)
	PN-4-SAN	23	47 (43.28)
	PN-5-SAN	19	56 (48.45)
	PN-6-SAN	32	60 (50.77)
	PN-7-SAN	20	54 (47.29)
	PN-8-SAN	30	41 (39.80)
	PN-9-SAN	29	43 (40.98)
	PN-10-SAN	28	43 (40.98)
	PN-11-SAN	23	47 (43.28)
	PN-12-SAN	20	45 (42.13)
	PN-13-SAN	24	45 (42.13)
	PN-14-SAN	19	41 (39.8)
	PR-1-SAN	21	39 (38.65)
	PR-2-SAN	18	39 (38.65)
	PR-3-SAN	21	32 (34.45)
C.D.		**1.61**	**1.39 (0.49)**

Values in parenthesis are arc sin transformed values

*Siderophore activity expressed in terms of mm diameter of pinkish/orange zone produced around the well on Chrome azurol-s (CAS) agar plate at $30^{o}C$ for 24h.

**The siderophore units (% SU) expressed as per cent reduction in blue colour as compared to reference i.e.

$$\frac{Ar-As}{Ar} \times 100 \qquad = \qquad \text{Siderophore unit (\% SU)}$$

Ar : Absorbance of reference at 630 nm (0.48)
As : Absorbance of test solution at 630 nm.

4.2.5 Phosphate solubilizing activity:

Almost all isolates of *Pseudomonas* sp. isolated from the rhizosphere soil of apple and pear that were screened out for production of phosphate solubilizing activity on Pikovskaya's agar plate for production of yellow zone around well at 28^{o}C for 24h as an indication of tricalcium phosphate solubilization. Phosphate solubilizing activity was also estimated by spectrophotometric method in terms of inorganic tricalcium phosphate solubilization (µg/ml) and release of phosphorus at 28^{o}C for 24.

Maximum phosphate solubilization in terms of mm diameter of yellow zone produced on Pikovskaya's agar plate was documented in Table 8, Plate 6. Maximum solubilization efficiency was shown by 39.9 % *Pseudomonas* isolates i.e., four from apple and seven from pear in the range of 29-33 mm diameter or 314-371% phosphate solubilising efficiency. Isolates number involved were AN-1-Nag, AN-4-Nag, AN-5-Nag, AN-6-Nag, PN-3-San, PN-4-San PN-10-San and PN-14-San followed by second group of isolates five from apple and five from pear showed between 25-28 mm diameter or 257-300% phosphate solubilising efficiency. AN-2-NAG (371) followed by *Pseudomonas* strain AN-4-NAG and AN-5-NAG (328). Third group of isolates were felled between the range of 19-23 mm diameter or 171 – 228.5% phosphate solubilising efficiency.

Eight isolates showed phosphate solubilization between 30 mg/ml- 330 mg/ ml while rest seven showed phosphate solubilization between 150 mg/ml – 235 mg/ml of available phosphate. Maximum phosphate solubilization of tricalcium phosphate in liquid medium has shown by two *Pseudomonas* isolates i.e., PN-2-San and PN-4-San in the range of 430 mg/ml – 445 mg/ml followed by 41.35% of isolates (i.e., twelve isolates) in phosphate solubilization between 370mg/ml to 375 mg/ml. all the isolates were statistically different from each other.

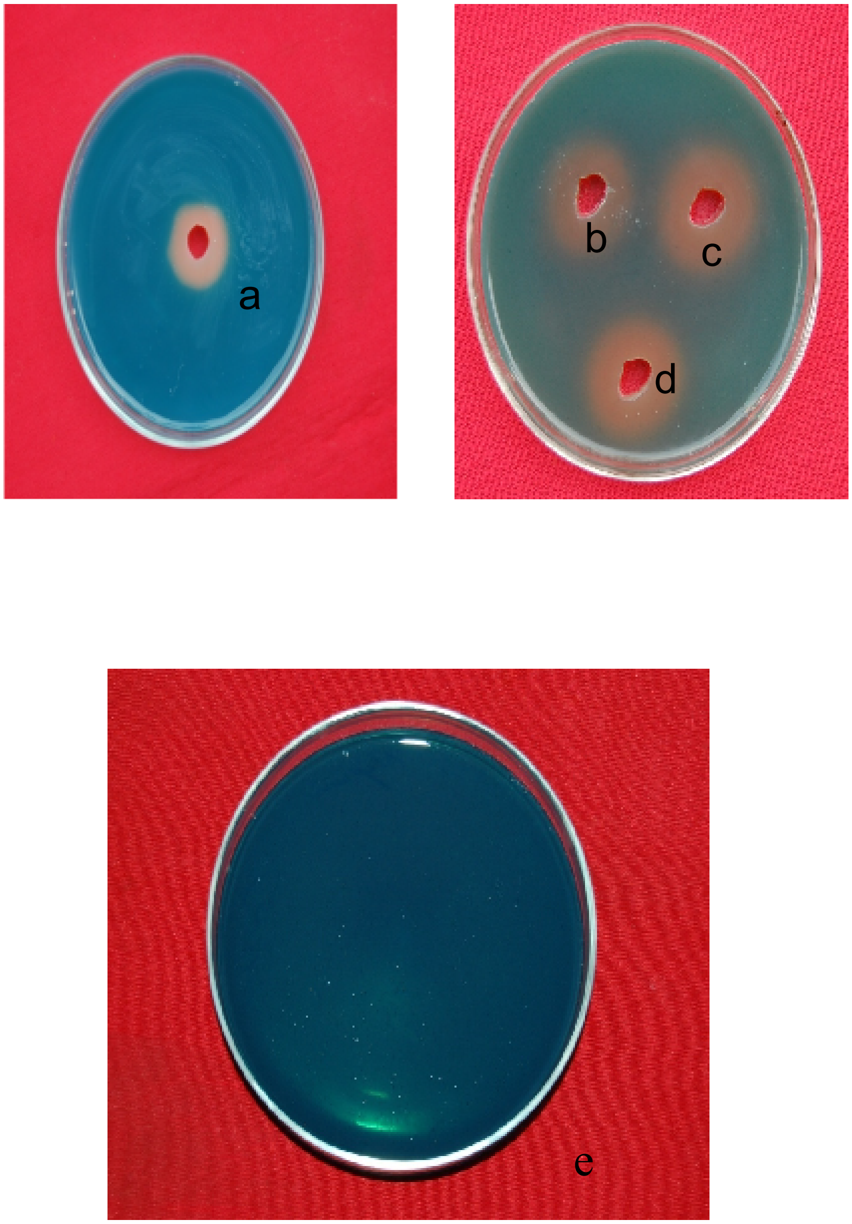

Plate 4: Siderophores production by strains of *Pseudomonas* species viz. An-2-NAG (a), PN-10-SAN (b) , PN-1-SAN **(c) PN-6-SAN (d) and control (e) on Chrome azurol-S agar at 30^{0}C after 24 hours**

Table 8.Phosphate solubilizing activity of fluorescent *Pseudomonas* sp. isolated from rhizospheric soil of apple and pear in normal and replant sites.

Plant	Bacterial isolates	Phosphate solubilization activity		
		Plate Yellow zone (mm dia)*	Phosphate solubilizing efficiency	Quantitative** Conc. (mg/ml)
Apple	AN-1-NAG	27	285.1 (2.45)	395
	AN-2-NAG	33	371 (2.57)	375
	AN-3-NAG	27	285.1 (2.45)	330
	AN-4-NAG	30	328 (2.52)	320
	AN-5-NAG	30	328 (2.52)	395
	AN-6-NAG	29	314 (2.50)	320
	AN-7-NAG	19	171 (2.23)	225
	AN-8-NAG	27	285.1 (2.45)	320
	AN-9-NAG	25	257 (2.41)	375
	AN-10-NAG	26	271 (2.43)	395
	AR-1-NAG	19	171 (2.230	320
	AR-2-NAG	25	257 (2.41)	225
	AR-3-NAG	20	185 (2.27)	320
C.D.		**1.61**	**1.43 (0.06)**	**1.63**
Pear	PN-1-SAN	26	271(2.43)	250
	PN-2-SAN	27	285.1(2.45)	430
	PN-3-SAN	30	328 (2.52)	395
	PN-4-SAN	31	342.8 (2.54)	445
	PN-5-SAN	27	285.1 (2.45)	375
	PN-6-SAN	24	242.8 (2.39)	300
	PN-7-SAN	28	300 (2.48)	320
	PN-8-SAN	23	228.5 (2.36)	355
	PN-9-SAN	26	271 (2.43)	370
	PN-10-SAN	32	357 (2.55)	235
	PN-11-SAN	29	314 (2.50)	150
	PN-12-SAN	30	328 (2.52)	225
	PN-13-SAN	31	342.8 (2.54)	375
	PN-14-SAN	31	342.8 (2.54)	395
	PR-1-SAN	23	228.5 (2.36)	225
	PR-2-SAN	21	200 (2.30)	375
	PR-3-SAN	19	171 (2.23)	225
C.D.		**1.61**	**1.79 (0.13)**	**1.33**

Phosphate solubilizing activity expressed in terms of mm diameter of yellow zone produced around the well on Pikovskaya agar plate at 28°C in *Pseudomonas* sp. respectively after 48h.

$$\text{Percent phosphate solubilizing efficiency (\%SE)} = \frac{Z - C}{C} \times 100$$

Z = Diameter of zone (mm diameter)

C = Diameter of well (mm diameter)

**Phosphate solubilizing activity expressed in terms of tricalcium phosphate solubilization which intern represent µg/ml of available orthophosphate as calibrated from the standard curve of KH_2PO_4 (10-100 µg/ml).

4.2.6 HCN and Ammonia production:

All the isolates of *Pseudomonas* species were screened out for production of HCN. Color change of filter paper (already dipped in picric acid) from yellow to brown has shown HCN production.

All the isolates of *Pseudomonas* species were screened out for production of ammonia in peptone broth after 4 days of incubation and using Nessler's reagent. Color change in broth tubes from yellow to brown showed production in ammonia. Results are presented in table 9.

Maximum production (+++++) of both the activities were shown by four isolates from apple i.e., AN-1-Nag, AN-2-Nag, AN-3-Nag and AN-5-Nag and two isolates from pear i.e., PN-3-San and PN-7-San (Table 9, Plate 7).

Two isolates from apple (AN-5-Nag, AN-10-Nag) and five isolates from normal site of pear (i.e., 2, 9, 10, 13 and 14) showed maximum production of ammonia (+++++).

4.2.7 Proteolytic activity

Proteolytic activity expressed in terms of mm diameter of clear zone produced on skim milk agar plate at 37^0C for 72h and also quantified for production of Proteolytic activity by quantitative liquid assay method.

All the *Pseudomonas* isolates from apple and pear showed production of Proteolytic activity on skim milk agar plates at 37^0C for 72h in the range of 16-32 mm diameter of clear zone and 39-70 µg/ml as units of activity.

Maximum production of zone of proteolysis (mm diameter) on milk agar plates was shown by *Pseudomonas* strain AN-10-Nag, PN-6-San (32mm) followed by AN-4-Nag (31 mm) (Table 10, Plate 8). On basis of quantitative assay PN-4-San showed maximum activity (73 µg/ml) followed by two isolates ie, PN-1-San and PN-9-San (70 µg/ml). Results depicted that almost all strains differ statistically.

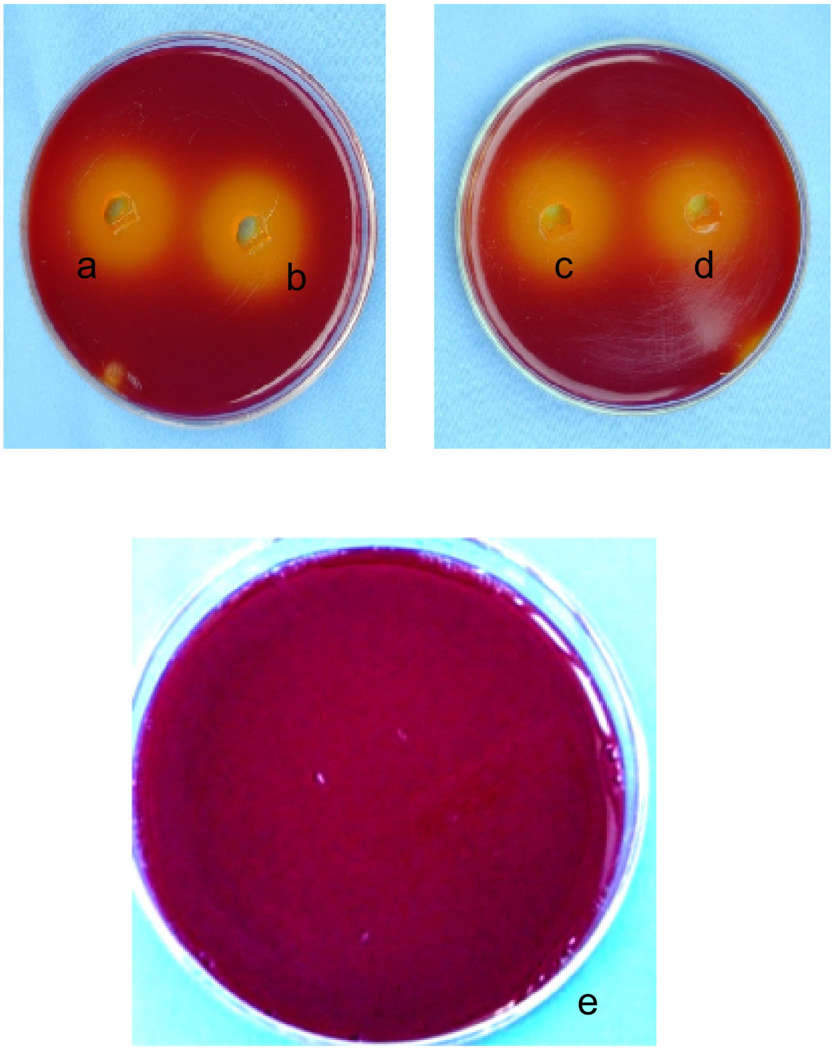

Plate 5: Phosphate solubilizing activity shown by strains of *Pseudomonas* species viz. PN-2-SAN (a) , PN-4-SAN (b), AN-1-NAG (c), AN-2-NAG (d) and control (e) on Pikovskaya's agar plate at 28 ^{0}C after 24 hours

Table 9. Production of HCN/Ammonia activity by *Pseudomonas* sp. isolated from rhizosphere soil of apple and pear.

Plant	Bacterial isolates	HCN production*	Ammonia Production**
Apple	AN-1-NAG	+++++	+++++
	AN-2-NAG	+++++	+++++
	AN-3-NAG	+++++	+++++
	AN-4-NAG	+++++	+++
	AN-5-NAG	+++	+++++
	AN-6-NAG	+++	+++
	AN-7-NAG	+++	+++
	AN-8-NAG	+++	+++
	AN-9-NAG	+++	+++
	AN-10-NAG	+++	+++++
	AR-1-NAG	+++	+++
	AR-2-NAG	+++	+++
	AR-3-NAG	+++	+++
Pear	PN-1-SAN	++	+++
	PN-2-SAN	+++	+++++
	PN-3-SAN	+++++	+++++
	PN-4-SAN	+++	+++
	PN-5-SAN	+++	+++
	PN-6-SAN	+++	+++
	PN-7-SAN	+++++	+++++
	PN-8-SAN	+++	+++
	PN-9-SAN	+++	+++++
	PN-10-SAN	+++	+++++
	PN-11-SAN	+	+++
	PN-12-SAN	+++	+++
	PN-13-SAN	+++	+++++
	PN-14-SAN	+++	+++++
	PR-1-SAN	+	+++
	PR-2-SAN	+++	+++
	PR-3-SAN	+++	+++

\- - Indicates yellow
+++ - Indicates light brown
++++ - Indicates dark brown
+++++ - Indicates orange brown

*HCN production on King's medium B agar plate expressed in terms of change of colour of picric acid paper strips from deep yellow (-) to orange brown (+++++) at 28°C by *Pseudomonas* sp. in 4 days.
**Ammonia production expressed in terms of change of colour of culture broth from faint yellow (-) to deep brown (+++++) at 28°C by *Pseudomonas* sp. for 4 days after addition of Nessler's reagent.

Table 10. Proteolytic activity of *Pseudomonas* sp. isolated from rhizosphere soil of apple and pear.

Plant	Bacterial isolates	Proteolytic activity	
		Plate	**Quantitative****
			Conc. (µg/ml)
Apple	AN-1-NAG	27	33
	AN-2-NAG	28	37
	AN-3-NAG	25	48
	AN-4-NAG	31	68
	AN-5-NAG	28	43
	AN-6-NAG	23	52
	AN-7-NAG	19	55
	AN-8-NAG	20	68
	AN-9-NAG	22	40
	AN-10NAG	32	48
	AR-1-NAG	19	37
	AR-2-NAG	24	22
	AR-3-NAG	23	39
C.D		**1.63**	**1.63**
Pear	PN-1-SAN	27	70
	PN-2-SAN	28	37
	PN-3-SAN	25	21
	PN-4-SAN	23	73
	PN-5-SAN	19	52
	PN-6-SAN	32	61
	PN-7-SAN	30	49
	PN-8-SAN	21	42
	PN-9-SAN	29	70
	PN-10-SAN	29	45
	PN-11-SAN	20	60
	PN-12-SAN	19	49
	PN-13-SAN	23	42
	PN-14-SAN	19	52
	PR-1-SAN	16	43
	PR-2-SAN	22	39
	PR-3-SAN	19	47
C.D.		**1.63**	**1.63**

- Proteolytic activity expressed in terms of mm diameters of clear zone produced around the well on skim milk agar plates at 37°C for 24h.
- One protease unit is the amount of enzymes required to solubilize 1mg of casein in Tris HCL buffer (0.05 M, pH 7) at 37^0 C with in five minutes.

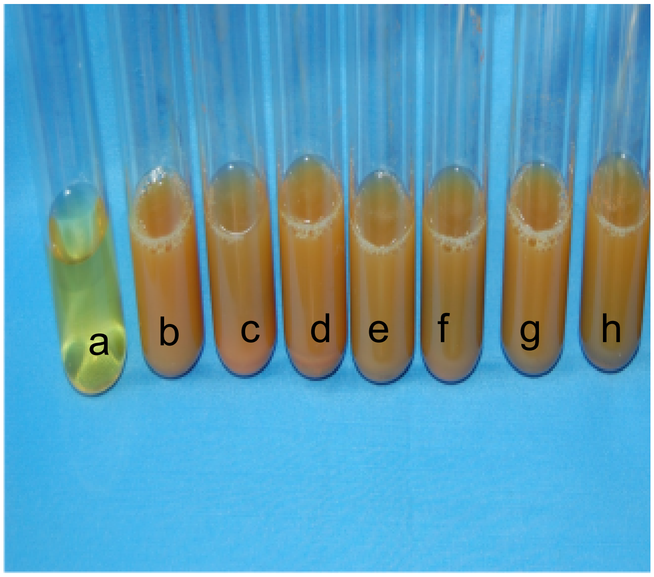

Plate 6: Production of ammonia shown by strains of *Pseudomonas* sp.(b - h) AN-2-NAG, AN-3-NAG, AN-4-NAG, AN-9-NAG, PN-3-SAN, PN-7-SAN, PN-10-SAN and control (a) in peptone water after 4 days at 28 ^{0}C

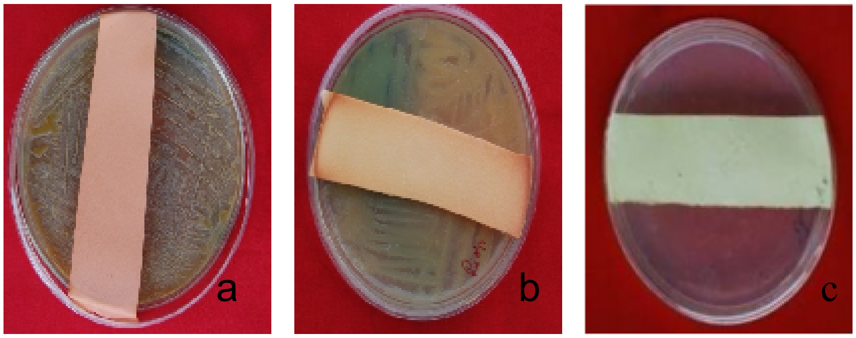

Plate 7: HCN production shown by strains of *Pseudomonas* species viz. PN-4-SAN (a), AN-4-NAG (b), control (c) at 28 ^{0}C for 4 days

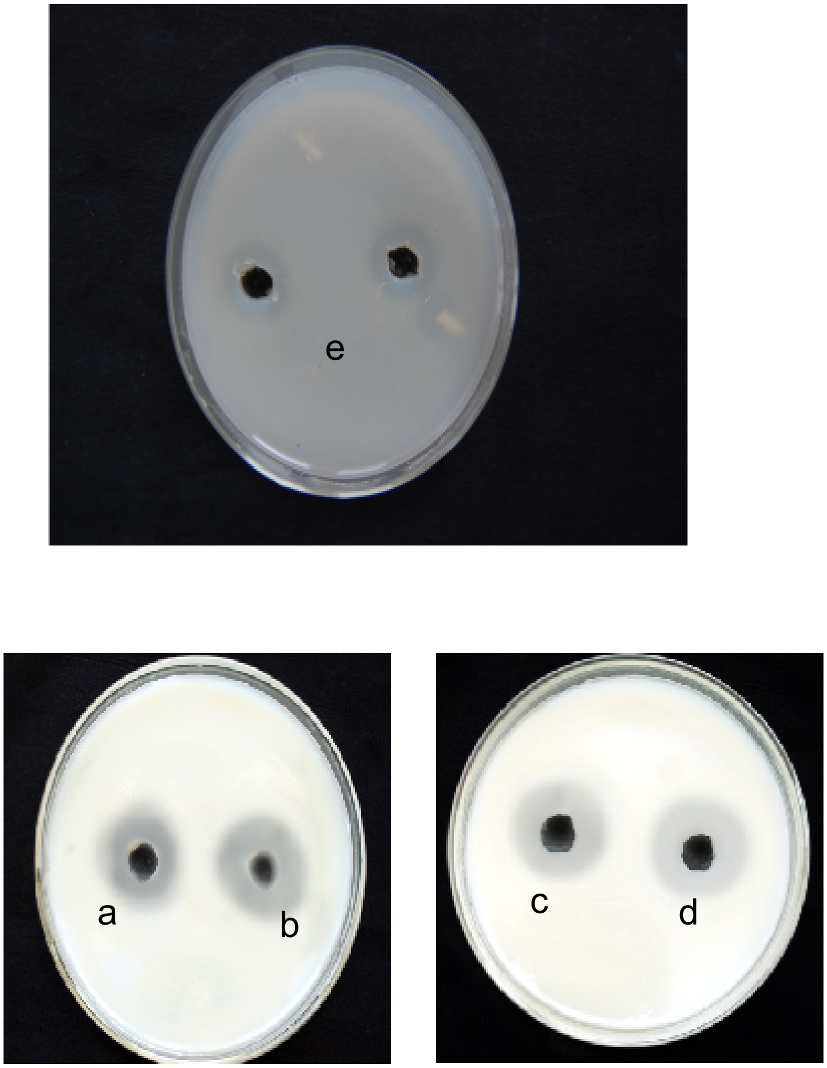

Plate 8: Proteolytic activity shown by strains of *Pseudomonas* species viz. AN-2-NAG (a), AN-4- NAG (b), PN-4-SAN(c), PN-10-SAN (d) and control (e) on skim milk agar plate at 37°C after 24 hours

4.2 Preliminary characterization, production of plant growth regulators ; auxins, gibberellins, cytokinins and selection of best media at different incubation period

4.2.1 Effect of different media on growth and production of plant growth regulators produced by fluorescent *Pseudomonas* PN-4-SAN at different incubation period.

4.2.1.1 Growth

The results (Table 11a) revealed that the average growth (O.D. 0.55) obtained was maximum in Nutrient medium and was statistically more significant than growth in other media. The interaction studies revealed that the incubation period increased gradually, the cell density also increased. The average growth was maximum at 72h (0.56).

4.2.1.2 Production of plant growth regulators:

The average production of auxins (Table 11b) by fluorescent *Pseudomonas* was maximum in Nutrient broth (22.33 µg/ml) and was statistically more significant than in other media. The average production of auxins in Succinate media (14 µg/ml), King's media (14.67 µg/ml) was statistically at par with each other.

There was significant increase in production of gibberellins (Table 11c) with increase in incubation period. The average production of gibberellins was maximum in nutrient broth (233.7 µg/ml) and at 72h (205.2 µg/ml) followed by peptone water (155.7 µg/ml). The interaction studied showed that difference in production obtained was significant.

The means revealed that the maximum production of cytokinins (Table 11d) was in succinate media and Nutrient broth (210.0µg/ml) followed by Trypticase soy broth (170.0µg/ml). The average production of cytokinins king's media and peptone water was statistically at par with each other. The interaction study revealed that with increase in incubation period there was significant increase in production of cytokinins.

Table 11 Effect of media on the growth and production of plant growth regulators by fluorescent *Pseudomonas* PN-4-SAN at different incubation period.

Media	**Time interval (h)**															
	a) Growth at 540 nm				**b) Auxins (µg/ml)**				**c) Gibberellins (µg/ml)**				**d) Cytokinins (µg/ml)**			
	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean
Succinate media	0.23	0.29	0.35	0.29	8	11	23	14.00	128	146	189	154.3	150	200	280	210.0
King's media	0.19	0.29	0.60	0.36	6	12	26	14.67	90	128	150	122.7	100	150	200	150.0
Nutrient media	0.27	0.39	1.00	0.55	10	22	35	22.33	154	211	336	233.7	150	200	280	210.0
Peptone water	0.28	0.30	0.40	0.33	9.5	19	22	16.83	120	147	200	155.7	100	150	200	150.0
T. soy broth	0.28	0.35	0.45	0.36	9.5	19.5	22.5	17.17	96	117	151	121.3	140	170	200	170.0
Mean	0.25	0.32	0.56		8.60	16.70	25.70		117.6	149.8	205.2		128.9	174.0	232.0	

Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$
Media	0.12	Media	0.86	Media	0.96	Media	0.96
Interval	0.09	Interval	0.67	Interval	0.74	Interval	0.75
Media × Interval	0.21	Media × Interval	1.46	Media × Interval	1.63	Media × Interval	1.63

4.2.2 Effect of different media on growth and production of plant growth regulators produced by fluorescent *Pseudomonas* PN-10-SAN at different incubation period.

4.2.2.1 Growth

Table 12 a showed that average growth was maximum in nutrient medium (0.44) and at 72h (0.46) and was statistically more significant than growth in any other media and incubation period. The average growth in different media and different incubation period was statistically different from each other. The interaction was significant

4.2.2.2 Production of plant growth regulators

The average production of auxins (Table 12 b) by *Pseudomonas* fluorescence was maximum in Nutrient broth (18.67 µg/ml) and was statistically at par with succinate medium (17 µg/ml). The average production was maximum at 72h (25.50 µg/ml) and was statistically more significant than other incubation period.

There was significant increase in production of gibberellins (Table 12c) with increase in incubation period. The average production of gibberellins was maximum in nutrient broth (242.7 µg/ml) and at 72h (192.6 µg/ml) and was statistically more significant and different from other. The interaction studied showed that difference in production obtained was significant.

The means revealed that the maximum production of cytokinins was in nutrient broth (243.3 µg/ml) followed by king's medium (176.7 µg/ml) (Table 12 d). the maximum average production of cytokinins was observed at 72h (222.0 µg/ml) and was statistically different from each other. The interaction study revealed that with increase in incubation period there was significant increase in production of cytokinins.

Table 12 Effect of media on the growth and production of plant growth regulators by fluorescent *Pseudomonas* PN-4-SAN at different incubation period

Media	Time interval (h)															
	a) Growth at 540 nm				b) Auxins (µg/ml)				c) Gibberellins (µg/ml)				d) Cytokinins (µg/ml)			
	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean
Succinate media	0.20	0.28	0.31	0.26	8	14	29	17.00	124	179	217	173.3	70	150	200	140.0
King’s media	0.23	0.30	0.45	0.33	5	10	29.5	14.83	74	117	140	110.3	100	150	280	176.7
Nutrient media	0.17	0.35	0.81	0.44	7	20	29	18.67	177	250	300	242.7	200	250	280	243.3
Peptone water	0.25	0.28	0.32	0.28	10	17	20	15.67	74	103	130	102.3	70	150	200	140.0
T. soy broth	0.28	0.35	0.45	0.36	9	17	20	15.33	115	124	176	138.3	70	100	150	106.7
Mean	0.23	0.31	0.46		7.90	15.60	25.50		112.7	154.6	192.6		102.0	160.0	222.0	

Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$
Media	0.009	Media	0.94	Media	0.96	Media	0.96
Interval	0.007	Interval	0.73	Interval	0.75	Interval	0.75
Media × Interval	0.02	Media × Interval	1.59	Media × Interval	1.63	Media × Interval	1.6

4.2.3 Effect of different media on growth and production of plant growth regulators produced by fluorescent *Pseudomonas* AN-2-NAG at different incubation period.

4.2.3.1 Growth

Table 13 a showed that average growth was maximum in nutrient medium (0.64) and at 72h (0.54) and was statistically more significant and statistically more different than growth in any other media and incubation period. The interaction was significant but the cell density at different incubation period in succinate media and King's media (0.35) was statistically at par.

4.2.3.2 Production of plant growth regulators

The average production of auxins (Table 13 b) by *Pseudomonas* sp. was maximum in Nutrient broth (23.17µg/ml) and was statistically more significant than in other media. The average production was maximum at 72h (25.20 µg/ml) and statistically more significant from other incubation period.

There was significant increase in production of gibberellins with increase in incubation period (Table 13 c). The average production of gibberellins was maximum in nutrient broth (185.30 µg/ml) and at 72h (172.2 µg/ml). All the values were statistically different from each other. The interaction studied showed that difference in production obtained was significant.

The means revealed that the maximum production of cytokinins was in nutrient broth (243.30µg/ml) followed by king's medium (210.00 µg/ml) (Table 13 d). The average production of cytokinins in succinate and peptone water (150.00 µg/ml) was same. The interaction study revealed that with increase in incubation period there was significant increase in production of cytokinins.

Table 13 Effect of media on the growth and production of plant growth regulators by fluorescent *Pseudomonas* AN-2-NAG at different incubation period.

Media	Time interval (h)															
	a) Growth at 540 nm				b) Auxins (µg/ml)				c) Gibberellins (µg/ml)				d) Cytokinins (µg/ml)			
	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean
Succinate media	0.26	0.30	0.50	0.35	9.5	17	25	17.17	145	169	190	168.0	100	150	200	150.0
King's media	0.28	0.35	0.42	0.35	6.5	10	21	12.50	60	90	120	90.00	150	200	280	210.0
Nutrient media	0.30	0.55	1.07	0.64	11.5	21	37	23.17	150	171	235	185.3	200	250	280	243.3
Peptone water	0.21	0.29	0.36	0.29	8	17	21	15.33	70	120	147	112.3	100	150	200	150.0
T. soy broth	0.28	0.31	0.37	0.33	9	18	22	16.33	84	120	169	124.3	70	150	220	140.0
Mean	0.27	0.36	0.54		8.90	16.60	25.20		101.8	134.0	172.2		124.0	180.0	236.0	

Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$	Effects	C.D.$_{0.05}$
Media	0.01	Media	0.89	Media	0.96	Media	0.96
Interval	0.008	Interval	0.69	Interval	0.75	Interval	0.75
Media × Interval	0.02	Media × Interval	1.50	Media × Interval	1.63	Media × Interval	1.6

4.2.4 Effect of different media on growth and production of plant growth regulators produced by fluorescent *Pseudomonas* AN-4-NAG at different incubation period.

4.2.4.1 Growth

Table 14 a showed that average growth was maximum in nutrient medium (0.43) and at 72h (0.44) and was statistically more significant than growth in any other media and incubation period. All the values were statistically different from each other.

4.2.4.2 Production of plant growth regulators

The average production of auxins (Table 14 b) by *Pseudomonas* sp. was maximum in Nutrient broth (19.33 µg/ml) and was statistically at par with peptone water (18.50 µg/ml). The average production was maximum at 72h (25.20 µg/ml) and statistically more significant from other incubation period.

There was significant increase in production of gibberellins with increase in incubation period (Table 14 b). The average production of gibberellins was maximum in nutrient broth (183.3 µg/ml) and at 72h (186.4 µg/ml). All the values were statistically different from each other. The interaction studied showed that difference in production obtained was significant.

The means revealed (Table 14 c) that the maximum production of cytokinins was in king's medium (243.30µg/ml) which was statistically more significant from other. The average production of cytokinins in succinate and peptone water and (150.00 µg/ml) was statistically at par with each other . The interaction study revealed that with increase in incubation period there was significant increase in production of cytokinins.

Table 14 Effect of media on the growth and production of plant growth regulators by fluorescent *Pseudomonas* AN-10-NAG at different incubation period

Media	**Time interval (h)**															
	a) Growth at 540 nm				**b) Auxins (µg/ml)**				**c) Gibberellins (µg/ml)**				**d) Cytokinins (µg/ml)**			
	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean
Succinate media	0.23	0.30	0.46	0.33	11	15	27	17.67	120	147	189	152.0	100	150	200	150.0
King’s media	0.26	0.32	0.35	0.31	6	10.5	22	12.83	70	120	170	120.0	150	200	280	210.0
Nutrient media	0.29	0.40	0.60	0.43	11	22	25	19.33	134	196	220	183.3	200	250	280	243.3
Peptone water	0.28	0.35	0.42	0.35	9.5	20	26	18.50	96	131	169	132.0	100	150	200	150.0
T. soy broth	0.21	0.29	0.37	0.29	8	17	21	15.33	96	147	184	142.3	70	150	220	146.7
Mean	0.25	0.33	0.44		9.10	16.90	24.20		103.2	148.2	186.4		124.0	180.0	236.0	

Effects	$\text{C.D.}_{0.05}$	Effects	$\text{C.D.}_{0.05}$	Effects	$\text{C.D.}_{0.05}$	Effects	$\text{C.D.}_{0.05}$
Media	0.009	Media	0.91	Media	0.96	Media	0.96
Interval	0.007	Interval	0.70	Interval	0.75	Interval	0.75
Media × Interval	0.01	Media × Interval	1.56	Media × Interval	1.63	Media × Interval	1.6

4.3 Extraction, separation and partial characterization of plant growth regulators viz. auxins, gibberellins and cytokinins

All the three plants growth regulators i.e. auxins gibberellins, cytokinins extracted and separated from 72 h old cell free culture supernatants. *Pseudomonas* sp. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG were grown in Nutrient media under shake condition (90 rpm) at 28°C for 72 h. All the four cell free culture supplemented harvested by centrifugation at 10000 rpm for 30 min at 4°C

Auxins

Acidified supernatant extracted with diethyl ether and partitioned with sodium bicarbonate. Extracted and concentrated fraction dissolved in methanol. Methanol fraction (100 µl) spotted on silica gel-G plates and developed in isopropanol: water (30:20 v/v) for 12-14 h and sprayed with Salper reagent.

The results of extraction and separation on TLC of extract showed that the most of the auxins could be extracted with diethyl ether and further concentrated by evaporation at 40°C.

The thin layer chromatography performed with methanol fraction using solvent systems for each PGRs. The results obtained showed (Table 15 and plate 9) that the solvent system isopropanol; water (30:20 v/v) was best for studying the homogeneity of auxins. It gave the maximum Rf value of 0.81. Pink spots corresponding to auxins or auxins like substances were visible when sprayed with Salper reagent.

Gibberellins

72 h old cell free supernatant was mixed with saturated sodium bicarbonate and extracted with ethyl acetate. Aqueous layer acidified and re-extracted with equal volume of ethyl acetate. The concentrated fraction dissolved in methanol. Methanol fraction (100 µl) spotted on silica gel-G plates and developed in solvent isopropanol: ammonium hydroxide: water (10:1:1 v/v/v) for 2-3 hours and silica gel-G plates heated at 120°C for 10 min and sprayed with water; concentrated sulphuric acid (30:70 v/v).

Thin layer chromatography performed with methanol fraction. The results obtained showed (Table 15 and plate 10) that the solvent system isopropanol: ammonium hydroxide: water (10:1:1 v/v/v) was best for studying the homogeneity of gibberellins. It gave the maximum Rf value of 0.80 and gray spots correspondence to gibberellins activity.

Cytokinins

Acidified supernatant extracted with diethyl ether and partitioned with n-butanol. Extracted and concentrated fraction dissolved in HCl and passed through Dowex 50 column chromatography in which 0.5 ml of extracted sample applied to column (25 x 1.5 cm) of Dowex 50 which was equilibrated with ethanol and distilled water and eluted with ammonium hydroxide buffer. 3ml of fractions were collected and was assayed for Cytokinins by radish cotyledon bioassay at 25^0 C under fluorescent light. Extracted and concentrated fraction evaporated and dissolved in distilled water. Water fraction (100 µl) spotted on silica gel-G plates and developed in n-butanol: 1N NH_4OH: water (7:1:2 v/v/v upper phase) for 2-3 hours and silica gel-G plates kept in closed iodine jar for 10 minute.

The thin layer chromatography performed with methanol fraction. The results obtained showed (Table 15 and plate 11) that the solvent system n-butanol: 1N NH_4OH: water (7:1:2 v/v/v upper phase) was best for studying the homogeneity of cytokinins. It gave the maximum Rf value of 0.75 correspondences to brown spots of cytokinins or cytokinins like substances.

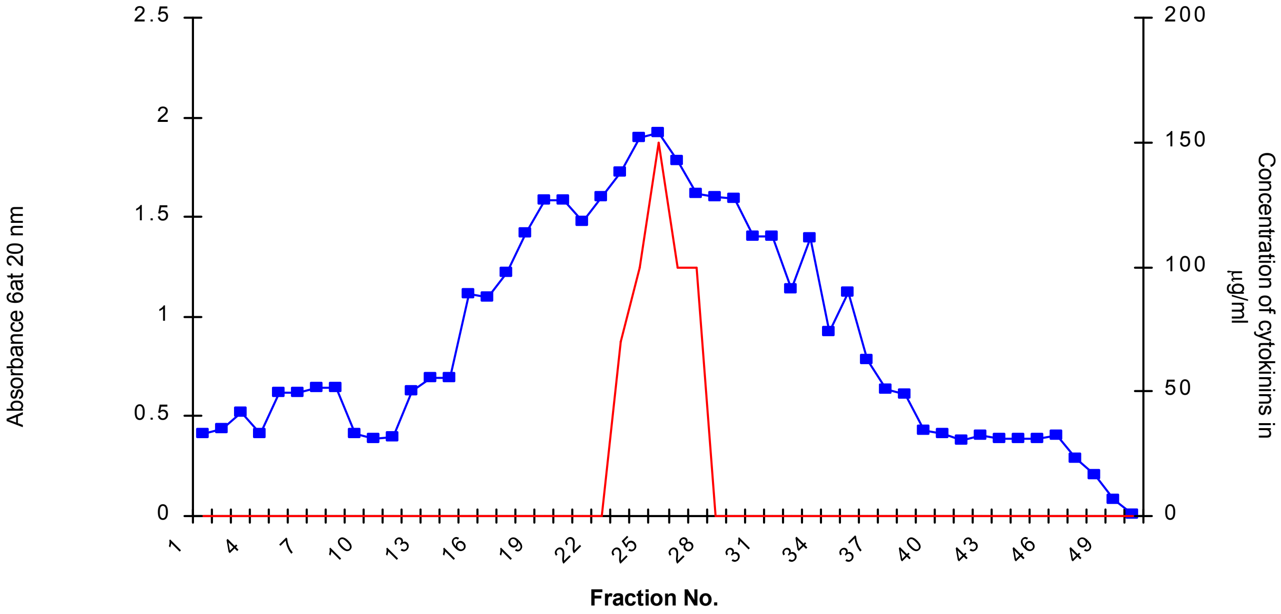

Fig No.5 Dowex 50 chromatographic profile of extracted fraction of cytokinins of fluorescent *Pseudomonas* sp. PN-4-SAN. 0.5 ml of 5 ml extracted sample applied to column (25 x 1.5 cm) of Dowex 50 which was equilibrated with ethanol and distilled water and eluted with ammonium hydroxide buffer. 3ml of fractions were collected and was assayed for cytokinins by radish cotyledon bioassay at 25^0 C under fluorescent light.

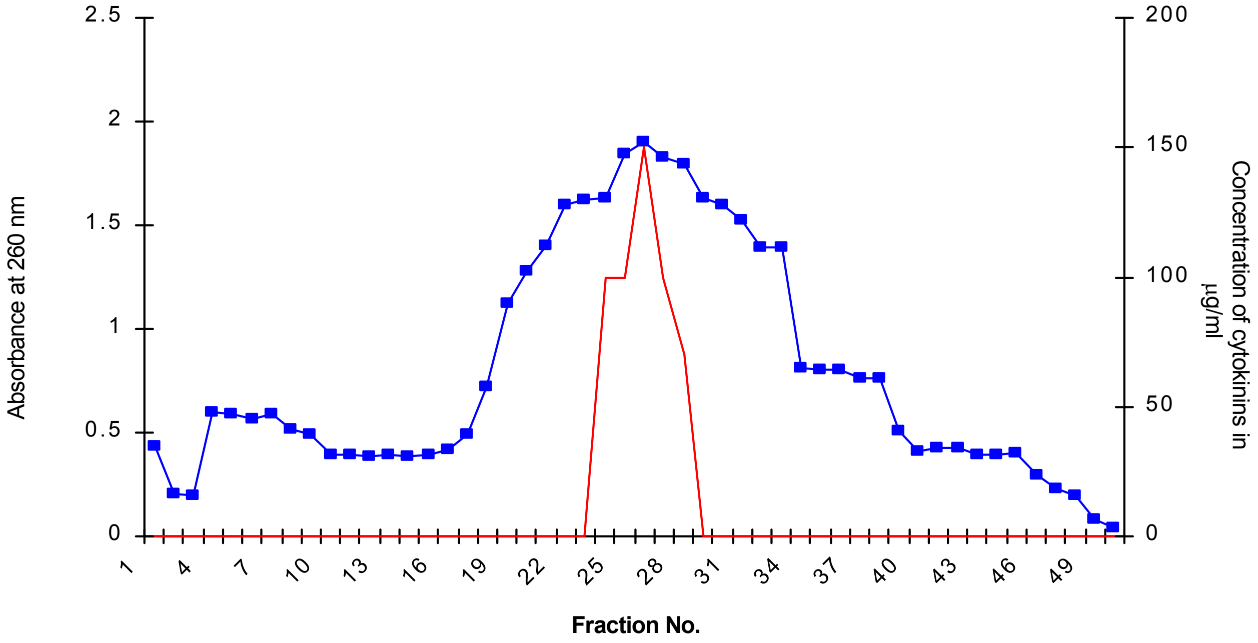

Fig No.6 Dowex 50 chromatographic profile of extracted fraction of cytokinins of fluorescent *Pseudomonas* sp. PN-10-SAN. 0.5 ml of 5 ml extracted sample applied to column (25 x 1.5 cm) of Dowex 50 which was equilibrated with ethanol and distilled water and eluted with ammonium hydroxide buffer. 3ml of fractions were collected and was assayed for cytokinins by radish cotyledon bioassay at 25^0 C under fluorescent light.

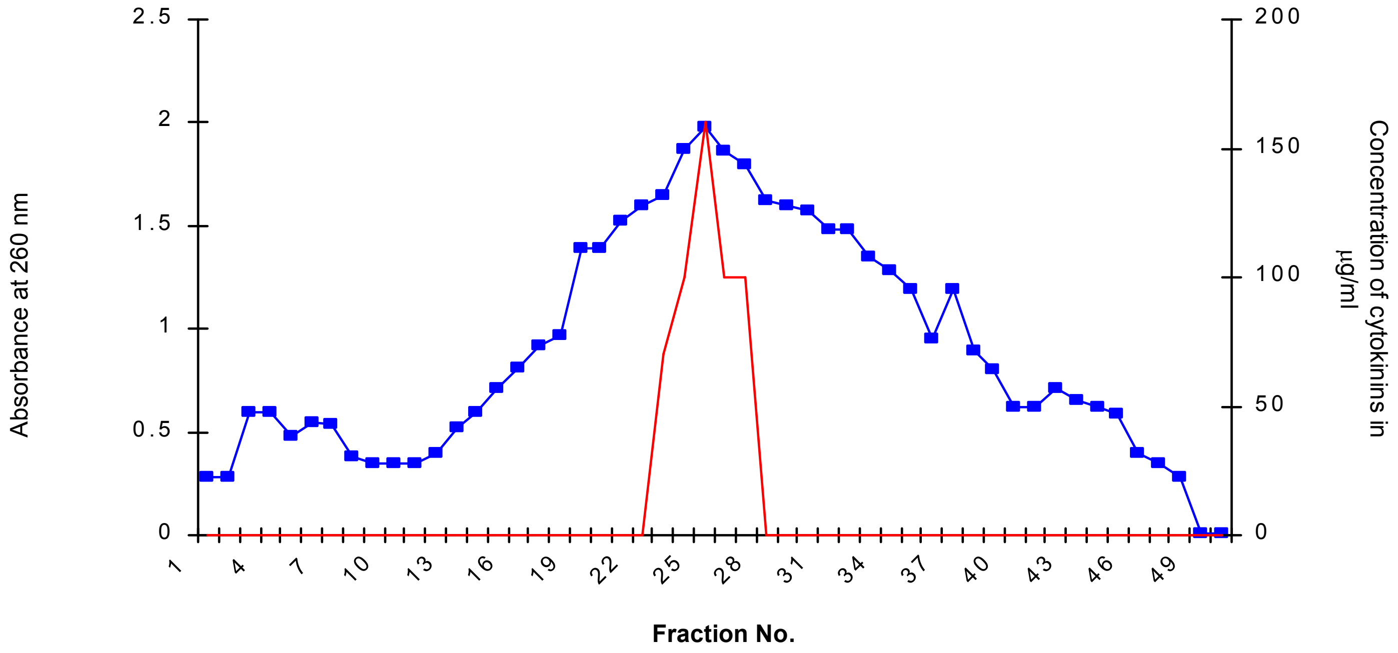

Fig No.7 Dowex 50 chromatographic profile of extracted fraction of cytokinins of fluorescent *Pseudomonas* sp.AN-2-NAG. 0.5 ml of 5 ml extracted sample applied to column (25 x 1.5 cm) of Dowex 50 which was equilibrated with ethanol and distilled water and eluted with ammonium hydroxide buffer. 3ml of fractions were collected and was assayed for cytokinins by radish cotyledon bioassay at 25^0 C under fluorescent light.

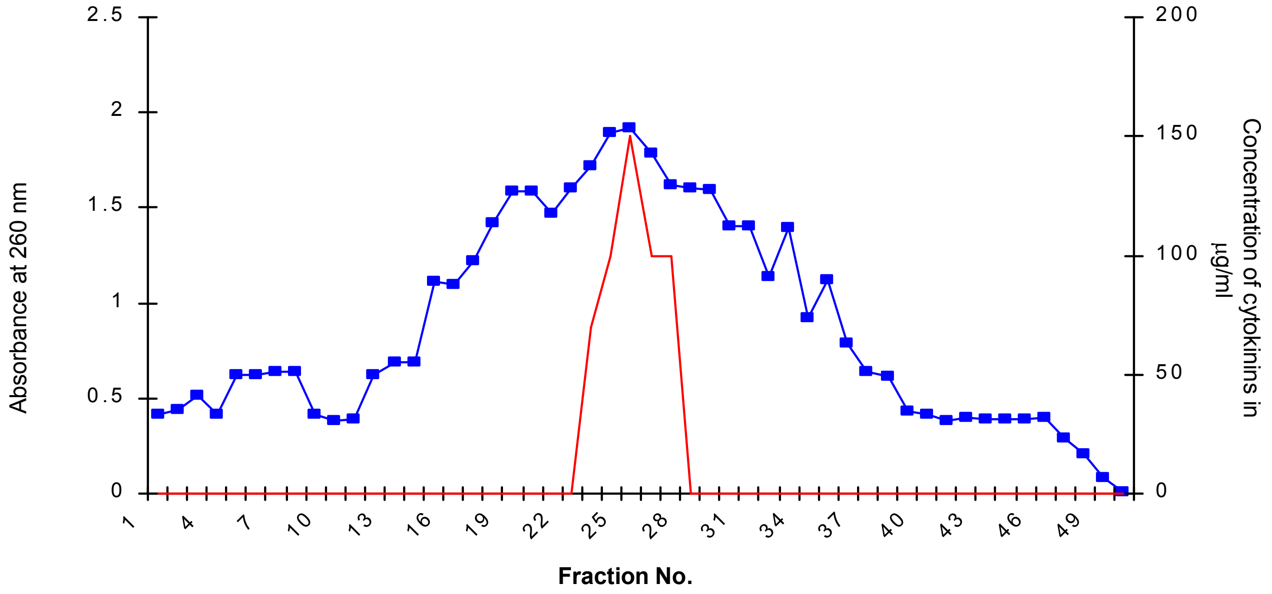

Fig No.8 Dowex 50 chromatographic profile of extracted fraction of cytokinins of fluorescent *Pseudomonas* sp.AN-4-NAG. 0.5 ml of 5 ml extracted sample applied to column (25 x 1.5 cm) of Dowex 50 which was equilibrated with ethanol and distilled water and eluted with ammonium hydroxide buffer. 3ml of fractions were collected and was assayed for cytokinins by radish cotyledon bioassay at 25^0 C under fluorescent light.

Table15Thin layer chromatographic analysis on Silica gel-G of partially purified bacterial plant growth regulators viz. auxins, gibberellins and cytokinins from *Pseudomonas* sp.

Plant growth regulators	Isolates	Solvent system	Spraying reagent	Color of spots	Rf value
Auxins	PN-4-SAN	Isopropanol: Water (30:20)	Salper	Pink	0.81
	PN-10-SAN	-do-	-do-	Pink	0.80
	AN-2-NAG	-do-	-do-	Pink	0.81
	AN-4-NAG	-do-	-do-	Pink	0.81
Gibberellins	PN-4-SAN	Isopropanol: NH_4OH: Water (10:1:1)	Water: H_2SO_4 (30:70)	Grey	0.80
	PN-10-SAN	-do-	-do-	Grey	0.79
	AN-2-NAG	-do-	-do-	Grey	0.79
	AN-4-NAG	-do-	-do-	Grey	0.80
Cytokinins	PN-4-SAN	n butanol: 1N NH_4OH: Water (7:1:2)	Iodine	Brownish	0.73
	PN-10-SAN	-do-	-do-	Brownish	0.75
	AN-2-NAG	-do-	-do-	Brownish	0.75
	AN-4-NAG	-do-	-do-	Brownish	0.72

$$\text{Rf value calculated as} = \frac{\text{Distance run by the compound}}{\text{Distance run by the solvent front}}$$

4.4 Evaluation of plant growth regulators by bioassay

Plant tissue culture bioassay:

The three plant growth regulators viz. auxins, gibberellins and cytokinins were evaluated by plant tissue culture bioassay.

Avena coleoptiles bioassays

The barley seedling were grown in sand in tray and used for the bioassays. The results showed that the purified and extracted samples of auxins like substances from selected strains of Pseudomonas were used for bioassay. The length of piece coleoptiles was measured and was treated with partially purified auxins. The increase in length was measured and expressed in terms of concentration of auxins (µg/ml).

Partially purified auxins have increased the length of avena coleoptiles and partially purified auxins extracted samples from *Pseudomonas* sp. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG (Table 16 Plate 12) the length of avena coleoptile piece by 0.2, 0.25, 0.25, 0.2 cm respectively. This increase in length of coleoptiles calculated from the dosage response curve of IAA.

Table 16 Effect of partially purified auxins of *Pseudomonas* species on the length of Avena coleoptile of barley.

Partially purified auxins	Growth of coleoptile	
	Increased length (cm)	Auxins (µg/ml)
PN-4-SAN	0.20	40.00
PN-10-SAN	0.25	50.00
AN-2-NAG	0.20	50.00
AN-4-NAG	0.20	40.00
Standard	0.28	56.00

* Auxins concentration expressed in terms increased length (cm) of coleoptile calculated from dose response curve of IAA (10-100 µg/ml).

- Each value is the mean of triplicate

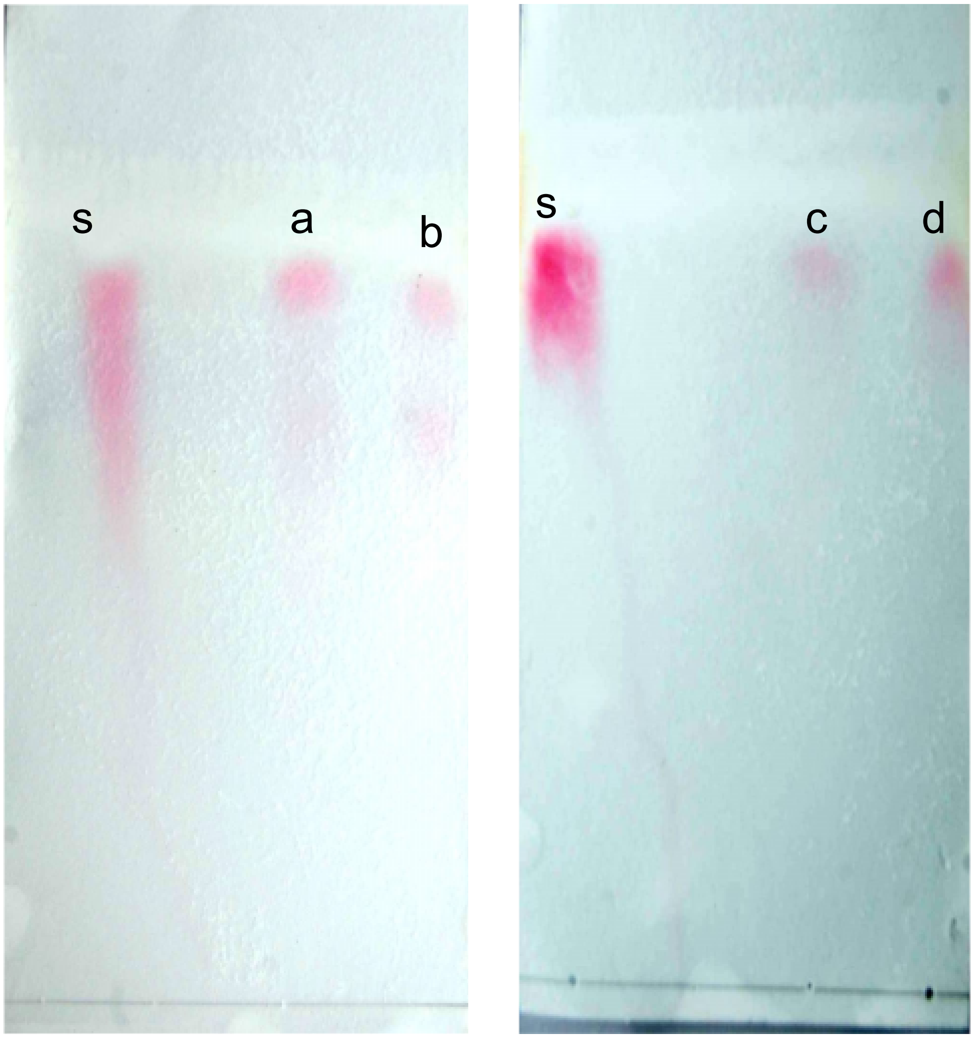

Plate 9. Thin layer chromatographic pattern on silica gel-G of partially purified auxins of *Pseudomonas* sp. PN-4-SAN(a), PN-10-SAN (b), AN-2-NAG (c), AN-4-NAG and standard (s) using Isopropanol: Water (30:20) solvent system and sprayed with Salper reagents.

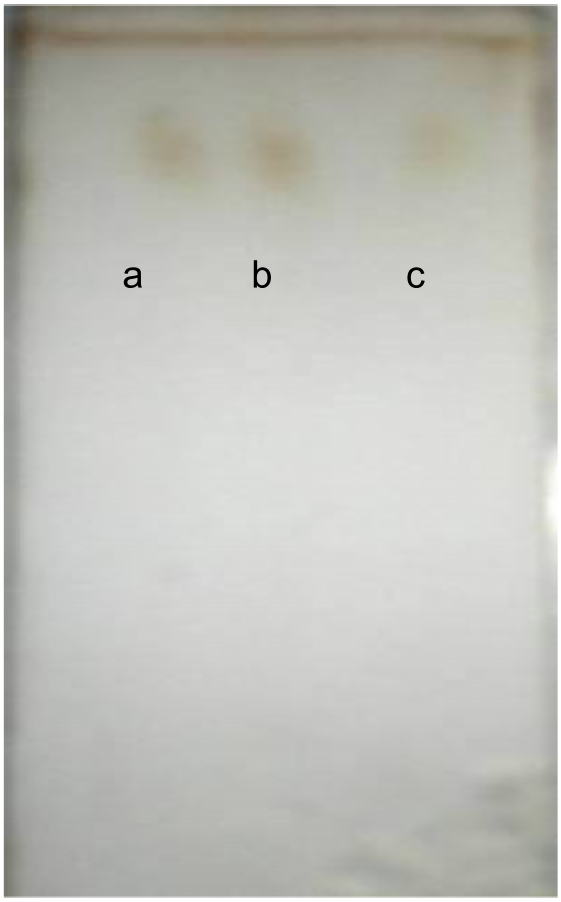

Plate.10 Thin layer chromatographic pattern on silica gel-G of partially purified gibberellins of *Pseudomonas* sp. PN-4-SAN (a), PN-10-SAN (b) and AN-4-NAG (c) using Isopropanol: NH_4OH: Water (10:1:1) solvent system and sprayed with Water: H_2SO_4 (30:70).

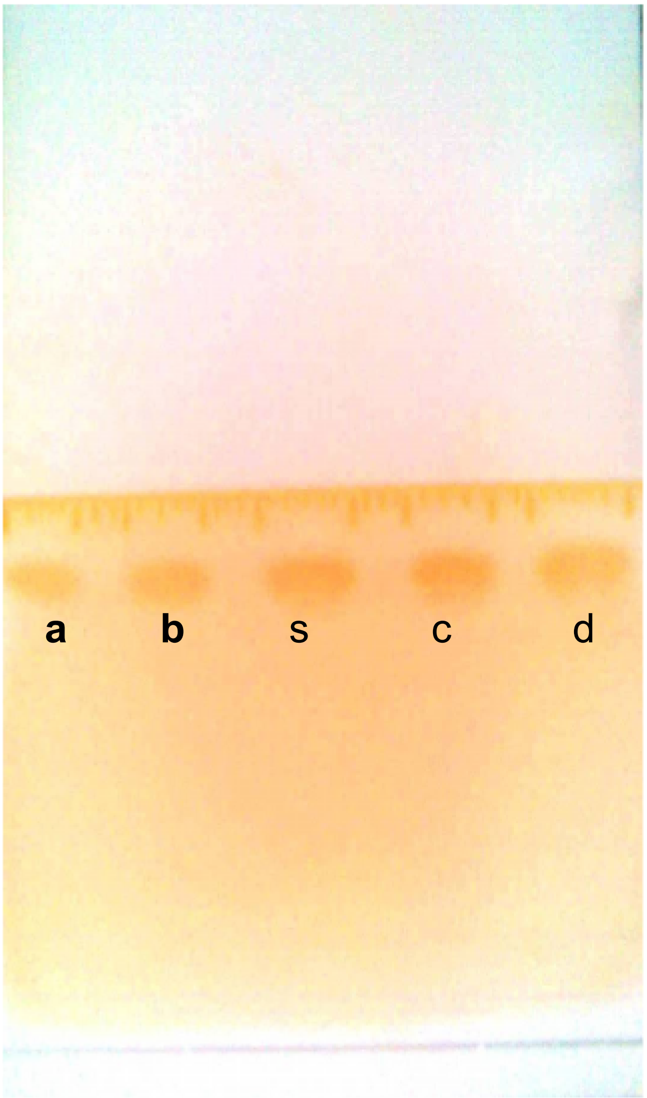

Plate11. **Thin layer chromatographic pattern on silica gel-G of partially purified cytokinins of *Pseudomonas* sp. PN-4-SAN(a), PN-10-SAN (b), AN-2-NAG (c), AN-4-NAG (d) and kinetin (s) using n-butanol: 1N NH4OH: Water (7:1:2) and developed in iodine chamber.**

Alpha-amylase release activity

The dehulled rice seeds without embryo were used for the α-amylase activity. The apical half of the embryo were sterilized with 1 % sodium Hypochlorite and weighed in the group of five. The partially purified and extracted gibberellins like substances used for the bioassay.

The results showed that partially purified extracted gibberellins like substances from *Pseudomonas* sp. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG respectively have increased the alpha-amylase activity in terms of % units/ gram of rice seed halve (Table 17). The increase in alpha-amylase activity directly related to the concentrations of gibberellins (µg/ml) present in the partially purified samples as calculated from the standard curve.

The maximum release of alpha-amylase activity detected in treatment with *Pseudomonas* sp AN-2-NAG, AN-4-NAG (51 %)

Table 17 Effect of partially purified gibberellins like substances from fluorescent *Pseudomonas* on of α-amylase release activity from rice seed without embryo.

Partially purified gibberellins	Gibberellins* (µg/ml)	α-amylase activity (% units)
PN-4-SAN	40.00	45.00
PN-10-SAN	37.00	48.00
AN-2-NAG	35.00	51.00
AN-4-NAG	35.00	51.00
O.D. of initial starch	0.386	

* Gibberellins of (µg/ml) of gibberellic (GA_3) as calibrated from standard curve (100-1000 µg/ml).

α- amylase activity measured by using following formulae

$$\% \text{ units} = \frac{C - E}{E} \times 100\ (\%)$$

C – O.D. of initial starch solution E – O.D. at the end of reaction

Radish cotyledon bioassay

Partially purified Cytokinins have increased the weight of radish cotyledons and partially purified Cytokinins extracted samples from *Pseudomonas* sp. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG (Table 18, Plate 13) the increase in weight of cotyledons were 0.02, 0.04, 0.04, 0.2 cm respectively. This increase in weight calculated from the dosage response curve of kinetin.

Table 18 Effect of partially purified cytokinins of fluorescent *Pseudomonas* on the weight of radish cotyledon seeds

Partially purified Cytokinins	**Growth of callus (g)**	
	Increased weight (g)	**Cytokinins* (µg/ml)**
PN-4-SAN	0.04	150.00
PN-10-SAN	0.04	150.00
AN-2-NAG	0. 02	70.00
AN-4-NAG	0. 03	100.00

* Cytokinins production expressed in terms of kinetin (µg/ml) as calibrated from the dose response curve (100-1000µg/ml).

- Each value is the mean of triplicate.

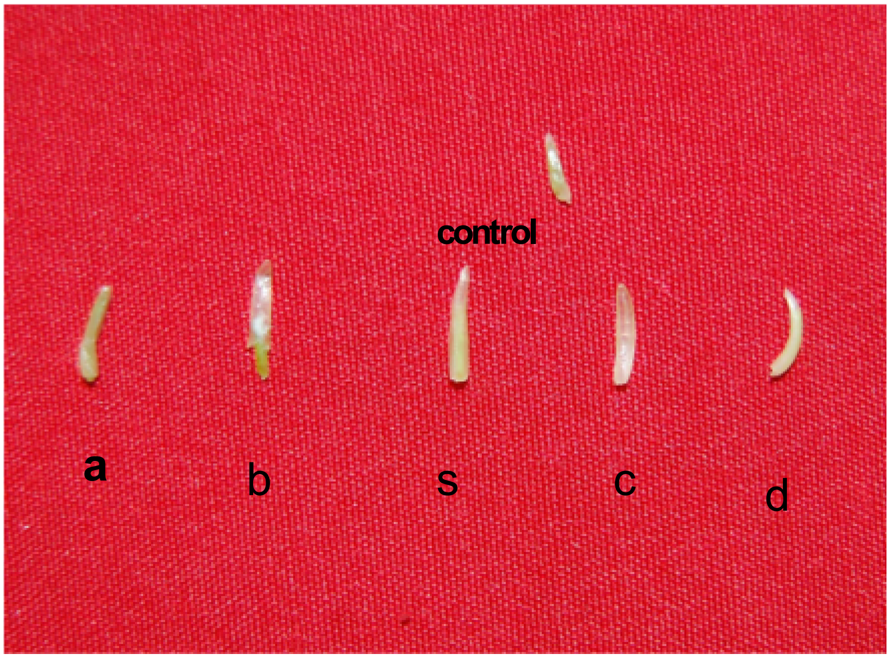

Plate 12. Effect of partially purified auxins on the length of avena coleoptile by *Pseudomonas* sp. PN-4-SAN (a), PN-10-SAN (b), AN-2-NAG (c) , AN-10NAG (d) and standard (s) after 48h

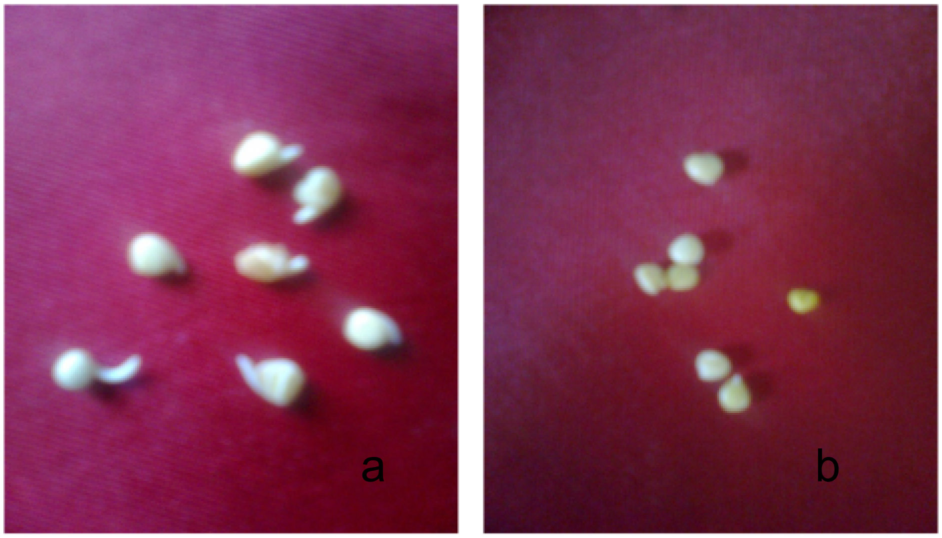

Plate 13. Effect of partially purified cytokinins on the weight of radish cotyledon seeds by *Pseudomonas* sp. PN-4-SAN (a) and control (b) after 3 days.

Chapter-V

DISCUSSION

Replant disease also termed apple replant disease (ARD) distributed worldwide and is often encountered in establishing new orchards on old sites. This disease occurs in other fruit crops also like pear etc. (Mai and Abawi, 1981). Huge losses in gross income over ten years could result from failure to control replant disease. The economic impacts of the soil borne disease complex are also serious (Arneson and Mai, 1976; Smith, 1995). The disease is a complex syndrome that affects young trees in replant orchard sites and reduces growth survival, growth and yield of replanted trees. Methyl bromide (MB) has been the fumigant used most widely to control this disease. But alternative to MB and cultural methods are needed (Yao *et al*., 2006) because MB is being phased out because of environmental problems (Mckenry *et al*., 1994; Yao *et al*., 2006). The etiology of replant disease is still unclear (Mai and Abawi, 1981; Mazzola, 1997; Merwin *et al*., 2001). Both biotic and abiotic factors are involved. Soil fumigation or steam pasteurization and methyl bromide treatment suppressed apple replant disease in 50 per cent of replanted orchards out side India (Merwin *et al*., 2001) indicating that abiotic factors are also involved in this disease. Soil bacteria, plant parasitic nematodes, fungi, actinomycetes, oomycetes especially *Pythium* and *Phytopthora*, have all been implicated individually or together as causative agent of this disease (Catska *et al*., 1982; Mai and Abawi, 1981 and Merwin *et al*, 2001). Thus scientists are searching for safer alternative to the use of MB. Alternative fumigants have been evaluated but none have provided as effective control to MB (Duniway, 2002; Martin, 2003). Mazzola (1997) have emphasized fungal involvement in disease and the important effects of *Pseudomonas* species in the suppression of this disease in apple. Cover crops have also been tested for their ability to suppress the disease in several orchards but results were inconsistent from site to site.

So this study was carried out to find some alternative by finding out best indigenous *Pseudomonas* species which may also be efficient for production of plant growth regulators and other direct, indirect plant growth promoting activities. We have studied the total bacterial population and *Pseudomonas* population present in the rhizosphere of apple and pear plants growing present in normal orchards and replant problem sites of Mandi district of Himanchal Pradesh. Isolated indigenous fluorescent *Pseudomonas* sp. and screened out for the production of various direct and indirect plant growth promoting and disease suppressing activities i.e. plant growth regulators, antifungal, antibacterial, siderophores, phosphate solubilizing, ammonia and HCN and identified the isolates upto genus level. The best fluorescent *Pseudomonas* strain was selected and optimized their cultural conditions for production of three plant growth regulators and partially purified the plant growth regulators so as to select potential strains that can be developed as bioagents and also in the form of consortium for field trial for management of replant problem in H.P.especially Mandi district.

The rhizosphere soil harbors variety of microorganisms with different type of metabolic and adaptive responses to the variable supply of water, oxygen, organic substrates and other available nutrients (Paul & Jalali, 1998). It serves a promising and very dynamic habitat for microorganisms (Srivastava et al., 1999 and Parmar & Dadarwal, 1997).

Pseudomonas species make up a diverse group of bacteria that are generally found in all geographical conditions. This fluorescent *Pseudomonas* stimulates plant growth by facilitating either uptake of nutrients from soil or by producing certain plant growth promoting substances. Besides these bacteria also prevent proliferation of phyto pathogens & there by support plant growth (Weller 1988, Glick 1995). However their efficiency often varies since the population is not distributed at random. A part from host plant soil factors such as, composition organic matter, pH, water and oxygen availability play significant role in selection of natural flora (Ross *et al*., 2000).

The prevailing soil and climatic conditions greatly influence the performance of an introduced effective strain. The results are not consistant and they vary from plant type and soil type. The preplant treatment that is effective in one site may not effective in other site, so the isolation and characterization of efficient and best indigenous PGPR is impotant for development of effective consortium for management of replant problem of fruit crops especially apple and pear. It is therefore essential to isolate indigenous organisms that can be used in same ecological niche. For these the structure, diversity and total number of bacterial community and specific plant growth promoting fluorescent *Pseudomonas* diversity in relation to replant problem of apple and pear in two different location of H.P. i.e. Sanyardi and Nagwain (Mandi district) in relation to site specific rhizosphere / soil are to be understood. So the present study was aimed at to assess the total bacterial and total *Pseudomonas* population present in replant and normal sites of apple and pear (Table1 and 2). The aim was to isolate and select indigenous fluorescent *Pseudomonas* sp. to produce different plant growth regulators i.e. auxins, gibberellins and cytokinins and other plant growth promoting activities like HCN, ammonia production, phosphate solubilization, protease and siderophore production for selection of efficient strains.

5.1 IDENTIFICATION, MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION OF BACTERIAL ISOLATES

As is evident from the table 3, 4a & 4b isolated bacteria from apple and pear rhizosphere soil are gram negative, non spore producing, cocobacillus shaped, catalase and oxidase positive and also producing fluorescent pigment belonging to genus *Pseudomonas*. It may attribute to specific choice of media employed for isolation i.e. nutrient agar and king’s media. Several others hence supported use of king’s medium (King’s *et al*, 1954) for isolation of fluorescent *Pseudomonas* sp. The identification of these strains could not be ascertained upto species level due to unusual biochemical, serological and immunological characteristics.

5.2 SCREENING OF FLUORESCENT PSEUDOMONAS FOR THE PRODUCTION OF DIFFERENT PLANT GROWTH PROMOTING ACTIVITIES

5.2.1 Production of plant growth regulators viz auxins, gibberellins and cytokinins.

The microbial synthesis of plant growth regulators cytokinins, gibberellins and auxins is an important factor in increasing soil fertility. They are secondary metabolites which are important biotechnological and economical products produced commercially from fungi for agricultural and horticultural industry (Deacan, 1984). Bacteria that inhibit the rhizosphere may influence plant growth by contributing to a host's plants endogenous pool of phytohormones such as auxins. Production of auxins i.e. indole acetic acid (IAA) is wide spread among plant associated bacteria (Patten and Glick, 2002). They (Bashan and Levanony 1990) induces additional root hair and/or lateral root formation. Thereby enhancing the plant ability to take up nutrients from soil and increased yield. Gibberellins are endogenous hormone functioning as plant growth regulators and influencing a range of developmental processes in plants including stem elongation, germination, dormancy, sex expression and fruit senescence (Elezar and Escamilla, 2000). Large scale application of best selected and efficient indigenous strains of fluorescent *Pseudomonas* sp. in specific replant site may be able to manage and control replant disease effectively by increasing the concentration of these hormones in plant rhizosphere that in turn may be effective in increasing root size and volume and also help the young plant to establish properly under these hard and harsh conditions.

Auxins

The mechanism that commonly involved explaining the various effect of PGPR on plants is the production of plant growth regulators (PGR's) especially auxins (Tien *et al.*, 1979). Auxins are class of phytohormones and well characterized as indole 3-acetic acid (IAA) which is known to stimulate the lateral roots (e.g. increase in cell elongation) and long term (e.g. cell division and differentiation) responses in plants. The growth of plant treated with IAA secreting PGPR is affected by the amount of IAA that the bacterium produces and the responses observed may vary from one species of plant to another. Thus

PGPR facilitate plant growth by altering the hormonal balance within the affected plant (Barbieri *et al*., 1986). The production of auxins also depends upon the strains and type of microorganisms and on their age. The maximum IAA was observed in the stationary phase of *Azotobacter* (Vancura and Macura, 1960; Barea and Brown, 1974) and other species of *Pseudomonas* species and *Bacillus* species (Katznelson and Cole, 1965). The auxins type substances were detected by means of paper chromatography methods.

In our studies the strain of *Pseudomonas* species isolated from the rhizosphere of apple and pear produced auxins like substances in the stationary phase of growth i.e. at 72 hour of incubation period at 28°C for *Pseudomonas* sp. The results (Table 5) showed that production of auxins like substances by all the strains of *Pseudomonas* sp. ranges from 1 to 30 µg/ml.

5.2.3.2 Gibberellins

Gibberellins are one of the major groups of growth hormones which play an essential role in the growth and development of plants. They are diterpenoid acids biologically derived from tetracyclic diterpenoid hydrocarbons produced in good quality by microorganisms. A number of microorganisms have been reported to produce GA_3 and GA_3 like substances. Fungal cultures are able to produce gibberellins in higher yields.

Many microorganisms producing phytohormones were able to form gibberellins and kinetins, either together with auxins or often alone. *Azotobacter* is the microorganisms, which is able to synthesize large amount of biologically active substances. Gibberellic acid (GA_3) and gibberellins like compounds were identified in cultures of various species of *Azotobacter, Pseudomonas* (Azcon and Barea, 1975; Vancura, 1960). Microbial synthesis of gibberellins and other metabolites is an important factor in soil fertility (Katznelson and Cole, 1965). *Azospirillum* sp. is plant growth promoting bacteria whose beneficial effect to be partially due to production of phytohormone including gibberellins (GA_3). The beneficial effect of *Azosprillum* spp. on growth and yield (Okon and Labandera - Gonzales, 1994) or water stress allevation (Creus *et al*., 1997) of graminaneous plant of GA production of the bacteria.

The GA_3 are naturally produced by higher plants, fungi and bacteria and regulate plant growth and development. They are typical secondary metabolites in microorganisms. Gibberellic acid is the main product of gibberellins in fungi and bacteria. Currently GA_3 is largely produced from fungus *Gibberellia fujikuroi* on an industrial scale (Santos *et al*., 2003). It is also synthesized from *Azotobacter* and *Azospirillum* in culture medium and from wild strain of fungi such as *Sphacelome* sp., *Phaeosphaeria* sp. and *Neurospora* sp. (Rademacher, 1994). *Azotobacter* sp. is capable of synthesis of plant growth promoting substances auxins, gibberellins and cytokinins.

GA_S are a class of phytohormones with many demonstrated effects on a number of physiological processes (Davies, 1995). Among the 130 GA_3 identified up to now from plant, fungi and bacteria are GA_1, GA_3 and GA_4, the three most commonly directly effective GA shoot elongation promoters. Their level in plant tissue appears to be regulated by three processes. Application of GA_3 in concentration similar to those produced by microorganisms or by inoculation with different strains of *Azospirillum* species can promote the growth of roots in corn seedlings (Fulchieri *et al*, 1990 & 1993).

All the strains of *Pseudomonas* species isolated from the rhizosphere of apple and pear plants (Table 5) were grown in nutrient broth for 72 h i.e. up to stationery phase of growth. In cell free supernatants maximum production of gibberellins like substances shown by strains of fluorescent *Pseudomonas* sp. AN-2-NAG, AN-3-NAG (60 µg/ml) and least production has been shown by strain PR-1-SAN and PR-3-SAN (19 µg/ml).

5.2.3.3 Cytokinins

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 (Kloepper, 1993; Glick, 1995). Information on the microbial production of PGR's (plant growth regulators) and their effect on plant growth have been limited and focused primarily only ethylene and auxins (Arshad and Frankenherger, 1992; Glick, 1995). Production of cytokinins by plant pathogenic bacteria *Agrobacterium* and *Pseudomonas syringae* is well known. However, PGPR including *Azotobacter, Azospirillum, Rhizobium, Bacillus* and *Pseudomonas* have been reported to synthesize cytokinins in pure culture. Cytokinins are a group of plant hormone essential for cell division and differentiation in plants most occurring cytokinin include isopentyladenine (IP) and trans-Zeatin (tZ) are derivatives of N_6 prenylated adenine.

Cytokinins are N_6 substituted aminopurines that act as PGR’s and influence physiological and developmental processes of plants such as cell division, seed germination, and root development, accumulation of chlorophylls, leaf expansion and delay of senescence (Salisbury and Ross, 1992). Endogenous cytokinins are synthesized in roots and translocated in the shoots. They play important role in root and shoot communication and their level may alter root function and soil conditions. Cytokinins produced by bacteria that live in close proximity to the root also may influence plant growth and development (Arshad and Frankenberger, 1993). Cytokinins also mediate interactions between microbes and their associated plants. Plant growth promoting *Pseudomonas fluorescens* synthesize three cytokinins (Gracia *et al*., 2001).

In our study most of the strains i.e. *Pseudomonas* species from rhizosphere of apple and pear has been found to produce cytokinins like substance in nutrient broth at 28^0C for *Pseudomonas* species respectively under shake conditions. Maximum production of cytokinins have been recorded in *Pseudomonas* species AR-1-NAG, PN2-SAN, PN-4-SAN and PN-8-SAN (150 µg/ml) followed by six isolates AN-1-NAG, AN-2-NAG, AN-

4-NAG, AN-10-NAG, PN-1-SAN and PN-6-SAN (100 µg/ml), and least production in AN-7-NAG, AR-3-NAG, PN-11-SAN and PN-14-SAN (70 µg/ml).

5.2.2 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 (Sharma *et al.,* 1994). They 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 food rot of black pepper caused by *Phytopthora capsici.* Application of rhizobacteria resulted in 38.9 percent enhanced growth of black pepper and 66.6 percent survival of ginger tillers. In successful cropping systems were 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 to the practical utility.

Microbial interaction in the rhizosphere of plants are of considerable importance to agriculture (Thomashow and Weller 2004; Howie and Suslow, 1991). The *Pseudomonas* isolates from apple and pear in our study were screened out for the production of antifungal activities (Table 6, Plate 1,2,3) in vitro against various fungal plant pathogens. Present isolates were found to be effective against fungi i.e. *Fusarium oxysporum Alternaria* and *Pythium* sp. Indicating that antagonistic metabolites may be broad spectrum in nature like antibiotics. Whether the *Pseudomonas* sp. Contain one or more than one type of secondary metabolites has not been ascertained. It may be due to that the microorganisms selected from the rhizosphere enrichment environment like apple and pear may be sol diversified and may be able to produce bioactive substances and biocatalysts due to environmental stress conditions (Cheetham, 1987).

5.2.3 Siderophore production

Siderophores are iron chelating compounds secreted by bacteria around the roots that affect the growth of the plants. They act differently depending upon the nature of the producer organisms, either deleterious rhizosphere bacteria or plant growth promoting rhizobacteria (PGPR). Nearly all the microorganisms depend upon the uptake of iron under aerobic conditions. This essential element is present in form of insoluble oxide hydrates ($Fe_2O_{3.\ n}\ H_2\ O$). Therefore its concentration is far too low to sustain the growth of microorganisms. One way to overcome this problem is the production of iron complexing compounds named siderophores. After complexation, iron (III) is available in water soluble form as ferric siderophore, which can be taken up by microorganisms (Neilands, 1981)

Duffy and Defago (1999) demonstrated that a myriad of environmental factor including pH, the level of iron and the form of iron ions, the presence of other trace elements and an adequate supply of carbon ,nitrogen and phosphorous that can modulate siderophore synthesis. Meyer and Abdullah (1978) studied the biosynthesis of yellow green fluorescent water soluble pigment by *Pseudomonas fluorescens.* The biosynthesis occurred only when the bacteria were iron deficient and was directly influenced by the nature of organic carbon source. The segment formed by a very stable Fe^{3+} complex and was purified in this form.

In our study all the fluorescent *Pseudomonas* isolates from apple and pear were screened out for the production of siderophore by qualitative and quantitative methods i.e. plate and liquid assay method. The results showed that in plate assay siderophores are produced in the range of 18mm – 32mm diameter pinkish / orange zone in chromeazurol – S agar plates by all the strains and in quantitative estimation siderophore production was observed in terms of reduction in blue color in the range of 29.00 – 56.00 percent siderophore units (% SU). (Table 7 and plate 4)

5.2.4 Phosphate solubilising activity

Phosphorous is a major nutrient for crop plant and is involved in many essential processes including cell division, photosynthesis, breakdown of sugar, energy transport and nutrient transfer with in the plant (Tandon, 1987). At the same time, the role of phosphorous in expression and maintenance of genetic material is also well established. Phosphorous is one of the major plant nutrients. It is second only to N as the most essential element of plant growth. Plant obtains phosphorous from the soil in the form of HPO_4^{2-} and $H_2 PO_4$.

The use of phosphate solubilising microorganisms as inoculants simultaneously increase the plant crop yield. Strains from the genus *Pseudomonas, Bacillus* and *Rhizobium* species are among the most powerful phosphate solubilizers (Rodriguenz and Fraga, 1999). Soil microorganisms play an important role in the solubilisation of bound phosphates, which are either in the form of organic phosphatic compounds or inorganic phosphates and make them available to higher plants (Kapoor *et al*, 1989).

In our studies, many indigenous strains of plant growth promoting *Pseudomonas* species isolated from the rhizosphere of apple and pear possess phosphate solubilising activity both on Pikovskaya's agar plate (Table 8, Plate 5) and solubilize in tricalcium phosphate in liquid culture as estimated by spectrophotometric method according to Lata and Saxena (2003).

5.2.5 HCN and ammonia production

There are number of different ways in which PGPR can inhibit phytopathogens. It has been suggested (Voisard *et al.,* 1989) that some *Pseudomonas* are able to synthesize hydrogen cyanide (HCN), to which these *Pseudomonas* are themselves resistant that may be linked to the ability of these strains to inhibit some pathogenic fungi. Although the role of HCN in disease supressionis not considered to be firmly established. It has been demonstrated that several different microrganisms including strains of *Cladosporium* and

Pseudomonas cepacia were able to hydrolyze fusaric acid (Toyoda and Utsammi, 1991). Fusaric acid is the causative agent of the damage of plant that occurs upon *Fusarium* infection.

In our study, various strains of *Pseudomonas* isolates from the rhizosphere of apple and pear showed production of HCN in vitro as evaluated from the color change of pre dipped picric acid paper strips from yellow to orange brown (Table 9, Plate 6).

There is indirect evidence of usefulness of *Azotobacter* free living nitrogen fixing bacteria in crop improvement under tropical and sub tropical conditions especially with strains excreting a high amount of ammonia in addition to a variety of growth promoting factor in presence of carbon rich root exudates (Narula and Gupta, 1986). The effect of ammonia excreting strains of *Azotobacter* AC_2 was studied on cereals and legumes and they were beneficially influenced by the inoculation of *Azotobacter*and increase in nitrogen fixation crops might be due to capacity of particular crop to utilize ammonia in the inoculated soil with ammonia excreting microorganisms was found to be more as compared to uninoculated control.

Almost maximum strains of fluorescent *Pseudomonas* in our study produce ammonia in liquid culture as observed from change in color from light yellow to brown. These bacterial strains are also free living in rhizosphere / soil of apple and pear (Table 9, Plate 7).

5.2.6 Proteolytic activity

Protease executes a large amount of functions and have important biotechnological applications. Protease represent one of the three largest group of industrial enzymes and find applications in detergents, leather industry, food industry, pharmaceutical industry and bioremediation process (Anwar and Saleemuddin, 1998; Gupta *et al.,* 1992). A number of eukaryotic and prokaryotic organisms are reported to secrete different types of extracellular hydrolytic enzymes as well as some non hydrolytic enzymes (Lipases, α and β amylase, cellulases, xylanose, penicillinase, invertase, m *Arthrobacter* sp. *Aspergillus* sp., *Bacillus cereus*, *Candida* sp., *Lactobacillus* sp., *Pseudomonas* sp. In agriculture these proteases producing microorganisms have role as biological control agents for eradication of some fungal plant pathogens.

The screening of isolates for protease production on skim milk agar plates showed that all Pseudomonas isolates were positive for proteolytic activity. Maximum protease production by *Pseudomonas* isolates were in the range of 16mm -32mm diameter of clear zone around the well and by quantitative estimation, activity was found in the range of 2.2 µg/ ml – 7.3 µg/ ml (Table 10, Plate 8). Maximum production was shown by PN-4-SAN.

One of the main function of the enzymes in the soil and rhizosphere may be their effect on the plant nutrition. They transform the organic matter in mineral constitutes, important for plant growth. The enzymes activity is merely always higher in rhizosphere soil than in free soil (urease, invertase, catalase, phosphates and amylase etc). The enzymes in the rhizosphere may be produced by plant root microorganisms (Vancura and Jandera 1986). Another function of enzyme may be in the formation and regulations of the microbial associations in the rhizosphere. The selections and accumulations of some microbial species take place on the roots and in their proximity. The regulation of synthesis and the activity of the enzymes may enable the microorganisms to utilize prefentially from the mixtures of substrates. The substrates most usable for their growth and thus to gain the superiority under the given conditions (Macura, 1971 and 1975; Lockwood 1960).

5.3 EFFECT OF MEDIA ON THE GROWTH AND PRODUCTION OF PLANT GROWTH REGULATORS VIZ AUXINS, GIBBERELLINS, CYTOKININS AT DIFFERENT INCUBATION PERIOD i.e. 24h, 48h, 72h.

Microorganisms are extremely adaptable and they are unrivalled in their capacity to respond to major fluctuations in the environment. This adaptability is found on a highly developed physiology that enable the microorganisms to change structurally and functionally in response to environmental change and thereby to maintain either totally or partially their growth potential (Bull and Brown, 1979; Tempest and Neijssel, 1978). Such phenotypic variability is exploitable in biotechnology and careful manipulation i.e. process optimization produces fermentations having precisely defined properties but during process scale up, environmental and cultural conditions should be exactly reproduced.

So, selection of a suitable medium for cultivation of microorganisms is very important to reveal their potential ability to produce secondary metabolites with biological activities. It has been pointed out by Demain (1973), that the production of secondary metabolites appeared to be affected by the same regulatory mechanism that control primary metabolism, induction feedback and catabolite regulation. The manipulation of fundamental procedures i.e. selection of carbon and nitrogen source, restriction of growth rate by control of pH or oxygen uptake, may also lead to significant improvement (Vandamme, 1981; Flickinger and Periman, 1979). Cultural conditions play an important role in cellular growth and also in production of biological activities by microorganisms (Kotake *et al*., 1992; Singh *et al*., 1983). 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.

For mass multiplication of inoculums for field trials and also for development of plant growth promoting bioagents i.e. biofertilizer for management of replant problem. It is very essential and important to study optimization and standardization of the physical and chemical conditions for optimum production of each plant growth regulators viz. auxins, gibberellins and cytokinins has been done. Biosynthesis of bioactive substances is a specific property of some species or even some strains of microorganisms. This property depends greatly upon the conditions of cultivation of microorganisms and to evaluate best medium and cultural conditions for high production of bacterial biomass and plant growth promoting activities (Issac *et al*., 1992).

Although good growth may occur in many media secondary metabolites may only be produced in a specific medium (Bentley and Keil, 1962). Some times 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 produce high yields of a desired secondary metabolite is still considerable (Demain, 1972 and 1973). Distribution of precursors into various branched pathways may vary and may depend on the growth environment and on the genotype of the organism. The flow of precursors can be increased in the desired direction by mutation or medium manipulation. The presence or absence of certain ions of carbon and nitrogen sources can inhibit, activate, induce and suppress certain enzymes perturbing the normal channeling of key intermediates that support balanced growth (Weinberg, 1978; Malik, 1982).

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 an organism and provide an information for possible parameters affecting biological activity production by an organisms in vitro. The present experiments (Tables 11 to 14) focused on the identification of a suitable growth medium for *Pseudomonas* species PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG that could induce higher levels of plant growth regulators during fermentation.

Growth and production of plant growth regulators viz. auxins, gibberellins and cytokinins by *Pseudomonas* species PN-4-SAN, PN-10-SAN and was studied by liquid fermentation in batch cultures in order to find out the growth phase at which production started and period at which maximum activity could be obtained. The best selected bacterial strains of *Pseudomonas* species i.e. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG were found to produce maximum plant growth regulators viz. auxins, gibberellins and cytokinins. 100 ml of succinate medium, King's medium, nutrient broth, peptone water, trypticase soy broth were used for production of all the three plant growth regulators viz, auxins, gibberellins and cytokinins by *Pseudomonas* sp. They were inoculated with 18 hours old respective culture of organism and incubated at 28°C under shake conditions (90 rpm) in triplicate flasks. Samples were withdrawn at different intervals i.e. 24h, 48h, 72h and analyzed for growth (absorbance at 540 nm) and for production of plant growth regulators. The production of plant growth regulators viz. auxins, gibberellins and cytokinins (Tables 11-14) was to be associated with gradual increasing growth. The maximum production of this extra cellular form of activity was noted towards the end of the log phase and during stationary phase after 48 h or 72 h. Maximum production of growth regulators by four isolates could be compared to each other and maximum production was optimized to 72 h of incubation as maximum growth was also observed at 72h in case of all the media.

The effect of different incubation period on the biosynthesis of auxins, gibberellins and cytokinins (Table 11 to 14) showed that increase in production started at 24-48 h of incubation and reached a maximum level at 72 h. Bacterial growth persistently increased upto 72 h and then it stabilized but the production of each regulator was much more as compare to growth of the organism

All the best selected isolates showed maximum average growth in nutrient broth after 72 and also average maximum production of auxins, gibberellins and cytokinins were observed in nutrient media.

This study corroborate with earlier report on the production of GA_3 by *Pseudomonas* sp. (Karakoc & Aksoz, 2006). The highest level of gibberellic acid (GA_3) production was obtained in nutrient broth at 30^0C for 72 h at pH 7 on a rotary shaker. But it is in contrast to previous reports (Rademacher 1994; Chiang and Aksoz, 1993) suggesting maximum synthesis of gibberellins after incubation of 2 to 4 h or 12-18 days. Our studies also point out the maximum production of all the three growth regulators at 72 h of incubation.

5.4 EXTRACTION, SEPARATION AND PARTIAL CHARACTERIZATION OF AUXINS, GIBBERELLINS AND CYTOKININS

Once plant growth regulators viz. auxins, gibberellins and cytokinins production are detected, the assay which detects and confirm the production of plant growth regulators and can be used to follow the purification of the compound at commercial level from strains that showed high production. Plant growth regulators may be isolated as organic complex or the ligand, depending on the nature of the particular compound.

Pseudomonas sp. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG were selected for plant growth regulators production viz. auxins, gibberellins and cytokinins for purification and partial characterization. The plant growth regulators viz. auxins, gibberellins and cytokinins produced extra cellular in Nutrient broth for *Pseudomonas* under best optimum conditions i.e. 28^0C under shake conditions (90 rpm) and for 72 hours. Auxins were extracted from 72 h old cell free culture supernatant with diethyl ether and pooled fraction were evaporated in rotary evaporator at 40^0C, gibberellins were extracted from 72 h old supernatant with ethyl acetate and all the pooled ethyl acetate fraction were evaporated at 40^0C on a rotary evaporator. Similarly cytokinins were extracted from the supernatant with diethyl ether and extracted with equal volume of n-butanol and the evaporated fractions were passed through the Dowex 50 column.

The homogeneity of plant growth regulators viz. auxins, gibberellins and cytokinins was tested on silica gel-G by thin layer chromatography plates. Result (Table 16) showed that auxins gave a pink spot with isopropanol: water (30:20) solvent system after spraying with salper reagent (Plate 9). The gibberellins gave a grey spot on silica gel plate with isopropanol: ammonium hydroxide: water (10:1:1) solvent system after heating at 110^0C for 10 minutes and spraying with water and sulphuric acid (30:70) (Plate 10). Cytokinins gave brown spot on silica gel plate with n -butanol: 1N NH_4OH: Water (7:1:2) solvent system in iodine chamber (Plate 11).

Strzelczyk and Pokojska-Burdziej (1984) studied the production of auxins and gibberellins like substances by mycorrhizal fungi, bacteria and actinomycetes was studied.

Chromatography and bioassays were used. The highest biological activity was exhibited in substances located at the R_f 0.2-0.4 the organisms produced minute amounts of gibberellins-like substances which appeared at different R_f values. Auxins production is much more common among the root zone organisms of pine than the production of gibberellins-like substances

Verma, *et al.* (2001) investigated that the plant growth regulators (PGR_S) like gibberellins, kinetin 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 strain produced all the three plant growth regulators (PGRs) while other produce one or two of these. The production of these PGRs was further confirmed by bioassays.

5.5 Evaluation of plant growth regulators by bioassays

5.5.1 Avena coleoptiles bioassays

In plant IAA generally occurs in concentration of few micrograms per kilogram of fresh plant tissue and this concentration is too minute for ordinary chemical tests. For measuring such low concentration of active material, a biological response frequently is found to be useful.

Avena coleoptiles response to IAA and this response has been used to develop a sensitive bioassay procedure for measuring concentration of IAA in extracts (Noggle and Fritz, 1976) As shown in (Table 17, Plate 12) partially purified extracts of supernatants for different bacteria i.e. *Pseudomonas* sp. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG, concentration of IAA is about upto 40, 50, 50, 40 µg /ml respectively and it has increased the length of Avena coleoptiles. This indicates that auxins or auxins like substances are present in the partially purified samples.

5.5.2 Alpha amylase release bioassays

Gibberellins induce the synthesis of enzyme alpha amylase and measurement of alpha-amylase activity is a direct method for assaying gibberellins because primary role of it to induce the synthesis of enzyme activity (Jones & Varner, 1967, Noggle & Fritz, 1976).

As the result showed (Table 18) partially purified and gibberellins like substances from 72 h old culture supernatants of *Pseudomonas* sp. PN-4-SAN, PN-10-SAN, aN-2-NAG and AN-4-NAG has increased the alpha –amylase activity. They released 45 to 51 % units of enzyme alpha-amylase from seeds half that correspondent to 35 to 40 µg/ml concentration of GA_3 as calculated from the standard curve.

5.5.3 Radish cotyledon test

The residue collected from Dowex 50 column chromatography were used for its bioassay. The residue produced from four selected strains of fluorescen*t Pseudomonas* were applied to twelve cotyledons of radish seeds and incubated at 25^0 C under fluorescent light for three days. The cotyledon were filtered and weighed. Change in weight represents the concentration of cytokinins calculated from standard dose response curve by using kinetin as standard (100-1000 µg/ml)

Similar production of cytokinins like compounds by *A. vinlandii* were reported by Gonzalez- Lopez *et al*. (1986). Cytokinins production by *Azotobacter* sp. ranges from 0.05 to 4.4 µg/ml kinetin equivalent/ml of liquid culture reported by Azcon and Barea, (1975).

Chapter-VI

SUMMARY AND CONCLUSIONS

Present study aims at the isolation and characterization of indigenous and efficient producer of plant growth regulators and plant beneficial fluorescent *Pseudomonas* species so as to select and develop more efficient native plant growth promoting and disease suppressing bioagents of specific soil type and specific plant type for fields trials and for management of replant problem in specific sites of orchards.

Attempts were made to isolate identify and characterize indigenous fluorescent *Pseudomonas* strains associated with apple and pear and screened out for production of auxins, gibberellins and cytokinins along with other direct and indirect plant growth promoting activities like, antifungal, siderophores, phosphate solubilization, lytic enzymes, ammonia and HCN production. Also optimized for media, their production production potential for auxins, gibberellins & cytokinins and other cultural conditions for mass multiplication of potential strains. Partially purified and characterized the three regulators and evaluated by TLC and bioassays.the salient features of the study are following

- Total bacterial and *Pseudomonas* viable count from rhizosphere soil of apple and pear in normal and replant sites was observed in terms of log cfu/g of soil. The density of fluorescent *Pseudomonas* sp. less in replant site as compared to normal site.

- On the basis of morphological, physiological and biochemical characterization, most of the isolates belong to genera *Pseudomonas* sp. sp. especially fluorescent group of *Pseudomonads*.

- All the isolates of *Pseudomonas* sp. were found to show the production of plant growth regulators viz. auxins, gibberellins and cytokinins.

- 52 per cent inhibition of *Fusarium oxysporum* has been shown by *Pseudomonas* strain PN-4-SAN, PN-8-SAN and PN-10-SAN isolated from rhizosphere of pear. Similar inhibition of *Pythium* sp. was shown by *Pseudomonas* strains AN-9-NAG and AR-1-NAG isolated from apple and PN-2-SAN, PN-11-SAN from pear. Antifungal activity against *Alternaria* sp. was shown by AN-6-NAG and PN-5-SAN.

- Maximum number of *Pseudomonas* strains was found to produce siderophores in nutrient medium ranging from 19-32 mm diameter of pinkish/orange colored zones of chromeazurol-S (CAS) plates. Maximum per cent siderophore units were recorded in *Pseudomonas* strains PN-6-SAN. (60 %SU) quantitatively.

- Phosphate solubilization on Pikovskaya's agar plate after 24 h in terms of yellow colored zone (mm) has been recorded maximum in *Pseudomonas* strain AN-2-NAG (33 mm) followed by PN-10-SAN (32 mm). While quantitatively maximum tricalcium phosphate solubilization has been recorded in *Pseudomonas* sp. PN-4-SAN (445 µg/ml available phosphate).

- Almost all the isolates have shown the production of toxic substances i.e. hydrogen cyanide (HCN) and ammonia.

- Maximum production of protease activity on skim milk agar plate was found in two strains i.e. AN-10-SAN and PN-6-SAN (32 mm).

- Four potential strains of fluorescent *Pseudomonas* sp. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG selected for studying the optimum cultural conditions for production and characterization of plant growth regulators.

- They showed maximum production of three regulators viz. auxins, gibberellins and cytokinins at 72 h of incubation period under shake conditions (90 rpm) in nutrient broth at 28^0C.

- The homogeneity of the extracted partially purified auxins, gibberellins and cytokinins by was checked by thin layer chromatography. Auxins gave pink spot with Salper reagent; gibberellins gave grey/blue spot on spraying with water: sulphuric acid and cytokinins gave brown spot in iodine chamber.

- Partially purified and extracted regulators evaluated by different bioassays i.e, avena coleoptile straight growth test, α-amylase release and radish cotyledon bioassay showed increased length, enzyme activity and increase in weight of cotyledons.

REFERENCES

Ahamad F, Ahamad I and Khan M S. 2005. Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. *Trunk J. Biol*. **29**: 29-37.

Alvares R, Nissen S J and Sutter E G. 1989. Relationship between indole-3- acetic acid levels in apple (*Malus pumila* Mill) rootstocks cultured *in vitro* and adventitious root formation in the presence of indole butyric acid. *Plant Physiol*. **89**:439-443.

Angelona Roco. 2001. *In vitro* biocontrol activity of *Trichoderma* on *Alternaria* in presence of growth regulators. *Journal of Biotechnology.* **4(2**)

Ansar Ali, Touqeer Ahmad, Nadeem Akhtar Abbasi and Ishfaq Ahmad Hafiz. 2009. Effect of different concentration of auxins on *in vitro* rooting of olive cultivar moraiola. *Pak. J. Bot.* **41 (3):** 1223-1231.

Anwar A and Saleemuddin M. 1998. Alkaline proteases. A review. *Bioresource Technology* **6**(3): 175-183.

Arkhipova T N, Veselov S U, Melentiev A I, Martynenko E V and Kudoyarova G R. 2005. Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. *Plant and soil*. **272**: 201-209.

Arneson P A and Mai W F. 1976. Root disease of fruit trees in New York State: VII Costs and returns of preplant soil fumigation in replanted apple orchards. *Plant Disease Reports* **60**: 1054-1057.

Arnon D I. 1956. Phosphorus metabolism and photosynthesis. *Annual Review of Plant Physiology* **7**: 325-354.

Arshad M and Frankenberger W T Jr. 1991. Microbial production of plant hormones. *Plant and Soil* **133**: 1-8.

Arshad M and Frankenberger W T jr. 1992. Microbial production of plant growth regulators. **In**: Soil Microbial. Ecol. Ed. Metting FB jr. Marcel Dekker Inc. New York. pp. 307-347

Arshad M and Frankenberger W T. 1989. Biosynthesis of ethylene by *Acremonium falciforme*. *Soil Biol. Biochem*. pp. 633-638

Azcon R and Barea J M. 1975. Synthesis of auxins, gibberellins and cytokinins by *Azotobacter vinelandii* and *Azotobacter beijerinckii* related to effects produced on tomato plants. *Plants and Soil* **43** : 609-619

Bandara W M M, Seneviratne G and Kulasooriya S A. 2006. Interaction among entophytic bacteria and fungi: effects and potential. *J. Bio. Sci.* **31** (5): 645-650.

Barbieri P, Zanelli T, Galli E and Zanetti G. 1986. Wheat inoculation with *Azospirillum brasilense* sp. 6 and some mutants altered in nitrogen fixation and indole-3, acetic acid production. *FEMS Microbiological Letters* **36**: 87-90.

Bardin S D, Huang H C and Meyer J R. 2004. Control of *Pythium* damping off of sugar beet by seed treatment with straw powders and biocontrol agent. *Biological control* **29** : 453-460.

Barea J M and Agilar A C. 1982. Production of plant growth regulating substances by the vesicular arbucsular mycorhizal-fungus *Glomus mosseae*. *Applied and Environmental Microbiology* **43** (4): 810-813.

Barea J M and Brown M E. 1974. Effect on plant growth produced by *Azotobacter paspali* related to synthesis of plant growth regulating substances. *Journal of Applied Bacteriology* **37**: 583-593.

Bashan Y and Levanony H. 1990. Alterations in membrane potential and in proton efflux in plant roots induced by *Azosprillum brasilense. Plant soil* **137**: 99-103.

Benson N R, Howell C R and Wild J R. 1973. Soil fumigation helps replants in old apple orchards. Proceedings of Washington State Horticulture Association **69**: 90-91, 93-95.

Bentley R and Keil J G. 1962. *J. Biol.chem*. 237-867.

Berdy J. 1974. Recent developments of antibiotic research and classification of antibiotics according to chemical structures. *Advances in Applied Microbiology* **18**: 309-406.

Beringer J E. 1990. Beneficial microorganisms. In: Agricultural Biotechnology. (eds.) Perley, G.J. 698p.

Bhatia S, Dubey R C and Mesheswari D K. 2005. Enhancement of plant growth and suppression of collar rot of sunflower caused by *Sclerotium roltsii* through fluorescent *Pseudomonas*. *Indian Phytopath*. **58** (1): 17-24.

Bottini R, Fulchieri M, Pearce D and Pharis R P. 1989. Identification of gibberellins A1, A3 and iso-A3 in cultures of *Azospirillum lipoferum*. *Plant Physiol*. **90**: 45-47.

Brandy N C. 2001. The nature and properties of soils. Macmillan Publishing Company, New York. 621p.

Braun V and Braun M. 2002. Active transport of iron and siderophore antibiotics. *Current Opinions in Microbiology* **5**: 194-201.

Bruckner B and Blecschmidt D. 1991. The gibberellin formation. *Reviews in Biotech.* **11**:163-192.

Bruckner B. 1992. Regulation of gibberellins formation by the fungus *Gibberellia fujikouri*. *Ciba Found Symp*. **171**: 129-143.

Buchanan R E and Buchanan E D. 1958. Bacteriology. Oxford and IBH Publishing Co. 5th ed. 678p.

Buckland B C, Dunnil P and Lilly M D. 1975. Continuous production of ethanol from fructose by immobilized growing cells of *Zygomonas mobilis*. *Biotechnology Bioengineering* **27**: 613-620.

Bull A T and Brown C M. 1979. Continuous culture applications to microbial biochemistry. IN: Intl. Rev. Biochem. (ed. J R. Quayle), University: Park Press Baltimore M. D. **21**: 177-226.

Bu'lock J D.1961. Secondary metabolism in fungi and its relationship to growth and development. In : *The filamentous fungi*. *Industrial Mycology*, Smith J.E. (ed). London Edward Arnold pp.35-58.

Burke M E and Pattee P A. 1967. Purification and characterization of staphylotic enzyme from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **93** : 860-865

Buvana R. 2002. effect of 2,4-D and Naringenin on induction of Paranodules of *Azorhizobium* colinodans in cereals. *Indian Journal of Microbiology.* **26**: 111-115

Castka V. 1993. Biological control of phytotoxic and phytopathogenic microorganism in the plant rhizosphere. In: Vancura V, Kunc F. (eds.) Inter-relationships between microorganisms and plants in the soil. pp. 463-468.

Catska V, Vancura V, Hudska G and Prikryl Z. 1982. Rhizosphere micro-organisms in relation to the apple replant problem. *Plant and Soil* **69**:187-197.

Cheetham P S J. 1987. Screening for novel biocatalysts. *Enzyme Microb. Technology*. **9**: 194-213.

Cheruth Abdul Jaleel. 2009. Changes in the photosynthetic characteristics of *Catharanthus roseus L.* as a result of exogeneous growth regulators. *Plant Omics Journal*. **2**(4): 169-174

Chet I. 1984. *Trichoderma* - Application, mode of action and potential as a biocontrol agent of soil borne plant pathogenic fungi. In: *Innovative approaches to plant disease control.* ed. Chet. I. New York. John Wileys Sons. pp.137-160.

Chiang N and Aksoz N. 1993. *Aspergillus niger*'den Gibberellik Asit Eldesi ve Uygun Fizyolojik Kosullarin Saptanmasi. Doga. *Turk Biyoloji Dergisi*.**17**:63-74.

Claude J. 1996. studies on growth regulators and improved avena coleoptile elongation test for auxins. *Plant Physiol.* **41**: 1308-1312

Creus C, Sueldo R and Barassi C. 1997. Shoot growth and water status in *Azospirillum* inoculated wheat seedlings grown under osmotic and salt stresses. *Plant Physiology Biochemistry* **35**: 939-944

D' Aoust J Y, Gerber N N. 1974. Isolation and purification of prodigiosin from *Vibrio sychroerythrus*. *J. Bacteriol*. **118**: 756-757.

Dave A and Patel H H. 1999. Inorganic phosphate solubilizing soil *Pseudomonas*. *Indian Journal of Microbiology* **39**(3): 161-164.

Davies P J. 1995. The plant hormones: their nature, occurrence and functions: In plant hormones ed. Davies. P J. Dordrecht. The Netherlands: *Kluwer Academic Publisher*. pp. 1-12

Deacan J W. 1984. Introduction to modern mycology. In: Wilkinson J F (ed). Blackwell Sci. Publ. Oxford London Edinburg.

Demain A L. 1973. Mutation and production of secondary metabolites. *Adv. App. Microbiol.* **16**: 177-202.

Demain A L. 1992. Regulation of secondary metabolism. **In**: Biotechnology of Filamentous Fungi: Technology and Products. (eds. D B. Finklestein and C. Ball). Butterworth-Heinemann, Boston.

Dilfuza. 2007. Plant growth promoting properties of rhizobacterial isolated from wheat and pea grown in loamy sand soil. *Turk J BIOL.* **32**: 9-15

Donderski W and Gluckowska M. 2000. Production of cytokinins like substances by Planktonic bacteria isolated from lake Jeziorak. *Journal of Environmental Studies* **9** (5): 369-376.

Dubey S K. 2000. Effectiveness of rock phosphate and superphosphate amended with phosphate solubilizing microorganisms in soybean grown on vertisols. *Journal of Indian Society of Soil Science* **48**(1): 71-75.

Duffy K Brion and Defago G. 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescence* biocontrol strains. *Applied and Environmental Microbiology* **65**(6):2429-2438.

Duniway J M. 2002. Status of chemical alternative to methyl bromide for pre-plant fumigation of soil. *Phytopathology* **92**: 1337-1343.

Durham D R, Stewart D B and Stellwag E J. 1987. Novel alkaline and heat stable proteases from alkalophilic *Bacillus* sp. strain GX 6638. *Journal of Bacteriology* **169** : 2762-2768.

Dutta J R, Dutta P K and Banerjee R. 2005. Kinetic study of low molecular weight protease from newly isolated *Pseudomonas* species using artificial neural network. *Indian Journal of Biotechnology* **4** : 127-133.

Elezar M, Escamilla S. 2000. Optimization of GA_3 production by immobilized *Gibberella fujikuroi* mycelium in fluidized bioreactors. *Journal of Biotechnology*. **76**: 147-155

Flickinger M C and Perlman D. 1979. Application of oxygen-enrichment aeration on production. **In**: *Comprehensive Biotechnology. The Principles, Applications and Regulations of Biotechnology in Industry, Agriculture and Medicine* (eds. A T Bull and H Dalton), Pergamon Press, New York. **1**: 251-279.

Frankenberger W T Jr and Brunner W. 1983. Methods of detection of auxins-indole acetic acid in soil by high performance liquid chromatography. *Soil Soc. Am. j.* **47**: 237-241.

Fulchieri M, Lucangeli C and Bottini R.1990. Effects of *A. Lipoferum* and GA_3 on root growth in corn. In : Nitrogen Fixation : Proceedings of fifth international symposium on N- fixation by non legumes, Polsinelli M, Materasii R and Vicenzini M (eds.), Kluwer Academic publishers, Dordrecht, The Netherlands pp.297-298

Fulchieri M, Lucangeli C and Bottini R.1993. Inoculation with *A. Lipoferum* affects growth and gibberellin status of corn seedlings roots. *Plant Cell Physiology* **134** : 1305-1309

Garden L, David C, Morel M, Glickmann E, Abu-Ghorrah M, Gluckmann E, Petit A and Dessaux Y. 1992. Evidence for a correlation between auxin production and host plant species among strains of *Pseudomonas syringa*e sub sp. Savastanoi. *Appl. Environ. Microbiol*. **58** (5): 1780-1783.

Gaur A C. 1990. Phosphate solubilizing microorganisms as biofertilizers. Omega Scientific Publishers, New Delhi, pp.149-151.

Gazala Nasim and Meemona Rehman. 2006. Effect of cytokinins on *in vitro* growth and biomass production of some soil fungi. *Mycopath.* **4(1)**: 37-43

Ghosh A C and Basu P S. 2002. Growth behaviour and bio-production of indole acetic acid by *Rhizobium* sp. isolated from root nodules of leguminous tree *Dalbergia lanceolaria*. *Indian Journal of Experimental Biology* **40**: 796-801.

Gilbert E J, Cornish A and Jones C W. 1991. Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF-2. *Journal of General Microbiology* **137** : 2223-2229.

Glick B R, D M. Karaturovic and P C Newell. 1995. A novel procedure for rapid isolation of plant growth promoting Pseudomonas. *Canadian Journal of Microbiology* **41**: 533-536.

Glick B R. 1995. The enhancement of plant growth by free living bacteria. *Can. J. Microbiol.* **41**:109-117.

Gom K and Matusoka M. 2003. Gibbrellin signailling pathway. *Current opinions in Plant Biol.* **6**:489-493.

Gonzalez-lopenz J, Salmeron V, Martinez-Toledo M V, Ballesteros F and Ramos-cormenzana A. 1986. Production of auxins, gibberellins and cytokinins by *Azotobacter vinelandii* ATCC 12837 in chemically defined medium and dialyzed soil media. *Soil. Biol. Biochem*. **18**: 119-120.

Gooday G A. 1986. Chitinase activities in animals, fungi and bacteria. In : *Chin in nature and technology*, Muzzarelli K, Jeunaux C and Goodey C W (eds.), New York, Planum Press, pp.241-261

Grabley S and Thiericke R. 1998. Bioactive agents from natural sources, Trends in discovery and application. *Ads. Biochem. Bioeng*. **64**: 101-154.

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

Gupta V and Satyanarayan T. 2002. Production of extracellular Siderophores by ectomycorrhizol fungi. *Indian Journal of Microbiology* **4**: 107-110

Hasan H A H. 1999. Fungal utilization of organophosphate pesticide and their degradation by *Aspergillus flavus* and *A. Syndowii* in soil. *Folia Microbiology* **44**:77-84

Hasan H A H. 2002. Gibberellin and auxin production by plant root-fungi and their biosynthesis under salinity-calcium interaction. *Rostlinna vyroba*. **48** (3): 101-106.

Hein K. 1974. Microorganisms as a cause of specific replant problem of fruit trees. Proceedings of XIX International Horticulture Congress, Warsawa. 127.

Hoestra H. 1968. Replant diseases of apple in the Netherland. *Meded Land-Bouwhogesch.* Wageningen. **68**: 1-105.

Holbrook A A, Edge W J W and Fermor T R. 1991. Spectrophotometric method for determination of gibberellic acid, in gibberellin. P. 159-167. A.C.S. Washington, D.C.

Horikoshi K.1971. Production of alkaline enzymes by alkalophilic microorganisms. Part II. Alkaline amylase produced by Bacillus No. A-40-2. *Agricultural Biology and Chemistry* **35** : 1783-1791.

Howell C R and Stipanovic R D. 1980. Suppression of *Pythium ultimum* induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and antibiotic Pyoluteorin. *Phytopathology* **70**: 178-182.

Howie W J and Suslow T V. 1991. Role of antibiotic biosynthesis in the inhibition of *Pythium ultimum* in the cotton spermosphere and rhizosphere by *Pseudomonas fluorescence*. *Molecular PlantMicrobe Interaction* **4**: 393-399

Hutzinger O and Kosuge T. 1968. Microbial synthesis and degradation of indole-3-acetic-acid. III. The isolation and characterization of indole-3-acetyl-E-L-lysine. *Biochemistry* **7**: 601-605.

Ishimaru C A. 1993. Biochemical and genetic analysis of siderophores produced by plant associated *Pseudomonas* and *Erwinia* species. In: Iron chelation in plants and soil microorganisms. Barton LB and Hemming BC (eds). Academic Press, Inc.

James D W, Suslow T V and Steinback K E. 1985. Effects of *Pseudomonas putida* and Synthetic iron chelators on induction of soil suppressiveness to *Fusarium* wilt pathogens. *Applied Environment al Microbiology* **50**: 392-396.

Johri B N, Rao Ch. V S and Goel R. 1997. Fluorescent *Pseudomonas* in plant disease management. **In**: *Biotechnology approaches in soil microorganisms for sustainable crop production* (ed. Dadarwal K R.) *Scientific Publishers, Jodhpur* 193 p.

Jones R L and J E Verner. 1967. The bioassays of auxins. *Planta* **72**: 155-167.

Joo G J, Kim Y M, Lee T J, Song K S and Rhee T K. 2001. Growth promotion of red pepper plugs seedlings and production of gibberellins by *Bacillus cerus*, *Bacillus macroides* and *Bacillus pumilus*. **26** (6): 487-491.

Kampert M, Strzelczyk E and Pokojska A. 1975. Production of auxins by bacteria isolated from pine roots (*Pinus syivestris* L.). *Acta Microbiol. Poll.* **7**: 135-143.

Kapoor K K, Mishra M M and Kukneja K. 1989. Phosphate solubilization by soil microorganisms – 1 review. *Indian Journal of Microbiology **29***(2): 119-127

Karakoc S and Aksoz N. 2006. Some optimal cultural parameters for gibberellin acid biosynthesis by *Pseudomonas sp*. *Turk J. Biol*. **30**: 81-85.

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

Katayama N, Nozaki Y, Okonogi K, Harada S and Ono H. 1992. Ferrocins new iron containing peptide antibiotics produced by bacteria, *J. Antibiot*. **46**: 65-69.

Katznelson H and Cole S E. 1965. Production of gibberellins like substances by bacteria and actinomycetes. *Canadian Journal of Microbiology* **11** : 733-741.

Kaur M, Gupta M, Tripatathi K A K and Gupta K G. 1989. Lytic effect of *Pseudomonas aeruginosa* elastase on gram positive and gram negative bacteria: entrablatt Bakt. *Intl. J. Microbiol*. **34**: 855-859.

Kaur M, Tripathi K K, Jain P K, Bansal M R and Gupta K G. 1988. Production and partial characteristics of elastase of B.subtilis isolated from the cervix of human females. *Can. J. Microbiol*. **34**: 855-859.

Khakhipour N, Khavazi K, Mojallali H, Pazira E and Asadirahmani H. 2008. Production of auxin hormone by fluorescent *Pseudomonas. American Eurasian J. Agric. And Environ. Sci.* **4**(6): 687-692.

Khalid A, Arshad M and Zahir Z A. 2004. Screening plant growth promoting rhizobacteria for improving growth and yield of wheat. *Journal of applied Microbiology* **96** (3): 473.

Khan S M, Zaidi A and Wani P A. 2006. Role of phosphate solubilizing microorganism in sustainable agriculture. A review.

King E O, Ward M K and Raney D E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Chemical Medicine* **44**: 301-307.

Kloepper J W, Leong J, Teintze M, Schroth M N. 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. *Nature* **286**: 885-886.

Kloepper J W. 1993. Plant growth promoting rhizobacteria as biological control agents. pp 225-274. **In**: *Soil Microbial Ecology: Application in agricultural.*

Knowles C J. 1976. Microorganisms and cyanide. *Bacteriological Review* **40**: 652-680.

Kotake C, Yamaski T, Moriyama T, Shinoda M, Komiyana N and Furumai T. 1992. *J. Antibiotic.* **45**: 1442-1450.

Kucey R M N, Janzen H H and Leggett M E. 1989. Microbially mediated increases in plant available phosphorus. *Advances in Agronomy* **42**: 199-228.

Kumar D B S and Dubey H C. 1993. Siderophore production by plant growth promoting fluorescent *Pseudomonad* R B T-13. *Indian Journal of Microbiology* **33**(1): 61-65.

Kumar P K R and Lonsane B K. 1986. Immobilized growing cells of *Gibberella fujikuroi* P-3 for production of gibberellic acid in batch and semi-continuous cultures. *Appl. Microbiol. Biotech*. **28**:537-542.

Kunz D A and Nagappan O. 1989. Cyanase-mediated utilization of cyanate in *Pseudomonas fluorescens* NCIB 11764. *Applied and Environmental Microbiology* **55**(1): 256-258.

Lankford C E. 1973. Bacterial assimilation of iron. *Critical Review of Microbiology* **2**: 273-331.

Lata and Saxena A K. 2003. Characterization of plant growth promoting rhizobacteria. In: Training manual on Biofertilizer Technology (eds.) A K Saxena. IARI Delhi pp. 24-25.

Lata G S and Verma J P. 1998. Role of fluorescent *Pseudomonads* in the suppression of rot and damping off of cotton. *Indian Phytopathol*. **49** (1): 3-8.

Lee S, Encarnacion M F, zentella M C, Flores LG, Fscamilla J E and Kennedy C. 2004. Indole-3-actic acid biosynthesis deficient in *Gluconacetobacter diazotrophicus* strains with mutation in cytochrome C biogenesis genes. *Journal of Bacteriology* **186** (16): 5384-5391.

Lifshitz R, Kloepper J W, Kozlowski M, Simonson C, Carlsonn J, Tuppign E M and Zaleska I. 1987. Growth promotion of canola (rapes seed) seedlings by a strain of *Pseudomonas putida* under gnotohiotic conditions. *Can. J. Microbial.* **33**: 390-395.

Lindow S E, Desurmont C, elikins R, McGourty g, Clark E and Maria T B. 1998. Occurrence of indole-3-acetic-acid producing bacteria on pear trees and their association with fruit russet. *J. American Phytopathology* **88** (11): 1150-1157.

Lockwood J L. 1960. Lysis of mycelium of plant pathogenic fungi by natural soil. *Pytopathology* **50**:787-789

Loper J E and Buyer J S. 1991. Siderophore in microbial interactions on plant surfaces. *Mol. Plant Microbe Interaction* **4**: 5-13.

Lorck H. 1948. Production of hydrocyanic acid by bacteria. *Physiologia Plantarum* **1**: 142-146.

Macura J. 1971. Some biological ecological aspects of the rhizosphere effects. *Folia Microbiology* **16**:328-336.

Macura J. 1975. Regulation of enzymes participating plant pathology, Mahadevan A and Sridhar R (eds.), Sivakari Publications, Madras.

Mahadvan A. and Sridhar R K 1986. Method In Physiology. 3rd ed. *Plant Physiology.*

Mai W F and Abwai G S. 1981. Controlling replant disease of pome and stone fruits in northeastern United-States by preplant fumigation. *Plant Disease* **65**: 859-864.

Malik V S. 1982. Genetic and biochemistry of secondary metabolites. *Adv. Appl. Microbio*l. **28**: 28-101.

Manero G F J, solan R B, Probanza A, Mehouachi J, Tadeo F R and Talon M. 2001. The plant growth promoting rhizobacteria *Bacillus pumilus* and *Bacillus lichiniformis*. Produce high amount of physiologically active gibberellins. *Physiologia Plantrum* **111** (2): 206-211.

Maor R, Haskin S, Kedmi H L and Sharon A. 2004. In plant production of indole-3-acetic acid by *colletotrichum gloeospoirioides* f. sp. aeschynomene. *Applied and Environmental Microbiology* **70** (3): 1852-1854.

Maria Guneth T R, Sandra A Valencia-Plata, Castillo J B and Patricia martienz-Nieto. 2000. Isolation of *Enterobacteria*, *Azotobacter* sp. and *Pseudomonas* sp. Production of indole-3-acetic acid and siderophore from Colombian rice rhizosphere. *Ravista Latinamircana De Microbiologia* **42**: 171-176.

Martin F N. 2003. Development of alternative strategies for management of soil borne pathogens currently controlled with methyl bromide. *Annual Review of Phytopathology* **41**: 325-350.

Marumaya A, Maeda M and Simidu U. 1986. Occurrence of plant hormone (Cytokinins) producing bacteria in the sea. *J. Applied Bacteriol*. **61**: 569- 574.

Mazzola M. 1997. Identification and pathogenicity of *Rhizooctonia* sp. isolated from apple roots and orchards soils. *Phytopathology* **87**: 582-587.

Mc Keller R C and Cholette H. 1986. Possible role of calcium in the formation of active extracellular proteinase by *Pseudomonas fluorescens. Journal of Applied Bacteriology* **60**: 37-44.

Mckenry M, Buzo T, Kretsch J, Kaku S, Otomo E, Ashcroft R, Lange A and Kelle K. 1994. Soil fumigation provides multiple benefits; alternatives give mixed results. *Microbial Ecology* **48**: 338-348.

Merwin I A, Byard R, Robinson T L, carpenter S, Hoying S A, Iungerman K A and Fargione M. 2001. Developing an integrated program for diagnosis and control of apple replant disease. *HortScience* **23**: 791.

Meuwely P and Pilet P E. 1991.Local treatment with indole-3-acetic acid induces differential growth responses in *Zea mays* L. *Planta* **185**: 58-64.

Meyer J M and Abdullah M A. 1978. The fluorescent pigment of *Pseudomonas fluorescens*. Biosynthesis purification and physio-chemical properties. *Journal Gen. Microbiol*. **107**: 319-328.

Morihara K. 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. *Journal of Bacteriology* **88**(3): 745-757.

Munjam S, Vasavi Girisham S and Reddy S M. 2002. Production of indole acetic acid by anoxygenic phototrophic bacteria under different conditions. *Indian Journal of Microbiology* **42**: 215-218.

Narula N and Gupta K G. 1986. Ammonia excretion by *Azotobacter chrooccum* In liquid culture and soil in the presence of manganese and clay minerals. *Plant and Soil* **93**: 205-209

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

Neilands J B. 1981. Iron absorption and transport in microorganism. *Annual Review of Nutrition* **1**: 27-46.

Neilands J B. 1981. Microbial iron compounds. *Annual Review of Biochemistry* **50**: 715-731.

Neilands J B. 1982. Microbial envelope proteins related to iron. *Annual Review of Microbiology* **36**: 285-309.

Nickell I. 1982. Plant growth regulators. Agricultural uses. Springer-verlag. New York. p. 173.

Nieto K F and Frankenberger W T. 1989 b. Biosynthesis of cytokinins by *Azotobacter chrococcum. Soil Sci. Soc. Am*. J. **53**: 735-740.

Nieto K F and Frankenberger W T. 1988. Determination of cytokinins by ion suppression-reverse phase high performance liquid chromatography. *J. Liq. Chromatogr*. **11**: 2907-2925.

Nieto K F and Frankenberger W T. 1989 a. Biosynthesis of cytokinins in soil by *Azotobacter chroococcum*. *Soil Biol. Biochem*. **21**: 967-972.

Noggle R. and Firtz J. 1976. Introductory Plant Physiology. Niall India Pvt. Ltd. Jay Print Press. New Delhi.

Okon and Labandera-Gonzalez C A. 1994. *Agronomic applications of Azospirillum.* In : Improving plant productivity with rhizosphere bacteria, Ryder M H, Stephens P M and Bowen G D (eds.). Commonwealth scientific and industrial research organization, Adelaide, Australia, pp.274-278.

Omura S and Nakagawa A. 1984. Macrolide antibiotics possessing new biological activities. *Farumashia* **209**: 1226-1232

Oxford A E, Raistrid H and Simorart P. 1935. *Biochem. J.* **29**: 1102.

Pandey A, Trivedi P, Kumar B and Palni L M S. 2006. Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B_0) isolated from sub alpine location in the Indian central himalaya. *Current microbiology* **53**(2):102-107

Parmar N and Dadarwal K R. 1997. Rhizobacteria form the rhizosphere and rhizoplane of Chick pea (*Cicer arietinum* L.). *Indian J. Microbiol*. **37**: 205-210.

Patten C L and Glick B R. 1996. Bacterial biosynthesis of indole acetic acid. *Can. J. microbial*. **42**: 207-220.

Patten L and Glick B R. 2002. Role of *Pseudomonas putida*: indole acetic acid in development of the host plant root system. *Applied and Environmental Microbiology* **68** (8): 3795-3801.

Paul N B and Sundara Rao W V B. 1971. Phosphate dissolving bacteria in the rhizosphere of some cultivated legumes. *Plant and Soil* **35**: 127-132. .

Paul V and Jalali I. 1998. Rhizosphere bacteria for biocontrol of plant diseases. *Ind. J. Microbiol*. **38**: 187-204.

Perrig D. ET al. 2007. Plant growth promoting compounds produced by two agronomically important strains of *Azospirillum* brasilense and implication for inoculant formulation. *Applied Microbial and Cell Physiology.*

Phillips D A and Torrey J G. 1972. Studies on cytokinins production by *Rhizobium*. *Plant Physiol.* **49**: 11-15.

Pierre W H, Chan E C S and Kreig N R. 1993. Microbiology. Tata Mc Graw Hill Publishing Company Limited, 918p.

Rademacher W. 1994. Gibberellin formation in microorganisms. *Plant Growth Regulation*. **15**: 613-616.

Rodriguez H and Fraga R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances* **17** : 319-339

Ross I L, Alami Y, Harvey P R, Achovak W, Ryder M H. 2000. Genetic diversity and biological control activity of novel species of closely related Pseudomonas isolated from wheat field soils in South Australia. *Applied and Environmental Microbiology* **66** : 1609-1616.

Rumberger A, Merwin I A and Thies J E. 2007. Microbial community development in the rhizosphere of apple trees at a replant disease site. *Soil Biology and Biochemistry* **39**:1645-1654

Rumberger A, Yao S, Merwin I A, Nelson E B and Thies J E. 2004. Rootstock genotype and orchard replant position rather than soil fumigation or compost amendment determine tree growth and rhizosphere bacterial community composition in an apple replant soil. *Plant and Soil* **264**: 247-260.

Sakakibara H, Kasahara H, Veda N, Kojima M, Takei K, Hishiyama S, Asami T, Okada K, Kamiya Y, Yamaya T. and Yamaguchi S. 2005. *Agrobacterium tumefaciens* increase cytokinins production in plastids by modifying the biosynthetic pathway in the host plant. *Proceedings of the National Academy of Sciences of the United States of America* **102** (28): 9972-9977.

Salamone K. 2001. Effect of plant growth regulators produced by rhizobacteria on the growth of plants. *Journal of Indian Society of Soil Science* **74**(1): 142-147

Salisbury B F and Ross W C. 1985. Gibberellins, Plant Physiology, Wadsworth Publishing Company, California, U.S.A. 309-329

Sang Mo Kang, Gil Jae Joo, Muhammad Humayun. 2009. Gibberellin production and phosphate solubilization of newly isolated strain of *Acitinobacter* and its effect on plant growth. *Biotechnol Lett.* **31**: 277-281

Santos M G E, Couto M C M, and Rebelo H L S. 2003. Ion-selective electrodes based on metalloporphyrins for gibberellic acid determination in agricultural products. *Anal. Bioanal. Chem*. **375**: 511-516.

Scher F M and Baker R. 1982. Effect of *Pseudomonas putida* and a synthetic iron chelators on induction of soil suppressiveness to *Fusarium* wilt pathogens. *Phytopathology* **72**: 1567-1573.

Schippers B, Bakker A W and Bakker P A H M. 1987. Interactions of deleterious and beneficial microorganism and the effect of cropping practices. *Annual Review of Phytopathology* **25**: 339-358.

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

Sharma Y R, Anandaraj M and Venugopal W and Woods D E. 1994. Disease of spice crops. Advanced in Horticulture, plantation and spice crops. Malhotra publishing house. New Delhi. 10:1014-1057.

Singh K, Shegal S N, Rakhi S and Vezina C. 1983. *J. Antibiot*. **36**: 1770-73.

Singh R and Sinha A P. 2005. Influence of time of application of *Pseudomonas fluorescens* in suppressing sheath blight of rice. *Indian Phytopathology* **58**(1): 30-34.

Smith T G. 1995. Orchards updates: Washington State University Cooperative Extension Bulletin 219, Cornell Cooperative Extension.

Sobieszczanski J. 1961. The share of rhizosphere bacteria in mobilizing, transporting and sorting phosphorus from the unavailable phosphate. *Soils and Fertilizers* **25**: 124p.

Sridevi M, Mallaiah K.V. 2007. Production of indole-3-acetic acid by *Rhizobium* isolates from Sesbania species. *African Journal of Microbiology Research.* **1(7**): 125-128

Srivastava A C, Ahamad S, Agarwal D K and Sarbhoy A K. 2003. Screening of potential gibberellin producing *Fusarium* strains for the hybrid rice production. *Food, Agriculture and Environment* **1** (2): 250-253.

Srivastava R, Johri B N and Sharma A. 1999. Colonization of wheat (*Triticum aestivum* L.) root by *Fluorescent Pseudomonas* (GRP3) and PRS3). *Indian J. Microbiol*. **39**: 205-210.

Steele D B and Stowers M D. 1991. Techniques for selection of industrially important microorganisms. *Annu. Rev. Microbiol*. **45**: 89-106.

Steenhoudt O and Vanderleyden J. 2000. *Azospirillum* a free living nitrogen fixing bacterium closely associated with grasses; genetic, biochemical and ecological aspects. *FEMS Microbiol. Rev*. **24**:487-506.

Streelczyk E and Burdzeij A P. 1984. Production of auxins and gibberellins like substances by mycorrhizal fungi, bacteria and actinomycetes isolated from soil and the mycorrhizosphere of pine (*Pinus Silvestris* L.). *Plant and soil* **81** (1): 185-194.

Swaby R J and Sperber J I. 1958. Phosphate dissolving microorganism in the rhizosphere of legumes In: E.G. Hallsworth (eds.) Nutrition of the legumes. Academic Press, New York. pp. 289-294.

Taller B J and Wong T Y. 1989. Cytokinins in *Azotobacter vinelandii* culture medium. *Applied and Environmental Microbiology*. **55**(1): 266-267.

Tandon H L S. 1987. Phosphorous Research and Agricultural Production in India. Fertilizer development and Consultation Organization, New Delhi, India

Tari P H and Anderson A J. 1988. *Fusarium* wilts suppression and agglutinability of *Pseudomonas putida. Applied and Environmental Microbiology* **54**: 2037-2041.

Tempest D W and Neijssel O M. 1978. Eco-physiological aspects of microbial growth in aerobic, nutrient-limited environments. *Adv. Microb. Ecol*. **2**: 105-153.

Thomashow L S and Weller D M. 2004. Role of antibiotics and siderophore in biocontrol of take-1ll disease of wheat. *Plant and soil.* **129** (1):93-94

Tien T M, Gaskin S and Hubbell D H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Applied Environmental Microbiology* **37**: 1016-1024.

Toyoda H and Utsumi R.1991. Methods for preservation of *Fusarium* disease and microorganisms used for the same. U.S. Patent No. 4988586.

Tsavkelova E A, Cherdyntseva T A and Netrusov A I. 2005. Auxin production by bacteria associated with orchid roots. *J. Microbiology* **74** (1): 55-62.

Van Peer R and Schippers B. 1989. Plant growth responses to bacterization and rhizosphere microbial development a hyrdoponic cultures. *Canadian Journal of Microbiology* **35**: 456-463.

Vancura V and Jandera A. 1986. Formation of biologically active metabolite by rhizosphere micro flora FEMS Symp. 33 **In**: *Microbial community in soil. Applied Science Publishers*. London and New York.

Vancura V and Macura J. 1960. Indole derivatives in *Azotobacter* cultures. *Fol. Microbiology* **5**: 293-298.

Vandamme E J. 1981. Environmental influence on the dynamics of gramicidin S fermentation. **In**: *Topics in Enzymes and Fermentation. Biotechnology* (ed. A. Wiseman), Ellis Horwood, Chichester, pp. 187-261.

Vasanthakumar Archana and Mcmanus Patricia S. 2004 Indole-3- acetic producing bacteria associated with cranberry stem gall. *J. Bacteriology* **94**: 1164-1171

Verma A, Kukreja K, Pathak D V, Suneja S and Narula N. 2001. In vitro production of plant growth regulators (PGRs) by *Azotobacter chroococcum. Indian Journal Microbiology* **41**: 305-307.

Vikram A, Hamzehzarghani H. and Alagawadi A.R. 2007. Production of plant growth promoting substances by phosphate solubilising bacteria isolated from vertisols. *Journal of Plant Sciences.* **2(3)**: 326-333

Vivekanathan R, Ravi M, Ramanathan A and Samiyappan R. 2004. Lytic enzymes induced by *Pseudomonas fluorescens* and other biocontrol organisms mediated defence against the anthracnose pathogen in mango. *World Journal of Microbiology and Biotechnology* **20**: 235-244.

Voisard C, Keel C, Hass D and Defago G. 1989. Cyanide producton by *Pseudomonas fluorescence* helps suppress black rot of tobacco under gnotobiotic conditions. *EMBOJ* **8**: 351-358

Weinberg D. 1978. *Folia. Microbiol*. **23**: 496.

Weller D M. 1988. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* **26**: 379-407.

Wichner S and E. Libbert. 1968. Interaction between plant and epiphytic bacteria regarding their auxin metabolism. Detection of IAA producing epiphytic bacteria and their role in long duration experiments on tryptophan metabolisms in plant homogenates. *Plant Physiology* **21**: 227-241.

Yao S, Merwin I A, Abawi G S and Thies J E. 2006. Soil fumigation and compost amendment alter soil microbial community composition but do not improve tree growth or yield in an apple replant site. *Soil Biology and Biochemistry* **38**: 587-599.

Yeole R D and Dube H C. 1997. Increased plant growth and yield through seed bacterization. *Indian Phytopathol*. **50** (3): 316-319.

Zakharova E A, Shcherbakov A A, Brudnik V V, Skripko N G, Bulkhin N and Ignatov V V. 1999. Biosynthesis of indole-3-acetic acid in *Azospirillum brasilense*. *Eur. J. Biochem*. **259**: 572-576.

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ABSTRACT

Under the present study, an attempt was made to study production of bacterial plant growth regulators viz. auxins, gibberellins and cytokinins produced by indigenous strains of *Pseudomonas* species isolated from the rhizosphere of apple and pear. They all were screened out for the production of different plant growth promoting activities like antifungal, siderophores, phosphate solubilization, HCN, ammonia and protease. Four strains each of *Pseudomonas* species (PN-4-SAN, PN-10-SAN, AN-2-NAG, AN-4-NAG) were selected for further studies with the aim to find out best indigenous plant growth regulators producing bacterial strains for application in field in order to increase yield and crop productivity and also to find out industrially important strains for production of plant growth regulators viz. auxins, gibberellins and cytokinins. The selected *Pseudomonas* isolates preferred nutrient broth for the growth and production of all three growth regulators. All the four strains produced maximum plant growth regulators at 72 h incubation period at pH 7.0 under shake condition while the optimum temperature for production of growth regulators was 28^{o}C. An attempt was also made to extract, purify and evaluate plant growth regulators by thin layer chromatography, by spectrophotometric and specific bioassay method. By TLC, auxins gave pink spot gibberellins gave grey spot and cytokinins gave brown spot upon spraying with Salper reagent, solvent water: sulphuric acid and iodine respectively. Specific bioassays viz. Avena coleoptile straight test, α-amylase release test and radish cotyledon bioassays showed increased length, enzyme activity and increase in weight of cotyledons.

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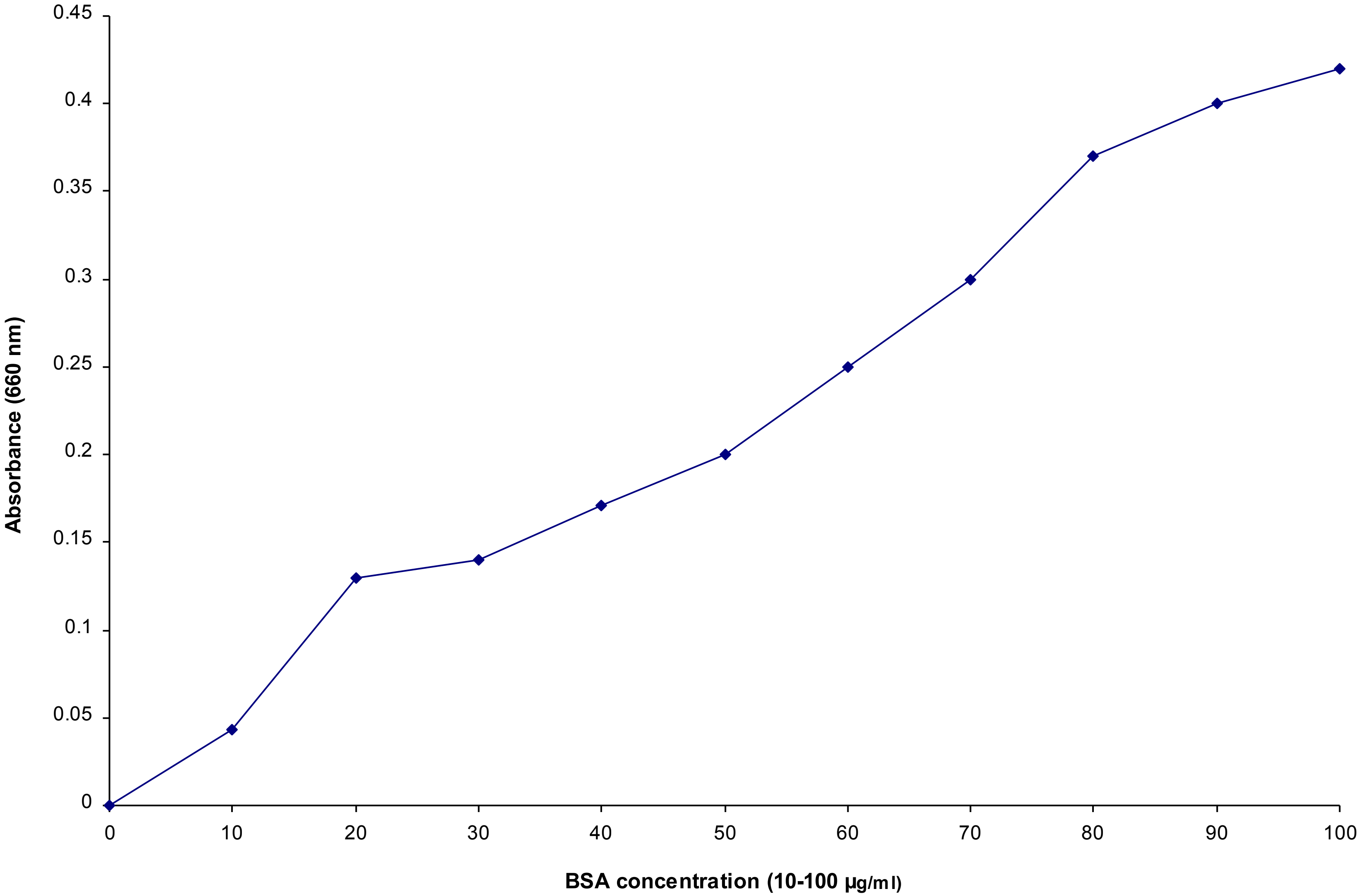
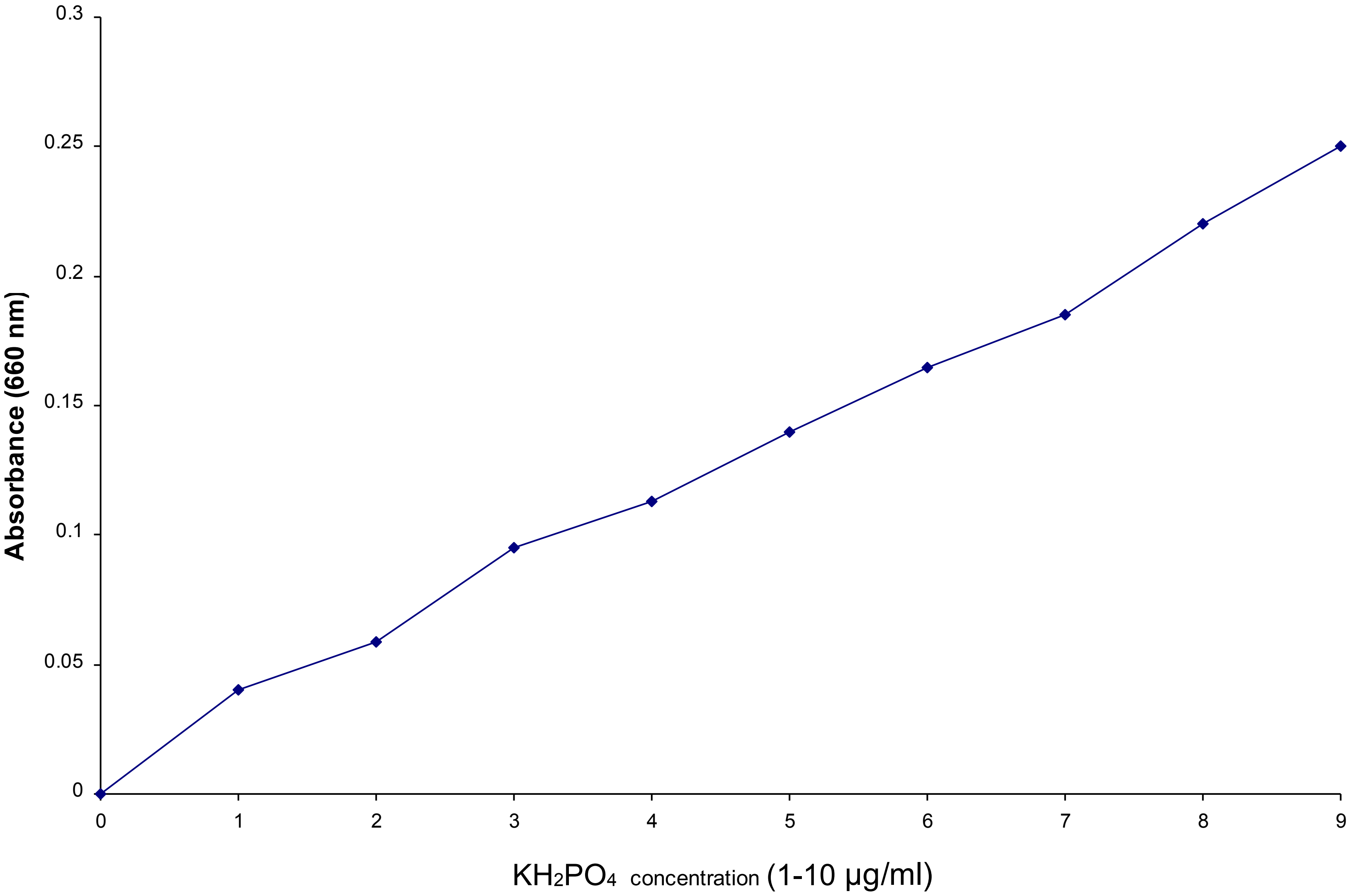
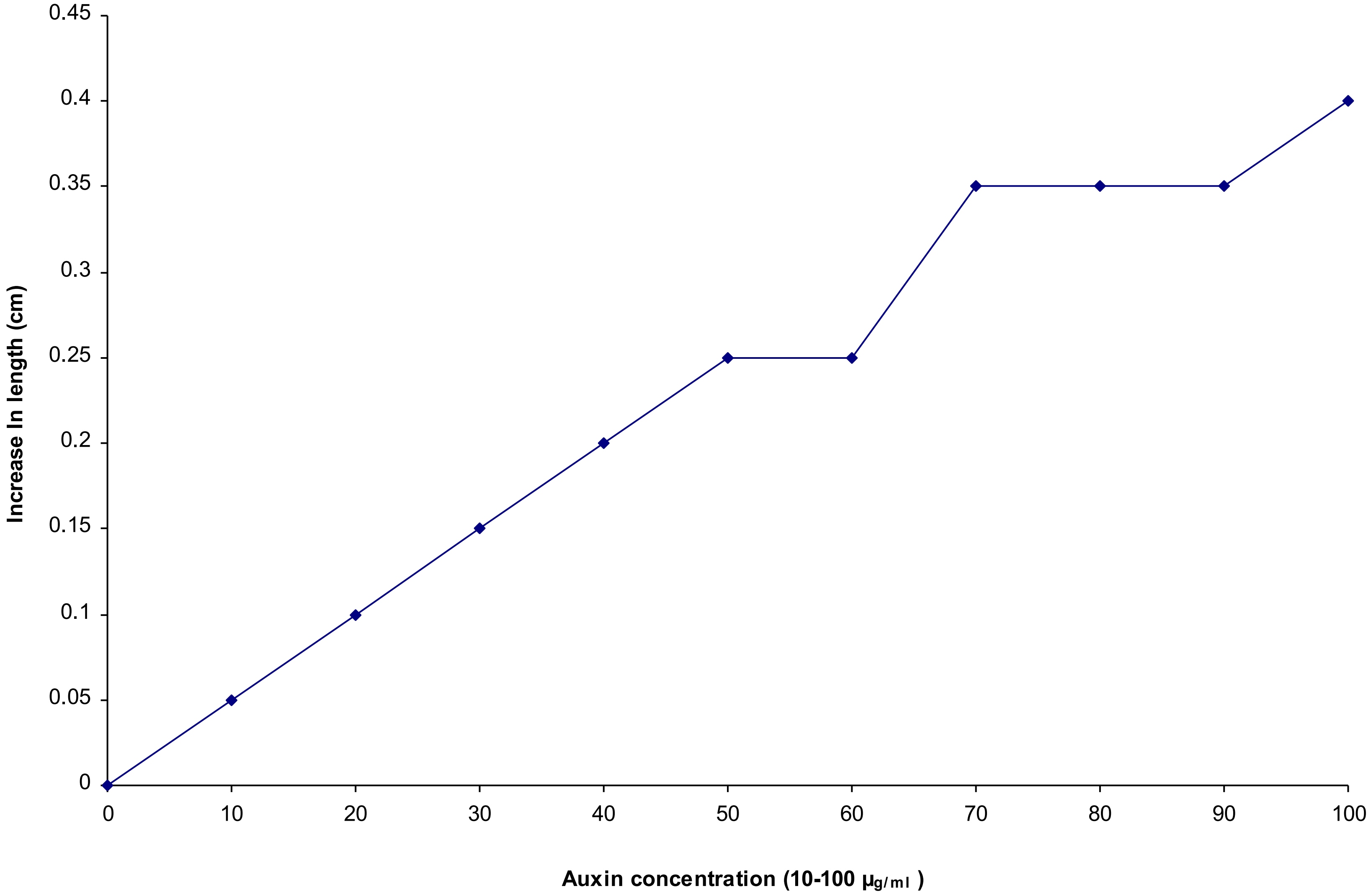


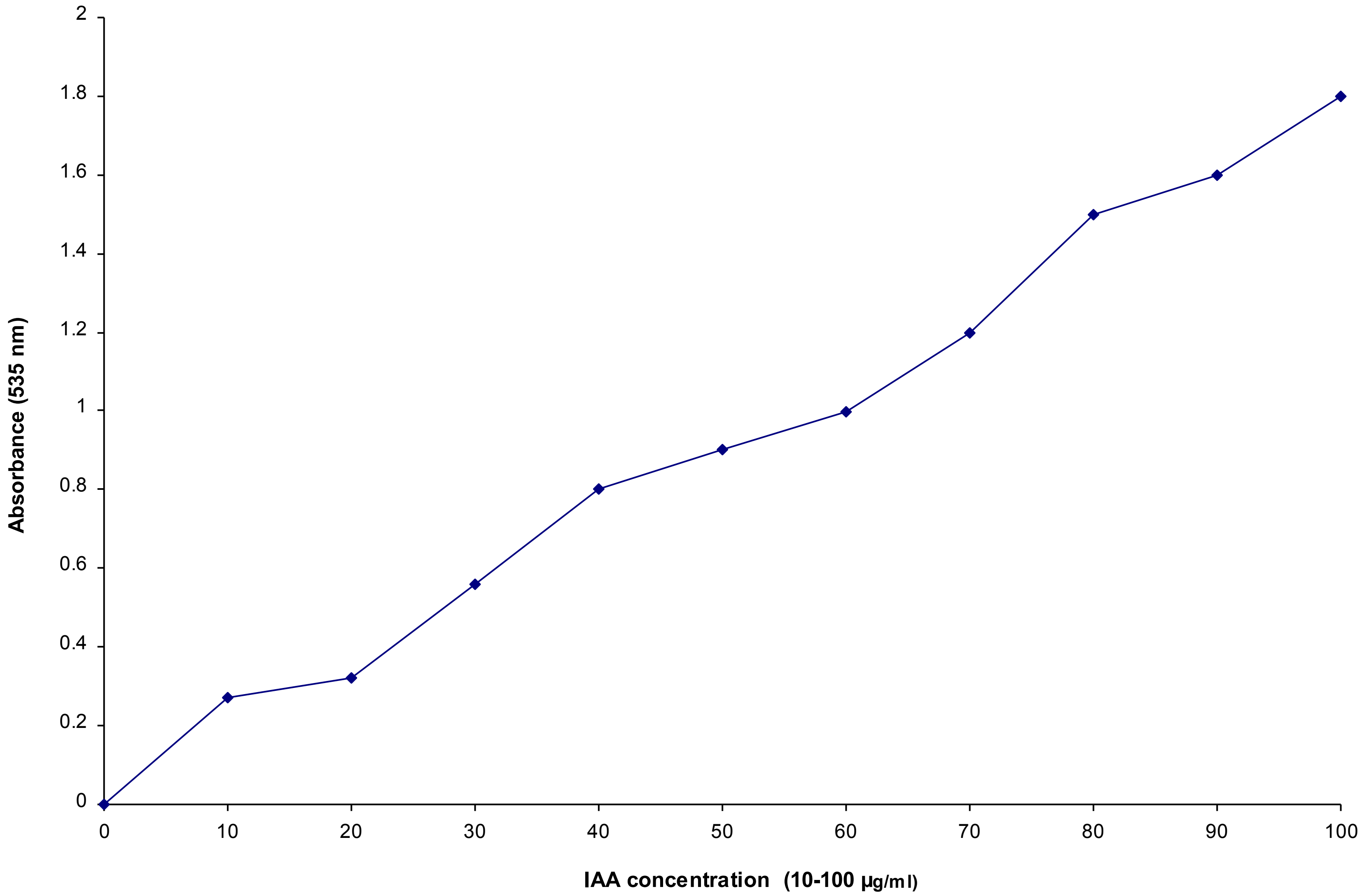
Estimation of bovine serum albumin (10-100 µg/ml) by Folin's phenol method



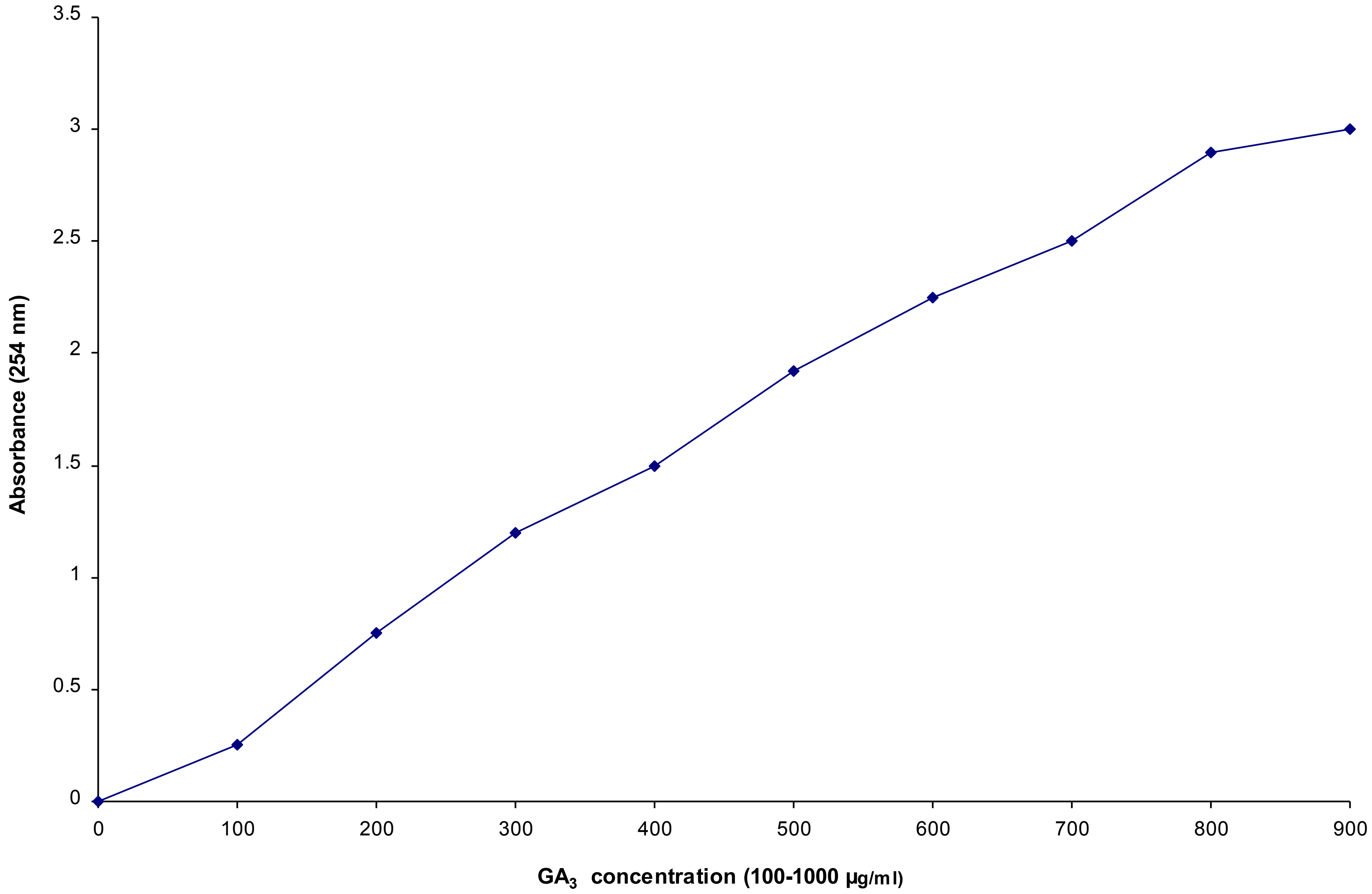
Estimation of available phosphate



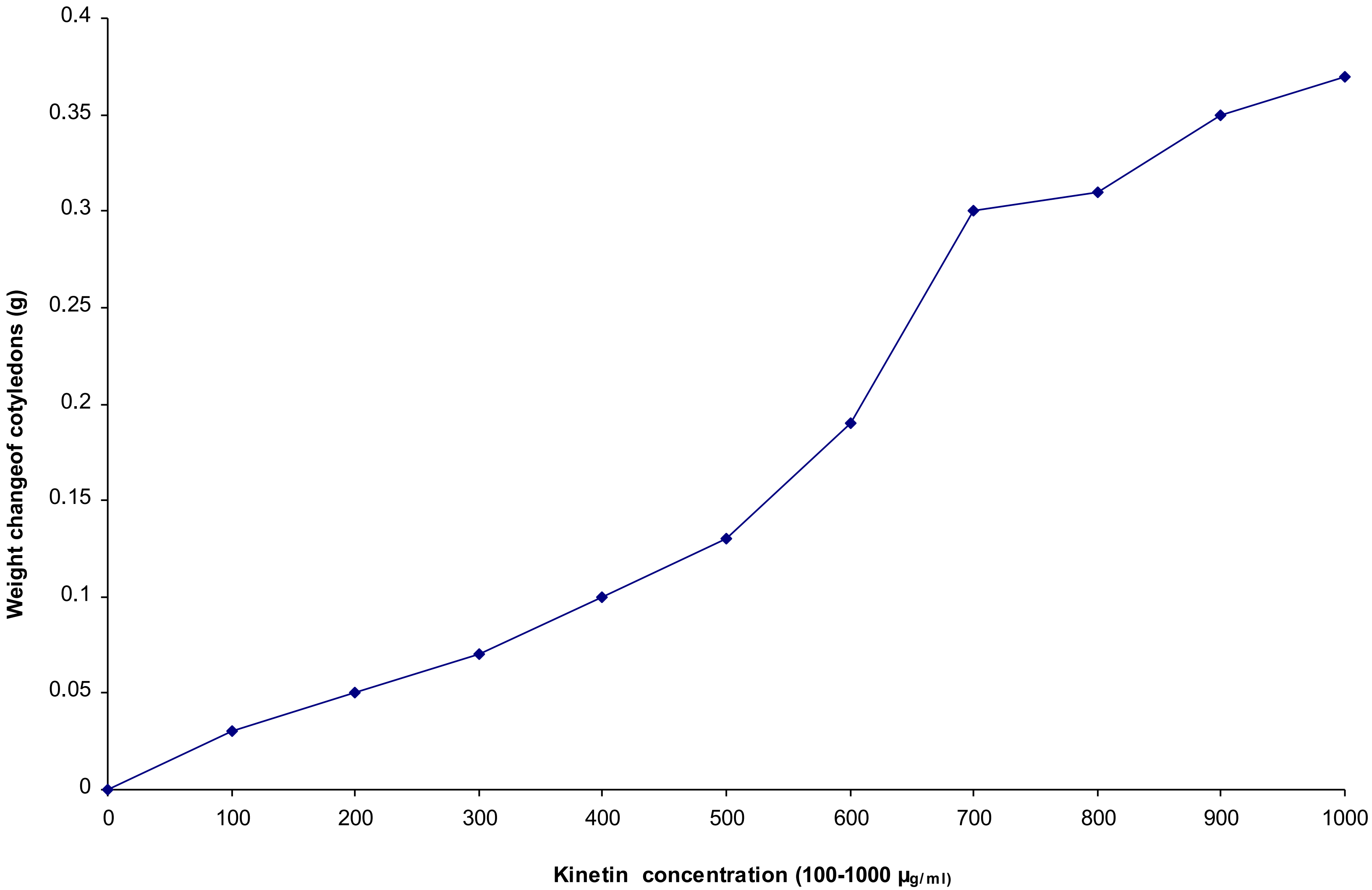
Dose response curve of auxins for Avena coleoptile straight growth bioassays (Noggle and Fritz 1976)



Estimation of indole acetic acid (10-100 µg/ml) by colorimetric method



Estimation of gibberellic acid (GA_3) of gibberellic acid by spectrophotometric method of Holbrook *et al,* 1961



Dose response curve of Kinetin (100-1000 μg/ml) for radish cotyledon bioassay by Letham, 1971.

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