DIVERSITY OF ACTINOMYCETES IN THE RHIZOSPHERE OF *Arnebia euchroma* (Ratanjot): A CRITICALLY ENDANGERED PLANT OF HIMACHAL PRADESH

Thesis

by

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(F-2017-33-M)

submitted to

Dr. YASHWANT SINGH PARMAR UNIVERSITY OF HORTICULTURE AND FORESTRY
SOLAN (NAUNI) HP – 173 230 INDIA

in

partial fulfilment of the requirements for the degree

of

MASTER OF SCIENCE
MICROBIOLOGY
DEPARTMENT OF BASIC SCIENCES
COLLEGE OF FORESTRY
2019

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CERTIFICATE - I

This is to certify that the thesis titled, **"Diversity of actinomycetes in the rhizosphere of *Arnebia euchroma* (Ratanjot): A critically endangered plant of Himachal Pradesh"**, submitted in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE** in the discipline of **MICROBIOLOGY** of Dr. Yashwant Singh Parmar University of Horticulture and Forestry, (Nauni) Solan (HP)-173230 is a bonafide research work carried out by **Ms Pooja Sharma (F-2017-33-M)** daughter of Sh. Keshav Ram Sharma 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 have been fully acknowledged.

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CERTIFICATE - II

This is to certify that the thesis titled, **"Diversity of actinomycetes in the rhizosphere of *Arnebia euchroma* (Ratanjot): A critically endangered plant of Himachal Pradesh"**, submitted by **Ms Pooja Sharma (F-2017-33-M)** daughter of Sh. Keshav Ram Sharma to the Dr. Yashwant Singh Parmar University of Horticulture and Forestry, (Nauni) Solan (HP) – 173230 India in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in discipline of **MICROBIOLOGY** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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ACKNOWLEDGEMENT

Putting all the things aside I would like to thank "God–The Almighty" for providing me the strength with patience to complete this research work.

I would like to express my very great appreciation to ***Dr (Mrs.) Sunita Devi*** *chairman of my advisory committee (Assistant Professor), Department of Basic Sciences for her expert guidance, present help, immaculate suggestions and live encouragement during the entire course of studies which led me on and on to achieve my destination successfully. Her willingness to give us valuable time from her busy schedule so generously has been very much appreciated.*

I emphatically owe my heartiest thanks to ***Dr (Mrs.) Nivedita Sharma, Dr Bhupender Dutt*** *and* ***Dr Anita Kumari*** *worthy members of my advisory committee for their full cooperation and valuable suggestions throughout the course of investigationand manuscript preparation.*

I owe profound debt to ***Miss Aditi Rana*** *(JRF) Department of Basic Sciences for her thoughtful assistance and encouragement during the course of investigation.*

To my Parents, I owe more than I can express. My special graditude is due to my ***parents****,* ***sisters*** *and* ***brother*** *for their loving support. Without their encouragement & understanding it would have been impossible for me to finish this work. Without them, I would be nowhere.*

I am thankful for the cooperation and affection which I received from my guidemate, ***Aishwarya chauhan****. I wish to extend my warmest thanks to my friends* ***Diksha, Dimple*** *and* ***Priyanka*** *for their all-time love and support.*

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

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

Place :
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LIST OF ABBREVIATIONS USED

%	: Per cent
% I	: Per cent increase
°C	: Degree celsius
µg	: Microgram
µL	: Microlitre
bp	: Base pairs
C.D.	: Critical difference
CFU	: Colony forming unit
cm	: Centimeter
Df	: Degree of freedom
Dia	: Diameter
D.W	: Distilled water
et al.,	: and others
g	: Grams
h	: Hour
i.e	: That is
AR	: Analytical grade
U	: Units
IU	: International Unit
M	: Molar
mg	: Milligram
min	: Minutes
mL	: Milliliter
mm	: Millimeter
mM	: Millimolar
MSS	: Mean sum of square
N	: Normal
nm	: Nano meter
O.D.	: Optical density
rpm	: Revolutions per minute
UV	: Ultra violet
V	: Volts
v/v	: Volume by volume
w/v	: Weight by volume

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Chapter-1

INTRODUCTION

India, particularly the Trans-Himalayan region, is a varietal emporium of medicinal plant species and has been a major part of India's rich medicinal plant heritage (Alluri *et al*., 2005; Bhadrecha *et al*., 2017; Devi and Kaundal, 2017). Medicinal plants are considered as the backbone of the traditional medicines being used worldwide. More than 50 per cent medicinal plants traditionally used in India come from the Himalayan region (Uniyal *et al.*, 2002; Verma and Tiwari, 2016). Owing to variations in altitude and topography, Himachal Pradesh, located in the lap of the Himalayas, has varied climatic conditions,which make this state ideal for the thriving of a wide diversity of medicinal plant species (Sharma and Mishra, 2009; Singh and Thakur, 2014). This biodiversity of medicinal plants and its sustainable utilization by the local people for various activities (traditional healthcare, cultural and religious) help in sustaining their health, medicinal, spiritual and other needs (Gautam *et al.*, 2011).

Earlier, only the local people used these plant resources for their own consumption, but in the recent decades, the demand for herbal products and plant based drugs in national and international markets has increased rapidly, led to their heavy exploitation. One of the prime reasons for their over exploitation is the dependence of such a huge population of human beings on these valuable and limited resources. Secondly, their unscientific harvesting during which the regenerative parts of a plant like roots, tubers, fruits, seeds, flowers and bark are indiscriminately collected, led to degradation, depletion and even extinction of particular species. Due to this ruthless exploitation, many important medicinal plant species are becoming rare and some of them have become critically endangered (Pandey *et al.*, 2005). *Arnebia euchroma* represents a classical example of a critically endangered plant that is found in the trans- Himalayan region of Himachal Pradesh, though mainly harvested for its roots; almost all parts of the plant are being used in medicines, food, dyes and beverages.

Arnebia euchroma belongs to family *Boraginaceae*, commonly known as "Ratanjot". It is a well- known traditional perennial, erect, caespitose and hairy herb with many stems arising from the axils of its basal leaves, distributed in dry regions of Asia and Northern

Africa (Liu *et al.*, 2012). The plant is able to grow in well-drained sandy and nutritionally poor soil but cannot grow in shade. It has sweet and bitter tastes and exhibits cold property while acting on the liver and heart channels. It regulates blood circulation and counteract on toxicity that is associated with measles, inflammation and sores. Traditionally, it is used in Indian and Chinese traditional medicinal systems in treatment of jaundice, burns, eczema, mild constipation, frostbite, dermatitis and various skin disorders (Pirbalouti *et al*., 2009).It is a major ingredient of ointment named Tzu-Yun-Kao used for the treatment of burns and wounds in Asia.

The roots contain a copious purple dye - a good source of red naphthoquinone pigment has anti-microbial, anti-inflammatory, anti-tumour and contraceptive properties. Natural shikonin compounds extracted from the roots of *A.euchroma* and their derivatives have cytotoxicity and antitumour effects (Damianakos*et al*., 2012). Beside these medicinal properties, these metabolites have commercial importance as natural colorants in food, cosmetics, textiles and exhibit various pharmaceutical properties (Kumar *et al.*, 2011). It is a usual practice in the Lahul and Spiti valley to sell and use its roots as edible dye and medicine (Aswal and Mehrotra, 1994). Local doctors (known as *Amchis* in western Himalayas) use its root extract in different indigenous medicines, prescribed for cough and blood purification.

Its reproductive cycle ranges from June to August. Out of total five species of genus *Arnebia*, only three are reported to be found in the Lahaul and Spiti region - the cold desert of trans-Himalayas (Aswal and Mehrotra, 1994). Out of these three species, *Arnebia euchroma* (syn. *Macrotomia perennis* Boiss) is the one that is used commercially on a large scale. Known as Dimok or Khamed in native language, the species is famous by its trade name Ratanjot (Singh *et al.*, 2012). During 1998, *euchroma* species was placed under the category of endangered plants (Vedand and Tandon, 1998). However, considering its medicinal importance, plants of *A. euchroma* have been overexploited and have, now been included in the list of critically endangered plants of Himachal Pradesh according to the latest International Union for Conservation of Nature (IUCN) categorization (Kala, 2004; Manjkhola *et al*., 2005; Singh *et al*., 2012).

All plant-associated microenvironments, especially the nutrient rich rhizosphere, is a major habitat for a plethora of microorganisms (bacteria, fungi and actinomycetes) that have imperative impact on plant health, nutrition and quality (Berg and Smalla, 2009; Mendes *et*

al., 2011). Actinomycetes constitute a significant component of rhizospheric microbial communities associated with different types of plants, particularly medicinal plants.

They are Gram-positive, filamentous bacteria that exhibit multifarious plant growth promoting traits and stimulate plant growth by various direct and indirect mechanisms. Actinomycetes directly stimulate growth by solublization of insoluble nutrients like phosphates, production of growth hormones, nitrogen fixation and indirectly by antagonizing pathogenic organisms or by the production of siderophores, lytic enzymes, antibiotics, florescent pigments and cyanides etc (Bhattacharya and Jha, 2012; Kamal and Sharma, 2014)

Rhizospheric soil of medicinal plants represents one of the best sources for the isolation of actinomycetes. It is considered as an enrichment environment because of addition of root exudates and litters of plants in it that serve as a nutritive source for the growth and biological activity of microorganisms including actinomycetes. Actinomycetes associated with the rhizosphere of medicinal plants play an important role in agriculture as biocontrol agents (Kennedy *et al*., 2004) besides producing enormous numbers of agroactive metabolites (Tanaka and Omura, 1993).

The study of rhizospheric actinomycetes from medicinal plants is very crucial, as they are well known to have impact on plant growth and productivity, and also produce industrially important metabolites (Bafana and Lohiya, 2013). *Arnebia euchroma,* the herb under study grows in the trans-Himalayan region of Himachal Pradesh, which is a unique habitat for its growth, the microbial diversity present in its rhizosphere, may help in the adaptation of plant under prevailing conditions. As such very little information is available on the diversity of actinomycetes associated with the rhizosphere of this plant. Therefore, the present study was proposed with the following objectives.

OBJECTIVES:

i) To assess the diversity of actinomycetes associated with the rhizosphere of *Arnebia euchroma* (Ratanjot).

ii) To screen the actinomycetes for multifarious plant growth promoting attributes.

iii) To identify and characterize the potential isolates.

Chapter-2

REVIEW OF LITERATURE

In spite of tremendous advances in modern science, there is an awakening all over the world regarding the use of traditional systems of medicines for the treatment of various diseases. It is estimated that 80 per cent of the population of the developing countries still relies on traditional plants based medicines for their primary health care needs (Bodeker and Kronenberg, 2002; Mishra *et al*., 2012).

Indian Himalayan region is a varietal emporium of medicinal plants which serve as sources of natural products for human utilization. Some of them are *Aconitum heterophyllum, Angelica glauca, Arctium lappa, Bergenia ligulata, B. stracheyi, Dioscorea deltoidea, Hedychium spicatum, Heracleum candicans, Picrorhiza kurrooa, Podophyllum hexandrum, Polygonatum cirrhifolium, P. verticillatum, Rheum australe, Saussurea costus, Selinum tenuifolium, Thalictrum foliolosum, Arnebia euchroma* and *Valeriana jatamansi* (Butola and Badola, 2008). Owing to indiscriminate use, increased demand in herbal based industries and unscenitific harvesting, these plant species are continuously depleating from their natural habitats while some are at the verge of extinction. Amongst these, *Arnebia euchroma* provides a classical example of commercially valuable and critically endangered plant growing in the Trans-Himalayan region of Himachal Pradesh.

The literature relevant to the present study is reviewed under the following sub headings.

2.1 *Arnebia euchroma*: Distribution and medicinal importance
2.2 Rhizosphere: A dynamic entity
2.3 Actinomycetes: General characteristics and their interactions with plant rhizosphere
2.4 Isolation and diversity of actinomycetes
2.5 Plant growth promoting attributes of actinomycetes
    2.5.1 Phosphate solubilization
    2.5.2 Phytase activity
    2.5.3 Siderophore production
    2.5.4 Nitrogen fixation

2.5.5 HCN and ammonia production
2.5.6 Lytic enzymes production
2.5.7 Production of plant growth regulators
2.6 Antagonistic activity of actinomycetes against soil-borne fungal phytopathogens.
2.7 Identification and characterization of actinomycetes
2.8 Effect of plant growth promoting actinomycetes on seed germination and seedling growth.
2.9 Pigments present in the roots of *Arnebia euchroma*

2.1 *Arnebia euchroma*: DISTRIBUTION AND MEDICINAL IMPORTANCE

Arnebia euchroma (Ratanjot) occurs principally in the alpine and sub alpine zones of Kashmir, Kumaon, Garhwal and Nepal on open slopes and shrubberies in the altitudinal range of 3000-4300 m. Some of the common localities are Har-ki-Doon, Himtoli, Kedarnath, Mana, Valley of Flowers, Gorson, Dronagiri, Malari in Uttarakhand;*Great Himalayan National Park* (GHNP), Pin Valley, Chamba, Lahaul and Spiti, Rohtang, Kinnaur Valley in Himachal Pradesh and Kurram Valley, Deosai, Makra, Kagan Valley, Bedori, Aliabad, Pir Panjal in Kashmir. In the sub alpine zone, Ratanjot occurs mostly in shaded areas amidst Betula, Rhododendron, oak and pine trees while, in the alpine zone, the species occurs in open sunny locations. It is likewise distributed in North Africa, Turkey and in the northern provinces of Iran especially in the mountain territories (Kosger *et al*., 2009).

Arnebia euchroma exhibits numerous medicinal properties. It is used in the treatment of measles, mild constipation, burns, frostbite, eczema, dermatitis, wound healing etc. For instance, the roots of *Arnebia euchroma*- commonly known as 'Havachoobeh' in folklore and Iranian traditional Medicine (ITM) has been used traditionally for centuries by the Iranian people for treatment of the burn wounds as "Marhame Havachoobeh" (Aliasl *et al*., 2014; Nasiri *et al*., 2016). *Arnebia euchroma* has some analgesic properties also (Soltanian *et al*., 2010).Its roots contain important chemical components such as *Naphthoquinone*, shikonin, alkanin and isohexenyl naphthazarin esters derivatives that have many pharmacological properties. These components have biological properties such as wound healing and antibacterial effects (Hosseini *et al.*, 2007; Pirbalouti *et al*., 2009). This herb was evaluated to test its efficacy as anti-inflammatory (Kaith *et al*., 1996; Singh *et al*., 2003), antimicrobial (Shen *et al*., 2002; Hosseini *et al*., 2007) antiamoebic, antitumor, and anticancer effects (Mehrabani *et al*., 2015) as depicted in Fig. 2.1.

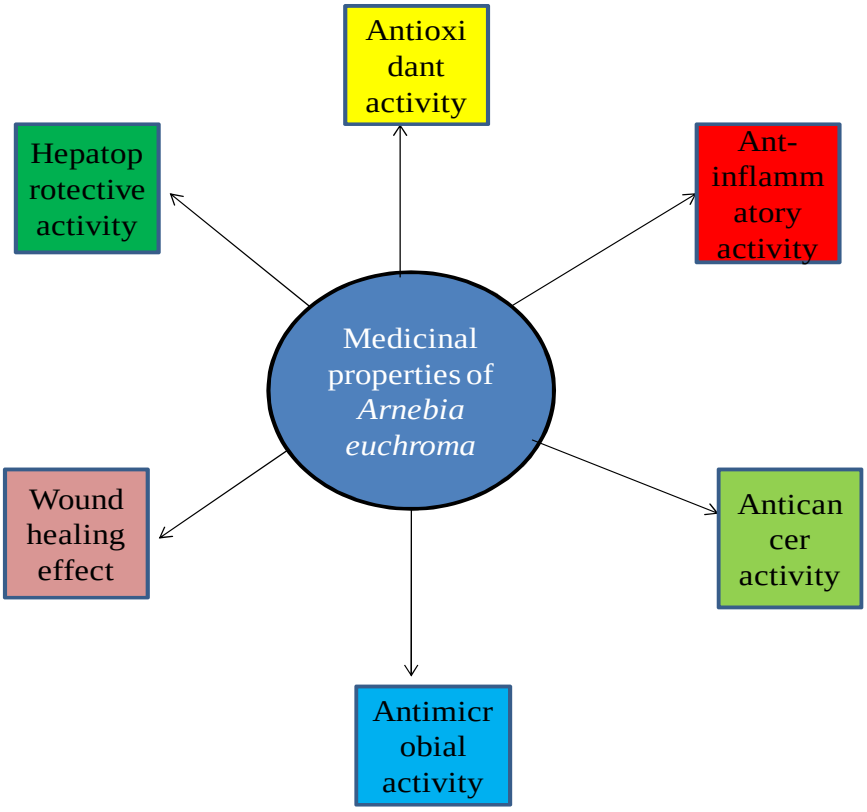

Fig. 2.1 Medicinal properties of *Arnebia euchroma* (Ratanjot)

Pirbalouti *et al*. (2009) examined the effectiveness of diethyl ether extracts of *Arnebia euchroma* roots and *Malva sylvestris* flowers at 200 mg/kg/day dose on reduction of burn wound area. Burns were induced in Wistar rats divided into six groups; Group-I was treated with cold cream, Groups-II and III with cold cream containing mixture of two extracts while, Group-IV was treated with goat lipid containing extract of *Arnebia euchroma* and *Malva sylvestri*. Group-V was treated with goat lipid whereas, Group-VI received the standard drug (silver sulphadiazine cream). The extracts treated animals showed significant reduction ($P < 0.05$) in the wound area when compared with other groups. Also, histological studies of the tissue obtained on days 6, 9 and 16 from the extract treated by goat lipid containing *Arnebia euchroma* and *Malva sylvestris* showed increased well organized bands of collagen, more fibroblasts, and few inflammatory cells.

Ashkani *et al.* (2012) investigated the wound healing effect of *Arnebia euchroma* (AE) on histomorphometrical parameters involved in the healing process of third-degree burn wounds. They divided 48 female Sprague-Dawley rats, each with a standard third-degree burn wound on the posterior surface of the neck, into four groups; AE10 and AE20 groups were treated with carboxymethylcellulose (CMC) gels which contained AE hydroalcoholic

extract at the concentration of 10and 20 per cent, respectively;the untreated burn (UB) group, received no treatment while, the last was gel-base treated group.They observed that wound closure rate, fibroblast population, volume density of collagen bundles, and length density of vessels were significantly improved in AE10 and AE20 groups in comparison with the gel-base and untreated burn (UB) groups (P value <0.05).

Ko Feng *et al*. (1995) observed that acetylshikonin, teracrylshikonin, acetylshikonin, teracrylshikonin, β,β-dimethylacrylshikonin and shikonin isolated from *Arnebia euchroma*, inhibited collagen (10 μg/mL)-induced aggregation of washed rabbit platelets in a concentration-dependent manner with IC_{50} values of 2.1 ± 0.2, 2.8 ± 0.3, 4.2 ± 0.5 and 10.7 ± 0.7 μM, respectively. Acetylshikonin also inhibited the aggregation and ATP release of washed rabbit platelets induced by arachidonic acid (AA, 100 μM), U46619 (1 μM), platelet-activating factor (PAF, 3.6 nM) and thrombin (0.1 U/mL) in a concentration-dependent manner. The IC_{50} values of acetylshikonin on the inhibition of these four agonists-induced platelet aggregation were 3.1 ± 0.4, 2.2 ± 0.2, 8.0 ± 0.6 and 12.7 ± 1.0 μM, respectively. The thromboxane B_2 formation caused by collagen, PAF and thrombin was inhibited by acetylshikonin, while formations of thromboxane B_2 and prostaglandin D_2caused by AA were not inhibited. Acetylshikonin did not inhibit cyclooxygenase activity since it did not attenuate prostaglandin E_2 formation after incubation of sheep vesicular gland microsomes with AA. Acetylshikonin suppressed both the rise of intracellular Ca^{2+} concentration and the generation of inositol monophosphate caused by these five aggregation inducers. Platelet cyclic AMP level was unaffected by acetylshikonin.

To investigate the healing effect of *Arnebia euchroma* ointment versus silver sulfadiazine on burn wounds in rats Nasiri *et al.* (2016) divided fifty rats into 4 equal groups receiving ointment base, normal saline (NS), standard 1 per cent silver sulfadiazine (SSD), and 5 and 10 per cent *Arnebia euchroma* ointments (AEO). Results concluded that average area of wound on the 10th day was 10.2±2.3, 8.4±2.6, 12.4±2.5, 5.9±2.2 and 5.7±2 cm^2 for ointment base, NS, 1 per cent SSD, and 5 per cent and 10 per cent AEO, respectively. Wound size was significantly lower in 10 per cent AEO than 1 per cent SSD and control groups on the 10th day post-burn injury. On day 11, the percentage of wound contraction in 5 and 10 per cent AEO was 53±14.7 per cent and 55.9±10.5 per cent which was more than 1 per cent SSD (15.3±10.8%).The collagen fibers were well formed and horizontally-oriented in 5 and 10 per cent AEO groups when compared with other groups.

2.2 RHIZOSPHERE: A DYNAMIC ENTITY

The rhizosphere is a core of biological and physical activities surrounding the living infrastructure of plant roots. It is generally considered to be a narrow zone of soil subject to the influence of plant roots where root exudates stimulate or inhibit microbial populations and their activities (Brimecombe *et al*., 2007). It is estimated that plants release between 20-50 per cent of their photosynthates through their roots (Buchenauer, 1998; Bottner *et al*., 1999). Plants select those bacteria which contribute most to their fitness by releasing organic compounds through exudates (Lynch, 1990) and creating an environment where diversity is high (Marilley and Aragno, 1999; Lucas *et al*., 2001). The bulk soil is generally a very poor, nutrient-diluted and therefore, hostile environment in which nutrient bioavailability is often hampered by the soil biochemistry. Within this nutritional desert, presence of plant roots serve as a mean to form true oasis with flourished microbial populations because all roots are able to actively secrete low and high molecular-weight molecules *viz.*, glucose, amino acids, polysaccharides, proteins, root debris, root border cells and root cap cells separated from the root apex during root growth in to the rhizosphere (Hawes *et al*., 2003).

Plants invest a lot of energy in root exudation which depends on light intensity, temperature, type and nutritional state of plants, stress factors, microbial activity in the rhizosphere and type of soil like soil texture and thus mechanical impedence. Higher microbial biomass and activity in the rhizosphere than in the bulk soil is attributed to the release of organic substances from roots in the rhizosphere soil. Not all compounds released from roots are organic because roots can also release proton, oxygen and water. Root products can be classified based on their perceived function in excretions (CO_2, bicarbonate ions, H^+, electrons, ethylene, etc.) and secretions (mucilage, H^+, electrons, enzymes, siderophores, etc.)The former one is thought to facilitate internal metabolism while, the latter facilitates external processes such as nutrient uptake (Uren, 2002). Low-molecular-weight exudates can diffuse to a longer distance as compared to high-molecular weight compounds but, they are more readily assimilated by soil microorganisms. Owing to the deposition of readily bioavailable low-molecular weight carbon compounds by plant roots, rhizosphere microbial load and activity are usually higher than the bulk soil (Anderson and Coats, 1995) that have major implications on soil functions. Rhizosphere is shown to have a well known influence on carbon, nitrogen, phosphorus cycling (Soomro, 2014), release of greenhouse gases (Korner, 2006) and pollutant breakdown in soils (Ahmad *et al*., 2012).

Most of actinomycetes are distributed in the rhizospheric soils of plants. The term rhizosphere, was coined by Hiltner (1904) and is defined as the zone of soil that surrounds plant roots influenced by root metabolism. This zone extends up to more than 5 mm from the root. Different kinds of microorganisms mainly bacteria, fungi and actinomycetes are found in rhizospheres and their density is higher in this zone than in bulk soils (Lynch, 1990). This difference could be ascribed to the secretion of root exudates that serve as sources of nutrients and energy for microbial growth (Soderberg *et al*., 2002). The composition and quantity of root exudates varies with respect to plant species and abiotic factors like temperature and soil moisture (Martin and Kemp, 1980).

Soil under medicinal plants provides an excellent example of an enrichment environment for the growth and activity of microorganisms due to the addition of root secretions and litters of plants in the rhizospheric soils. Actinomycetes are well known to play an important role in rhizosphere soil as they have the ability to degrade plant, animal and microbial polymers present in soil and litters (Suzuki *et al*., 2000; El-Tarabilya and Sivasithamparam, 2006; Norovsuren *et al*., 2007; El-Tarabily, 2008).

Pandey and Palni (2007) studied the rhizosphere effect exerted on the microbial communities by ten representative and important tree species *viz., Abies pindrow, Cedrus deodara, Aesculus indica, Alnus nepalensis, Betula utilis,Pinus rouxburghii, P. wallichiana, Quercus semecarpifolia, Rhododendron campanulatum and Taxus baccata L*. of the Indian Himalayan region. The rhizosphere to soil (R:S) ratio was found to range from 0.2 to 3.5, 0.3 to 2.9, and 0.3 to 3.4, for bacteria, actinomycetes and fungi, respectively.

2.3 ACTINOMYCETES: GENERAL CHARACTERISTICS AND THEIR INTERACTIONS WITH PLANT RHIZOSPHERE

Actinomycetes are a group of gram positive, filamentous organisms belonging to subclass *Actinobacteridae* and order *Actinomycetales*. All members of this order are reported to exhibit high G+C content (>55 mol %) in their DNA (Stackbrandt *et al*., 1997). Actinomycetes produce two kinds of branching mycelium, aerial and substrate mycelia. The aerial mycelium is important as it is that part of the organism that produces spores. For this reason they have been considered as fungi, as their name implies *akitino* means ray and *mykes* means mushroom/fungus. Hence, are named as ray fungi (Takizawa *et al*., 1993).

Actinomycetes are omnipresent in nature and exihibit unique metabolic diversity along with diverse enzymatic capabilities. They produce secondary metabolites which are valuable from agricultural, industrial and pharmaceutical point of view and the enzymes themselves are likewise significant. A variety of actinomycetes inhabit a diverse range of plants either as symbionts or endophytes like *Streptomyces scabies*, *S. galbus* and *S.hygroscopicus* (Hasegawa *et al*., 2006). An enormous number of actinomycetes isolated from soil and rhizosphere have been used as biocontrol agents against fungal root and seed pathogens *viz.*, *Alternaria brassicicola, Collectotrichum gloeosporioides*, *Fusarium oxysporum, Penicillium digitatum* and *Sclerotium rolfsii* (Bressan *et al*., 2003; Gadelhak *et al*., 2005; Goudjal *et al*., 2014). One of the prime function of actinomycetes is the production of commercially important clinical antibiotics like Abyssomicins, Bonactin, Chloro-dihydroquinones, Diazepinomicin, Frigocyclinone, Essramycin andTirandamycin (Manivasagan *et al*., 2014).

Actinomycetes are also well known saprophytic soil inhabitants (Takizawa *et al*., 1993) and produce a volatile compound called geosmin, which literally means “earth smell” (Gust *et al*., 2003). The strong odour that occurs in the air when rain falls after a dry spell of weather is due to the production of this organic compound. *Streptomyces* are common and usually a major component of the total actinomycetes population in natural habitats. Few actinomycete genera like *Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium, Kineospora,Microbispora, Micromonospora, Nonomuraea* which are often very difficult to isolate and grow because their growth rate is slow, hence, are called rare actinomycetes (Hayakawa, 2008).Other habitats for majority of actinomycetes include rice paddy, agricultural fields, tropical forests and natural caves (Gomes *et al*., 2000; Lee and Hwang, 2002; Shirokikh, 2006; Ilic *et al*., 2007).

Some special media are required by actinomycetes in order to differentiate and develop of characteristic spores and pigments. The chemical composition of media and agar quality has good impact on the expression of taxonomic characteristics by *Streptomycetes.* Highly purified agar, devoid of growth promoters like salts of magnesium, iron, manganese, and zinc, can slow down proper growth and differentiation (Okami and Hotta, 1988). Some medium ingredients are reported to enhance the melanin pigment production (Lakshmipathy *et al*., 2010). Actinomycetes produce a variety of pigments like yellow, orange, red, purple, blue, olive green etc. Some investigators have studied actinomycetes for commercial

production of pigments and *Streptomyces coelicolor* that produces blue pigment is a well-studied member of this group (Sanchez *et al*., 1954).

Hayakawa and Nonomura (1987) developed an isolation medium in order to reduce the growth of competing soil microoganisms without any adverse effect on the growth of actinomycetes. They formulated a Humic acid-vitamin (HV) agar, mediumwith soil humic acid as the sole source of carbon and nitrogen for recovery and optimum growth of *Streptomyces* along with various rare actinomycetes, while restricting growth of non-filamentous bacterial colonies. HV agar 10 was supplemented with synthetic antibacterial agents such as nalidixic acid and cycloheximide in order to eliminate bacterial and fungal contaminants (Hayakawa *et al*., 1996). Moreover, the addition of some antibiotics like Novobiocin, Tunicamycin, Rifamycin, Chlortetracycline and Macrolide or aminoglycoside in HV medium enabled to recover some rare genera of actinomycetes like *Actinoplanes*,*Kitasatospora*, *Micromonospora*, *Actinomadura* and *Nocardia.* Minimal Agar medium (MM) containing glucose, yeast extract and mineral salts was formulated likewise to recover actinomycetes genera like *Actinomadura*, *Amycolatopsis* from desert soils (Hozzein *et al*., 2008).

Tokala *et al.* (2002) studied the novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant. The root-colonizing soil actinomycete *S. lydicus* WYEC108 influenced pea root nodulation by increasing root nodulation frequency. *S. lydicus* also colonized and then sporulated within the surface cell layers of the nodules. Colonization led to an increase in the average size of the nodulesand improved the vigor of bacteroids within the nodules by enhancing nodular assimilation of iron and possibly other soil nutrients. Bacteroid accumulation of the carbon storage polymer, poly-β-hydroxybutyrate, is reduced in colonized nodules.

Abdel-Fattah and Mohamedian (2000) studied the effect of interaction between vesicular-arbuscular (VA) *mycorrhiza* and *Streptomyces coelicolor* on the growth response, nutrition and metabolic activities of *Sorghum* (Sorghum bicolor) plants grown in non-sterilized soil amended with chitin waste in a greenhouse over 8 weeks. Chitin amendment resulted in an increase in the microbial population and chitinase activity in soils. VA mycorrhizal inoculation significantly increased the growth, photosynthetic pigments, total soluble protein and nutrient contents of Sorghum as compared to non-mycorrhizal Sorghum. Inoculation with *S. coelicolor* significantly increased the intensity of mycorrhizal root

colonization and arbuscular formation, but the levels of mycorrhizal infection and their beneficial effects were significantly reduced with the addition of chitin waste to the soil. Analysis of total amino acids and ammonia contents in leaves based on dry matter production showed that, in most instances, total amino acids of mycorrhizal plants were significantly higher than those of non-inoculated plants. The microflora of the rhizosphere was highly affected by mycorrhizal inoculation.

2.4 ISOLATION AND DIVERSITY OF ACTINOMYCETES

Soil is a complex environment providing nutritional, physical and biological variability. It serves as a vital source of nutrients for the survival of microbes. Actinomycetes play a crucial role in this environment because of their wide range of metabolic processes and biotransformation which includes degradation of insoluble remains of other organisms, like lignocellulose and chitin, hence actinomycetes as central organisms in carbon recycling. Although actinomycetes have huge adaptability to survive in different environments but their number varies with season. In some parts of the world, they are more prevalent in dry season as compared to rainy season for instance in lakes of the Middle Plateau, Yunnan, China (Xu and Jiang, 1996)

Wordwide investigators have isolated actinomycetes for various applications and they have found immense potential and diversity in all the continents. These filamentous bacteria have been able to colonize geographically distinct locations like plain lands, agricultural soils, compost soil, river waters, estuaries, oceans, mountains, snow covered Arctic regions etc. (Lyutskanova *et al.*, 2009; Dhanasekaran *et al*., 2009).

It is pertinent to select methodologies to isolate the uncommon and rare actinomycetes from their natural habitats so that reisolation of strains that produce known bioactive metabolites can be avoided besides improving the quality of natural products screened (Takahashi and Omura, 2003; Berdy, 2005).Different media, methods and techniques that enhance the growth of desirable actinomycetes in their natural habitats (enrichment) or eliminate undesirable actinomycetes including *Streptomyces* and other contaminants from the isolation plate media (pretreatment) for isolating novel actinomycetes from natural habitats, especially from various types of soil, were improved and developed.

Numerous actinomycetes were isolated from rhizosphere of medicinal plants *viz*., *Leucaena leucocephala,Madhuca induca, Eclypta alba, Gliricidia maculate, Lawsonia*

inermis, *Azadirachta indica*, *Pongamia globra* and *Chromolana oderata* grown in Kolli hills of Salem district, Tamilnadu, India and were evaluated for the detection of antimicrobial substances. These antimicrobial compounds were and tested against seven microbial pathogens *viz.*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus amyloliquefaciens*, *Staphylococcus aureus*, C*andida albicans* and *Cryptococcus neoformans*. The results showed that out of seven, five isolates were found to be potential antagonists against tested pathogens through the production of secondary metabolites, which are characterized as potent chemicals to control diverse kinds of pathogens (Thangapandian *et al*., 2007).

Gangwar *et al.* (2014) investigated the diversity and biopotential of endophytic actinomycetes isolated from three medicinal plants, *viz.*, *Aloe vera*, *Mentha arvensis* and *Ocimum sanctum* from herbal garden, Punjab Agricultural University, Ludhiana and they observed that maximum isolates belongs to genus *Streptomyces* (60%) followed by *Micromonospora* (25%), *Actinopolyspora* (7.5%), and *Saccharopolyspora* (7.5%). The highest number of endophytic actinomycetes was observed to be associated with *Ocimum sanctum* (45%). Out of twenty two isolates tested, twelve showed the ability to solubilize phosphate ranged from5.4-16.5 mg/100 mL, while sixteen isolates produced indole-3 acetic acid (IAA) ranged between 8.3-38.8 µg/mL. Nine isolates produced hydroxamate- type of siderophore whose amount varied from 5.9-64.9 µg/mL while, only four isolates were able to produce catechol-type of siderophore in the range of 11.2-23.1 µg/mL.

A total of sixty six actinomycetes were isolated from rhizosphere of mangroves in Andhra Pradesh, India. The population count of actinomycetes fluctuated from $19 \times 10^4$to 80 $\times 10^4$ CFU/g soil. Out of sixty six, only eight isolates exhibiting plant growth promoting potential, were further characterized by physiological and biochemical characteristics and identified as different species of *Streptomycetes* genera using 16S rRNA gene sequencing (Shrivastava *et al*., 2015).

Rani *et al*. (2016) studied the diversity of root associated microorganisms of medicinal plant *Alpinia galanga* of Tamil Nadu. A total of twenty six isolates were obtained, out of which, nineteen bacteria, five fungi and two were actinomycetes. *Aspergillus* sp. showed higher distribution (30%), followed by *Rhizopus* sp., *Penicillium* sp., *Trichoderma* sp. (20%) and *Fusarium* sp. (10%). Actinomycetes were identified as *Streptomyces* sp. and *Frankia* using 16S rRNA gene sequencing technique.

Devi and Kaundal (2017) assessed the microbial diversity associated with the rhizospheric soil samples of *Arnebia euchroma*, collected from two villages namely Nako and Chango in Kinnaur and one i.e. Gue in Lahaul & Spiti district of Himachal Pradesh. They observed that bacterial and actinomycetes loads were significantly higher at Gue followed by Nako and Chango. However, evenness (E1) values were comparatively low at Gue than Chango and Nako, which implies that Gue has a greater diversity of microbial species, which however was not evenly distributed.

Mohan *et al.* (2018) isolated twenty four (five actinomycetes, ten bacteria and nine fungi) plant growth promoting microorganisms from the rhizosphere of *Gmelina arborea* in Tamil Nadu, India and observed that occurrence and distribution of bacterial population (25 x 10^6 CFU/g soil) in rhizosphere of *Gmelina arborea* was maximum, followed by actinomycetes (20 x 10^4 CFU/g soil) and fungi (12 x 10^3 CFU/g soil). Likewise, actinomycetes isolate A3 showed maximum IAA production, followed by the fungal isolate F9 and bacterial isolate B3. These potential isolates were identified as (A3)-*Streptomycetes* sp. (F9)–*Pencillum* sp. and (B3)-*Rhizobium* sp. based on morphological and biochemical characteristics.

Gong *et al.* (2018) assessed the actinobacterial diversity from eight coastal salt marsh rhizosphere soils collected from Jiangsu Province, China, using culture-based and 16S rRNA gene high throughput sequencing (HTS) methods. Actinobacterial sequences represented 2.8–43.0 per cent of rhizosphere bacterial communities, as determined by HTS technique. The actinobacteria community comprised 34 families and 79 genera. Additionally, 196 actinobacterial isolates were obtained, of which 92 representative isolates were selected for further 16S rRNA gene sequencing and phylogenetic analysis. The 92 strains comprised seven suborders, 12 families, and 20 genera that included several potential novel species.

2.5 PLANT GROWTH PROMOTING ATTRIBUTES OF ACTINOMYCETES

2.5.1 Phosphate solubilization

Phosphorous (P) the second most important and growth limiting macronutrients for plants. It is an important constituent of nucleic acids, phospholipids, phytins, co-enzymes, phosphorylated sugars and nucleotides, and is involved in many essential processes like transfer of energy, cell division, sugar breakdown, photosynthesis, flower and seed formation, stalk and stem strength, root development, nodule formation in legumes, crop maturity,

resistance in plant diseases, and nutrient uptake (Miriko *et al*. 2018). In the soil, it is typically found in insoluble forms, unavailable to plants. Some microorganisms are capable of solubilizing inorganic phosphate and making it available to plants (Ahemad and Kibret, 2014). High proportion of phosphate solubilizing microorganisms (PSM) exists in the rhizosphere, and they are metabolically more active than from other sources (Vazquez *et al*., 2000). Some bacterial species have the potential to mineralize and solubilize organic and inorganic phosphorus, respectively (Khiari and Parent, 2005).

There is no biological atmospheric source for phosphorus as in case of nitrogen (Ezawa *et al*., 2002). A survey on Indian soils revealed that 98 per cent of these need phosphorus fertilization either in the form of chemical or biological fertilizers. It is estimated that only, 0.1 per cent of the total P present in soil is available to the plants because of its chemical fixation and low solubility. Soil P dynamics is characterized by physicochemical (sorption-desorption) and biological (immobilization mineralization) processes. Large amount of phosphorus applied as fertilizer enters into the immobile pools through precipitation reaction with highly reactive Al^{3+} and Fe^{3+} in acidic, and Ca^{2+} in calcareous or normal soils (Gyaneshwar *et al*., 2002). Under such conditions, microorganisms offer a biological rescue system as they are capable of solubilizing the insoluble inorganic P of soil and make it available to the plants (Tilak *et al*., 2005). For example, phosphate-solubilizing bacteria (PSB) found in majority of soils are reported to solubilize the insoluble inorganic P of soil and make it available to the plants (Satyaprakash *et al*., 2017 Rachmansyah *et al*., 2017).

An enormous number of microorganisms including bacteria, fungi and actinomycetes are known to produce acidic metabolites. These metabolites either by lowering the soil pH or by direct chelation with metal cations, release fixed or insoluble phosphorus in available form (Narsian and Patel, 2000; Reyes *et al*., 2002). Many species of actinomycetes are able to solubilise phosphates *in- vitro* and most of them live in the plant rhizosphere.

Actinobacteria isolated from the rhizosphere are capable of increasing availability of phosphorus to plants either by mineralization of organic phosphate or through solubilisation of rock phosphate accompanied by acids production (Hinsinger *et al*., 2003). Hamdali *et al.* (2008) observed that among the six selected actinomycetes, only three *viz., Streptomyces griseus*, *Streptomyces cavourensis*, *Micromonospora aurantiaca* strains significantly improved wheat plant growth in test tubes as well as in rock phosphate amended soil. The

strains showing the best phosphate releasing abilities were also having most important stimulatory effect on shoot and root growth of the plant.

Kataria (2012) screened and characterized two hundred and two actinomycetes from rhizospheric soil of five medicinal plants *viz*., *Curcuma longa* L., *Mentha arvensis*, *Ocimum sanctum*, *Zingiber officinalis* and *Cymbopogon citrates* from Punjab Agriculture University, Ludhiana which were furher evaluated as plant growth promoters and antifungal agents. The highest number and diversity of actinomycetes were observed to be associated with rhizosphere of *Curcuma longa* L. The potential isolates showed phosphate solubilization, siderophore and IAA production. *Streptomyces griseofuscus* Os-10, from *Ocimum sanctum*, produced maximum amount of IAA (93.8 µgmL-1) while, *Streptomyces aureus* Mt-10 from *Mentha arvensis*,showed maximum phosphate solubilization activity (68.51 mg/100mL). *Streptomyces globisporus* Mt-8, an isolate from Mentha *arvensis* showed maximum catechol–type of siderophore production (51.6 μgmL^{-1}) while, *Streptomyces flavus* Cc-10 from *Cymbopogon citrates* produced maximum hydroxamate-type of siderophore (144.2 μgmL^{-1}).

Damam *et al.* (2016) isolated and screened a total of sixty two actinomycetes obtained from rhizosphere of some forest medicinal plants*viz.*, *Calycopteris floribunda* (CFB), *Maeruga oblongifolia* (MO), *Lantana camara* (LC), *Zingiberofficinale* (ZO) and *Schleichera oleosa* (SO) grown in the Pakhal forest, Pakhal wild life sanctuary, Warangal, Telangana, India, for their plant growth promoting activities. Among these, forty seven isolates (75%) showed ammonia production, forty five (72%) indole acetic acid (IAA) production, thirty two (51%) hydrogen cyanide (HCN) production and eighteen (29%) phosphate solubilization.

Therefore, it is evident that biological mechanisms of microbial induced phosphate solubilization represent an attractive solution for making P available. Phosphate solublizing microorganisms (PSM's) have the potential to enhance dissolution of indigenous sources of rock phosphorus and transform them into more agronomically effective P-fertilizers.

2.5.2 Phytase activity

Phytate is one of the major storage forms of phosphorus in plants. Although, phytate is present in soil as plant based organic matter, it remains unavailable for plants. (Shivange *et al.*, 2012; Lazali *et al.*, 2013). Phytate has strong binding capacity to mono or divalent cations

in soil (Cerino *et al.*, 2012). Due to its unusual molecular structure phytate can also form complexes with other nutrients such as metallic ions (Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} etc.) rendering them unavailable to plants (Shim and Oh, 2012). Phytase is an enzyme that can hydrolyze the complex organic forms of phosphorous such as phytate into simpler forms by sequential hydrolysis of phosphate ester bonds and release the usable form of inorganic phosphoros (Shin *et al.*, 2001; Shivange *et al.*, 2012). It is a natural enzyme often used for breaking down and increasing the nutritional quality of phytate containing compounds. Phytase is widely distributed among plants,animal tissues and microorganism. Bioaugmentation of soil by phytase producing bacteria as biofertilizers can increase the bioavailability of phosphorous to plants, hence indirectly increase plant growth and decrease phosphorous pollution (Kumar *et al.*, 2013; Miao *et al.*, 2013). Moreover, uptake of other essential metal ions also increases by adding phytase producing microbes in soil (Pontoppidan *et al.*, 2012).

Arif *et al*. (2018) screened a total of 90 *Lactobacilli*, isolated from indigenous poultry droppings and homemade fermented milk products for phytate hydrolyzing ability and enzyme activities. Out of 62 isolates showing phytate hydrolysis, 16 were confirmed by cobalt chloride staining. Out of 16, three isolates i.e. PDP10, PDP24 and FYP38 were identified as *Lactobacillus gallinarum, Lactobacillus reutri* and *Lactobacillus fermentum* respectively, and further selected for physico-chemical optimization for phytase production. PDP10, PDP24 and FYP38 showed highest phytase activity at 35°C (6.86±0.15, 5.12±0.12 and 5.65±0.13 IU/mL, respectively), pH 5.0 (6.86±0.15, 5.12±0.11 and 5.50±0.13 IU/mL respectively), 1 per cent NaCl (4.78±0.14, 4.18±0.13 and 5.58±0.02 IU/mL respectively) and 0.3 per cent bile salts (3.76±0.12, 1.12±0.11 and 2.21±0.15 IU/mL respectively). Glucose was optimum carbon source for phytate hydrolysis. Optimum nitrogen source was peptone for PDP10 and PDP24 while tryptone for FYP38.

Hosseinkhani and Hosseinkhani (2009) isolated phytase producing bacteria using phytase specific medium amended with phytin and glucose as the only sources of phosphate and carbon respectively from soil and poultry faeces. Among five isolated strains, one identified as *Pseudomonas* sp. with highest phytase activitiy with 23 mm of clearance zone on solid medium and 800 U/mL in phytase specific broth medium. The enzyme was produced extracellularly in the late stage of exponential growth and the maximum phytase production was observed after 72 h of incubation at 28˚C and 180 rpm shaking conditions.

Singh *et al.* (2013) isolated a total of thirty two phytase producing bacterial strains from the soil samples collected from pulse fields, poultry farms and cattle sheds using phytase screening medium and observed that one of the best strains i.e. DR6 showed a clearance zone of 39 mm and was identified as *Bacillus subtilis*. Optimization studies of this strain revealed that maximum enzymatic activity of 378U/mL was shown at 50°C; pH 5.5 with glucose + sucrose as carbon sources and Yeast extract as nitrogen source, thereby, indicating the use of this enzyme as feed supplement in animal diet and for reducing phosphorus pollution problem in areas of livestock production.

Ghorbani-Nasrabadi *et al*. (2012) isolated a total of sixty-seven phytate-degrading rare actinomycetes from different soil ecosystems on Modified Glycerol Arginine Agar (MGAA) medium. The potential to dephosphorylate phytate was confirmed in liquid culture for 46.3 per cent of the isolates. Only 12 strains were selected for determining their phytate-degrading capacity directly. The results concluded that the enzymes had maximum phytate-degrading capability at 55˚C and pH 5.0 for isolate No. 43 and 37 ˚C and pH 7.0 for isolate No. 63. These isolates i.e 43 and 63 were identified as *Streptomyces alboniger* and *S. ambofaciens,* respectively using 16S rRNA gene sequencing technique.

Aly *et al.* (2015) isolated 50 actinomycetes from heated soil, sand, wastewater, animal faces and some plant ecological wastes on Starch nitrate agar medium. Out of 50, 20 isolates (40%) produced extracellular phytase enzyme on solid medium containing wheat bran as carbon source. In liquid medium, the phytase activity was measured in terms of U/mL and the most active isolate in phytase production was R10. Using morphological, physiological and biochemical studies, it was identified as *Streptomyces sp.* using 16S rDNA analysis, it was identified as *S. luteogriseus* R10. Growth of *S. luteogriseus* R10 in medium containing 1 per cent Na-phytate (pH 6.5) at 40°C for 7days increased phytase production. The maximum phytase activity was achieved using wheat bran, straw, rice husk and hay after seven days of incubation at 40°C but low activity was obtained using Sawyer and baggage as a carbon source.

2.5.3 Siderophore production

Iron is considered as an important nutrient for almost all life forms. In anoxic conditions, it occurs in form of ferric ion (Fe^{3+}) and combined with oxygen to produce insoluble hydroxides and oxyhydroxides. Due to this, iron became unavailable to both plants

and microorganism (Rajkumar *et al*., 2010). Siderophores produced by plant growth promoting rhizobacteria (PGPR) in the rhizosphere under iron deficient environment could efficiently chelate environmental iron and inhibit the growth of native microflora including root pathogens (Lim *et al*., 1991).

Sidrophores are iron chelating compounds which have very high affinity for ferric ions. These bacterial iron chelators are thought to sequester the limited supply of iron available in the rhizosphere, making it unavailable to pathogenic fungi, thereby restricting their growth (Loper and Henkel, 1999). Rhizobacterial siderophores were helpful in reducing the stresses imposed on plants by high levels of heavy metals in soil. Most of siderophore soluble in water and categorized into intracellular and extracellular siderophore (Ahemad and Kibret, 2014).

Siderophores are the compounds that have ability to bind with Fe^{3+}transport it back to the microbial cell and make it available for their growth. The soil actinomycetes, especially species of *Streptomyces*, have been reported to produce siderophores (Tokala *et al.,* 2002). Siderophores were used for agriculture and medical treatment. Microbial siderophores may also be utilized by plants as an iron source (Wang *et al.,* 1993). Rhizosphere soil actinomycetes have to compete with other rhizosphere bacteria and fungi for iron supply and therefore, siderophore production may be very important for their growth. Competition for iron is also a possible mechanism to control the phytopathogens in agriculture. Actinomycetes produce these compounds in order to compete for iron with phytopathogenic fungi (Tarkka and Hampp *et al*., 2008)**.**In recent decades, siderophores from actinomycetes were used for clinical application. For instance, siderophores like desferrioxamine from *Streptomyces pilosus* and oxachelin from *Streptomyces* sp. GW9/1258 were used for treatment of iron overload and removal of other toxic metals from human tissue (Sontag *et al.,* 2006). *Streptomyces lydicus* WYEC108 originally isolated from the rhizosphere soil of linseed was also found to produce hydroxamate type siderophore (Hamby, 2001) that had the ability to convert nitrate into nitrite and ammonia. Nitrate reducing strains were enumerated from the rhizosphere of *Glyceria maxima* that ranged from 3.2 x 10^6 to 3.3x 10^8cfu/ g and total number of potential nitrate reducing strains in the rhizosphere significantly increased with NO_3^- addition in the rhizosphere (Nijburg *et al.,* 1997). It was reported by El-Tarabily and Sivasithamparam (2006) that *Microbispora rosea, Micromonospora chalcea* and *Actinoplanes philippinensis* isolates,produced X-1,3,X-1,4 and X-1,6-glucanases that caused

lysis of *Phytophthora aphanidermatum* hyphae *in- vitro* and reduced damping-off disease of cucumber under controlled glasshouse conditions. These isolates were able to suppress damping-off in soil amended with or without cellulose.

Nimnoi *et al.* (2010) isolated a total of 10 endophytic actinomycete strains from healthy shoots and roots of *Aquilaria crassna* Pierre ex Lec (eaglewood). Analysis of 16S rDNA sequences of these isolates showed that they belong to members of the genera *Streptomyces* (2 isolates), *Nonomuraea* (1 isolate), *Actinomadura* (1 isolate), *Pseudonocardia* (1 isolate) and *Nocardia* (3 isolates). The remaining 2 isolates were unidentified. Among 10 isolates tested, the amount of hydroxamate-type siderophore produced by 2 isolates was undetectable. While, the remaining 8 isolates produced the amount of hydroxamate-type ranging between 3.21 ± 0.12 and 39.30 ± 0.40 μg mL^{-1}. Also, catechols-type siderophore produced by 9 isolates was undetectable. *Actinomadura glauciflava* was the only isolate that produced catechols-type of siderophores amounting to 4.12 ± 0.90 μg mL^{-1}.

A total of four hundred and forty five actinomycetes obtained from the rhizosphere of sixteen medicinal plants *viz.*, *Acanthus ebrateatus, Achyranthes aspera L., Amaranthus gracilis, Bariena lunulina L., Boesenbergia pandurata, Curcuma mangga, Cymbopogon citratus ,Cymbopogon nardus, Cyperus rotundus L., Imperata cylindrical, Languas galangal, Ocimum sanctum L., Pandanus amaryllifolius, Rhinacanthus nasutus, Stemona tuberosa, Zingiber officinale* of Thailand, were screened for the production of indole-3-acetic acid and siderophores. Morphological and chemotaxonomic studies indicated that 89 per cent of the isolates belonged to the genus *Streptomyces,* 11 per cent were non-*Streptomycetes*, *viz.*, *Actinomadura* sp., *Microbispora* sp., *Micromonospora* sp., *Nocardia* sp, *Nonomurea* sp. while, three isolates remained unclassified. The highest number and diversity of actinomycetes were observed to be associated with the rhizosphere of *Curcuma mangga*. Out of four hundred and forty five, thirty six actinomycetes isolates showed abilities to produce indole-3-acetic acid (IAA) while, seventy five isolates produced siderophores. *Streptomyces* CMU-PA101 and *Streptomyces* CMU-SK126 possesed highest ability to produce antifungal compounds, IAA and siderophores (Khamna *et al*., 2009).

Poomthongdee *et al.* (2014) isolated a total of three hundred and fifty-one actinomycetes from rhizospheric soil samples using acidified media with pH 5.5 and were further evaluated for their antifungal, siderophore production and phosphate solubilization

activities. The total count of actinomycetes growing on acidified starch casein agar and Gause no. 1 agar were below 2.48×10^4 CFU g^{-1} soil. Two hundred and twelve isolates were assigned to acidophiles and the remaining 139 isolates were neutrophiles. Of these actinomycetes, 57.8, 32.5 and 50.4 per cent, showed antagonistic activity against three rice pathogenic fungi; *Fusarium moniliforme, Helminthosporium oryzae* and *Rhizoctonia solani*, respectively. More than half of the isolates (68.1%) inhibited at least one tested pathogenic fungus, whereas 25.9 per cent exhibited antifungal activities against all tested fungi. Three hundred and thirty-eight isolates (96.3%) produced siderophore and 266 isolates (75.8%) solubilized phosphate. Three hundred and twenty-five isolates (92.6%) were classified as *streptomycetes* based on their morphological characteristics and the presence of the L-isomeric form of diaminopimelic acid in whole-cell hydrolysates.

2.5.4 Nitrogen fixation

N is one of the most common nutrients required for plant growth and productivity. It forms an integral part of proteins, nucleic acids and other essential biomolecules. More than 78 per cent of N is present in atmosphere in free state, but is unavailable to the plants. Atmospheric N needs to be converted into ammonia which can be readily taken up by the plants. The major part of N that is present in soil is due to its fixation by certain specialized group of microorganisms called N-fixers or diazotrophs (Kim and Rees, 1994). Biological N fixation is the process of the conversion of atmospheric di-nitrogen (N_2) to the non-gaseous N compound ammonium (NH_4^+) by N-fixing bacteria. It fixes about 60 per cent of the earth's available N and represents economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al.*, 1997). The reaction involved in biological N fixation is:

$$N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\text{ inorganic P}$$

Globally, biological nitrogen fixation is of tremendous importance to the environment and agriculture. The fixed nitrogen that is provided by biological nitrogen fixation is less prone to leaching and volatilization as it is utilized *in-situ* and therefore, the biological process contributes an important and sustainable input into agriculture (Dixon and Kahn, 2004).

Biological nitrogen-fixation is a complex process that involves a number of functional and regulatory gene products (Triplett *et al*., 1989). The actual reduction of N_2 is performed by the nitrogenase protein complex which consists of two metallo proteins: the nitrogenase or

nitrogenase molybdenum-iron protein (MoFe protein) and the nitrogenase reductase or nitrogenase iron protein (Fe protein).

In total, twenty three genera of woody plants in eight families are capable of forming symbiotic N_2 –fixing associations with soil actinomycetes of the genus *Frankia* (Bond, 1976). *Frankia* are nitrogen-fixing actinomycete symbionts that cause the formation of perennial nodules on the roots of a botanically diverse group of plants. The association is referred to as "actinorhizal" (Lechevalier, 1994).These actinorhizal species are important components of many natural ecosystems and some may fix N2 at rates comparable to agricultural legumes (Torrey, 1978).Two strains, namely D ll and G2, were isolated from root nodules of *Casuarina equisetifolia* (Diem *et al*., 1982) which was the first report of nitrogen fixation by free-living actinomycetes isolated from nodules of a nitrogen-fixing non-legume.

The increasing cost of petroleum products required for N fertilizer production has focused attention on biological system for N fixation. Aggarwal *et al.* (1986) found that inoculation of wheat and barley with ammonia excreting strains caused increased dry weight and enzyme activity. Whitehead and Cotta (2004) isolated a total of 40 bacterial cultures, which were capable of producing at least 40 mM ammonia in peptone-amino acid medium, concentrations similar to those produced by hyper-ammonia producing (HAP) bacteria isolated from the lumen of cattle.

Nitrogen fixation by symbiotic associations between actinomycetes and root systems of a diversified group of woody dicotyledonous plants is generally less recognized than legume-*Rhizobium* symbiosis. The actinomycete induced nodulation of plants like Alders (*Alnus*), bog plants like sweet gale (*Myricagale*), sweet fern (*Comptonia peregrina*), bayberry (*Myrica pensylvanicum*) and various species of *Ceanothus* and their role in N_2 fixation have been be recognized as one of the largest sources of biological fixation of atmospheric dinitrogen. Even the ability of *Frankia* to fix nitrogen is not apparently unique but may be shared by certain *Streptomyces* (Yamanaka *et al*., 2003). About 160 species in 15 genera among 7 families have been reported worldwide to have actinomycete induced nodulated nitrogen fixation. Furthermore, few of these plants have been of direct agricultural importance in the commerce of man (Torrey, 1978).

A total of three hundred twenty one isolates were recovered, from rhizospheric and non-rhizospheric soils of different plants grown in Punjab and Himachal Pradesh regions. Out

of these, sixty two isolates were found to be endophytic, one hundred fifty six rhizospheric while one hundred three were non-rhizospheric isolates. Studies on plant growth promoting activities revealed that twelve isolates showed abilities to produce indole acetic acid, ten produced siderophores and 12 isolates showed ammonia production. Phosphate solubilization was observed in five isolates and four fixed atmospheric nitrogen. Hence these isolates may be used as biocontrol and plant growth promoting agents. Morphological and chemotaxonomic studies identified and characterized all the active isolates belonged to the genus *Streptomyces* (Kumar *et al*., 2013).

2.5.5 HCN and ammonia production

HCN is a volatile secondary metabolite that suppresses the growth and development of competing microorganisms (Akhtar and Siddiqui, 2006). It is a broad-spectrum antimicrobial compound involved in biological control of root disease which is produced by plant associated rhizobacteria (Ramette *et al*., 2003). Bacteria produce a wealth of volatile compounds mainly HCN which are responsible for limiting the fungal growth and contribute towards the antagonism (Rezzonico *et al*., 2007). Some rhizobacteria, including species of *Alcaligenes, Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* are capable of producing HCN (Ahmad *et al*., 2008). HCN is formed from glycine through the action of HCN synthetase enzyme which is associated with the plasma membrane of certain rhizobacteria (Martínez *et al*., 2010). It is a powerful inhibitor many metal enzymes, especially copper containing cytochrome c oxidases (Hassanein *et al*., 2009). Marques *et al.* (2010) recommended that bacteria can synthesize ammonia and supply nitrogen to the host plant. Additionally, over production of ammonia serve as a prompting factor for the virulence of opportunistic plant pathogens. Hence, ammonia and HCN production play an essential role in suppression of plant disease.

Almost all the rhizospheric actinomycetes are able to produce ammonia and hydrogen cyanide. For instance, Anwar *et al.* (2016), reported production of ammonia and HCN from genus *Streptomyces*. According to Kremer and Souissi (2001) an enormous number of rhizobacteria were able to produce volatile compounds such as ammonia and hydrogen cyanide, which are reported to play an important role in biocontrol. HCN produced in the rhizosphere seedlings by the selected rhizobacteria is a potential and environmentally compatible mechanism for biological control of weeds and minimizing harmful effects on the growth of desired plants.

Joseph *et al.* (2012) isolated a total of one hundred fifty bacterial isolates belonging to *Bacillus, Pseudomonas, Azotobacter and Rhizobium* from different rhizospheric soil of chick pea in the vicinity of Allahabad. Ammonia production was detected in 95 per cent of isolates of *Bacillus* followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter (*45.0%). Selvakumar *et al.* (2008) reported that two isolates *viz*., KR-3 and KR-4 were able to grow in N-free medium and excreted extracellular ammonia which is an indicative of their ability to fix atmospheric N. Karthikeyan and Sakthivel (2012) observed ammonia production in 96 per cent of isolates of *Bacillus* followed by *Pseudomonas* (92%), *Azospirillum* (65%) and *Azotobacter* (50%) obtained from different plant rhizospheres.

The production of ammonia is also reported to be involved in antagonistic interactions that result in disease control (Saraf *et al*., 2008). Howell *et al*. (1988) reported that volatile compounds such as ammonia produced by *Enterobacter cloacae* were involved in the suppression of *Pythium ultimum*-induced damping-off of cotton.

Passari *et al.* (2015) recovered forty two endophytic actinomycetes from medicinal plants which were further evaluated for their antagonistic potential and plant growth-promoting abilities. Out of these, only twenty two antagonistic isolates were used to evaluate phosphate solubilization, siderophores, HCN, ammonia, chitinase, indole-3-acetic acid production, as well as antifungal activities. Out of twenty two isolates, the amount of indole-3-acetic acid (IAA) ranged between 10–32 µg/mL was produced by twenty isolates while, all isolates were positive for ammonia production ranged from between 5.2 to 54 mg/mL. Among twenty two isolates tested, the amount of hydroxamate-type siderophores were produced by sixteen isolates ranged from 5.2 to 36.4 µg/mL, while catechols-type siderophores produced by five isolates ranging from 3.2 to 5.4 µg/mL. Fourteen isolates showed P-solubilization ranged from 3.2 to 32.6µg/mL. Chitinase and HCN production was shown by nineteen and fifteen different isolates, respectively. These results clearly suggested the possibility of using endophytic actinomycetes as bioinoculant for plant growth promotion, nutrient mobilization or as biocontrol agent against fungal phytopathogens for sustainable agriculture.

Viraj and Meenu (2015) isolated one hundred and sixty three PGPR isolates from *Jathropha curcas* and only ten isolates were screened on the basis of their fast growing ability and motility for the production of IAA, HCN, ammonia and phosphate solubilization. DN2, DP3, SP1 and SAz1 showed good potential ability for plant growth promotion. Anwar

et al. (2016) isolated ninety eight rhizospheric actinomycetes from different wheat and tomato fields of Punjab, Pakistan. The isolates were characterized morphologically, biochemically, and genetically and were subjected to a comprehensive *in-vitro* screening for various plant growth promoting (PGP) traits. All rhizobacterial isolates were positive for ammonia, and hydrogen cyanide production.

2.5.6 Lytic enzymes production

Enzymes are the organic catalysts produced generally by microorganisms, and are different from other catalysts. Various kinds of enzymes are produced by microorganisms. The antagonistic activity against different type of microbes may also be due to the production of lytic enzymes that are produced *in-vitro* or *in-vivo* by microorganisms. Chitinases and proteases produced by actinomycetes are considered as factors which may participate in the biological control of plant pathogens, mainly fungal pathogens (Strzelczyk *et al*., 1990). Proteinases are the most common hydrolytic enzymes in nature. Localized in cells or secreted outside, they play an important role both in the process of degradation of trophic proteins and in the changes in cellular proteins. The enzymes chitinase and chitobiase produced by some fungi and bacteria like *Mucor*, *Trichoderma* and *Pseudomonas* species, respectively possessed lytic effect which was related to antagonistic behaviour (Uloha and Peberdy, 1991).

Lytic enzymes such as proteases, cellulases, chitinases, pectinases and lipases are involved in the biological control of phytopathogens (Dunn *et al*., 1997; Gajera *et al*., 2013). Several enzymes produced by the actinomycetes are becoming industrially important for either product manufacture or chemical diagnosis. Pronase is a proteolytic enzyme produced by *Streptomyces gratiae* shown to have mixture of several exopeptidases and endopeptidases valued for analytical processing (Neihans *et al*.,1999). Likewise, protease is an important class of industrial enzymes. A variety of extracellular hydrolytic enzymes such as cellulase, chitinase and amylase produced by actinomycetes are reported to be applicable in agriculture. (Beyer and Diekmann, 1985; Hopwood and Sherman, 1990; Taechowisan *et al*., 2003). These enzymes have the ability to degrade cell wall and spore of fungal pathogen so fungus lost its ability to infect the host plant. Beside from agricultural usages, some enzymes from actinomycetes were applied for medical treatment. For instance, L-asparaginase (L-asparagine amido hydro-lase, E.C.3.5.1.1) has been widely used as a therapeutic agent in the treatment of certain human cancers, mainly in acute lymphoblastic leukemia (Gallagher *et al*.,

1989; Verma *et al*., 2007). This enzyme acts as a catalyst in the breakdown of asparagine to aspartic acid and ammonia. Tumor cells, especially lymphatic tumor cells, require L-asparagine for growth. When this amino acid was converted into aspartic acid and ammonium ion by this enzyme, the malignant cells died and destroyed. The production of L-asparaginase has been studied in actinomycetes by various researchers (Mosafa and Salama, 1979; Narayana *et al*., 2007).

It was reported by El-Tarabily (2006) that *Microbispora rosea*, *Micromonospora chalcea* and *Actinoplanes philippinensis* isolates produced X-1,3, X-1,4 and X-1,6-glucanases *in-vitro* that caused lysis of *Phytophthora aphanidermatum* hyphae and reduced damping-off disease of cucumber under controlled glasshouse conditions. These isolates were able to suppress damping-off in soil amended with or without cellulose.

Jeffrey *et al.* (2011) isolated and screened a total of forty actinomycetes from the soil samples collected from MARDI peat land research station at Sessang, Sarawak, Malaysia using multi-stage Dispersion and Differential Centrifugation (DDC) method for their ability to secrete bioactivities such as cellulase, mannanase, xylanase, lipase, protease and antimicrobial compounds. Pre-screening of forty actinomycetes showed that 45 per cent produced cellulase, 5 per cent mannanase, 12.5 per cent xylanase, 12.5 per cent protease and 32.5 per cent lipase. These actinomycetes also showed antimicrobial activities against various test pathogens such as *Ralstonia solanacearum* (10%), *Pantoea stewartii* (20%) and *Bacillus subtilis* (10 %). Eight of the best producers of bioactivities were identified using 16S rRNA gene sequencing technique. Seven of the actinomycetes were identified to be from the genus *Streptomyces* while one was from the genus *Kitasatosproria.*

Singh and Padmavathy (2014) assessed the comparative amylase, lipase and cellulase production abilities of twenty two endophytic actinomycetes isolated from both fresh and fallen leaves of *Emblica officinalis* in Western Ghats. Among the endophytic actinomycetes from fresh leaves, 75 per cent exhibited amylase activity, 41.6 per cent lipase activity and 50 per cent cellulase activity whereas, in fallen leaves, 80 per cent exhibited amylase activity, 60 per cent lipase activity and 60 per cent cellulase activity. Hence, endophytic actinomycetes isolated from the fallen leaves exhibited remarkable lipase, cellulase and amylase producing capacity which mediates them as biocatalysts in bioremediation and biofuel production, as compared to fresh leaves.

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

Among different lytic enzymes, chitinases are particularly useful in agriculture as biocontrol agents against fungal pathogens because of their ability to hydrolyse the chitinous fungal cell wall (Maisuria *et al.,* 2008; Wahyudi *et al.,* 2011). Actinomycetes with potent chitinolytic activity have been used as biocontrol agents in agriculture. Examples include *Streptomyces somaliensis, S. somaliensis, S. somaliensis, Streptomyces purpurascens* and *Streptomyces griseus* (Quecine *et al.,* 2008).

Saadoun *et al*. (2007) screened a total of 110 isolates of *Streptomyces* obtained from Ajlun forests in northern Jordan, Arab for their ability to produce cellulase, pectinase and xylanase enzymes. Results showed that 79 per cent of the isolates were able to produce at least one of the enzymes with cellulase being the highest (71.8%). Thirty five isolates (32%) were able to produce all the three enzymes. Out of them, 24 were characterized morphologically and physiologically as *Streptomyces*.

Bruhlmann *et al.* (1994) screened isolates of actinomycetes obtained for the production of pectinolytic enzyme activities when grown on pectin-containing solid and liquid media. Pectinolytic enzymes, detected by using plate diffusion tests with a medium containing ramie (Boehmeria nivea) plant material as the sole carbon source, were mainly pectate lyases, but low activities of pectin esterases were also observed. Polygalacturonases and polymethylgalacturonases were not produced. Multiple forms of pectate lyases were detected in the culture supernatants of some of the strains by using the zymogram technique of isoelectric focusing gels. Xylanolytic and cellulolytic activities were always found to be associated with pectinolytic activities. None of the pectinolytic enzyme was notproduced in a medium with glucose as the sole carbon source.

Sonya *et al.* (2015) screened a total of 23 *Streptomycetes*-like isolates (SLI) obtained from different locations *viz*., Al- Qaiem, Al-Qamaryiah, Al-Sail Al-Kabeer, Al-Sail Al-Sagheer, Al-Sawtah, Gabrah (1), Gabrah (2), Tarabah, Qaiah and Wadi-Bowa at Taif region of Kingdom of Saudi Arabia (KSA) for the production of lytic enzymes. All isolates were found to produce amylase and 17 (73.9%) out of the 23 isolates, showed excellent growth on starch casein agar medium. In case of cellulase, lipase and gelatinase, the growth was shown by 15 (65.2%), 21(91.3%) and 17 (73.9%) isolates, respectively. Three isolates i.e 10, 12 and 13 were found to be potent tested enzymes producers and were therefore, further selected for identification. The isolate 10, 12 and 13 were identified as *Streptomyces lateritius,S. cacao* and *S. phaeopurpureus*, respectively.

Ghodsalavi *et al.* (2013) isolated 40 bacterial strains from the rhizosphere of *Valeriana officinalis* using various enriched and selective media and were further tested for the production of siderophore, indoleacetic acid (IAA), hydrogen cyanide (HCN), lipase and protease. The isolates were grouped into *Pseudomonas, Klebsiella, Xanthomonas, Bacillus, Erwinia, Agrobacterium* and *Arthrobacter* on the basis of cultural, morphological and biochemical characteristics. Results revealed that up to 98, 80 and 38 per cent of the isolates were able to produce siderophore, Auxin (IAA) and HCN production respectively. The results of lytic enzymes production revealed that 95 per cent of isolates produced lipase enzyme while only 78 per cent of the isolates were able to produce protease enzyme.

Rhizobacteria possessing multiple plant growth promoting activities were isolated from the rhizospheric soils of plants growing in semi arid region. These isolates were screened for their plant growth promoting activities like phosphate solubilization, production of indole- acetic acid, ammonia, hydrogen cyanide (HCN), catalase, amylase, cellulase, and chitinase. All the isolates solubilized phosphate. Among all isolates, only 33% produced ammonia while, 27, 53 and 50 per cent isolates showed lipase, amylase and chitinase activities, respectively. Forty per cent of the isolates produced indole-acetic acid whereas, only a single isolate produced HCN (Bhatt *et al*., 2014).

Sharma *et al.* (2017) isolated a total of fifty rhizospheric and forty three endorhizospheric bacteria from the rhizosphere of *Podophyllum hexandrum* grown in high hills of Churdhar, Himachal Pradesh, India and subjected to replica plating for one step screening of plant growth promoting (PGP) traits. Of the total, 80.48 per cent isolates produced indole-3-acetic acid, 12.20 per cent HCN, 53.65 per cent exhibited chitinase

while, 41.46 per cent exhibited protease activity. *In- vitro* per cent inhibition of fungal pathogens *viz., Alternaria solani, Fusarium oxysporum, Dematophora necatrix, Sclerotium rolfsii* and *Phytophthora* spp. by these bacteria ranged from 44 to 88 per cent. Twenty most efficient PGPR under *in-vitro* conditions were evaluated for growth promotion of tomato in growth chamber. Significantly highest root length (43.00 mm), shoot length (73.00 mm), plant dry weight (12.90 mg plant^{-1}) and vigour index (10,567.60) were recorded for isolate 2a1. Remarkable increase was also recorded in nitrogen (11.85 %), phosphorus (23.08 %) and potassium (36.84 %) content of shoot biomass over control. Strain 2a1 was identified as *Bacillus subtilis* through biochemical and phylogenetic analysis. These results demonstrated that strain 2a1 from *Podophyllum hexandrum* exhibited multiple PGP attributes for development as a biofertilizer to enhance soil fertility and promote plant growth.

2.5.7 PLANT GROWTH REGULATORS

2.5.7.1 Auxin production

Many soil and plant associated rhizobacteria are able to synthesize phytohormones (Bastian *et al*., 1998). Plant morphogenic effects may also be a result of different ratios of plant growth hormones produced by roots as well as by rhizospheric bacteria (Muller *et al*., 1989). Phytohormones like Indole acetic acid and Gibberellins are plant growth regulators, which have stimulatory effects on plant growth. IAA (indole-3-acetic acid) is the member of the group of phytohormones and is generally considered the most important native auxin (Ashrafuzzaman *et al*., 2009).

Indole acetic acid (IAA) is a common natural auxin. It is a product of L-tryptophan metabolism in microorganisms. The auxins are a group of indole ring compounds which have the ability to improve plant growth by stimulating cell elongation, root initiation, seed germination and seedling growth (El- Tarabily, 2008). Approximately 80 per cent of rhizosphere bacteria can secrete IAA (Bhavdish *et al* 2003). *Streptomyces* sp., inhabiting the rhizospheres of various plants, also serves as good source of IAA. Generally microorganisms isolated from the rhizosphere and rhizoplane of various crops have more potential of producing auxins than those from the root free soil (Arshad and Frakenberger, 1998). In the rhizospheric soils, root exudates are the natural source of tryptophan for rhizosphere micro-organisms, which may enhance auxin biosynthesis in the rhizosphere. Several *Streptomyces* species such as *S.olivaceoviridis*, *S. rimosus* and *S. rochei* were obtained from the tomato

rhizosphere, exhibited the ability to produce IAA and improved plant growth by increased seed germination, root elongation and root dry weight (Aldesuquy *et al* 1998; Tokala *et al.*, 2002 and El-Tarabily, 2008).

Rhizospheric soils of medicinal plants may be attractive sources of *Streptomyces* sp. capable of producing bioactive compounds related to plant growth promotion (Thangapandian *et al.*, 2007). Nowdays, some rhizospheric actinomycetes are exploited for the development of commercial product(s). For example, Mycostop, which was developed by using two IAA producing actinomycete strains i.e.*S. grieseoviridis*K61and *S. lydicus* WYEC 108 in order to promote plant growth (Mahadevan and Crawford, 1997). Several researchers like El-Tarabily and Sivasithamparam (2006) and Tsavkelova *et al.* (2006) reported that *Streptomyces* isolated from many crop rhizosphere soils showed IAA production and thereby promoted plant growth.Likewise high tryptophan concentration in root exudates of lemongrass enhanced IAA biosynthesis in *Streptomyces* CMUH009.

A total of two hundred-seventy *Streptomyces* isolates, obtained from rhizospheric soil samples of fourteen medicinal plants of Thailand, were evaluated for production of Indole-3-acetic acid. Out of these, only thirty isolates were able to produce Indole-3-acetic acid. Only one isolate i.e. CMU-H009 recovered from soil associated with lemongrass (*Cymbopogon citratus*) was very effective in producing IAA. Based on the 16S rDNA sequence analysis, this strain showed maximum homology (99.3%) with *Streptomyces viridis* NBRC 13373T (Khamna *et al*., 2010).

2.5.7.2 Gibberelin and Cytokinin production

Gibberellins are biologically active and endogenous hormones of higher plants that are involved in their varient developmental processes like stem elongation, germination, seed dormancy and sex expression (Gelmi and Perez, 2000).

Solans *et al.* (2011) evaluated phytohormone production by symbiotic and saprophytic actinomycetes obtained from the actinorhizal plant *Ochetophila trinervis*. Three saprophytic strains *viz.*, *Streptomyces* sp. (BCRU-MM40), *Actinoplanes* sp. (BCRU-ME3) and *Micromonospora* sp. (BCRU-MM18) out of 122, with multiferous enzymatic activities were selected for plant growth experiments in pots. Phytohormone production was evaluated in supernatant of non-inoculated and inoculated culture media in exponential growth phase. The levels of the three phytohormones produced by the saprophytic

rhizoactinomycetes were observed to be higher thansymbiotic *Frankia* strain. Zeatine biosynthesis was recorded to be higher (μg mL^{-1}) than IAA and GA_3 (ng mL^{-1}). *Micromonospora* strain showed the production of these phytohormones upto the highest levels. Although *O. trinervis* has been shown to be intercellularly infected by *Frankia* without mediation of root hair deformation, when plants were co-inoculated with actinomycetes culture, some root hair deformation was observed. This is the first report on identification of IAA, GA_3 and Z in saprophytic actinomycetes and their potential role in plant–microbe interaction.

El-Tarabily *et al.* (2009) evaluated the potential of *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* endophytic actinomycetes in cucumber roots, to promote plant growth and protect seedlings and mature plants of cucumber from diseases caused by *Pythium aphanidermatum*, under greenhouse conditions. TLC analysis of culture extracts of these three isolates revealed that *Streptomyces spiralis*produced IAA, IPYA, GA3 and iPa,*Actinoplanes campanulatus* IAA, IPYA and GA3, whereas *Micromonospora chalcea* produced only IAA and IPYA. None of these three isolates produced Zeatin. The other four isolates did not produce any PGP traits.

A commercial soil amendment containing a mixture of four PGPR (*Azospirillum lipoferum, Azotobacter chroococcum, Pseudomonas fluorescens and Bacillus megaterium*) was evaluated for impact on germination, initial growth and nutrient content of *Catharanthus roseus*. Root inoculation of PGPR strains significantly increased germination rate, vigour index and chlorophyll content (5.34 mg g-1 plant-1 in 120 DAS) compared with the control. In addition, nutrient element contents (P- 2.34, N- 0.34, K-2.20 per cent in 120 DAS) was investigated and significantly affected by bacterial applications compared with the control. Hence, it is evident that these four PGPRs in combination have the potential to increase the yield, growth and nutrient content of *Catharanthus roseus* (Lenin and Jayanthi, 2012).

Sharma *et al.* (2014) isolated the fluorescent *Pseudomonas* and *Azotobacter* from rhizospheric soil of *Swertia chirayata*. These PGPR were co-cultured with micropropagated shoots of *S. chirayata* on tissue culture medium and tested the influence of *in-vitro* caulogenesis and rhizogenesis along with different phytohormones independently and in combination. A significant plant growth promotion was observed which not only increased the survival rate up to 90 per cent, but also the multiplication rate at low auxin concentration

compared to non-inoculated plantlets. Both strains showed tryptophan dependent synthesis on MS basal medium supplemented with 200 µ/mL tryptophan with maximum IAA production

2.6 ANTAGONISTIC ACTIVITY OF ACTINOMYCETES AGAINST SOIL-BORNE FUNGAL PHYTOPATHOGENS

Besides producing enormous numbers of agroactive metabolites (Tanaka and Omura, 1993), actinomycetes also play an important role in agriculture as biocontrol agents. Antagonism against a diverse range of phytopathogens has been reported by various workers (Chamberlain and Crawford 1999; Trejo-Estrada *et al.,* 1998).

Crawford *et al.* (1993) observed that twelve actinomycete strains isolated from *Taraxicum officinale* rhizosphere were active against *Pythium ultimum.* Ten isolates of actinomycetes obtained from therhizospheric soils of medicinal plant were tested for antifungal activity against *Candida albicans* and *Candida tropicalis* and it was found that all *Streptomyces* isolates had antifungal activity (Barakate *et al*., 2002). Four actinomycete isolates obtained from the rhizospheric soil of cotton plant were found to have potent antagonistic activities against *Rhizoctonia solani* and were identified as *Streptomyceserumpens, S. purpureus, S. aurantiacus* and *S. microflavus* (Hassanin *et al.,* 2007).

Khamna *et al.* (2009) isolated a total of four hundred forty five actinomycetes from sixteen rhizospheric soils of medicinal plant grown in Lumphun Province. The highest number and diversity of actinomycetes were found in *Curcuma mangga* rhizosphere soil. Out of four hundred forty five isolates twenty three *Streptomyces* isolates showed activity against at least one of the five phytopathogenic fungi: *Alternaria brassicicola, Collectotrichum gloeosporioides, Fusarium oxysporum, Penicillium digitatum*and Sclerotium *rolfsii.* Two isolates, *Streptomyces* CMU-PA101 (accession number FJ025786) from *P. amaryllifolius* rhizosphere and *Streptomyces* CMU-SK126 (accession number FJ217218) from *C. mangga* rhizosphere, strongly inhibited all pathogenic fungal strains.

Three medicinal plants namely *Aloe vera, Mentha arvensis* and *Ocimum sanctum* were explored for assessing the diversity of endophytic actinomycetes along with their plant growth promoting and antimicrobial potential. Overall forty endophytic actinomycete were obtained from the root, stem and leaf tissues of these three medicinal plants. Endophytic actinomycetes were most commonly recovered from roots (70% of total isolates) followed by

stems (17.5%) and leaves (12.5%). The highest numbers of endophytic actinomycetes were obtained from *Ocimum sanctum* (45%). Out of 22 isolates tested, 12 showed the ability to solubilize phosphate ranged from 5.4 to16.5 mg/100 mL, while 16 isolates produced indole-3 acetic acid (IAA) in the range of 8.3 to 38.8 µg/mL. Nine isolates produced the amount of hydroxamate- type of siderophore ranged from 5.9 to 64.9 µg/mL while only four isolates were able to produce catechol-type of siderophore in the range of 11.2-23.1 µg/mL. Of the nine, interestingly, eight endophytic actinomycetes (88.9%) showed a significant antagonistic activity against one or more phytopathogenic fungi thereby, indicating their possible role as biocontrol agents **(**Gangwar *et al*, 2014).

Chaurasia *et al.* (2005) isolated an antagonistic strain of *Bacillus subtilis*, from the rhizosphere of established tea bushes, which was observed to cause structural deformities in six pathogenic fungi under *in- vitro* cultural conditions. This effect was attributed to the production of diffusible and volatile antifungal compounds. Out of selected test fungi, four were phytopathogenic while, remaining two were of clinical importance. The bacterial strain successfully restricted the growth of all test fungi in dual cultures, and induced morphological abnormalities such as mycelial and conidial deviations. The inhibitory effect caused by volatiles was found to be greater than that by diffusible compounds.

Tenorio-Salgado *et al.* (2013) evaluated the abilities of 15 different strains of *Burkholderia tropica* to inhibit the growth of four phytopathogenic fungi *viz.*, *Colletotrichum gloeosporioides*, *Fusarium culmorum*, *Fusarium oxysporum* and *Sclerotium rolfsii* using diverse analytical techniques including plant root protection, dish plate growth assays and gas chromatography-mass spectroscopy.Results showed that the fungal growth inhibition was due to volatile compounds produced by *B. tropica* and, in particular, two bacterial strains (MTo293 and TTe203) exhibited the highest radial mycelial growth inhibition. Morphological deformities such as disruption of fungal hyphae associated with these compounds, were identified using photomicrographic analysis. Eighteen volatile compounds including α-pinene and limonene involved in the growth inhibition were identified using gas chromatography-mass spectroscopy technique. Additionally, a high proportion of bacterial strains that produced siderophores during growth with different carbon sources, such as alanine and glutamic acid were also found; however, their roles in the antagonism remained unclear.

Cordovez *et al*. (2015) isolated and characterized 300 isolates of rhizospheric Actinobacteria from the *Rhizoctonia*-suppressive soil. *Streptomyces* species were the most abundant, representing approximately 70 per cent of the total isolates. Volatile Organic compounds (VOC) profiling of 12 representative *Streptomyces* isolates by *Solid Phase Microextraction* Gas Chromatography–Mass Spectrometry. (SPME-GC-MS) provided a more refined phylogenetic description of the *Streptomyces* isolates than the sequencing of 16S rRNA and the house-keeping genes *atpD* and *recA* only. VOCs of several *Streptomyces* isolates inhibited hyphal growth of *Rhizoctonia solani* and significantly enhanced plant shoot and root biomass. Coupling of *Streptomyces* VOC profiles with their effects on fungal growth, pointed to VOCs potentially involved in antifungal activity. Subsequent assays with five synthetic analogs of the identified VOCs showed that methyl 2-methylpentanoate, 1, 3, 5-trichloro-2-methoxy benzene and the VOCs mixture have antifungal activity.

Wang *et al*. (2013) isolated *Streptomyces* sp. TD-1 from soil surrounding a granary in Tianjin, China and was identified as *Streptomyces alboflavus* based on its morphological, physiological characteristics and 16S rDNA gene sequence analysis. The antifungal activity of the volatile compounds producing *S. alboflavus* TD-1 was investigated which showed that volatiles generated by S. *alboflavus* TD-1 inhibited storage fungi *Fusarium moniliforme Sheldon, Aspergillus flavus, Aspergillus ochraceus, Aspergillus niger,* and *Penicillum citrinum in-vitro*. Twenty seven kinds of volatile organic compounds were identified usingGas chromatography–mass spectrometry (GC/MS) from the volatiles of *S. alboflavus* TD-1 mycelia and2-methylisoborneol was found to be most abundant compound among them. *In-vitro* fumigation studies revealed that Dimethyl disulfide had antifungal activity against *F. moniliforme.*

Wu *et al*. (2015) isolated an actinomycete isolate from the forest soil sample of Tzu-chin Mountain (Nanjing China) which was identified as *Streptomyces. albulus* NJZJSA2 based on its morphological, physiological and biochemical characteristics alongwith 16S rDNA gene sequence analysis. This strain was evaluated for the production of antifungal volatile organic compounds (VOCs) against two fungal pathogens *viz*., *Sclerotinia sclerotiorum* and *Fusarium oxysporum* and results concluded that the VOCs generated by *S. albulus* NJZJSA2 inhibited mycelial growth of *Sclerotinia sclerotiorum* (SS) and *Fusarium oxysporum* (FO) by 100 and 56.3%, respectively. The germination of SS sclerotia and FO

conidia was completely inhibited in the presence of VOCs produced by *S. albulus* NJZJSA2 *in-vitro*. In soil, the VOCs delayed the germination of SS sclerotia and inhibited the germination of FO conidia for 45 days. Three relatively abundant VOCs, 4-methoxystyrene, 2-pentylfuran and anisole were prossessed strong antifungal activity. Microscopy analysis showed that the pathogen hyphae were shriveled and damaged after treatment with 4-methoxystyrene.

2.7 IDENTIFICATION AND CHARACTERIZATION OF ACTINOMYCETES

The various methods by which actinomycetes can be identified and classified are Chemotaxonomic characterization, morphological observation, biochemical characterization and molecular techniques. Chemotaxonomy is the study of chemical variation in cell compositions in microorganisms. One of the quickest methods for preliminary identification of actinomycetes to genus level is the demonstration of the presence of diaminopimelic acid isomers (DAP) and sugar composition in the whole-cell hydrolysates (Hasegawa *et al.*, 1983). The presence of DAP isomers is one of most important cell-wall properties of actinomycetes and gram positive bacteria. These amino acids, mostly located in the peptidoglycan of bacterial wall envelope are generally contained as one of the isomers, L-form or *meso*-form. Cells envelope of the actinomycetes contain glucosamine and muramic acid, the monomers of peptidoglycan. The DNA base compositions of most actinomycetes ranges from 63-78 mol per cent G+C. The value of mol per cent G+C content has been used to classify group of actinomycetes (Madigan and Martinko, 2006). Moreover, compositions of phospholipids, fatty acids and menaquinones in the cell have also been used by other workers (Boone and Pine, 1968; Lechevalier and Lechevalier, 1980).

Morphology has been an important characteristic to identify actinomycetes. It was this characteristic that was used in the first descriptions, especially of *Streptomyces* species. This study was made best using a variety of standard culture media, including those recommended for the International *Streptomyces* Project (Shirling and Gottlieb, 1966). For non-*Streptomyces* or rare actinomycetes, strains were cultivated on ATCC Medium No.172 (NZ-amine glucose starch agar) (American Type Culture Collection, 1982). Morphological observations including germination of spores, elongation and branching of vegetative mycelium, formation of aerial mycelium, color of aerial and substrate mycelia and pigment production have been used to identify actinomycetes (Holt *et al*, 1994). Actinomycetes produce a variety of pigments like from yellow, orange, red, purple, and blue, olive green etc.

Investigators have studied actinomycetes for commercial production of pigments. A well-studied member of this group is *Streptomyces coelicolor* that produces blue pigment(Sanchez-Marroquin *et al*., 1954).Formation of aerial and substrate mycelia were studied by light microscopy while the spore surface and structure by scanning electron microcopy.

Another approach by which actinomycetes can be identified and characterized is their basis of biochemical characterization. Various biochemical tests which are used for this approach are catalase, oxidase, geletinase, citrate utilization, carbohydrate fermentation, oxidative fermentation, MR-VP, indole tests etc (Singh *et al*., 2013).

Presently, molecular techniques based on 16S rDNA sequences are of utmost importance for actinomycetes identification. The phylogenetic tree constructed from 16S rDNA sequences provides information about the actinomycetes evolution. Based on the 16S rRNA gene sequencing, actinomycetes are separated into over 100 genera (Yokota, 1997).

Gautham *et al.* (2012) isolated and characterized *Streptomyces* species, major antibiotic producing filamentous bacteria from Western Ghats soils of Karnataka, India. Their characterization based on morphological and biochemical characteristics revealed the diversity of *Streptomyces* in the form of spore colour varying from white, gray, blue, red and pink. The colonies formed were discrete, lichenoid, leathery, powdery and butyrous colonies. The aerial and substrate mycelia varied in their colour from white, grey to blue series. The spore chain morphology studies revealed different arrangements of rectus, flexibilis, and retinaculum aperatum – open loops, hooks and spira – simple spirals, short and compact spirals. The pigments were dark red, green, and yellow, brown and brownish red in colour.

Shrivastava *et al*. (2015) isolated a total of 66 actinomycetes from rhizosphere of mangroves in Andhra Pradesh, India, using various enrichment techniques and pretreatments. Out of 66, 8 isolates possessing plant growth promoting potential were further characterized by physiological and biochemical traits and identified as different species of *Streptomycetes* genera using 16S rRNA gene sequencing technique.

Zhang *et al*. (2011) isolated a novel actinobacterial strain, designated CPCC 201356^{T}, from a rhizospheric of a medicinal plant- *Astragalus membranaceus* and subjected to polyphasic taxonomic analysis. Results showed that good growth occurred at 20–32°C, at pH

7.0–7.5 and with 0–1 per cent (w/v) NaCl. Colonies on R2A and ISP 2 agar were light red to red, round and lacked aerial mycelium which indicated that cells adhered to the agar surface only. The G+C content of the genomic DNA was 72.8 mol per cent. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain CPCC 201356^T belonged to the family *Geodermatophilaceae* and consistently formed a distinct sub-branch with *Geodermatophilus obscurus* DSM 43160^T. DNA–DNA hybridization between strain CPCC 201356^T and *G. obscures* DSM 43160^T was 17.4 per cent. On the basis of evidence from this polyphasic taxonomic study, a novel species, *Geodermatophilus ruber* sp. nov., was proposed; the type strain is CPCC 201356^T (=DSM 45317^T =CCM 7619^T).

Rama krishnan *et al*. (2009) isolated *Streptomycetes* sp. SCBT strain (Accession number EU143270) from the rhizosphere of medicinal plants grown in the Kolli hills of Tamil Nadu, India. The strain *Streptomyces* was identified and characterized in School of Chemical and Biotechnology (SCBT), capable of inhibiting the growth of a wide range of Gram-negative and Gram-positive bacteria. An almost complete 16S rRNA gene sequence of the isolate was generated and compared with sequences of representative *Streptomyces* spp. The 16S rRNA data supported the classification of the strain within the genus *Streptomyces*, and showed that the SCBT strain was closely related to *Streptomyces albogriseolus* ATCC AJ494865 with a sequence similarity of 99%. Despite the high sequence similarity, SCBT was phenotypically different from *S. albogrieseolus* ATCC AJ494865.

2.8 EFFECT OF PLANT GROWTH PROMOTING ACTINOMYCETES ON SEED GERMINATION AND SEEDLING GROWTH

Seed lots hold the potential to produce new, healthy crop plants which are necessary for food security. Seed potential was determined by the quality of seeds, which forms the foundation for successful grain crop production (Ellis *et al*., 1993). Seed quality is a good indicator of the marketability (Milosevic *et al.,* 2010) and potential performance of a seed lot in the field (Powell *et al*., 2009). The seed stock chosen by the grain producer can have a significant detrimental effect on crop yield if seed quality is low (Msuya and Stefano, 2010). The germination capacity of a seed lot is a key indicator of quality (TeKrony, 2003; Powell and Matthews, 2005). Germination tests give an indication of the maximum germination potential of a seed lot since seeds are subjected to ideal environmental conditions (ISTA, 2014). The acceptable level of germination may vary between countries. In general, however, for maize seed to be certified, a minimum percentage germination of 90 per cent is required

(MacRobert *et al*., 2014). Studies involving bacterial strains of, for example, *Pseudomonas, Azotobacter, Azospirillum* (Shaukat *et al*., 2006) and *Bacillus* (Niranjan *et al*., 2003; Kloepper *et al*., 2004), have indicated that these bacteria can improve seed germination, rate of germination, vigour, seedling growth and yield of important crops such as wheat (Shaukat *et al*., 2006), pearl millet (Niranjan *et al*., 2004) and maize (Nezaret and Gholami, 2009). Such bacteria are termed Plant Growth Promoting Rhizobacteria (PGPR) (Martínez-Viveros *et al*., 2010) and are described as a group of free-living rhizosphere-inhabiting bacteria that stimulate plant growth either directly by enhancing the plant's nutrient status, or indirectly by inhibiting plant pathogens (Vessey, 2003; Kumar *et al*., 2011). Some of the actinomycetes for example, *Streptomyces nobilis* are also reported to have influence on the seed germination and plant growth of wheat (Anwar *et al.*, 2016)

Gholami *et al.* (2009) evaluated the effect of six plant growth-promoting rhizobacteria (PGPR) *viz.*, *Pseudomonas putida* strain R-168, *P.fluorescens* strain R-93, *P.fluorescens* DSM 50090, *P.putida* DSM291, *A.lipoferum* DSM 1691, *A.brasilense* DSM 1690 on seed germination, seedling growth and yield of field grown maize in three experiments. Results of first study showed seed Inoculation significantly enhanced seed germination and seedling vigour of maize. In second experiment, leaf and shoot dry weight and also leaf surface area significantly were increased by bacterial inoculation in both sterile and non-sterile soil. The results showed that inoculation with bacterial treatments had a more stimulating effect on growth and development of plants in non sterile than sterile soil. In the third experiment, inoculation of maize seeds with all bacterial strains significantly increased plant height, 100 seed weight, number of seed per ear and leaf area .The results also showed significant increase in ear and shoot dry weight of maize.

Anwar *et al.* (2015) isolated 98 rhizospheric actinomycetes from different wheat and tomato fields of Punjab, Pakistan. These isolates were characterized based on their morphological, biochemical and molecular characteristics and were further subjected to seed germination bioassay and plant growth experiments. Significant increases in shoot length were observed with *Streptomyces nobilis* WA-3 (65%), increased root length was recorded in case of *S. nobilis* WA-3 (81%) as compared to water treated control plants. Maximum increases in plant fresh weight were recorded with *S. nobilis* WA-3 (84%), increased plant dry weight was recorded in case of *S. nobilis* WA-3 (85%) as compared to water treated control plants. In case of number of leaves, significant increase was recorded with *S.*

nobilis WA-3 (27%) while, significant increase in case of number of roots were recorded in case of strain *S. nobilis* WA-3 (30%) as compared to control plants.

2.9 PIGMENTS PRESENT IN THE ROOTS OF *Arnebia euchroma*

The roots of *Arnebia euchroma* are a good source of red naphthoquinone pigments. These metabolites have commercial importance as natural colorants in food, cosmetics, and textiles and exhibit various medicinal and pharmaceutical properties (Papageorgiou *et al*., 1999; Babula *et al*., 2009). The naphthoquinone pigments extracted from *Arnebia* species have antimicrobial, antiinflammatory, anti-viral, anti-tumor, cardiotonic and contraceptive properties (Chen *et al*., 2002; Shen *et al*., 2002; Singh *et al*., 2003). These derivatives also exhibit insulin like activity by inhibiting phosphatase and tensin homologue deleted on Chromosome 10 (PTEN) and protein tyrosine phosphatases (Nigorikawa *et al*., 2006). Studies have also revealed specific *in-vivo* and *in-vitro* antitumor effects of acetylshikonin (Xiong *et al*., 2009). It was found that this metabolite initiate tumor cell apoptosis processes. Acetylshikonin has been regarded as a wide spectrum therapeutic agent combating cancer.

Kumar *et al*. (2011) investigated *in-situ* extraction methods on naphthoquinone pigment yield in *A.euchroma* cell suspension cultures and observed that extraction resulted in significantly higher yield of naphthoquinone derivatives (637.15 mg L^{-1}) than control (369.38 mg L^{-1}), along with higher volumetric and specific productivities. The density of liquid paraffin also influenced the *in- situ* extraction. High Performance Liquid Chromatography (HPLC) chromatograms showed acetylshikonin as the major naphthoquinone derivative in the quantified fraction.

Haghbeen *et al*. (2011) collected the seeds of the Iranian *Arnebia euchroma* from Dena altitudes in the central Zagross, Iran. Chemical analysis showed that the dried root of the plant contained about 8.5 per cent (w/w) shikalkin pigment. The root explants of the young plantlets, obtained from the germinated seeds, were used for establishing callus. Further, the parameters effective on proliferation and pigment production of the resulting calli were also studied in detail. Accordingly, two modified media called mLS and mM9 were optimized for propagation and pigment production, respectively. Using these media, the biomass of the *A. euchroma* calli was increased to 600 per cent, and the pigment production reached to a maximum of 16.3 mg per gram of the wet biomass in a period of a subculture (21 days). Besides this, the antimicrobial activity of shikalkin pigment was examined on

some fungi and Gram-positive and Gram-negative bacteria. Results indicated that the pigment was almost ineffective on fungi and Gram-negative bacteria, but it was meaningfully effective against *Micrococcus luteus.*

Katoch *et al.* (2010) developed a simple, cost-effective and reliable method (Normal Phase-High Performance Thin-Layer Chromatography Method)for quantification of compounds present in *Arnebia benthami*.The developed method showed good band separation for shikonin (Rf, 0.37) and β,β-dimethylacryl shikonin (Rf, 0.58). The linearity ranged between 100 and 8,000 ng spot−1 with an average recovery of (>97%) in both cases. The High Performance Thin Layer Chromatography (HPTLC) fingerprints of different samples revealed maximum visibility of shikonin and β,β-dimethylacryl shikonin in ethyl acetate fractions (EAF and EACP). The results showed a higher content of both shikonin derivatives in EACP (24.84%) than that in EAF (8.41%), with β,β-dimethylacryl shikonin leading row.

Chapter-3

MATERIALS AND METHODS

The present study was undertaken in the Microbiology Research laboratory, Department of Basic Sciences, College of Forestry, Dr. Yashwant Singh Parmar, University of Horticulture and Forestry, Nauni- Solan (HP) India. The chemicals and media components used were of analytical grade (AR) obtained from Merck limited - India, Sigma-Aldrich Inc. USA and Hi Media Laboratories, Bombay, India. The materials used and the methods employed are presented below under the following sub-headings:

3.1 Study Area

3.2 Collection of soil sample

3.3 Determination of actinomycetes load in the rhizospheric soil samples of *Arnebia euchroma* (Ratanjot)

3.3.1 Standard Plate Count technique

3.4 Elucidation of actinomycetes diversity

3.5 Maintenance and preservation of actinomycete isolates

3.6 Difference in the growth behavior of same actinomycete isolate on different media

3.7 Screening of actinomycetes for multifarious plant growth promoting attributes

3.7.1 Phosphate-solubilization

3.7.1.1Qualitative estimation

3.7.1.2Quantitative estimation

3.7.2 Phytase activity

3.7.2.1 Qualitative estimation

3.7.2.2 Quantitative estimation

3.7.3 Siderophore Production

3.7.3.1 Qualitative estimation

3.7.3.2 Quantitative estimation

3.7.4 HCN Production

3.7.5 Ammonia Production

3.7.6 Nitrogen fixing ability

3.8 Determination of lytic enzymes production by actinomycete isolates

3.8.1 Chitinase activity

3.8.1.1 Preparation of colloidal chitin

3.8.1.2 Qualitative estimation

3.8.1.3 Quantitative estimation

3.8.2 Protease activity

3.8.2.1 Qualitative estimation

3.8.2.2 Quantitative estimation

3.8.3 Cellulase activity

3.8.3.1 Qualitative estimation

3.8.3.2 Quantitative estimation

3.8.4 Pectinase activity

3.8.4.1 Qualitative estimation

3.8.4.2 Quantitative estimation

3.8.5 Lipase activity

3.8.5.1Qualitative estimation

3.8.5.2 Quantitative estimation

3.9 Screening of actinomycetes for the production of plant growth regulators

3.9.1 Auxins production

3.9.2 Gibberellins production

3.9.3 Cytokinins production

3.10 Antagonistic activity of potential actinomycetes against soil-borne fungal phytopathogens

3.10.1 Antagonistic activity of the volatile metabolites secreted by the potential bacterial isolates on the growth of fungal pathogens

3.11 Identification and characterization of potential isolates

3.11.1 Morphological, physiological and biochemical characterization

3.11.2 Molecular characterization of potential actinomycetes isolates

3.12 Seed germination Bioassay

3.13 Detection of pigments present in the roots of *Arnebia euchroma*

3.1 STUDY AREA

Extensive survey was done for locating *Arnebia euchroma* -a valuable medicinal plant in two districts of Himachal Pradesh *viz.*, Kinnaur and Lahaul & Spiti. Kinnaur is located in

the North-eastern part of Himachal Pradesh and it shares its eastern boundary with Tibet. Most of the areas in Kinnaur have a temperate climate due to its high elevation. The lower parts receive monsoon rains whereas the upper areas of Kinnaur fall in the rain shadow area. Lahaul & Spiti is an integral part of Indian cold desert area of Northern- western Himalayan region. Located in the alpine arid zone of Himachal Pradesh, it is comprised of two subdivisions - Lahaul & Spiti. Despite the prevalence of extreme climatic conditions, Kinnaur and Lahaul –Spiti areas harbor valuable medicinal plant wealth. Accordingly, two villages namely Nako and Chango in Kinnaur and two i.e. Gue and Kibber in Lahaul and Spiti districts of Himachal Pradesh (Fig. 3.1) were selected for the collection of rhizospheric soil samples of *Arnebia euchroma.*

3.2 COLLECTION OF SAMPLES

The rhizospheric soil samples of *Arnebia euchroma* growing at four selected sites *viz*., Chango, Nako, Gue and Kibber, were collected in clean and sterilized polythene bags (Plate 3.1). Some samples were processed immediately while, other were kept in refrigerator at 4˚C for further use.

3.3 DETERMINATION OF ACTINOMYCETES LOAD IN RHIZOSPHERIC SOIL SAMPLES

Standard plate count technique was employed to determine actinomycetes load present in the collected rhizospheric soil samples of *Arnebia euchroma* (Ratanjot).

3.3.1 Standard Plate Count technique

All the rhizospheric soil samples were serially diluted to 10 folds by transferring 1 mL of sample to 9mL sterile dilution blank under aseptic conditions. Four different media *viz*., Nutrient Agar (NA), Actinomycetes Isolation Agar (AIA), Starch Casein Agar (SCA) and Kenknight Media (KM)(Appendix I) were used to determine the actinomycetes load by plating serial dilutions of samples on these media (Wollum, 1982). The plates were incubated at 28±2˚C for 7-15 days. Following incubation, number of colonies on each plate were counted and results were expressed in terms of log CFU/mL.

3.4 ELUCIDATION OF ACTINOMYCETES DIVERSITY

To elucidate the actinomycetes diversity present in the rhizospheric soil samples, different morphotypes of actinomycetes, appeared on NA, AIA, SCA and KM were selected on and purified on respective media.

3.5 MAINTENANCE AND PRESERVATION OF ACTINOMYCETES ISOLATES

The actinomycete isolates were grown on NA slants and preserved at 4°C in refrigerator for further use. The glycerol stocks of actinomycetes isolates were prepared in 30 per cent (v/v) glycerol and preserved at - 20 °C.

3.6 DIFFERENCE IN THE GROWTH BEHAVIOR OF SAME ACTINOMYCETE ISOLATE ON DIFFERENT MEDIA

To observe difference in the growth behavior of same actinomycetes isolate on different media *viz.*, NA, AIA, SCA and KM, the same actinomycete was streaked and grown on these media.The plates were incubated at 28±2°C for 7-15 days. Following incubation, difference in the colony characteristics, colour of aerial and substrate mycelia, diffusible pigment etc was observed (Kheiralla *et al*., 2016).

3.7 SCREENING OF ACTINOMYCETES FOR MULTIFARIOUS PLANT GROWTH PROMOTING ATTRIBUTES

3.7.1 Phosphate-solubilization

3.7.1.1Qualitative estimation

All actinomycete isolates were spot inoculated on Pikovskaya's (PVK) agar plates (Pikovskaya, 1948) supplemented with 0.5 per cent tri-calcium phosphate (TCP) (Appendix I). The plates were incubated at 28 ± 2°C for 7-15 days and the zones of P solubilization around the colonies were recorded in mm. The P solubilizing efficiency of the actinomycetes was calculated using the following formula:

Per cent solubilization efficiency (SE) = [(Z-C)/C] * 100, where

Z = P- solubilization zone (mm)

C = Colony diameter (mm)

The P- solubilization index (PSI) of isolates was calculated using following formula

PSI= (CD + HZ)/CD, Where CD = Colony diameter (mm) and HZ= Halo Zone diameter (mm)

3.7.1.2 Quantitative estimation of P- solubilization

Quantitative estimation of inorganic phosphate solubilization was done by employing Vanado-molybdate-yellow color method (Jackson, 1973). The actinomycete isolates were grown in 50 mL PVK broth supplemented with 0.5 per cent TCP (Appendix I). The broth

Fig. 3.1 Location map showing study area along with sample collection sites

Plate 3.1 ***Arnebia euchroma*** **(Ratanjot) growing at different sites (a) Nako (b) Chango (c) Kibber (d) Gue**

inoculated with 500 µL inoculum at 5×10^8CFU mL^{-1} was incubated for 15 days at 28±2°C at 180 rpm in an incubator shaker (Scigenics Biotech Orbitex). The cultures after incubation were harvested by centrifugation at 10,000 rpm for 10 min. The uninoculated autoclaved broth supplemented with the inorganic phosphate substrate was kept under the similar set of conditions to serve as control. To a 2.5 mL aliquot of Barton's reagent (Appendix II) was added 0.5 mL aliquot of the culture supernatant, and the final volume made to 50 mL with de-ionized water. The absorbance was read at 430 nm after 10 min in a UV/Vis. Spectrophotometer (6800, JENWAY). Total soluble phosphorus was estimated from the regression equation of standard curve of KH_2PO_4. P-liberated was expressed as µg mL^{-1} over the control. The pH of supernatants was measured using pH700 pH/mV/˚C/˚F Meter (Eutech Instruments). The standard curve was prepared by dissolving 0.2195 g KH_2PO_4 in 1 liter de-ionized water. A total of 100 mL de-ionized water was added to 150 mL of the stock solution to make the final concentration of 30 µg P mL^{-1}. To 1, 2, 3, 4, 5, 6, 8, 10, 15, 20 and 25 mL aliquots of the diluted stock, taken separately in 50 mL volumetric flasks, were added 2.5 mL Barton's reagent and the final volume made to 50 mL with de-ionized water. The absorbance was read at 430 nm after 10 min. The curve was plotted as concentration of KH_2PO_4 Vs optical density (Appendix IV). The regression equation was used to calculate the P-liberated.

3.7.2 Phytase activity

3.7.2.1 Qualitative estimation

All the actinomycete isolates were screened for the production of extracellular phytase by spot inoculated them on phytase specific medium plates (Chunshan *et al*., 2001) (Appendix I). The plates were incubated at 28 ± 2°C for 7-15 days and the zones of organic P- solubilization around the colonies were recorded in mm.

3.7.2.2 Quantitative estimation

Crude culture filtrate was used as an enzyme sample for the quantitative estimation of phytate using ammonium molybdate method (Quan *et al*., 2001). An aliquot of 150 µl of enzyme solution was incubated with 950 µl of substrate solution (Sodium phytate 4 mM, sodium acetate buffer 0.25M (Appendix III) and 1000 mL of distilled water, pH 4.5) at 37±2°C for 30 min. The reaction was stopped by adding 1 mL of 10 per cent Trichloro acetic acid (TCA). The released inorganic phosphate was analyzed by adding 2 mL of a coloring reagent (Ammonium molybdate 1 g, sulfuric acid 3.2 mL, ferrous sulfate 7.2 g and 100 mL of

distilled water) and the absorbance was read at 700 nm in UV/Vis. Spectrophotometer (6800, JENWAY).

A standard curve was plotted with Phytate standard which served as the reference for phytase activity. A 100 mL stock solution of KH_2PO_4 (10 µg/mL) was prepared in distilled water. A dilution series ranging from 2.5-25 µg/2.5 mL was prepared from the stock solution. A calibration graph (standard curve) was prepared with different phosphate levels according to the method of Fiske and Subbarow (1925) .This standard curve was used for the detection of the amount of inorganic phosphate (Pi) liberated due to the action of phytase enzyme by modified Fiske and Subbarow method. The procedure involved the addition of 750 µl of 5% TCA to 1 mL of dilution. This was followed by the addition of colour reagent (750 µl), prepared daily by mixing four volumes of 1.5 per cent (w/v) ammonium molybdate in a 5.5% (v/v) sulfuric acid solution and one volume of a 2.7% (w/v) ferrous sulfate solution. The reaction involved the production of phosphomolybdate, which was measured spectrophotometrically at 700 nm.

3.7.3 Siderophore production

3.7.3.1 Qualitative estimation

All glassware was cleaned with 6M HCl to remove iron. The initial screening for siderophore production by the actinomycete isolates was done on Chrome Azurol Sulphonate agar (CAS) plates (HiMedia, India) as described by Alexander and Zuberer,1991 (Appendix I). The plates were spot inoculated with 24 h cultures and incubated at 28±0.1°C for 30 days. The yellow-orange halo zones around the actinomycetes colonies revealed the production of siderophores. Siderophore production zone was calculated by subtracting the colony diameter from the diameter of total zone inclusive of the colony.

3.7.3.2 Quantitative estimation

Culture vials with 10 mL succinate broth (SB) was inoculated with 500 µl of actinomycete cultures grown in nutrient broth NB (Appendix I) for 7-15 days at 28 ± 2°C. The culture vials were incubated at 28 ± 2°C at 180 rpm for 7-15 days. The cultures were centrifuged at 13,000 rpm for 10 minutes. The quantification of siderophores was done by CAS-shuttle assay. To 0.5 mL aliquot of the culture supernatant was added 0.5 mL of CAS reagent (Appendix II) and the absorbance read at 630 nm in UV/Vis Spectrophotometer (6800, JENWAY).The absorbance was read against the reference consisting of 0.5 mL

aliquot of uninoculated SB and 0.5 mL of CAS reagent. Siderophore units were calculated using the formula:

% siderophore units = (Ar-As/Ar) x 100, where Ar = absorbance of the reference and As =absorbance of the sample

3.7.4 HCN production

The actinomycete isolates were screened for the production of HCN by adopting the method given by Bakker and Schippers (1987). The actinomycete isolates were streaked on NA amended with 4.4 g L^{-1} glycine. A Whatman filter paper No. 1 soaked in 2 per cent Na_2CO_3 prepared in 0.5 per cent picric acid solution was placed on the top of the plate. Plates were sealed with parafilm and incubated at 28 ± 2 °C for 15-30 days. The change in the colour of filter paper from orange to brown indicated HCN production by the actinomycete isolates.

3.7.5 Ammonia production

Ammonia production by actinomycete isolates was detected by the method given by Dye (1962).The actinomycete cultures were inoculated in 10 mL of peptone broth (Appendix I) and incubated at 28 ± 2°C for 3-7 days. After incubation, 1 mL of Nessler's reagent (Appendix II) was added, and the production of varying intensity of yellow (++) to brown (++++) colour in the test tubes indicated ammonia production by the actinomycete isolates

3.7.6 Nitrogen fixing ability

All the actinomycetes isolates were streaked on Jensen's medium (Appendix I). Plates were incubated at 28 ± 2˚C for 3-7 days and the isolates showing growth on plates were nitrogen fixers (Jensen, 1987).

3.8 DETERMINATION OF LYTIC ENZYMES PRODUCTION BY ACTINOMYCETES

3.8.1 Chitinase activity

3.8.1.1 Preparation of colloidal chitin

Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff (1988). Five grams of dried chitin (Hi-media) was suspended in 60 mL concentrated hydrochloric acid (HCl) and kept overnight at room temperature with vigorous stirring. To this mixture,

200 mL ice cold 95 per cent ethanol was added in order to precipitate the chitin and incubated overnight at 4ºC. The mixture was then centrifuged at 10,000 rpm for 20 min at 4ºC and the pellet of colloidal chitin was washed repeatedly with distilled water until it became neutral (pH 7.0). The colloidal chitin thus obtained was stored in a dark place at 4°C and further used as a substrate.

3.8.1.2 Qualitative estimation of chitinase activity

All actinomycete isolates were spot inoculated on Colloidal Chitin Agar (CCA) plates (Appendix I) containing 0.5 per cent colloidal chitin and incubated at 28 ± 2°C for 7 days (Rodriguez *et al*., 1993). After following incubation, chitinase activity was measured by subtracting the diameter of colony (mm) from the diameter of halo zone formed around the colony (mm).

3.8.1.3. Quantitative estimation of chitinase activity

Dinitrosalicyclic acid (DNSA) method was used for the determination of chitinase activity by the actinomycete isolates (Jha and Modi, 2017). The reaction mixture consisted of 2.5 mL buffer, 2.5 mL 1 per cent colloidal chitin and by 0.5 mL of crude enzyme. Tubes were incubated at 37± 2°C for 1 hr. The reaction was stopped by adding 3.0 mL of 10 per cent DNSA and heating in boiling water bath for 15min. The colored solution was centrifuged at 8000×g for 5 mins at 4°C. The optical density of supernatant was measured at 540 nm and reducing sugars were estimated from standard curve of glucose (Appendix IV).

The 10 mM stock solution of glucose was prepared by dissolving 1 mg of glucose in 100 mL of distilled water and the working solution of 100 µM concentration was prepared by diluting 1 mL of stock solution to 100 mL with distilled water. The standard curve was prepared by taking 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mL of working solution of glucose in different test tubes and final volume was made to 1.0 mL with sodium phosphate buffer (pH 6.8). The blank was constituted of 1 mL of sodium phosphate buffer (pH 6.8). Thereafter, 3.0 mL of dinitrosalicyclic acid (DNSA) reagent (Appendix II) was added to each tube and the samples were boiled for 5 min. in a water bath. After boiling, the samples were transferred immediately to a cold water bath. After 10 min, 20 mL of distilled water was added to the samples and the absorbance was read at 540 nm using UV/Vis. Spectrophotometer (6800, JENWAY).

3.8.2 Protease activity

3.8.2.1Qualitative estimation

All actinomycete isolates were spot inoculated on skim milk agar plates (Appendix I). Separately sterilized 1 per cent skim milk was added to nutrient agar before pouring in the plates and incubated at 28 ± 2°C for 6-7 days. Following incubation, the formation of a clear halo zone around the colony indicated positive proteolytic activity (Dunn *et al*., 1997). Protease activity was measured by subtracting the diameter of colony (mm) from the diameter of halo zone formed around the colony (mm).

3.8.2.2 Quantitative estimation

The cultures showing proteolytic activity were selected and grown in 100 mL of nutrient broth medium amended with 1 per cent skim milk. After 7 days of incubation at 28 ± 2°C, the cultures were centrifuged at 10,000 rpm for 10 min at 4°C. The cell-free supernatant was collected and passed through 0.22 µM nylon syringe filters and used as a crude enzyme in protease assay (Park *et al*., 2003).The protease activity was determined by caseinolytic assay method as described by Kanekar *et al.* (2002) with slight modifications. The reaction mixture constituted of 0.60 mL 50 mM sodium phosphate buffer (pH 7.0) (Appendix III) containing 2.0 per cent (w/v) of casein and 0.30 mL of enzyme solution. After incubating at 37± 2 °C for 1 h, the reaction was stopped by adding 1.2 mL of 10 per cent (w/v) TCA. The mixture was incubated at room temperature for 1 h. Following incubation, the reaction mixture was centrifuged at 12,000 g for 5 min. The supernatant was collected and the amount of casein hydrolyzed (by the release of tyrosine residues released) by the enzyme solution was measured using Folin-Ciocalteu Reagent (FCR) method. The supernatant was collected and the amount of casein hydrolyzed (by the release of tyrosine residues released) by the enzyme solution was measured using Folin-Ciocalteu method. To 2 mL of supernatant, 5 mL of sodium carbonate was added, followed by the addition of 1 mL of 1N Folin-Ciocalteau Reagent in test tubes. The test tubes were incubated for 30 min in dark at the room temperature and the absorbance of the samples was measured spectrophotometrically at 660 nm. The blank constituted of 0.30 mL of sodium phosphate buffer instead of 0.30 mL of enzyme solution. Control samples were assayed in the same manner as the test samples, except that the substrate was added at the end of the incubation period and after the enzyme had been inactivated by the addition of TCA. The amount of tyrosine residues released in the reaction mixture was calculated from the standard curve of tyrosine (Appendix IV).

The 10 mM stock solution of tyrosine was prepared by dissolving 181 mg of tyrosine in 100 mL of distilled water and the working solution of 100 µM concentration was prepared by diluting 1 mL of stock solution to 100 mL with distilled water. The standard curve was prepared by taking 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mL of stock solution of L-tyrosine in test tubes and the final volume of the samples was made 1 mL with distilled water. Thereafter, 2.5 mL of sodium carbonate was added, followed by the addition of 0.5 mL of 1N Folin-Ciocalteau Reagent in test tubes. The test tubes were incubated for 30 min in dark at the room temperature and the absorbance of the samples was measured spectrophotometrically at 660 nm using UV/Vis. Spectrophotometer (6800, JENWAY). Standard curve was prepared by plotting absorbance Vs concentration of L-tyrosine in µg mL^{-1}. Regression equation was used to calculate the amount of L-tyrosine residues liberated in the reaction mixtures by the crude enzyme.

3.8.3 Cellulase activity

3.8.3.1 Qualitative estimation

All actinomycete isolates were screened for their ability to solubilize cellulose in carboxymethyl cellulose (CMC) agar plates amended with 0.2 per cent of carboxymethyl cellulose (CMC) (Appendix I). The isolates were spot inoculated on the medium and after 7 days of incubation at 28 ± 2°C, plates were flooded with Gram's iodine (1g iodine in 100 mL of 2 per cent potassium iodide solution). A decolorized halo zone around the colony indicated the CMC degrading activity by the actinomycete isolates. Cellulase activity was measured by subtracting the diameter of colony (mm) from the diameter of halo zone formed around the colony (mm).

3.8.3.2 Quantitative estimation

Dinitrosalicyclic acid (DNSA) method was used for the determination of cellulase activity by the actinomycete isolates (Miller, 1959). The cultures showing cellulolytic activity were selected and grown in 100 mL of CMC broth medium amended with 0.2 per cent of CMC. After 7 days of incubation at 28 ± 2°C, the cultures were centrifuged at 10,000 rpm for 20 min at 4°C. The cell-free supernatants were collected and passed through 0.22 µM nylon syringe filters. The cell-free filtrates were used as a crude enzyme in cellulase assay. The reaction mixture comprising of 500 µM of 1 per cent CMC solution prepared in 50 mM sodium phosphate buffer (pH 6.8) and 500 µL of crude enzyme was incubated at 50°C for 30

min. After incubation, the reaction was terminated by adding 3.0 mL DNSA reagent and placed in a boiling water bath for 5 min. After boiling, the samples were transferred immediately to a cold water bath for 10 min. Thereafter, 20 mL of distilled water was added to the reaction mixture and the colour change in the samples was measured spectrophotometrically by taking absorbance at 540 nm. The blank comprised of 500 µL of sodium phosphate buffer (pH 6.8) in place of enzyme aliquot in the reaction mixture. In control, 500 µL enzyme solution was added to 500 µL of 1 per cent CMC solution prepared in 50 mM sodium phosphate buffer (pH 6.8) after the reaction has been stopped by the addition of DNSA reagent followed by the boiling of samples in a water bath for 5 min. The amount of glucose released in the reaction mixture was calculated from the standard curve of D-glucose (Appendix IV).

The 10 mM stock solution of glucose was prepared by dissolving 180 mg of glucose in 100 mL of distilled water and the working solution of 100 µM concentration was prepared by diluting 1 mL of stock solution to 100 mL with distilled water. The standard curve was prepared by taking 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mL of working solution of glucose in test tubes and making the volume to 1.0 mL with sodium phosphate buffer (pH 6.8). The blank constituted of 1 mL of sodium phosphate buffer (pH 6.8). Thereafter, 3.0 mL of dinitrosalicyclic acid (DNSA) reagent was added to each tube and the samples were boiled for 5 min. in a water bath. After boiling, the samples were transferred immediately to a cold water bath. After 10 min, 20 mL of distilled water was added to the samples and the absorbance was read at 540 nm using UV/Vis. Spectrophotometer (6800, JENWAY). Standard curve was prepared by plotting absorbance at 540 nm Vs concentration of glucose in $\mu g\, mL^{-1}$.

3.8.4 Pectinase activity

3.8.4.1 Qualitative estimation

The pectinolytic activity of actinomycetes was checked on Pectinase screening media (Appendix I) (Kumar and Sharma, 2012). The plates were spot inoculated with actinomycetes isolates and incubated at 28 ± 2°C for 6-7 days. After 7 days of incubation, plates were flooded with Gram's iodine. The formation of a clear zone around the colony indicated the pectinolytic activity by the isolates. Zone of pectin hydrolysis was measured by subtracting colony diameter (mm) from the diameter of zone formed around the colony (mm).The colonies forming more than 1.0 mm halo zone size were selected for quantitative estimation.

3.8.4.1 Quantitative estimation

The pectinase activity in the culture supernatants was estimated using the method given by Miller (1959). The actinomycetes isolates were grown in nutrient broth amended with 2.0 per cent pectin and 0.1 per cent yeast extract, and incubated at 28 ± 2°C at 150 rpm in an incubator shaker. After 7 days of incubation, the cultures were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cell-free supernatant was collected, passed through 0.22 µM nylon syringe filter and used as a crude enzyme. One hundred microlitre of enzyme solution was incubated at 37± 2°C for 1 h with 400 µL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.25 per cent pectin. After incubation, the reaction was stopped by adding 1.5 mL DNSA reagent. The reaction mixture was then placed in a boiling water bath for 5 min. After boiling, the samples were transferred immediately to a cold water bath for 10 min. Thereafter, 20 mL of distilled water was added to the reaction mixture and the colour change in the samples was measured spectrophotometrically by taking absorbance at 540 nm. The blank comprised of 400 µL of 50 mM sodium phosphate buffer (pH 7.0) in place of enzyme aliquot in the reaction mixture. In control, 100 µL enzyme solution was added to 400 µl of 50 mM sodium phosphate buffer (pH 7.0) containing 0.25 per cent pectin after the reaction has been stopped by the addition of DNSA reagent followed by the boiling of samples in a water bath for 5 min. The amount of galacturonic acid released in the reaction mixture was calculated from the standard curve prepared using glucose as a standard ($\mu g\, mL^{-1}$) (Appendix IV).

The standard curve was made from the stock solution of glucose. The enzyme activity was expressed in terms of U/mL. Unit of enzyme activity represents µ moles of glucose released/min/mL of enzyme. The 10 mM stock solution of glucose was prepared by dissolving 181 mg of glucose in 100 mL of distilled water and the working solution of 100 µM concentration was prepared by diluting 1 mL of stock solution to 100 mL with distilled water. The standard curve was prepared by taking 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mL of working solution of glucose in test tubes and making the volume to 1.0 mL with sodium phosphate buffer (pH 6.8). The blank constituted of 1 mL of sodium phosphate buffer (pH 6.8). Thereafter, 3.0 mL of dinitrosalicyclic acid (DNSA) reagent was added to each tube and the samples were boiled for 5 min. in a water bath. After boiling, the samples were transferred immediately to a cold water bath. After 10 min, 20 mL of distilled water was added to the samples and the absorbance was read spectrophotometrically at 540 nm using

UV/Vis. Spectrophotometer (6800, JENWAY). Standard curve was prepared by plotting absorbance at 540 nm Vs concentration of glucose in $\mu g\, mL^{-1}$.

3.8.4 Lipase activity

3.8.4.1 Qualitative estimation

All actinomycete isolates were spot inoculated on tributyrin agar medium (Appendix I) (Cardenas *et al*., 2001) and incubated at 28 ± 2 °C for 7 days. The formation of a clear zone around the colony indicated positive lipase activity by the actinomycetes.

3.8.4.2 Quantitative estimation

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis **(**Lawrence *et al*., 1967). One mL of the culture supernatant was added to the reaction mixture containing 1 mL of 0.1 M Tris– HCl buffer (pH 8.0)(Appendix III), 2.5 mL of deionized water and 3 mL of olive oil and incubated at 37° ± 2 °C for 30 min. Both test (in which all the reaction constituents were added with enzyme) and blank (in which all the reaction mixture were added without enzyme) were performed. After 30 min, the test solution was transferred to a 50 mL Erlenmeyer flask and 3 mL of 9 per cent ethanol was added to stop the reaction. Liberated fatty acids were titrated against 0.1 M sodium hydroxide (NaOH) (Appendix II) using phenolphthalein as an indicator. End point was an appearance of pink color. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per min under specified assay conditions. Lipase activity was expressed in terms of IU/mL using the following formula.

Enzyme activity (IU/mL) = (Volume titer NaOH)×(Molarity of NaOH)×(1000)×(2)×(df)/(1)

3.9 SCREENING OF ACTINOMYCETES FOR THE PRODUCTION OF PLANT GROWTH REGULATORS

3.9.1. Indole Acetic Acid (Auxins) production

Indole acetic acid production was quantitatively measured by the method given by Gordon and Weber (1951). All actinomycete isolates were grown in a Nutrient broth amended with tryptophan (0.1%) at 28±2°C for 6-7 days. Cultures were centrifuged at 10,000 rpm for 20 min. Two mL of supernatant was mixed with two drops of orthophosphoric acid and 4 mL of Salkowski reagent (Appendix II). Tubes were incubated at room temperature for 25 minutes. The intensity of pink color was read at 530 nm spectrophotometrically and the

amount of IAA produced was extrapolated from the standard curve. Standard curve was prepared by taking 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mL of standard IAA solution in each test tubes. The volume was made to 2 mL with distilled water and 4 mL of Salkowski reagent was then added in all tubes. Tubes were incubated for 25 minutes at room temperature and optical density was read at 530 nm. Standard curve was prepared by plotting absorbance at 530 nm Vs concentration of IAA solution in $\mu g\, mL^{-1}$(Appendix IV).

3.9.2 Gibberellins production

The production of gibberellins was estimated calorimetrically by the method of Holbrook *et al*. (1961) with slight modifications. To 15 mL of supernatant, 2 mL of zinc acetate reagent was added. After 2 min, 2 mL of potassium ferrocyanide (10.6% of potassium ferrocyanide in distilled water) was added and centrifuged at low speed (2000 rpm) for 15 min. To 5 mL of supernatant, 5 mL of 30 per cent HCI was added and mixture was incubated at 20±2°C for 75 min. For blank, 5 mL of 5 per cent HCI was used and absorbance was read at 254 nm. Concentration of gibberellins was calculated from the standard curve (Appendix IV) of gibberellic acid (GA_3, Hi-media).

3.9.3 Cytokinins production

Radish cotyledons expansion bioassay test was employed for estimation of cytokinins like substances (Letham, 1971). The radish seeds (*Raphanus sativus* L. cv Japanese white) which were procured from the Directorate of Extension, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni-Solan (HP) were germinated in total darkness for 48 h at 28±2°C. After removing the seed coat, smaller cotyledons were transferred to sterilized petridishes containing the 7 mL of test solution/ distilled water (control)/standard (kinetin) on filter paper strips. Twelve cotyledons were placed in each petri dish and were incubated at 25°C under fluorescent light for 3 days. Then cotyledons on filter paper strips in petridishes were blot dried and weighed. The dose response curve (final weight-initial weight) was plotted and expressed as increase in weight of cotyledons. Concentration of cytokinins present in the extract was calculated from standard curve (Appendix IV) of kinetin (10-100 µg/mL).

3.10 ANTAGONISTIC ACTIVITY OF POTENTIAL ACTINOMYCETES AGAINST SOIL-BORNE FUNGAL PHYTOPATHOGENS

The antagonistic activity of actinomycete isolates against fungal plant pathogens was measured using dual culture technique (Berg *et al*., 2000). The 5 mm mycelia disk from the

periphery of five days old cultures of each fungal phytopathogen *viz., Fusarium oxysporum, Rhizoctonia solani and Sclerotium rolfsii* grown on Potato Dextrose Agar (PDA) (Appendix I) was placed in the centre of NA plates. The actinomycete cultures were streaked on the four sides of plate, 2.5 cm away from the centre. Fungal growth without microbial inoculums was used as control. Plates were incubated at 28±2°C for 6-7 days and the growth of the fungal pathogen in both test and control experiments was recorded. The percent growth inhibition of fungal pathogens by actinomycete isolates over control was calculated as described by Whipps (1987).

Per cent inhibition = [(C-T)]/C × 100, where

C = Radius of fungal pathogen in control (mm) and T= Radius of fungal pathogen in treatment (mm)

3.10.1 Antagonistic activity of the volatile metabolites secreted by the potential actinomycete isolates on the growth of fungal pathogens

The effect of volatile metabolites released by actinomycete isolates on the mycelial growth of fungal phytopathogens was evaluated by 'inverted plate technique' as described by Fiddman and Rossall (1993). The actinomycetes isolates to be tested for their antagonistic activity against the fungal phytopathogens were streaked on Nutrient Agar (NA) (Appendix I) plates and incubated at 28 ± 2°C. The uninoculated NA plate was taken as control. At the end of incubation period, the top of each petri plate was replaced with the bottom of the petri plate inoculated in centre with the 5 mm mycelial disk of each fungal pathogen *viz., Rizoctonia solani, Sclerotium rolfsii* and *Fusarium oxysporum.* The two petri plates were then sealed together with the parafilm which avoided the escape of volatile compounds produced by the bacterial isolates and ensured their inhibitory effect on the growth of fungal plant pathogen. The plates were incubated at 28 ± 2ºC for 5 days and the per cent radial growth inhibition of the fungal phytopathogen by the volatile metabolites secreted by the bacterial isolates; relative to control was calculated as described by Whipps (1987) as described in the section 3.10.

3.11 IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL ISOLATES

3.11.1 Morphological, physiological and biochemical characterization

All the potential isolates were identified and characterized on the basis of their morphological, physiological and biochemical characteristics according to Standard methods described in Bergey's Manual of Systematic Bacteriology (Vos *et al*., 2009).

3.11.1.1 Colony morphology and pigment production

Colony characteristics of potential isolates *viz*., colour, elevation, margin and form, Gram's reaction and pigment production were observed (Sreevidya *et al*., 2016).

3.11.1.2 Physiological characterization

Optimum temperature and pH for the growth of actinomycete isolates were observed by inoculating them in 10 mL of Nutrient Broth (Appendix I) tubes. The tubes were then incubated at different temperatures *viz.*, 28˚, 37˚, 45˚, and 55˚C and pH *viz.*,4, 7, 9 and 12. The growth was observed at 600 nm in terms of optical density after 7 days of incubation using UV/Vis. Spectrophotometer (6800, JENWAY) (Akond *et al*., 2016)

3.11.1.3 Biochemical characterization

Biochemical characteristics of isolates including citrate utilization, Methyl red, Vogues Proskauer (VP), indole test, catalase, oxidase, carbohydrate fermentation test, oxidative- fermentation test etc were investigated (Chaudhary *et al*., 2013).

3.11.1.3.1 Catalase test

Small amount of culture was mixed with drops of 3 per cent hydrogen peroxide (H_2O_2) on clean glass slide. Catalase positive culture produced bubbles of gas within two seconds after mixing with H_2O_2.

3.11.1.3.2 Gelatin liquefaction test

Nutrient gelatin (Appendix I) deep tubes were prepared and stab inoculation of each isolate was made. All uninoculated deep tube was kept as a control. All inoculated tubes and uninoculated tubes were incubated at 28±2˚C for 5-7 days. After incubation, the tubes were placed in refrigerator at 4°C for 15 min. Deep gelatin tubes that remain liquefied indicated positive test for gelatin hydrolysis.

3.11.1.3.3 Indole test

Tryptone broth (Appendix I) was prepared and transferred into test tubes. The tubes were then inoculated with cultures while one tube was kept uninoculated as control. Tubes were then incubated at 28±2ºC for 5 days. After incubation, 1 mL of Kovac's reagent (Appendix II) was added to each test tube including control. Tubes were shaked gently after

intervals for 10-15 min and allowed to stand. Development of cherry red color in the top layer of the tube indicated positive result.

3.11.1.3.4 Methyl red and Voges - Proskauer Test (MR-VP)

MR-VP broth (Appendix I) was inoculated with actinomycetes isolates and uninoculated tube was kept as control. Tubes were incubated at 28±2ºC for 5 days.

In MR test, methyl red indicator was added, (Appendix II) changing the color from yellow to red indicated positive MR test.

In case of VP test, VP reagent was added (Appendix II) to VP broth inoculated with culture. Appearance of strong red color which changed into crimson red in 30 mins indicated positive test.

3.11.1.3.5 Citrate Utilization Test

Simmon's citrate agar (Appendix I) slants were prepared. After sterilization, slants were inoculated with the culture by means of stab and streak inoculation and one tube was kept uninoculated as control. These tubes were incubated at 28±2ºC for 5 days. Change of color from green to blue indicated positive result and no change in color indicated negative result.

3.11.1.3.6 Carbohydrate fermentation Test

Tubes of fermentation media (pH 7.3) were inoculated with different isolates separately followed by the incubation at 28±2ºC for 5 days. Tubes were then examined for the change in colour to yellow on acid production and formation of gas bubble on gas production.

3.11.1.3.7 Oxidase Test

Pieces of filter paper soaked in 1 per cent aqueous tetra methyl- p- phenylenediamine dihydrochloride. Fresh young cultures were taken with a clean platinum wire or tip of glass rod and rubbed on filter paper. Appearance of blue colour within ten seconds indicated positive oxidase test.

3.11.1.3.8 Oxidative fermentation (OF) test

Two tubes of OF test medium (Appendix I) were inoculated with the test organism using a straight wire by stabbing "half way to the bottom" of the tube. One tube of each pair

was covered with 1 cm layer of sterile mineral oil or liquid paraffin (it creates anaerobic condition in the tube by preventing diffusion of oxygen), leaving the other tube open to the air. Both tubes were incubated at 28±2ºC for 5-7 days. Acid production was detected in the medium by the appearance of a yellow color. In the case of oxidative organisms, color production may be first noted near the surface of the medium.

3.11.1.3.9 Triple Sugar Iron (TSI) Agar

The culture to be tested was inoculated in the butt of TSI agar (Appendix I) by stabbing and slants by streaking. The slants were then incubated at 28±2ºC for 4-5 days. Colour change of slants from red to yellow indicated the positive TSI test.

3.11.2 Molecular characterization of potential isolates

3.11.2.1 Isolation of genomic DNA

Genomic DNA of potential isolates was extracted using commercial Quick-DNA™ Fungal/Bacterial Microprep kit (Zymo) by following the manufacturer's instructions.

3.11.2.2 Gel electrophoresis

The electrophoresis of the genomic DNA was carried out in 1.0 per cent agarose gel stained with ethidium bromide (0.5 mg/mL), under submerged conditions using 1×TAE (Tris-Acetate EDTA) buffer (Appendix III) as tray buffer. To each 7 µl of genomic DNA sample, 3 µl of 1 X loading dye was added. 100-1500 bp DNA ladder marker was used as standard and the gel was run at 100 V until the loading dye reached the gel front. The genomic DNA of each isolates were viewed under the BIO-RAD gel documentation system in the form of sharp band used for further molecular studies and stored at -20°C. Isolated DNA samples were sent for sequencing at National Centre for Cell Sciences (NCCS), National Centre for Microbial Resource (NCMR) Pune, Maharashtra (India).

3.11.2.3 Sequence and phylogenetic analysis

The 16S rRNA sequences of different actinomycete isolates were analyzed for homology with library sequences using NCBI BLASTN program (http://www.ncbi.nih.gov/blast; Altschul *et al*., 1997). Phylogenetic analysis was constructed using MEGA 7.0 software by neighbour-joining method (Tamura *et al*., 2007). Confidence limits on grouping were done by the bootstrapping technique (1,000 repeats).

3.12 SEED GERMINATION BIOASSAY

Effect of actinomycete isolates on root elongation was evaluated by seed germination bioassay (Dey *et al*., 2004). Seeds of Maize and Pea were surface sterilized with 4% sodium hypochlorite (NaOCl) for 3 min, washed thrice with sterile de-ionized water and incubated at 25 ± 2°C on 1 per cent water agar plates for 48 hour for germination. The cultures were grown in NB at 28 ± 2°C for 7 days. UniformLy germinated seeds were dipped in 20 mL actinomycetes cultures for 1 h. The seeds were transferred to petriplates lined with wet filter paper and incubated at 28 ± 0.1°C in dark. The root length of the treated seedlings was recorded after 5 d of incubation as compared to the seedlings treated with uninoculated NB.

3.13 DETECTION OF PIGMENTS PRESENT IN THE ROOTS OF *Arnebia euchroma* (Ratanjot)

Detection of shikonin and its derivatives present in roots of *Arnebia euchroma* (Ratanjot) was done using Ultra Performance Liquid Chromatography (UPLC) performed at Institute of Himalayan Bioresource Technology (IHBT), Council of Scientific and Industrial Research (CSIR), Palampur (HP).

3.13.1 Sample preparation

Root extract sample of *Arnebia euchroma* was dissolved in 70 per cent methanol (LC-MS grade) to make up the concentration of 20 mg/mL and filtered with 0.22µ PTFE syringe filter. The samples were subjected for Ultra Performance Liquid Chromatography- Mass spectrometer (UPLC-MS) analysis.

3.13.2 Standard preparation

The standards Shikonin, Deoxyshikonin, and ββ-dimethylacrylshikonin were used for the qualitative and quantitative analysis by Ultra Performance Liquid Chromatography- Photo Diode Array (UPLC-PDA). Standard stock solutions were prepared in 1 mg/mL concentration. The standards were dissolved in Methanol (MeOH) (LC-MS grade) and filtered through 0.2µm filter and stored at 4˚C for further analysis. Working solutions (10ppm – 200ppm) were prepared by six serial dilutions of working solution of calibration curve. Identification of standard on UPLC was done on the basis of UV wavelength, retention time, while the quantification was on the basis of UV by comparing the area under the peak in UPLC (Table 3.1).

Table 3.1 Analysis of *Arnebia euchroma* (Ratanjot) root pigments by HPLC/UPLC-PDA/UPLC-MS using Isocratic method

Sample concentration	20 mg/mL
Standard concentration	1 mg/mL
Solvent system	A: Acetonitrile (90%)
	B: 0.1% Formic acid in water (10%)
Column	Agilent RRHD C18, 1.8µ 2.1*150mm
UV detection	274nm
Flow rate	0.220 mL/min
Run time	8min

Chapter-4

RESULTS AND DISCUSSION

The experimental results obtained from the present investigation are presented and discussed here in light of existing literature under the following subheadings:

4.1 Actinomycetes load in the rhizospheric soil samples of *Arnebia euchroma* (Ratanjot)

4.2 Actinomycetes diversity in the rhizosphere of *Arnebia euchroma* (Ratanjot)

4.3 Difference in the growth behavior of same actinomycete isolate grown on different media

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- 4.4.1 Phosphate-solubilization
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  - 4.4.2.1 Qualitative estimation
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4.8 Seed germination Bioassay
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4.10 Conservation of potential actinomycete isolates

4.1 ACTINOMYCETES LOAD IN THE RHIZOSPHERIC SOIL SAMPLES OF *Arnebia euchroma* (RATANJOT)

The rhizosphere of medicinal plants harbors a distinctive plethora of microorganisms due to their unique and structurally divergent bioactive secondary metabolites that are most likely responsible for the high specificity of the associated microorganisms (Qi *et al.,* 2012). Although, higher density of microbes in the rhizosphere has been reported in many plants (Timmusk *et al.,* 2011; Berendsen *et al.,* 2012; Gaiero *et al.,* 2013), there is only one study conducted by Devi and Kaundal (2017) on the number and composition of microbial communities associated with critically endangered medicinal plant i.e. *Arnebia euchroma* (Ratanjot).

The perusal of data depicted in Table 4.1 reveals a statistically significant difference in the actinomycetes load observed at four different sites *viz*., Nako, Chango, Gue and Kibber

and on four different media *viz.*, Starch Casein Agar (SCA), Actinomycetes Isolation Agar (AIA), Nutrient Agar (NA) and Kenknight and Munaier's Medium (KM) used for their isolation. The actinomycetes load was found to be significantly higher at Chango (Log CFU/mL=1.44), followed by Nako (Log CFU/mL=1.40) and Gue (Log CFU/mL=1.38) and lowest at Kibber (Log CFU/mL=1.33) as depicted in Fig. 4.1 and Plate 4.1. Likewise, four different media showed statistical difference in the actinomycetes load. Amongst all, a significantly higher actinomycetes load was observed on SCA (Log CFU/mL=1.48) followed by AIA (Log CFU/mL=1.41), NA (Log CFU/mL=1.29) and KM (Log CFU/mL=0.88) proving SCA as best medium for the isolation of actinomycetes. The interaction between different sites and media on actinomycetes load was found to be statistically significant thereby indicating that type of media as well as sites had significantly affected the actinomycetes population.

It is evident from these results that rhizosphere of *A.euchroma* anchorages a quite good population density of actinomycetes which may be attributed to the deposition of various root exudates like sugars, amino acids, organic acids, aromatics, polysaccharides, proteins and various other secondary metabolites in the rhizosphere. However, overall population of actinomycetes was low in comparison to the actinomycetes obtained from the rhizosphere of other plants like *Kochia indica* (Yakoob *et al.*, 2013). Low population density of actinomycetes may be explained in the light of facts that area under study falls in the cold temperate zone of the Himachal Pradesh where the climatic conditions are harsh. Secondly, there may be some selective pressure exhibited by the root exudates of *A. euchroma* which permitted only selective types of organisms to thrive in the rhizospheric soil.

Table 4.1 Total viable count of actinomycetes in the rhizosphere of *Arnebia euchroma* (Ratanjot) obtained from different sites on different media

Districts	Sites	Isolation media				
		NA* (Log CFU/mL)	KM** (Log CFU/mL)	SCA*** (Log CFU/mL)	AIA**** (Log CFU/mL)	Mean
Kinnaur	Chango	1.47	1.14	1.51	1.67	1.44
	Nako	1.25	1.48	1.46	1.50	1.40
Lahaul & Spiti	Gue	1.27	1.49	1.44	1.17	1.38
	Kibber	1.20	1.30	1.53	1.30	1.33
	Mean	1.29	0.88	1.48	1.41	
CD (0.05) M* : 0.017 ; S* : 0.017; S×M: 0.034			M*= Type of media S*=Sampling sites			

*- Nutrient Agar; **- Kenknight and Munaier's Medium; ***- Starch Casein Agar; ****- Actinomycetes Isolation Agar

Our results are also in accordance with Karthikeyan *et al.* (2008) who reported comparatively higher actinomycetes population of 12.22×10^5 CFU/g in *Ocimum sanctum*, 10.44×10^5 CFU/g in *Catharanthus roseus*, 8.44×10^5 CFU/g in *Aloe vera* and 6.22×10^5 CFU/g

in *Coleus forskholii*. Similarly, an investigation conducted by Tamilarasi *et al.*(2008), highest actinomycetes population (7.6 x 10^3 CFU/g) was reported to be associated with *Coleus amboinicus* while, minimum (8 x 10^2 CFU/g) with *Annona squamosa* and *Eclipta alba*.

4.2 ACTINOMYCETES DIVERSITY IN THE RHIZOSPHERE OF *Arnebia euchroma* (RATANJOT)

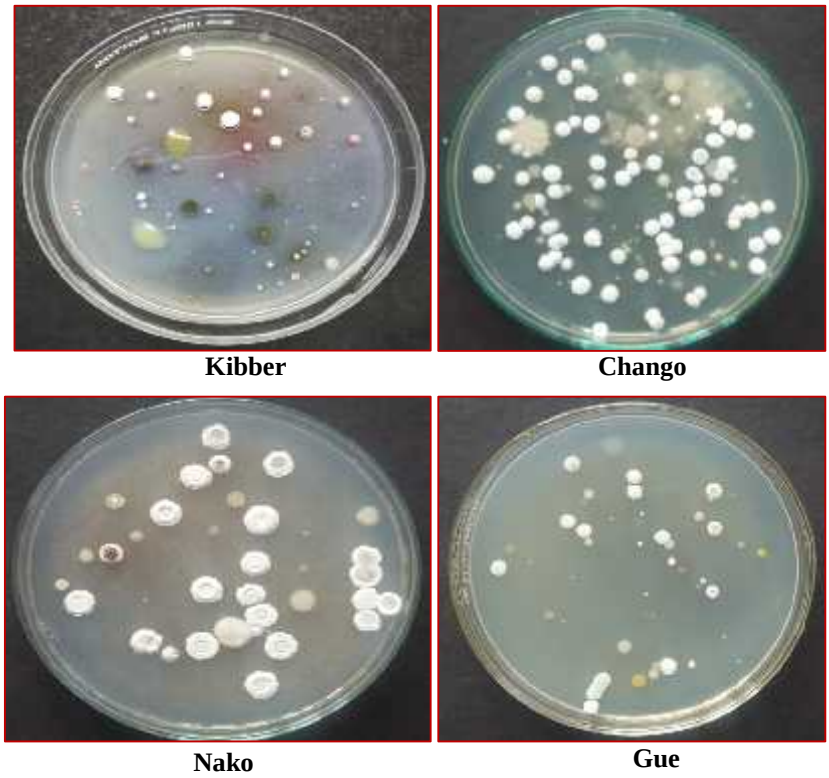
Microbial diversity studies are important in order to understand the microbial ecology in soil and other ecosystems (Joshi *et al*., 2011). All the rhizospheric soil samples of *A. euchroma* were processed for elucidating actinomycetes diversity present in them. In total, 33 different morphotypes of actinomycetes were obtained from all samples based on differences in their colony morphological features. Among these, 15 morphotypes were obtained from Nako, 7 from Chango, 6 from Gue and 5 from Kibber. Hence, percentage of actinomycetes morphotypes was observed to be highest at Nako (45.4%), followed by Chango (21.2%) and Gue (18.1%), and comparatively lowest (15.1%) at Kibber (Table 4.2, Fig 4.2 and Plate 4.2). Thus, it can be inferred from these results that in the rhizosphere of *Arnebia euchroma* exhibits overall a good diversity of actinomycetes. This could be ascribed to the facts that actinomycetes possess an unusual metabolic potential which enables them to survive under a variety of harsh environmental conditions like aridity and low water activity, generally, prevailing in the cold temperate zone of Himachal Pradesh. Secondly, their ability to produce motile spores makes their dispersal and survival easy in the arid regions (Okoro *et al*., 2009; Nawani *et al*., 2013; Dunckan *et al*., 2015; Buedenbender *et al.*, 2017).

Table 4.2 Elucidation of actinomycetes diversity in the rhizosphere of *Arnebia euchroma* (Ratanjot)

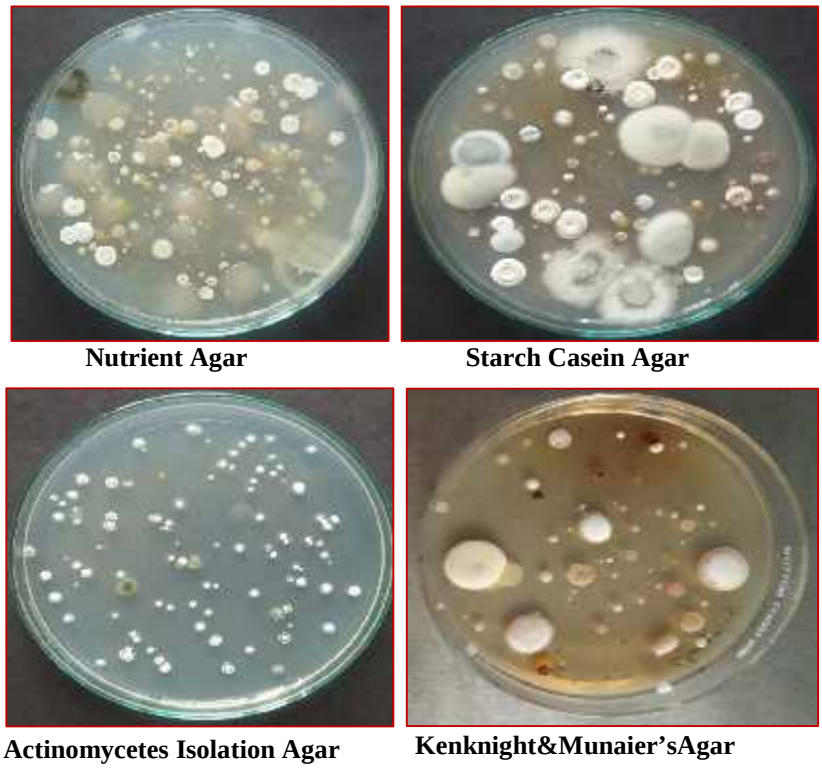
District	Sites	Number of different morphotypes
Kinnaur	Nako	15
	Chango	7
Lahaul & Spiti	Gue	6
	Kibber	5
	Total	**33**

4.3 DIFFERENCE IN THE GROWTH BEHAVIOR OF SAME ACTINOMYCETE ISOLATE GROWN ON DIFFERENT MEDIA

The data presented in Table 4.3 and Plate 4.3 reveals that same actinomycete isolate showed variations in their morphological features like shape, size, elevation etc along with pigments production on different media used for their enumeration. Moderate to excessive pigment production was observed among the isolates.



(a)



(b)

Plate 4.1 Difference in the actinomycetes load from *Arnebia euchroma* obtained at (a) different sites and (b) on different media

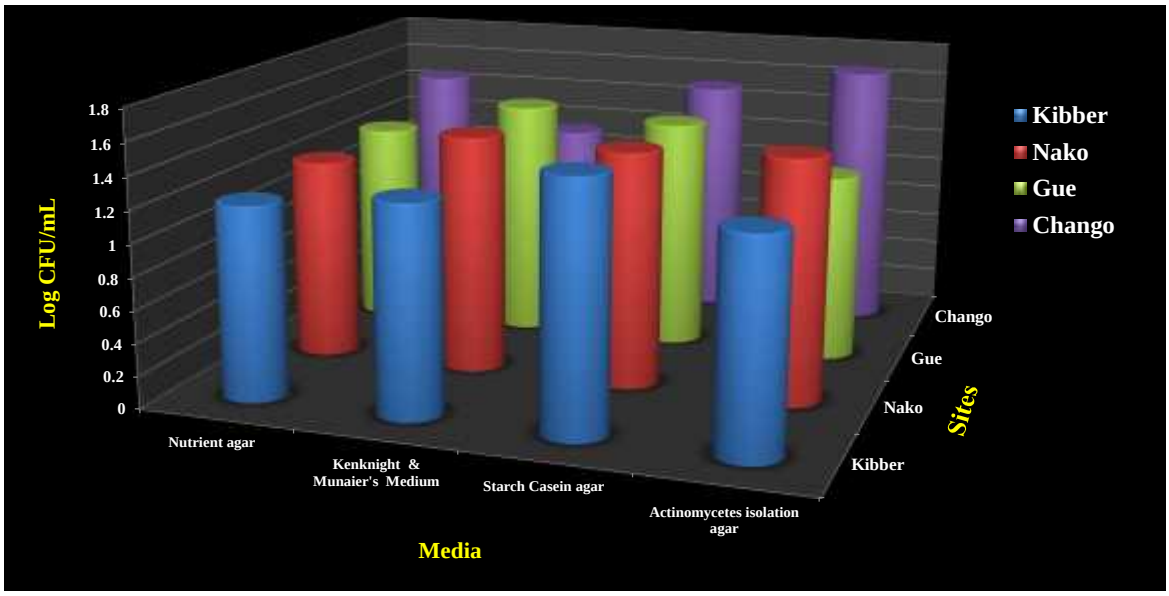


Fig. 4.1 Difference in the total viable counts of actinomycetes in the rhizosphere of *Arnebia euchroma* (Ratanjot) obtained from different sites on different media

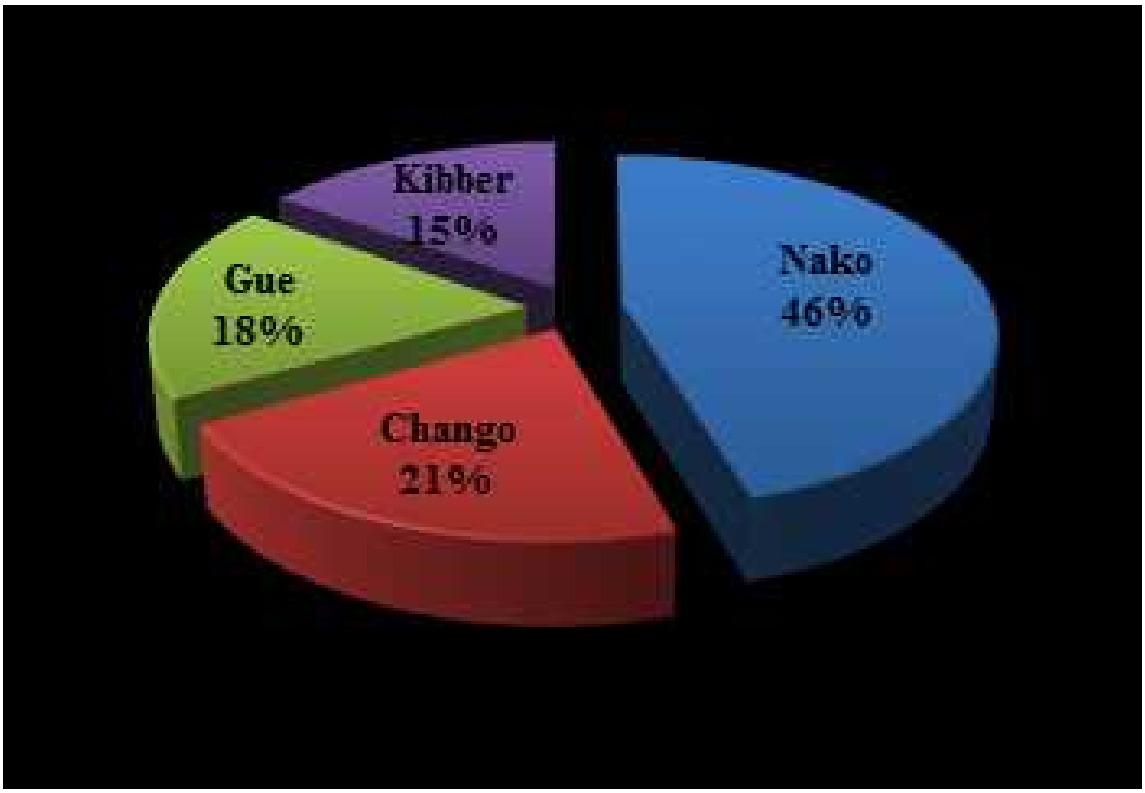


Fig. 4.2 Difference in the percentages of actinomycetes isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Plate 4.2 Diverse morphotypes of actinomycetes obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Table 4.3 Difference in the growth behavior of same actinomycete isolate on different media

Sr. No	Isolates	Nutrient Agar					Actinomycetes isolation agar					Starch casein agar					Kenknight and Munaier's Medium				
		Colour of aerial mycelium	Colour of substrate mycelium	Diffusible pigment	Elevation	Surface	Colour of aerial mycelium	Colour of substrate mycelium	Diffusible pigment	Elevation	Surface	Colour of aerial mycelium	Colour of substrate mycelium	Diffusible pigment	Elevation	Surface	Colour of aerial mycelium	Colour of substrate mycelium	Diffusible pigment	Elevation	Surface
1	NA1	Cream	Pale yellow	-	Flat	Smooth	Cream	Light yellow	-	Convex	Smooth	Grey	Light yellow	-	Flat	Smooth	White	Light yellow	-	Raised	Smooth
2	NA2	Yellow	Light yellow	-	Flat	Smooth	Cream	Light yellow	-	Flat	smooth	Light yellow	Cream	-	Raised	Smooth	White	Light yellow	-	Flat	Smooth
3	NA3	Yellow	Yellow	-	Umbonate	Smooth	Cream	Light yellow	-	Umbonate	Rough	Yellow	Yellow	-	Umbonate	Smooth	Cream	Cream	-	Raised	Smooth
4	NA4	Brown	Light brown	-	Umbonate	Smooth	Crem	Cream	-	Raised	smooth	Cream	Cream	-	Raised	Rough	White	White	-	Convex	Rough
5	NA5	Grey	White	-	Convex	Rough	Grey	Grey	-	Convex	Rough	Grey	Grey	-	Raised	Rough	Light grey	Light grey	-	Convex	Rough
6	GA1	White	Yellow	-	Undulate	Rough	Cream	Light yellow	-	Umbonate	smooth	White	White	+	Undulate	Smooth	Cream	Cream	-	Undulate	Smooth
7	GA2	White	Yellow	-	Undulate	Rough	White	White	-	Umbonate	Rough	Grey	Light yellow	-	Flat	Smooth	Light yellow	White	-	Raised	Smooth
8	GA4	White	Yellow	-	Flat	Rough	White	White	-	Umbonate	Rough	Grey	Grey	-	Umbonate	Rough	White	Cream	-	Flat	Smooth
9	GA5	orange	White	-	Convex	Smooth	Cream	Light yellow	-	Convex	Slimy	Cream	Cream	-	Convex	Smooth	Light orange	Cream	-	Convex	Smooth
10	GA6	White	Yellow	-	Undulate	Smooth	White	Yellow	-	Raised	smooth	White	White	-	Flat	Smooth	White	White	-	Umbonate	Smooth
11	GA7	White	Cream	-	Flat	Rough	White	White	-	Umbonate	Rough	Grey	Grey	-	Flat	Smooth	White	White	-	Convex	Smooth
12	CA1	White	Cream	-	Convex	Rough	White	White	-	Convex	Rough	White	White	-	Raised	Smooth	White	Cream	-	Flat	Smooth
13	CA3	White	Cream	-	Convex	Smooth	White	White	-	Convex	Rough	White	Yellow	+	Raised	Smooth	White	White	-	Flat	Smooth
14	CA4	Brown	Brown	+	Convex	Rough	Brown	Light brown	-	Raised	Rough	Brown	Cream	+	Raised	Rough	Brown	Cream	+	Raised	Smooth
15	KA1	Yellow	White	+	Raised	Rough	White	White	+	Convex	smooth	Yellow	cream	-	Raised	Smooth	Light grey	Grey	-	Raised	Rough
16	KA2	Yellow	White	-	Umbonate	Rough	Brown	Cream	-	Umbonate	smooth	Brown	White	-	Raised	Convex	White	White	-	Convex	Smooth
17	SCANI	Orange	Orange	-	Raised	Wrinkled	Light pink	Cream	-	Umbonate	Rough	Orange	Orange	-	Raised	Rough	Orange	Orange	-	Undulate	Rough
18	SCAK1	Grey	Grey	-	Raised	Smooth	Cream	Light yellow	-	Raised	Rough	Grey	Grey	+	Convex	Smooth	grey	Grey	-	Flat	Smooth
19	KMN2	Yellow	Cream	-	Convex	Smooth	White	Light yellow	-	Raised	smooth	Cream	Cream	-	Flat	Smooth	Light grey	White	-	Raised	Rough
20	SCAN4	Brown	Brown	-	Raised	Wrinkled	Brown	Cream	-	Raised	Wrinkled	Cream	Cream	-	Flat	Rough	Yellow	Brown	-	Raised	Smooth
21	SCAK5	Cream	Pale yellow	+	Flat	Smooth	White	White	-	Raised	Smooth	White	White	-	Flat	Smooth	White	White	-	Flat	Rough
22	SCAC3	Brown	Cream	-	Flat	Rough	Cream	Cream	-	Flat	Rough	Yellow	Brown	-	Raised	Rough	Yellow	Brown	-	Umbonate	Rough
23	SCAK3	Cream	cream	+	Flat	Rough	Cream	Cream	+	Flat	Rough	White	Cream	-	Umbonate	Rough	White	Cream	-	Convex	Rough
24	KMN1	Cream	Cream	-	Raised	smooth	Pale yellow	Cream	-	Flat	smooth	Cream	Cream	-	Umbonate	Smooth	White	White	+	Convex	Smooth
25	NAN2	Cream	Cream	+	Convex	Rough	White	Light yellow	+	Raised	smooth	Brown	Light brown	-	Raised	Rough	Brown	Cream	+	Raised	Rough
26	NA8	Cream	Cream	-	Convex	Rough	Cream	Cream	-	Raised	Rough	Purple	White	+	Flat	Rough	Pink	Pink	+	Raised	Rough
27	CA5	Red	Cream	-	Convex	Rough	Red	White	-	Raised	Rough	red	cream	+	Raised	Rough	Orange	Orange	-	Raised	Rough
28	CA6	Cream	Cream	+	Convex	smooth	White	White	+	Convex	Smooth	Brown	Cream	+	Undulate	Rough	Brown	Cream	+	Flat	Smooth
29	CA7	Orange	Orange	+	Raised	smooth	Orange	Light orange	+	Raised	Rough	Orange	Orange	+	Raised	Rough	Orange	Orange	-	Undulate	Rough
30	NA9	White	White	-	Raised	smooth	White	White	-	Raised	smooth	Cream	Cream	-	Flat	Smooth	White	White	-	Flat	Smooth
31	NA7	White	cream	-	Convex	smooth	White	White	-	Raised	Rough	Cream	Cream	-	Flat	Smooth	Grey	Grey	-	Flat	Smooth
32	NA10	Cream	cream	+	Raised	Rough	White	Light yellow	-	Raised	smooth	Light brown	Cream	+	Raised	Rough	White	White	-	Flat	Rough
33	NA6	Cream	cream	+	Undulate	Rough	Cream	Cream	-	Raised	smooth	Yellow	white	-	Flat	Smooth	Cream	Cream	-	Flat	Smoth

The same actinomycete isolates were able to produce pigments on one medium while failed to do so on another medium. This may be attributed to the difference in the composition and pH of isolation media. Our findings are in agreement with the findings of other researchers who observed that medium composition and pH influence the pigment production by actinomycetes (Sanchez-Marroquin and Zapata, 1954).

4.4 SCREENING OF ACTINOMYCETES FOR MULTIFARIOUS PLANT GROWTH PROMOTING ATTRIBUTES

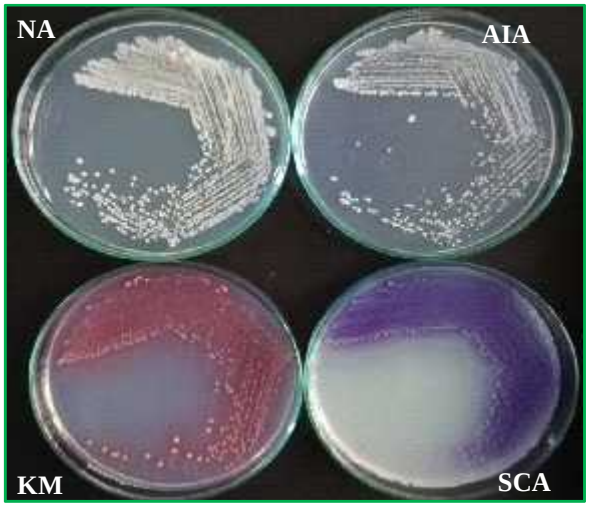
The actinomycete isolates obtained from the rhizosphere of *A. euchroma* were screened for various plant growth promoting attributes as discussed below.

4.4.1 Phosphate solubilization

4.4.1.1 Qualitative estimation

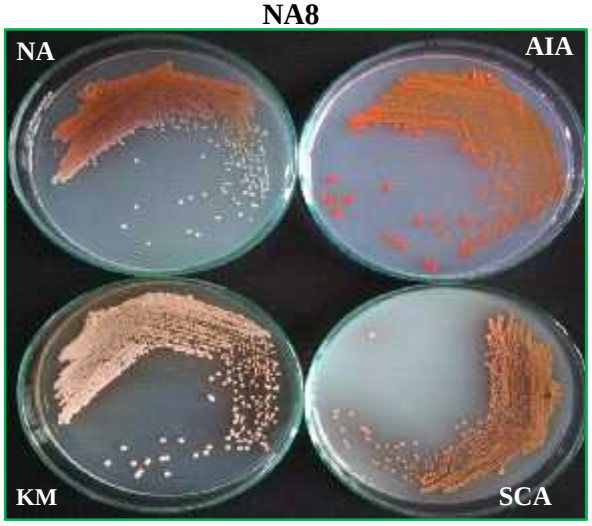
A total of 33 actinomycete isolates were examined for their P-solubilizing ability on Pikovskaya's agar medium. Only 11, out of 33, exhibited the ability to solubilize phosphate while, 22 isolates were found to be negative for this activity (Table 4.4 and Plate 4.4 a). The maximum halo zone size of 4 mm was shown by only four isolates *viz*., NA4, CA6, KA2 and NA8 while, for rest of the isolates, halo zone size ranged between 2-3 mm. The phosphate solubilization efficiency of 11 phosphate solubilizing actinomycetes varied from 33 to 300 per cent and highest efficiency was shown by SCAN1 (300%) while, lowest by CA7 (33%). Eight, out of 11 P-solubilizing actinomycetes *viz*., NA1, NA4, KA2, SCAN1, KMN2, NA8, CA6 and NA10 showed P- solubilizing efficiency of more than 50 per cent. Likewise, P-solubilization index of these 11 isolates ranged between 1.3-4.6 and highest value was shown by CA7 while, lowest by CA4. This could be ascribed to the fact that P-solubilization, which is measured in terms of diameter of halo zone, mainly depends upon nature of the phosphatic compounds released, organisms used, quantity and different rate of organic acids produced by microorganisms in to the surrounding medium (Kapoor *et al.*, 1989; Vessay, 2003).

Our results are in accordance with the observations of Passari *et al.,* (2015) who reported that phosphate solubilization efficiency of actinomycetes isolated from seven medicinal plants *viz*., *Eupatorium odoratum*, *Musa superba*, *Mirabilis jalapa*, *Curcuma longa*, *Clerodendrum colebrookianum*, *Alstonia scholaris* and *Centella asiatica* varied from 35 to 73 per cent. Similarly, Anwar *et al.* (2016) reported that P-solubilization index (SI) of *Streptomyces* spp. recovered from the rhizosphere of wheat (*Triticum aestivum*) ranged

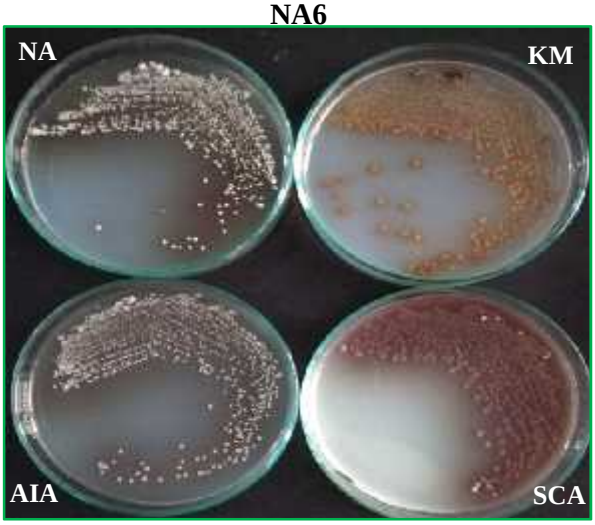


NA8

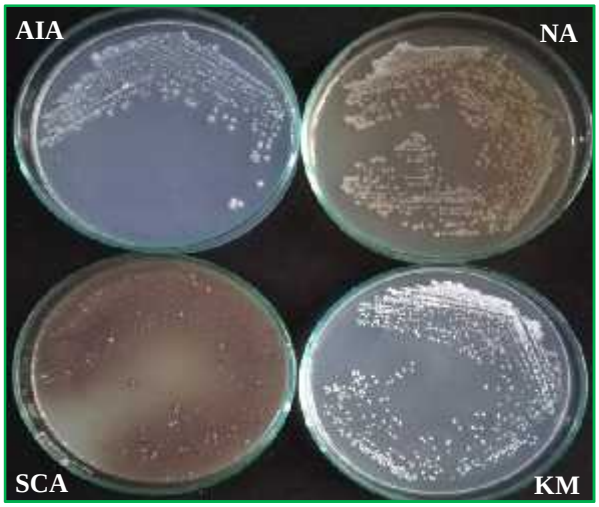
NA6



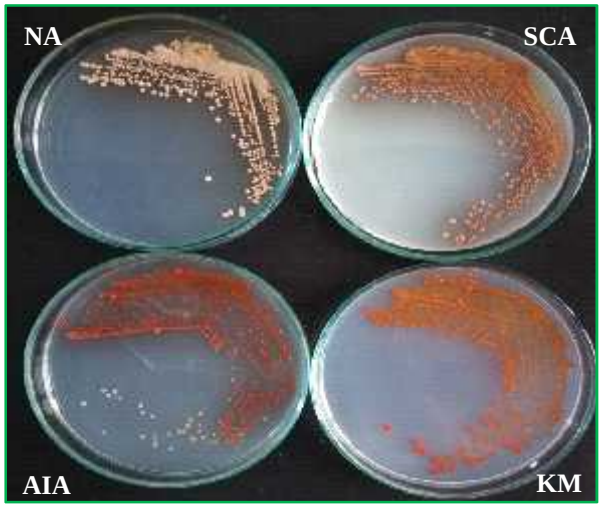
CA7



CA6



NA10



CA5

Plate 4.3 Difference in the growth behavior of same isolate on different media

between 2.20 to 2.33. Highest SI value of 2.33 was shown by *Streptomyces* sp. WA-1 followed by *S. djakartensis* TB-4 (2.27), *S. enissocaesilis* TA-3 (2.25), *S. nobilis* WA-3 (2.22), *S. mutabilis* WD-3 (2.21) and *S. kunmingensis* WC-3 (2.20).

Table 4.4 P-solubilization efficiency of actinomycetes isolated from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Halo zone diameter (HZD) = Zone diameter (ZD) – Colony Diameter(CD)					Phosphate solubilization Efficiency (SE%)*	Phosphate solubilization index (SI)**
		Days of incubation						
		6th	8th	10th	12th	14th		
1	NA1	1	1	2	3	3	60	1.6
2	NA2	-	-	-	-	-	-	-
3	NA3	-	-	-	-	-	-	-
4	NA4	1	2	3	4	4	67	1.6
5	NA5	-	-	-	-	-	-	-
6	GA1	-	-	-	-	-	-	-
7	GA2	-	-	-	-	-	-	-
8	GA4	-	-	-	-	-	-	-
9	GA5	-	-	-	-	-	-	-
10	GA6	-	-	-	-	-	-	-
11	GA7	-	-	-	-	-	-	-
12	CA1	-	-	-	-	-	-	-
13	CA3	-	-	-	-	-	-	-
14	CA4	1	1	2	2	2	33	1.3
15	KA1	-	-	-	-	-	-	-
16	KA2	1	2	3	4	4	100	2.0
17	SCAK1	-	-	-	-	-	-	-
18	SCAN1	1	1	2	3	3	300	4.0
19	KMN2	1	1	2	3	3	60	1.6
20	SCAN4	-	-	-	-	-	-	-
21	SCAK5	-	-	-	-	-	-	-
22	SCAC3	-	-	-	-	-	-	-
23	SCAK3	-	-	-	-	-	-	-
24	KMN1	-	-	-	-	-	-	-
25	NAN2	-	-	-	-	-	-	-
26	NA8	1	2	3	4	4	100	2.0
27	CA5	1	2	2	2	2	33	4.0
28	CA6	1	2	3	4	4	80	4.2
29	CA7	1	2	2	3	3	27	4.6
30	NA9	-	-	-	-	-	-	-
31	NA7	-	-	-	-	-	-	-
32	NA10	1	2	2	3	3	60	1.6
33	NA6	-	-	-	-	-	-	-

'-' Sign is the indication of no activity ;*SE= ZD-CD/CD × 100 and **SI= CD+HZD/CD

4.4.1.2 Quantitative estimation

Out of eleven P-solubilization actinomycetes, only eight isolates were found to be potent phosphate solubilizers qualitatively. Hence, only these isolates were further subjected for quantitative estimation of P-solubilization using PVK broth supplemented with 0.5 per cent TCP as it was reported to be a better source of insoluble phosphate under laboratory conditions in comparison to other sources of rock phosphate (Gaur *et al.*, 1973; Chambial, 1998; Dave and Patel, 1999).

The persual of data depicted in Table 4.5 reveals a statistical difference in the TCP solubilization by potential actinomycetes. The TCP solubilization was found to be significantly higher (53.66 µg mL^{-1}) for NA8 whereas, lowest (6.44 µg mL^{-1}) for SCAN1.

These results are in agreement with the observations of Hamdali *et al*. (2008) who reported high amount of phosphate solubilization in *Streptomyces cavourensis* (83.3 mg/100mL) followed by *Streptomyces griseus* (58.9 mg/100mL) and *Micromonospora aurantica* (39 mg/100mL). Acidification of external medium (Whitelaw, 2002) or the production of chelating substances(Welch *et al*., 2002; Hamdali *et al*., 2008) which in turn increase phosphate solubilization could be the probable reasons for mineral phosphate solubilization.

These results demonstrated that although actinomycetes exhibited low P-solubilizing activities, these might be helpful in making one of the essential nutrients available to the plants for improving their growth.

A decline in the pH of PVK broth was also recorded during P- solubilization, thereby, suggesting the secretion of organic acids by these actinomycetes as evident from Table 4.5. The solubilization of inorganic P by microorganisms has been attributed mainly to the production of low molecular weight organic acids like gluconic, α-ketogluconic, glycolic, oxalic, lactic, acetic, formic, malonic, malic and succinic acids which dissolves the insoluble P at low pH (Chen *et al*., 2006; Park *et al*., 2009; Vyas and Gulati, 2009). However, solubilization of insoluble phosphates could not be always linked with the acidity of medium and quantum of phosphate solubilization (Vassilev *et al*., 2006; Vyas and Gulati, 2009) because pH decline could also be ascribed to the chelation of phosphate anions by organic acids produced by microorganisms (Nautiyal *et al*., 2000).

Table 4.5 Quantitative estimation of phosphate solubilizing activity exhibited by actinomycetes obtained from *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolate	Halo zone Diameter in mm	Phosphate solubilization (µg/mL)	Pikovskaya's broth	
				Initial pH	Final pH
1	NA1	3	47.33[b]	7.0	5.72
2	NA4	4	20.77[e]	7.0	6.42
3	SCANI	3	6.44[h]	7.0	6.76
4	KMN2	3	29.20[c]	7.0	5.40
5	NA8	4	53.66[a]	7.0	5.70
6	CA6	4	15.11[f]	7.0	6.53
7	CA7	3	13.88[g]	7.0	6.50
8	NA10	3	23.77[d]	7.0	6.34
	CD(0.05)		0.28		

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

4.4.2 Phytase activity

4.4.2.1 Qualitative estimation

A total of 33 actinomycete isolates obtained from the rhizosphere of *A. euchroma* were screened for phytase activity using Phytase screening medium. As depicted in Table 4.6 and Plate 4.4 b, out of total 33, only 15 isolates (45.45%) showed positive phytase activity while, 18 (54.54%) were found negative for this activity. Diameter of halo zones for different isolates varied from 8 to 13 mm. The maximum phytase activity was shown by three isolates *viz.*, NA8, CA6 and CA7 (13 mm) while, minimum was exhibited by KMN2 and CA4 (8 mm).

4.4.2.2 Quantitative estimation

Out of 33, only 11 actinomycete isolates showed good ability to produce phytase qualitatively. Therefore, only these eleven isolates were further subjected for quantitative estimation as depicted in Table 4.7. The phytase activity was found to be significantly higher (134.44 U/mL) for CA7 whereas lowest (15.33 U/mL) for NA10. The phytase activities observed in the present investigation were found to be comparatively higher than those observed by Aly *et al.* (2015) who reported phytase activity exhibited by *Streptomyces* spp. isolated from decaying wood samples in the range of 0.08-7.90 U/mL. This may be attributed to the fact that phytase activity varies with respect to the type of actinomycete strains used and media composition.

Table 4.6 Qualitative estimation of phytase activity exhibited by actinomycetes obtained from *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Zone diameter (ZD) in mm	Colony diameter (CD) in mm	Halo zone diameter (HZD)* in mm
1	NA1	15	5	10
2	NA2	-	-	-
3	NA3	-	-	-
4	NA4	13	4	9
5	NA5	-	-	-
6	GA1	-	-	-
7	GA2	-	-	-
8	GA4	-	-	-
9	GA5	-	-	-
10	GA6	-	-	-
11	GA7	-	-	-
12	CA1	-	-	-
13	CA3	-	-	-
14	CA4	14	6	8
15	KA1	-	-	-
16	KA2	16	7	9
17	SCANI	15	4	11
18	SCAK1	13	3	10
19	KMN2	10	2	8
20	SCAN4	13	4	9
21	SCAK5	-	-	-
22	SCAC3	-	-	-
23	SCAK3	-	-	-
24	KMN1	15	6	9
25	NAN2	18	6	12
26	NA8	20	7	13
27	CA5	20	9	11
28	CA6	19	6	13
29	CA7	20	7	13
30	NA9	-	-	-
31	NA7	-	-	-
32	NA10	14	4	10
33	NA6	-	-	-

'-' Sign is the indication of no activity; *HZD= ZD-CD

Table 4.7. Quantitative estimation of phytase activity exhibited by actinomycetes obtained the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolate	Halo Zone Diameter (HZD) in mm	Phytase activity (U/mL)
1	NA1	10	45.77[i]
2	SCANI	11	114.12[f]
3	SCAK1	10	31.55[j]
4	NAN2	12	87.22[h]
5	NA8	13	126.33[b]
6	CA5	11	115.66[e]
7	CA6	13	124.88[d]
8	CA7	13	134.44[a]
9	NAN2	12	89.22[g]
10	NA8	13	125.33[c]
11	NA10	10	15.33[k]
	CD (0.05)		0.42

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

4.4.3 Siderophore production

Iron is a growth limiting factor for the majority of microorganisms (Arachibald, 1983). In many soils, iron is often not in an abundant form that plants can readily uptake and use. Siderophores are produced by rhizospheric bacteria to enhance plant growth by increasing the availability of iron near the roots (Alexander *et al*., 1991).

4.4.3.1 Qualitative estimation

All actinomycete isolates obtained from *A. euchroma* were screened for siderophore production using chrome azurol-S (CAS) agar plates. Out of total 33 isolates, only 15 (45.45%) were found to be positive for siderophore production while, 18 (34.54%) were found to be negative for this activity. Maximum siderophore production was shown by SCAK5 with halo zone size of 9 mm and minimum by six isolates *viz*., NA5, GA5, GA7, SCAN1, CA5 and NA9 with halo zone size of 4mm (Table 4.8 and Plate 4.4 c).

These results are supported by the Gangwar *et al*., (2013) who reported that out of 40 endophytic actinomycetes recovered from three medicinal plants *viz*., *Aloe vera, Mentha arvensis* and *Ocimum sanctum,* only 9 (22.5%) isolates showed siderophore production that ranged between 11.2-23.1 µg/mL.

Table 4.8. Siderophore production shown by actinomycetes obtained from *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolate	Zone Diameter (ZD) in mm	Colony Diameter (CD) in mm	Halo Zone Diameter (HZD)* in mm
1	NA1	-	-	-
2	NA2	10	5	5
3	NA3	-	-	-
4	NA4	14	9	5
5	NA5	13	9	4
6	GA1	-	-	-
7	GA2	-	-	-
8	GA4	-	-	-
9	GA5	8	4	4
10	GA6	19	11	8
11	GA7	9	5	4
12	CA1	-	-	-
13	CA3	-	-	-
14	CA4	8	3	5
15	KA1	-	-	-
16	KA2	-	-	-
17	SCAK1	-	-	-
18	SCANI	7	3	4
19	KMN2	-	-	-
20	SCAN4	-	-	-
21	SCAK5	15	6	9
22	SCAC3	16	10	6
23	SCAK3	-	-	-
24	KMN1	-	-	-
25	NAN2	-	-	-
26	NA8	-	-	-
27	CA5	9	5	4
28	CA6	-	-	-
29	CA7	15	8	7
30	NA9	15	11	4
31	NA7	11	5	6
32	NA10	-	-	-
33	NA6	17	12	5

'-' Sign is the indication of no activity; *HZD= ZD-CD

4.4.3.1 Quantitative estimation

Qualitatively, only 15 out of 33 actinomycetes were found to possess good ability to produce siderophore therefore, only these isolates were further subjected for quantitative estimation.

The persual of data depicted in Table 4.9 reveals a significant difference in the per cent siderophore units (% SU) produced by potential actinomycetes ranging from 6.13 to 17.24 per cent. Isolates SCAK5 and SCAN1 showed significantly higher (17.24 %) and lower siderophore production (5.46% SU), respectively whereas, three isolates *viz.*, NA5, GA5 and NA9 were found to be statistically at par.

On contrary, Khamna *et al*., (2009) observed comparatively higher siderophore production than present investigation in *Streptomyces* spp. isolated from rhizospheric soil of *Curcuma mangga.* Among these, strain *Streptomyces* CMU-SK exhibited highest ability to produce both catechol type (16.19 µg/mL) as well as hydroxamate type (54.9 µg/mL) siderophores. Low siderophore production by actinomycete isolates could be ascribed to the fact that rhizospheric actinomycetes have to compete with other rhizosphere bacteria and fungi for iron supply (Tokala *et al.,* 2002).

Table 4.9. Quantitative estimation of siderophore production by actinomycetes obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolate	Halo zone Diameter (HZ) in mm	% Siderophore units
1	NA2	5	7.67^{g}
2	NA4	5	7.48^{h}
3	NA5	4	6.13^{k}
4	GA5	4	6.21^{k}
5	GA6	9	16.25^{b}
6	GA7	4	7.85^{f}
7	CA4	5	7.30^{i}
8	SCAN1	4	5.46^{l}
9	SCAK5	9	17.24^{a}
10	SCAC3	6	7.24^{i}
11	CA5	4	9.41^{e}
12	CA7	7	12.35^{c}
13	NA9	4	6.18^{k}
14	NA7	6	10.23^{d}
15	NA6	5	6.72^{j}
	CD(0.05)		0.15

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

4.4.4 HCN Production

In the present study, all the actinomycetes isolates were screened for HCN production on nutrient agar plates supplemented with 0.14 per cent glycine. Among all, only one isolate i.e GA6 showed HCN production with orange- brown colouring of filter paper impregnated with sodium carbonate in picric acid (Table 4.10. and Plate 4.5 a).

HCN is produced by several microorganisms as a product of secondary metabolism that affects sensitive organisms by inhibiting the synthesis of ATP-mediated cytochrome oxidase (Knowles, 1976). HCN is postulated to play an important role in the biological control of various phytopathogens (Dey *et al.*, 2004), mainly by inhibiting electron transport chain which subsequently prevents the supply of energy to the cell and ultimately leads to death of the pathogen (Kundan *et al*., 2015).

Ahmed *et al.* (2014) reported that HCN production shown by actinomycetes helped in disease suppression hence, is an important mechanism of bioprotection. Jalaluldeen *et al.* (2014) found *Streptomycetes indiaens* is recovered from the rhizosphere of chilli plant as a potent HCN producer.

4.4.5 Ammonia production

Ammonia is considered as one of the plant growth promoting and antimicrobial substances produced by microorganisms. In the present study, twenty two out of total 33 actinomycete isolates obtained from rhizosphere of *Arnebia euchroma* showed ammonia production in peptone broth with production of varied intensity of yellow-brown colour upon adding Nessler's reagent (Table 4.10 and Plate 4.5 c).

Two actinomycete isolates *viz.*, CA7 and NA5 produced brown colour thereby indicated strong ammonia production whereas, 5 isolates *viz.*, GA1, CA5, CA6, NAN2 and SCAK3 produced yellow- brown colour indicated moderate ammonia production. Rest of the isolates showed light yellow colour hence, indicated weak ammonia production.

Ammonia and extracellular proteins are the nitrogenous secretions of N- fixers in nitrogen free or deficient medium (Behl *et al*., 2007) and amidases enzymes catalyze the hydrolysis of the carboxylic acid and ammonia (Asano and Lubbehusen, 2000). Generally, bacteria synthesize ammonia in order to supply nitrogen to the host plant (Marques *et al*.,

2010). But over production of ammonia may also serve as a prompting factor in the virulence of opportunistic phytopathogens. The production of ammonia along with HCN in the growth medium by *Streptomyces* contributed towards disease suppression among plants (Anwar *et al*., 2016).

Table 4.10. Nitrogen fixation, HCN and ammonia production by actinomycete isolates obtained from rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Ammonia production	HCN production	Nitrogen fixation
1	NA1	+ +	-	+++
2	NA2	-	-	+++
3	NA3	-	-	+++
4	NA4	++	-	+++
5	NA5	++++	-	+++
6	GA1	+++	-	++
7	GA2	-	-	+++
8	GA4	++	-	+++
9	GA5	+ +	-	++
10	GA6	-	+++	+++
11	GA7	++	-	+++
12	CA1	++	-	+++
13	CA3	++	-	+++
14	CA4	-	-	++
15	KA1	++	-	+++
16	KA2	-	-	++
17	SCAN1	++	-	+++
18	SCAK1	-	-	+++
19	KMN2	-	-	+++
20	SCAN4	-	-	++
21	SCAK5	-	-	+++
22	SCAC3	+ +	-	+++
23	SCAK3	+++	-	++
24	KMN1	-	-	+++
25	NAN2	+++	-	+++
26	NA8	+ +	-	++
27	CA5	+++	-	+++
28	CA6	+++	-	+++
29	CA7	++++	-	++
30	NA9	++	-	+++
31	NA7	++	-	+
32	NA10	++	-	+
33	NA6	++	-	+

'-' Sign is the indication of no activity; + += Weak activity, + + += Moderate activity, + + + += Strong activity

4.4.6 Nitrogen fixing ability

Nitrogen fixing ability possessed by actinomycete isolates obtained from the rhizosphere of *A. euchroma* was detected on N- deficient Jensen's medium. As evident from Table 4.10 and Plate 4.5 b, all isolates exhibited nitrogen fixing ability. Our findings are supported by Kaur *et al*. (2013) who observed that actinomycetes of genus *Streptomyces* isolated from different plants exhibited reasonable N- fixing ability. Nitrogen and phosphorus were reported to have influence on the greater yield of *Thymus vulgaris* (Omidbaigi and Arjmandi, 2002).

Nitrogen (N) is an essential nutrient for improved growth and yield in plants. Biological N_2 fixation is carried out by a variety of prokaryotic microbes (Nitrogen fixers) which mediate the conversion of unavailable atmospheric nitrogen (N_2) to available form (NH^{4+}) for plants. The nitrogenase enzyme catalyzes the reductive breakage of the very strong triple bond of N_2 to generate NH_3 (Rubio and Ludden, 2005) besides reducing a wide range of substrates (Burns *et al*., 1975).

The rhizosphere of *Arnebia euchroma* harbors a good proportion of nitrogen fixers which might probably be helpful in the perpetuation of this plant under prevailing harsh environmental conditions of trans-Himalayan region of Himachal Pradesh.

As depicted in Fig. 4.3, it is evident that only 33.33 per cent of the total isolates were phosphate solubilizers, whereas, 45.45 per cent were found to be positive for siderophore production and phytase activity. All isolates exhibited nitrogen fixing ability. However, only 66.66 and 3.03 percent of total isolates showed ammonia and HCN production, respectively

4.4.7 EXTRACELLULAR PRODUCTION OF LYTIC ENZYMES

The actinomycete isolates obtained from the rhizosphere of *A. euchroma* were examined for the production of different lytic enzymes.

4.4.7.1 Chitinase activity

4.4.7.1.1 Qualitative estimation

The perusal of data depicted in Table 4.11 revealed that majority of the isolates (90.92%) exhibited good chitinase activity on CCA medium amended with 0.05 per cent colloidal chitin, with the production of clear halo zones of chitin degradation ranging between 4-33 mm (Plate 4.6 d). Only three isolates (9.09%) were found to be negative for this activity.

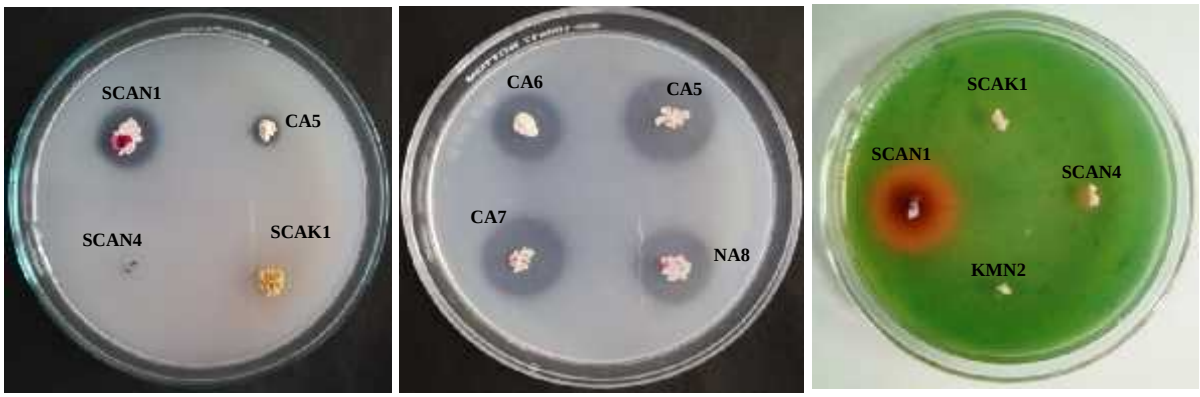


Plate 4.4 Multifarious plant growth promoting attributes exhibited by actinomycetes obtained from the rhizosphere of *Arnebia euchroma* (a) Phosphate solubilization (b) Phytase activity (c) Siderphore production

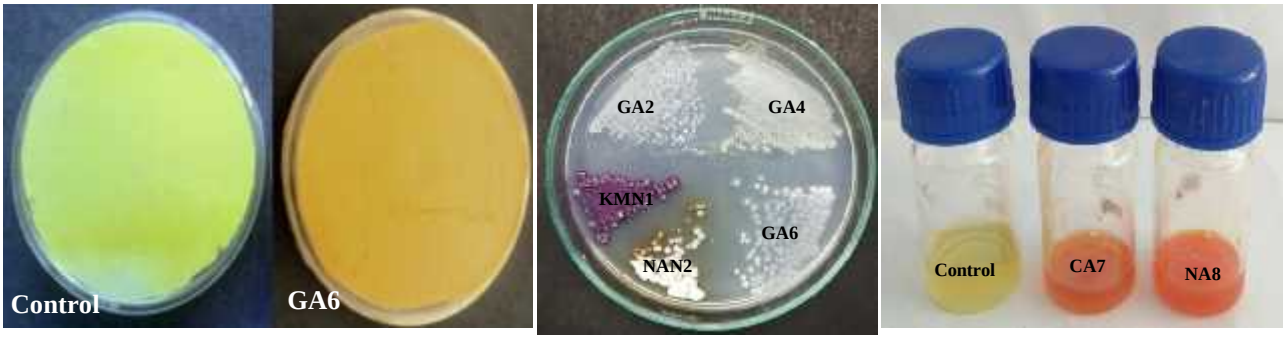


Plate 4.5 Actinomycetes isolate showing (a) HCN production (b) Nitrogen fixing ability and (c) Ammonia production

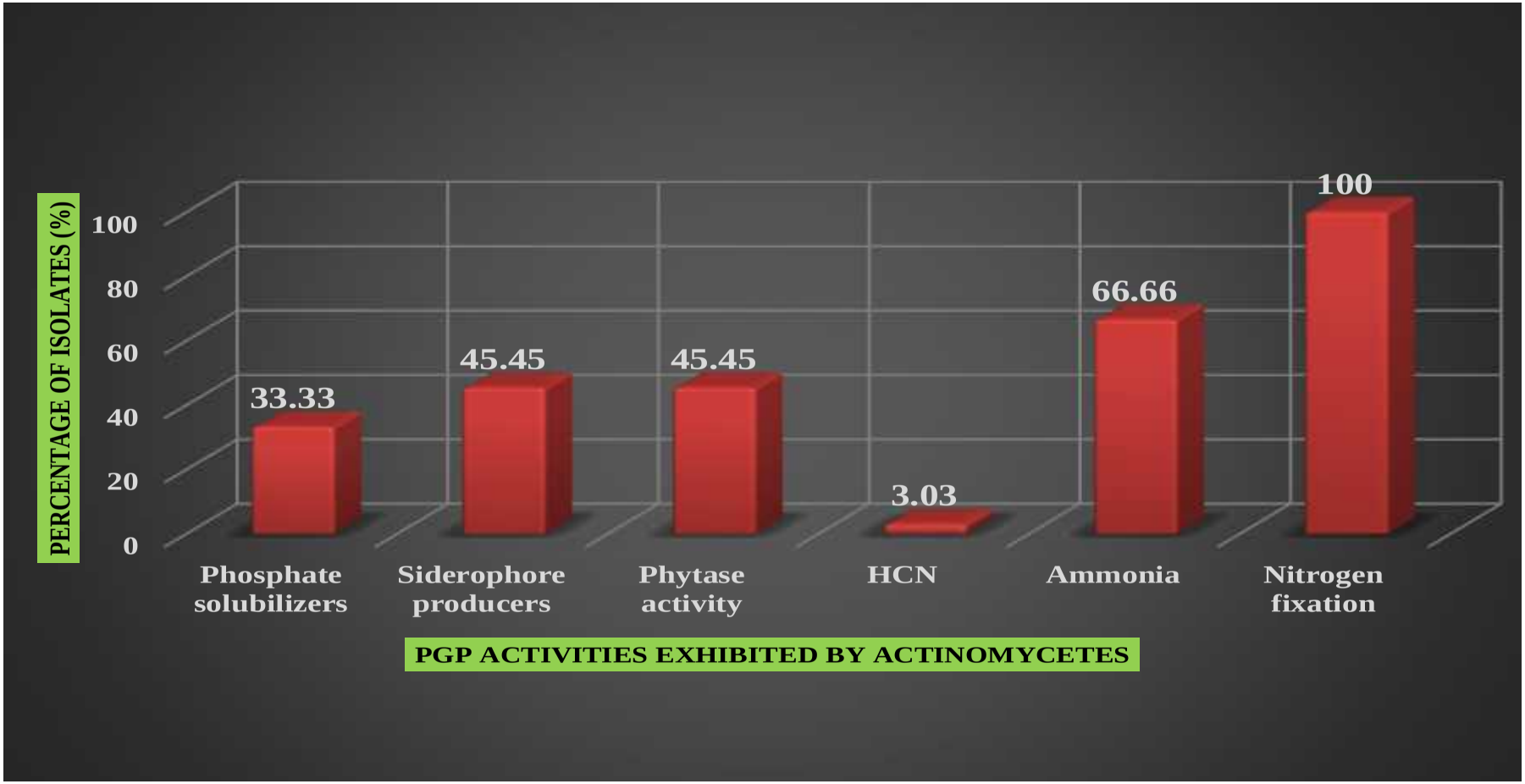


Fig 4.3 Percentages of different PGP activities exhibited by actinomycetes from *Arnebia euchroma* (Ratanjot)

Highest chitinase production was shown by isolate NA6 (33 mm) and lowest (4 mm) by CA1. Our results are in accordance with Passari *et al.* (2015) who observed high chitin degrading activity in two endophytic actinomycetes i.e. *Leifsonia xyli* 24 and *Microbacterium* sp. 21 recovered from medicinal plants with a colloidal chitin degradation zone of 15 and 17 mm, respectively.

Table 4.11. Chitinase activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Zone Diameter (ZD) in mm	Colony Diameter (CD) in mm	Halo Zone Diameter (HZD)* in mm
1	NA1	21	6	15
2	NA2	28	7	21
3	NA3	21	5	16
4	NA4	30	6	24
5	NA5	31	10	21
6	GA1	26	10	16
7	GA2	19	12	12
8	GA4	26	11	15
9	GA5	27	5	22
10	GA6	-	-	-
11	GA7	15	10	5
12	CA1	11	7	4
13	CA3	-	-	-
14	CA4	20	4	16
15	KA1	21	3	18
16	KA2	30	5	25
17	SCANI	27	5	22
18	SCAK1	35	9	26
19	KMN2	29	6	23
20	SCAN4	29	6	23
21	SCAK5	15	8	7
22	SCAC3	29	4	25
23	SCAK3	21	5	16
24	KMN1	35	4	31
25	NAN2	28	7	21
26	NA8	31	12	19
27	CA5	31	8	23
28	CA6	29	29	23
29	CA7	30	5	25
30	NA9	-	-	-
31	NA7	15	7	8
32	NA10	20	7	13
33	NA6	38	5	33

'-' Sign is the indication of no activity; *HZD= ZD-CD

4.4.7.1.2 Quantitative estimation

As depicted in Table 4.12, a total of 30 actinomycete isolates which were found to be potent chitinase producers qualitatively, were further subjected for quantitative estimation using colloidal chitin broth. Chitinase activity values ranged between 0.42-6.81 U/mL. Isolates NA6 and CA1 showed statistically highest (6.81 U/mL) and lowest (0.42U/mL) chitinase activities, respectively. Whereas, isolates NA7, SCAN1, CA6, KMN2, KMN1 and CA5 were found to be statistically at par.

Table 4.12. Quantitative estimation of Chitinase activity exhibited by actinomycetes obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolate	Halo Zone Diameter (mm)	Chitinase activity (U/mL)
1	NA1	15	3.52^{b}
2	NA2	21	4.36^{b}
3	NA3	16	3.53^{b}
4	NA4	24	3.76^{b}
5	NA5	21	5.03^{b}
6	GA1	16	3.42^{c}
7	GA2	12	2.86^{c}
8	GA4	15	3.31^{c}
9	GA5	22	3.26^{c}
10	GA7	5	0.53^{d}
11	CA1	4	0.42^{d}
12	CA4	16	3.83^{b}
13	KA1	18	3.93^{b}
14	KA2	25	2.73^{c}
15	SCANI	27	5.26^{a}
16	SCAK1	26	4.56^{b}
17	KMN2	23	6.46^{a}
18	SCAN4	23	3.43^{c}
19	SCAK5	7	2.46^{c}
20	SCAC3	25	4.43^{b}
21	SCAK3	16	3.46^{c}
22	KMN1	31	6.71^{a}
23	NAN2	21	3.83^{b}
24	NA8	19	4.93^{b}
25	CA5	23	6.52^{a}
26	CA6	23	5.76^{a}
27	CA7	25	4.53^{b}
28	NA7	8	5.26^{a}
29	NA10	13	4.86^{b}
30	NA6	33	6.81^{a}
	CD(0.05)		1.56

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

Our results are consistent with Nagpure *et al*. (2014) who observed that chitinase activity exhibited by *Streptomyces* sp. (MT7) recovered from the soil of Manipur, India was 3.24 U/mL and this enzyme contributed vigorously towards antagonism by degrading the chitin containing cell wall of fungal phytopathogens. Among 27 different actinomycete strains isolated from Coringa mangrove forests, Strain A (*Streptomyces* sp.) showed good chitinase production (Deepthi *et al*., 2012).

Chitinases produced by several microorganisms have been reported to play a vital role in degradation and recycling of carbon and nitrogen which remain entrapped in insoluble chitin (Hoang *et al*., 2011). The role of chitinolytic actinomycetes in the biological control of several disease causing phytopathogens has been recognized widely. Actinomycetes are broadly used to control the population of mosquitoes and different pests as biopesticides (Kumaran *et al.,* 2012).

4.4.7.2 Protease activity

4.4.7.2.1 Qualitative estimation

In total, 33 actinomycete isolates obtained from the rhizosphere of *A. euchroma* were screened for protease activity on Skimmed milk agar amended with 1% skim milk. Among these, only 14 isolates possessed protease activity while, nineteen were found to be negative for this activity (Plate 4.6 b). The persual of data depicted in Table 4.13 revealed that values of protease activity varied from 9 to 33 mm. The highest protease activity of 33 mm was shown by isolate NA7 while, minimum (9 mm) by SCAK3.

Several studies reported production of proteases from actinomycetes like members of genera, *Streptomyces, Nocardia and Nocardiopsis* (Wietzorrek and Bibb, 1997). Kaur *et al*. (2013) recovered proteolytic actinomycetes that belonged to genus *Streptomyces* from the rhizospheres of different plants. As reported by Gurielidze *et al.* (2010), protease activity exhibited by three alkaliphilic actinomycete isolates *viz*., *Streptomyces globisporolactis* 203A, *S. streptomycini* 204A, *Nocardia polaris* 206A recovered from the soils of Georgia ranged between 0.85 and 1.2 U/mL.

Table 4.13. Protease activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Zone Diameter (ZD) in mm	Colony Diameter (CD) in mm	Halo zone Diameter (HZD)*
1	NA1	-	-	-
2	NA2	-	-	-
3	NA3	-	-	-
4	NA4	-	-	-
5	NA5	-	-	-
6	GA1	35	16	19
7	GA2	20	10	10
8	GA4	20	10	10
9	GA5	30	14	16
10	GA6	18	4	14
11	GA7	23	10	13
12	CA1	-	-	-
13	CA3	-	-	-
14	CA4	-	-	-
15	KA1	-	-	-
16	KA2	-	-	-
17	SCANI	-	-	-
18	SCAK1	18	6	12
19	KMN2	-	-	-
20	SCAN4	-	-	-
21	SCAK5	23	8	15
22	SCAC3	-	-	-
23	SCAK3	13	4	9
24	KMN1	13	3	10
25	NAN2	-	-	-
26	NA8	-	-	-
27	CA5	-	-	-
28	CA6	-	-	-
29	CA7	-	-	-
30	NA9	35	15	20
31	NA7	45	12	33
32	NA10	23	12	11
33	NA6	40	10	30

'-' Sign is the indication of no activity; *HZD= ZD-CD

4.4.7.2.2 Quantitative estimation

For the quantitative estimation of protease activity, fourteen actinomycetes which showed positive activity on Skimmed Milk agar were further selected and grown in nutrient broth amended with 1 per cent skim milk. The persual of data in Table 4.14 revealed that

value of protease activity ranged between 1.22-28.13 U/mL. Isolate NA7 and NA10 showed statistically highest (28.13 U/mL) and lowest (1.22 U/mL) protease activities, respectively whereas, isolates NA9 and GA4 were found to be statistically at par.

Thumar and Singh (2007) reported that a salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain MIT-1 isolated from Mithapur, India possessed the ability to produce alkaline protease (130 U/mL). Also, a moderately halophilic, alkaliphilic and thermotolerant indigenous actinomycete isolate HA4, has been reported to exhibit protease activity with 2.7 mm of halo zone diameter (Ningthoujam *et al.*, 2009). Saini *et al*. (2016) observed that the protease activity values exhibited by actinomycete isolates ranged from 0.11 to 0.16 U/mL.

Besides chitin, cell walls of fungi consist of proteins and proteases are known to play a vital role in the biological control of fungal phytopathogens either through the lysis of fungal cell-wall or by competing with fungal pathogens for protein substrates (Hunsley and Burnett, 1970; Elad and Kapat, 1999). Some proteases producing microorganisms were also reported to possess antagonistic activity against diverse Gram negative and Gram positive bacteria (Kaur *et al*. 2001), which might be contributing towards their survival under natural competitive environment.

Table 4.14. Quantitative estimation of protease activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolate	Halo Zone (mm)	Protease activity (U/mL)
1	GA1	19.0	7.40^{g}
2	GA2	10.0	13.42^{d}
3	GA4	10.0	21.68^{b}
4	GA5	16.0	8.58^{f}
5	GA6	14.0	6.24^{h}
6	GA7	13.0	12.66^{d}
7	SCAK1	12.0	7.20^{g}
8	SCAK5	15.0	5.21^{i}
9	SCAK3	9.0	1.25^{j}
10	KMN1	10.0	17.20^{c}
11	NA9	20.0	22.10^{b}
12	NA7	33.0	28.13^{a}
13	NA10	11.0	1.22^{j}
14	NA6	30.0	11.15^{e}
	C.D(0.05)		0.99

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

4.4.7.3 Cellulase (CMCase) activity

4.4.7.3.1 Qualitative estimation

All the actinomycete isolates were screened for cellulase (CMCase) activity on carboxymethyl cellulose (CMC) agar plates (Plate 4.6c). As shown in Table 4.15, majority of the isolates (96.96%) exhibited cellulase activity and only one isolate (KA1) was found to be negative for this activity.

Table 4.15. Cellulase activity exhibited by actinomycetes isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Zone Diameter (ZD) in mm	Colony Diameter (CD) in mm	Halo zone Diameter (HZD)* in mm
1	NA1	15	7	8
2	NA2	26	7	19
3	NA3	14	6	8
4	NA4	21	5	16
5	NA5	26	8	24
6	GA1	28	9	19
7	GA2	27	5	22
8	GA4	33	6	27
9	GA5	26	6	20
10	GA6	10	4	6
11	GA7	20	7	13
12	CA1	30	10	20
13	CA3	27	10	17
14	CA4	15	5	10
15	KA1	-	-	-
16	KA2	20	4	16
17	SCANI	25	4	21
18	SCAK1	30	6	24
19	KMN2	14	5	9
20	SCAN4	16	5	11
21	SCAK5	19	5	14
22	SCAC3	23	4	19
23	SCAK3	16	5	11
24	KMN1	33	4	29
25	NAN2	25	7	18
26	NA8	25	7	18
27	CA5	31	6	25
28	CA6	24	7	17
29	CA7	30	4	26
30	NA9	29	10	19
31	NA7	25	7	18
32	NA10	20	4	16
33	NA6	26	7	19

'-' Sign is the indication of no activity; *HZD= ZD-CD

Qualitatively, cellulase activity varied from 6 to 29 mm. Highest activity zone of 29 mm was shown by KMN1 while, lowest (6 mm) by GA6. These results are in accordance with Jeffery (2008) who reported that two actinomycetes strains *viz*., *Streptomyces aureofaciens* and *Streptomycetes* sp. recovered from the soil showed cellulase activity with halo zones size varied from 13-30 mm.

4.4.7.3.2 Quantitative estimation

Out of thirty three, only 22 isolates which qualitatively found potent cellulase producers were further subjected for quantitative estimation using CMC broth. The persual of data depicted in Table 4.16 showed that values of cellulase activity ranged from 24.18 -31.21 U/mL. Isolates NA5 and GA6 showed statistically highest (31.21 U/mL) and lowest (24.18 U/mL) cellulase activities, respectively. Whereas, isolates NA2, NA3, GA5, SCAC3, KMN1 and CA5 were found to be statistically at par.

Several reports have revealed the cellulolytic potential of different actinomycetes obtained from various ecological niches. Alam *et al.* (2011) reported that CMC-ase activity exhibited by actinomycete isolates obtained from the soil ranged from 1.51 U/mL to 1431.81 U/mL. Jeffrey and Azrizal (2007) isolated cellulolytic actinomycetes from different soil samples that belonged to *Streptomyces* group. Bui (2014) successfully isolated *Streptomyces* and *Actinomyces* exhibiting high cellulolytic potential from coffee exocarps. Das *et al.* (2014) isolated cellulose degrading *Streptomyces griseochromogenes*, *Streptomyces rochei*, *Streptomyces plicatus* and *Streptomyces enissocaesilis* from diverse habitats.

4.4.7.4 Pectinase activity

4.4.7.4.1 Qualitative estimation

Pectinases are produced by various microorganisms including bacteria, yeasts and filamentous fungi (Whitaker, 1990).

In the present investigation, all actinomycete isolates were screened for pectinase activity on nutrient agar medium amended with 2.0 per cent pectin and 0.1 per cent yeast extract (Plate 4.6 a). The data depicted in Table 4.17 revealed that 84.8 per cent of the total isolates exhibited pectinase activity while, 15.2 per cent were found to be negative for this activity. The value of halo zone diameter ranged between 2 to 29 mm. The highest (29 mm) and lowest (2 mm) halo zone diameter was shown by isolates NA6 and GA6, respectively.

Our results are consistent with Ashokvardhan *et al.* (2014) who recovered pectinolytic potential actinomycete strains from the rhizosphere of *Capsicum annuum L.*

Table 4.16. Quantitative estimation of cellulase activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolate	Halo Zone Diameter (HZD) in mm	Cellulase activity (U/mL)
1	NA1	8	26.96[c]
2	NA2	19	28.92 [b]
3	NA3	8	27.74 [b]
4	NA4	16	26.82 [c]
5	NA5	24	31.21[a]
6	GA1	19	27.12[c]
7	GA2	22	27.51[c]
8	GA4	27	27.56[c]
9	GA5	20	28.41[b]
10	GA6	6	24.18 [d]
11	GA7	13	26.72 [c]
12	CA1	20	25.94 [c]
13	CA3	17	26.44 [c]
14	CA4	10	25.12 [d]
15	KA2	16	26.58 [c]
16	SCANI	21	26.62 [c]
17	SCAK1	24	25.64[d]
18	KMN2	9	26.78 [c]
19	SCAN4	11	24.50[d]
20	SCAK5	14	24.52[d]
21	SCAC3	19	28.82 [b]
22	SCAK3	11	25.72[d]
23	KMN1	29	29.36[b]
24	NAN2	18	25.12 [d]
25	NA8	18	24.92 [d]
26	CA5	25	28.36 [b]
27	CA6	17	27.52[c]
28	CA7	26	25.28[d]
29	NA9	19	27.55[c]
30	NA7	18	27.48[c]
31	NA10	16	26.72[c]
32	NA6	19	27.12[c]
	CD(0.05)		1.64

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

Table 4.17. Pectinolytic activity exhibited by actinomycetes isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Zone Diameter (ZD) in mm	Colony diameter (CD) in mm	Halo zone Diameter (HZD)* in mm
1	NA1	23	5	18
2	NA2	9	3	6
3	NA3	6	2	4
4	NA4	11	3	8
5	NA5	28	5	23
6	GA1	11	6	5
7	GA2	11	6	6
8	GA4	24	5	19
9	GA5	30	6	24
10	GA6	13	11	2
11	GA7	16	7	9
12	CA1	-	-	-
13	CA3	-	-	-
14	CA4	23	4	19
15	KA1	-	-	-
16	KA2	25	7	18
17	SCANI	25	4	21
18	SCAK1	28	5	23
19	KMN2	7	4	3
20	SCAN4	20	4	16
21	SCAK5	21	7	14
22	SCAC3	23	6	17
23	SCAK3	15	6	9
24	KMN1	30	4	26
25	NAN2	23	4	19
26	NA8	24	3	19
27	CA5	30	4	26
28	CA6	21	3	18
29	CA7	25	5	20
30	NA9	-	-	-
31	NA7	-	-	-
32	NA10	20	9	11
33	NA6	37	8	29

'-' Sign is the indication of no activity; *HZD= ZD-CD

4.4.7.4.1 Quantitative estimation

Qualitatively, out of 33, only 29 isolates were found to be efficient in terms of pectinase activity, therefore, were further subjected for quantitative estimation. The persual of data depicted in Table 4.18 revealed that values of pectinse activity varied from 1.60 to 48.33 U/mL. Isolates NA6 and GA6 showed significantly higher (48.33 U/mL) and lower (1.60 U/mL) pectinase activities, respectively. Whereas, isolates NA3, GA1, NA1 and GA4 were found to be statistically at par.

Table 4.18. Quantitative estimation of pectinase activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolate	Halo Zone Diameter (HZD)	Pectinase activity (U/mL)
1	NA1	18	19.33^{c}
2	NA2	6	9.00^{g}
3	NA3	4	21.66^{b}
4	NA4	8	8.33^{h}
5	NA5	23	12.66^{e}
6	GA1	5	20.33^{b}
7	GA2	6	6.66^{i}
8	GA4	19	19.34^{c}
9	GA5	24	7.66^{h}
10	GA6	2	1.60^{k}
11	GA7	9	15.00^{d}
12	CA4	19	16.00^{d}
13	KA2	18	4.32^{j}
14	CA4	19	16.00^{d}
15	SCANI	21	4.66^{j}
16	SCAK1	23	2.30^{k}
17	KMN2	3	2.00^{k}
18	SCAN4	16	7.62^{h}
19	SCAK5	14	7.81^{h}
20	SCAC3	17	6.67^{i}
21	SCAK3	9	7.33^{h}
22	KMN1	26	16.12^{d}
23	NAN2	19	10.00^{g}
24	NA8	19	11.66^{f}
25	CA5	26	6.62^{i}
26	CA6	18	10.02^{g}
27	CA7	20	13.12^{e}
28	NA10	11	2.61^{k}
29	NA6	29	48.33^{a}
	CD(0.05)		1.42

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

On contrary, Golinska and Dham (2011) observed comparatively less pectinase activities that ranged from 0.03 to 1.4 U/mL than present investigation which could be ascribed to the fact that actinomycetes possess high potential to degrade a diverse range of polysaccharides including pectin from plants under prevailing environmental conditions.

Numerous bacteria and fungi (pathogenic, saprophytic and ectomycorrhizal) have been reported to exhibit pectolytic activity (Dahm and Strzelczyk, 1995; Dahm *et al*. 1997; Redlak *et al*., 2001). Besides participating in the decomposition of dead organic plant matter, pectinases play an indispensable role in establishing microbial interactions with plants. They also take part actively in the establishment of both pathogenic as well as symbiotic relationships between macro and micro microorganisms (Golinska and Dahm, 2011).

4.4.7.5 Lipase activity

4.4.7.5.1 Qualitative estimation

Lipases are the members of hydrolases family that act on carboxylic ester bonds. The natural function of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Lipases are widely distributed throughout the plant and animal kingdoms, as well as in molds and bacteria (Aiyer, 2004).

All actinomycete isolates obtained from the rhizosphere of *A. euchroma* were screened for lipase activity on Tributyrin agar medium (Plate 4.6 e), out of which, only 72.7 per cent isolates exhibited lipase activity while, 27.3 per cent were found to be negative for this activity. The perusal of data depicted in Table 4.19 revealed that, lipase activity values ranged from 6 to 28 mm, with highest activity (28 mm) was shown by isolate NA6 (6 mm) and lowest (6 mm) by isolate NA5. Our results are in agreement with Ashokvardhan *et al*. (2014) who reported that actinomycete strains isolated from rhizospheric soil possessed good lipase activity. Another study conducted by Jeffrey (2008) reported that *Streptomyces* exhibited in range of 15-30 mm of halo zone diameter.

Actinomycetes exhibit high potential to produce diverse range of hydrolytic enzymes including lipase which could be ascribed to results of natural selection of microbes in order to thrive in the competing environment (Arjit *et al*., 2012). Apparently, the rhizosphere of *A. euchroma* may serve as an imperative resource for screening valuable enzymes. Srividya *et al*. (2012) reported that plant growth promoting and broad spectrum biocontrol potential of

Streptomyces sp. 9p isolated from rhizospheres of Brinjal, Capsicum and Chilli grown in Bangalore and Assam is due to the simultaneous production of lipases, proteases, IAA and siderophores along with antifungal activity.

Table 4.19 Lipase activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Zone Diameter (ZD) in mm	Colony Diameter (CD) in mm	Halo Zone Diameter (HZD)*
1	NA1	35	10	25
2	NA2	-	-	-
3	NA3	-	-	-
4	NA4	14	6	8
5	NA5	10	4	6
6	GA1	24	12	12
7	GA2	22	10	12
8	GA4	20	10	10
9	GA5	15	4	11
10	GA6	17	6	11
11	GA7	13	5	8
12	CA1	-	-	-
13	CA3	14	4	10
14	CA4	-	-	-
15	KA1	-	-	-
16	KA2	-	-	-
17	SCANI	23	7	16
18	SCAK1	30	7	23
19	KMN2	13	6	7
20	SCAN4	21	9	12
21	SCAK5	19	5	14
22	SCAC3	9	2	7
23	SCAK3	9	2	7
24	KMN1	-	-	-
25	NAN2	20	11	9
26	NA8	16	7	9
27	CA5	13	5	8
28	CA6	13	6	7
29	CA7	14	6	8
30	NA9	-	-	-
31	NA7	-	-	-
32	NA10	25	8	17
33	NA6	40	12	28

'-' Sign is the indication of no activity; *HZD= ZD-CD

4.4.7.5.2 Quantitative estimation

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Twenty isolates, out of 33 showed lipase activity qualitatively, hence were further subjected for quantitative estimation using Tributyrin broth. As depicted in Table 4.20 lipase activity exhibited by actinomycetes ranged between 1000-2040 IU/mL. The isolate NA5 showed significantly higher lipase activity (2040 IU/mL) among all isolates, while, six isolates *viz*., GA1, NA10, NA6, NA8, NAN2 and SCAK5, were found to be statistically at par.

Table 4.20 Quantitative estimation of lipase activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Halo Zone Diameter (HZD) in mm	Lipase activity (IU/mL)
1	NA1	25	1240[c]
2	NA4	8	1200[c]
3	NA5	6	2040[a]
4	GA1	12	1000[d]
5	GA2	12	1120[c]
6	GA4	10	1240[c]
7	GA5	11	1280[c]
8	GA6	11	1160[c]
9	GA7	8	1240[c]
10	CA3	10	1400[b]
11	SCANI	16	1160[c]
12	SCAK1	23	1040[c]
13	KMN2	7	1120[c]
14	SCAN4	12	1520[b]
15	SCAK5	14	1000[e]
16	SCAC3	7	1120[c]
17	SCAK3	7	1120[c]
18	NAN2	9	1000[e]
19	NA8	9	1000[e]
20	CA5	8	1240[c]
21	CA6	7	1160[c]
22	CA7	8	1160[c]
23	NA10	17	1000[e]
24	NA6	28	1000[e]
	CD(0.05)		174.66

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

Various workers reported the lipolytic potential of actinomycetes. Selvam *et al*. (2011) observed that actinomycetes isolated from marine sediments showed lipase activity varied from 580-700 U/mL. Poovarasan *et al*. (2015) isolated ten actinomycetes from guava rhizosphere soil, out of which highest lipase activity (30.27 U mg $^{-1}$) was shown by *Leifsonia poea* followed *Streptomyces canus* (28.42±0.01 U mg $^{-1}$) and *Streptomyces scabiei* (24.67 U mg-^{1}). *Streptomyces griseus* was investigated by Vishnupriya *et al*. (2010) for the production of lipase using different substrates. Maximum lipolytic activity was recorded with Sunflower and Palm oil at pH ranging between 6-9 after 48 hrs of incubation.

It is evident from these results that collectively 84.84, 90.90, 96.96, 72.72 and 42.42 per cent of the total isolates exhibited pectinase, chitinase, cellulase, lipase and protease activities, respectively (Fig. 4.4).

4.5 SCREENING OF ACTINOMYCETES FOR THE PRODUCTION OF PLANT GROWTH REGULATORS

4.5.1 Auxins production

Auxin (IAA) is a very important plant hormone that regulates various aspects of plant growth and development like cell division, cell elongation and differentiation, root initiation, apical dominance etc (Guilfoyle *et al*., 1998). Tryptophan acts as a precursor for IAA synthesis (Koga *et al*., 1994).

The persual of data depicted Table 4.21 and Fig. 4.5 revealed that actinomycete isolates showed auxin production ranging from 11.55 to 44.52 µg/mL. Isolates NA10 and NA3 showed statistically highest (44.52 µg/mL) and lowest (11.55 µg/mL) auxins production, while isolates NA1 and CA6 were found to be statistically at par.

Our results are in accordance with those of Khamna *et al.* (2009) who reported that *Streptomyces* showed IAA production in range of 5.5–144 µg/mL. *Streptomyces* CMU-H009 isolated from lemongrass (*Cymbopogon citrates*) and *Streptomyces* from crop rhizosphere possessed the ability to produce IAA and promote plant growth. It could be inferred that IAA, a plant growth hormone can promote plant growth in rhizosphere soils (El-Tarabily and Sivasithamparam, 2006; Tsavkelova *et al.*, 2006)

Kataria (2012) also reported that actinomycete isolates produce IAA in range from 10.2 to 93.8 µg/mL. *Streptomyces griseofuscus* (Os-10), isolated from the rhizospheric soils

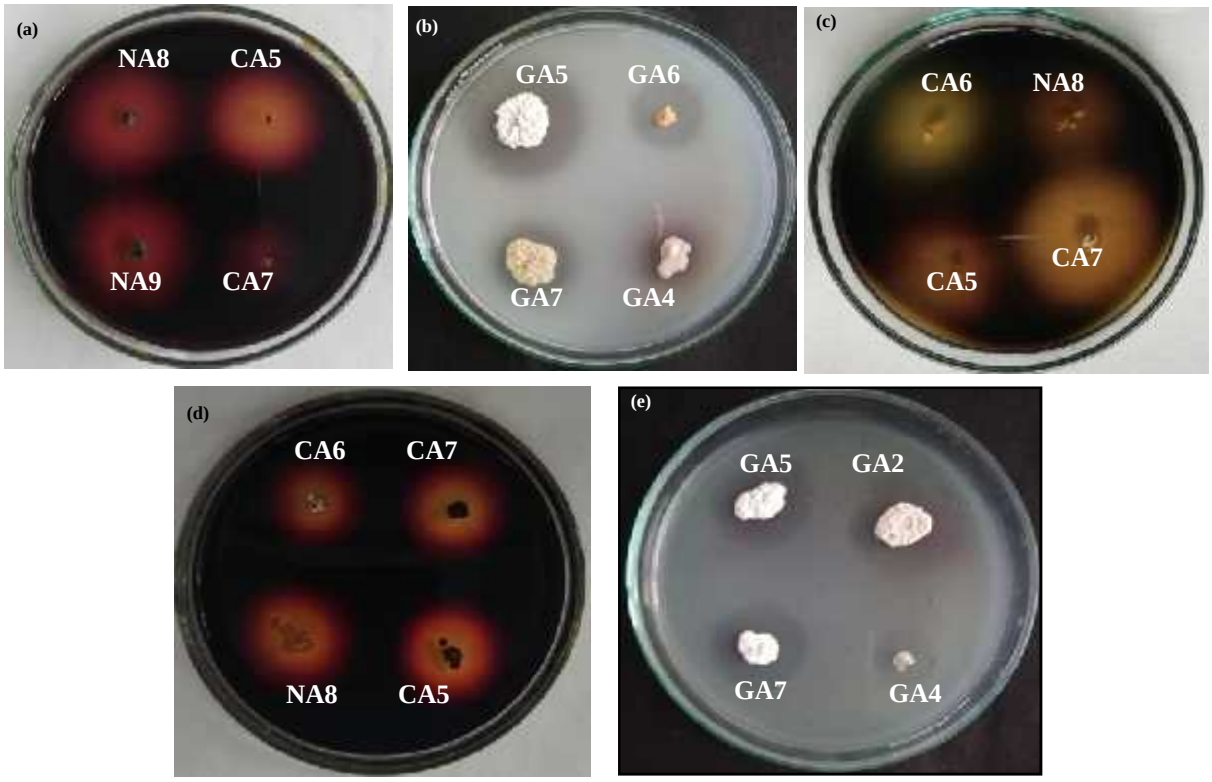


Plate 4.6 Actinomycetes isolates showing extracellular production of different lytic enzymes (a) Pectinase (b) Protease (c) Cellulase (d) Chitinase and (e) Lipase

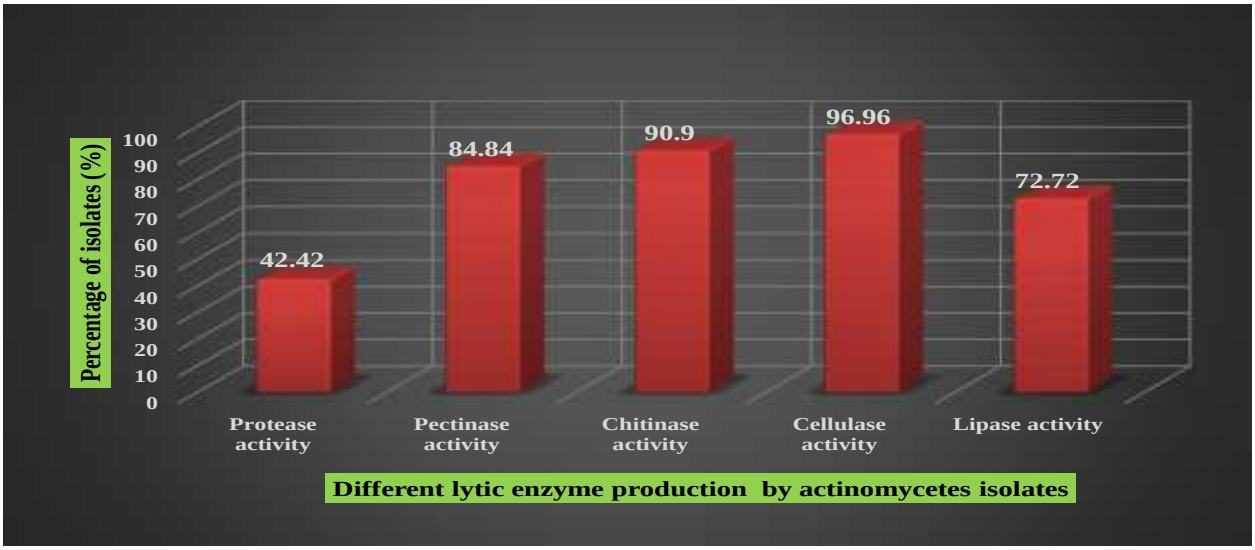


Fig. 4.4 Percentage of different lytic enzymes produced by actinomycete isolate obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

of *Ocimum sanctum* was observed to produce highest amount of IAA i.e 93.8 µg/mL while, *S.globisporus* Cc-3,a *Cymbopogon citrates* isolate produced minimum amount of IAA production i.e. 10.2 µg/mL.

4.5.2 Gibberellins production

Gibberellins are biologically active, endogenous hormones of higher plants that are involved in a range of developmental processes in higher plants including stem elongation, germination, seed dormancy and sex expression (Gelmi and Perez, 2000).

In the present study, gibberellins production by actinomycete isolates obtained from the rhizosphere of *A. euchroma* varied from 4.52 to 41.72 µg/mL as depicted in Table 4.21 and Fig 4.5. Isolates CA3 and KMN2 showed statistically highest (41.72µg/mL) and lowest (4.52 µg/mL) gibberellins production, respectively. Whereas, isolates NA10 and NA6 were found to be statistically at par. Our results are consistent with the results of Patil and Patil (2012) who reported gibberellins production by actinomycetes recovered from the soil ranging from 10 to 80 µg/mL.

4.5.3 Cytokinins production

Cytokinins mediate interactions between microbes and their associated plants. In present investigation, cytokinins production, expressed in terms of µg/mL was measured indirectly by radish cotyledon expansion bioassay. All the actinomycete isolates showed cytokinins production ranging from 15.26 to 34.24µg/mL (Table 4.21 and Fig 4.5) significantly higher cytokinins production (34.24 µg/mL) was shown by isolate CA5 while, minimum (15.26 µg/mL) by GA7. Isolates NA1, GA6, CA3, KA2, and KMN1 were found to be statistically at par.

Our results are in agreement with those of Solans *et al.* (2011) who evaluated three saprophytic strains of actinomycetes *viz*., *Streptomyces* sp., *Actinoplanes* sp. and *Micromonospora* sp. isolated from the rhizosphere of *Ochetophila trinervis* for phytohormones production (Auxins, Gibberellins and Zeatin) and observed that Zeatin biosynthesis was higher than IAA and GA_3, and only *Micromonospora* strain among these produced the highest levels of these phytohormones.

Cytokinins production has been documented in various actinomycetes like *Streptomyces olivaceoviridis*, *S. rimosus* and *S. rochei* (Aldesuquy *et al*., 1998). As plant

growth regulators, cytokinins (N_6 substituted aminopurines) influence plant's physiological and developmental processes like cell division, root development and seed germination, accumulation of chlorophylls, delay of senescence and leaf expansion (Werner *et al*., 2001). Herzong and Geisler (1976) recorded improved yield of wheat due to higher levels of endogenous cytokinins.

Table 4.21 Production of plant growth regulators by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

Sr. No.	Isolates	Plant growth regulators		
		Auxins (µg/mL)	Gibberellins (µg/mL)	Cytokinins (µg/mL)
1	NA1	39.42^{b}	14.51^{i}	32.65^{a}
2	NA2	27.50^{f}	10.95^{k}	27.67^{c}
3	NA3	11.55^{k}	8.02^{l}	18.45^{g}
4	NA4	20.07^{j}	13.32^{j}	28.23^{c}
5	NA5	21.11^{i}	21.31^{g}	22.15^{f}
6	GA1	26.47^{g}	35.47^{b}	26.62^{d}
7	GA2	29.80^{e}	13.57^{j}	15.26^{h}
8	GA4	21.77^{i}	17.42^{h}	25.34^{d}
9	GA5	24.67^{h}	6.92^{m}	28.32^{c}
10	GA6	11.72^{k}	26.75^{e}	31.12^{b}
11	GA7	24.21^{h}	15.97^{h}	25.31^{d}
12	CA1	38.27^{c}	7.10^{l}	21.67^{f}
13	CA3	25.85^{g}	41.72^{a}	30.87^{b}
14	CA4	31.75^{d}	8.52^{l}	27.65^{c}
15	KA1	12.07^{k}	5.32^{n}	28.82^{c}
16	KA2	25.37^{g}	28.67^{d}	31.24^{b}
17	SCANI	26.07^{g}	24.67^{f}	22.64^{f}
18	SCAK1	23.85^{h}	10.82^{k}	17.52^{g}
19	KMN2	25.30^{g}	4.52^{n}	27.64^{c}
20	SCAN4	27.21^{f}	11.45^{k}	27.86^{c}
21	SCAK5	24.40^{h}	14.15^{i}	24.65^{e}
22	SCAC3	25.33^{g}	21.23^{g}	21.12^{f}
23	SCAK3	26.02^{g}	15.62^{i}	22.32^{f}
24	KMN1	26.32^{g}	6.12^{m}	30.41^{b}
25	NAN2	27.77^{f}	24.91^{f}	29.21^{c}
26	NA8	27.82^{f}	10.85^{k}	28.12^{c}
27	CA5	27.67^{f}	34.02^{b}	34.24^{a}
28	CA6	40.22^{b}	26.57^{e}	25.26^{d}
29	CA7	25.15^{g}	27.05^{d}	17.54^{g}
30	NA9	23.57^{h}	30.37^{c}	18.48^{g}
31	NA7	28.55^{e}	40.60^{a}	25.41^{d}
32	NA10	44.52^{a}	40.05^{a}	28.42^{c}
33	NA6	27.23^{f}	20.21^{g}	31.21^{b}
	CD(0.05)	1.67	1.54	1.65

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

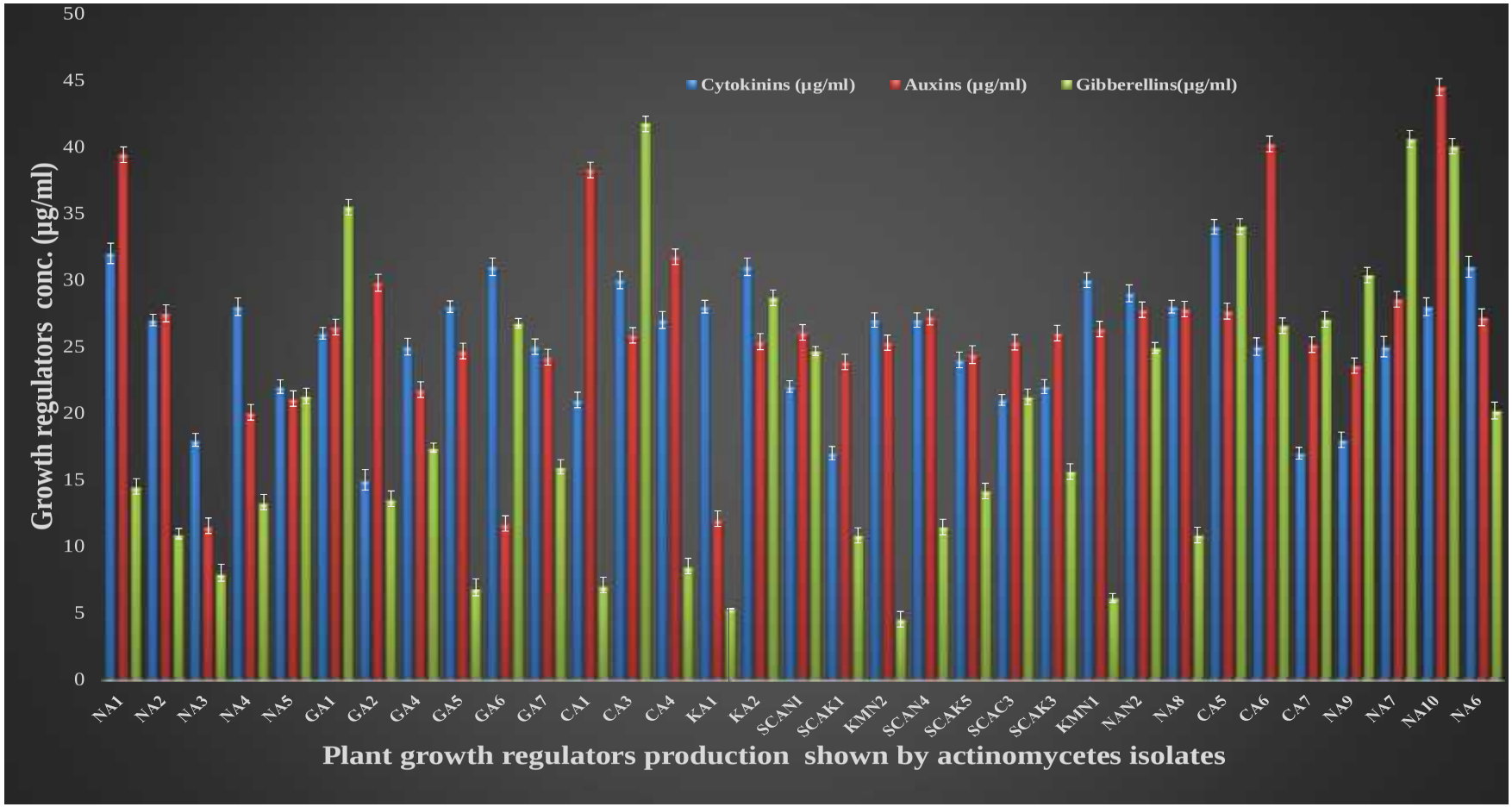


Fig. 4.5 Production of plant growth regulators by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

4.6 ANTAGONISTIC ACTIVITY OF POTENTIAL ACTINOMYCETES AGAINST SOIL-BORNE FUNGAL PHYTOPATHOGENS

The synthesis of antibiotics is one of the most effective mechanisms that actinomycetes can employ to prevent growth of phytopathogenic fungi. It is an indirect method of plant growth promotion.

In the present study, all the eight potential actinomycetes isolates showed antagonism against three selected soil borne fungal phytopathogens *viz., Fusarium oxysporum, Sclerotium rolfsii and Rhizoctonia solani* (Plate 4.7). The persual of data depicted in Table 4.22 reveals a significant difference in the per cent growth inhibition of phytopathogenic fungi. Amongst all, the isolate NA4 showed significantly higher inhibition in the growth of *F. oxysporum* (37.5%) while, lowest (31.2%) by GA7 over control. The significantly higher inhibition (42.2 %) in the growth of *Sclerotium rolfsii* was shown by isolate SCAN1 followed by KA2 and NA6 (33.3%). Isolates SCAN1 and NA8 showed statistically highest (40.0 %) and lowest (31.2%) per cent inhibition in the growth of *Rhizoctonia solani*, respectively. The antagonism displayed by the actinomycetes is ascribed to the extracellular production of different antifungal substances such as lytic enzymes *viz.*, chitinases, proteases, cellulases, pectinases, antibiotics, HCN and siderophores (Chung *et al.*, 2008; Singh *et al*., 2008).

Table 4.22. Antagonistic activity of efficient actinomycetes against soil-borne fungal phytopathogens

Actinomycetes isolate	Percent growth inhibition of fungal pathogens					
	Fusarium oxysporum (C=80mm)		*Sclerotium rolfsii* (C=90mm)		*Rhizoctonia solani* (C=80mm)	
	mm diameter	% Inhibition	mm diameter	% Inhibition	mm diameter	% Inhibition
NA8	54	32.5^{e}	57	36.6^{e}	59	26.2^{f}
CA5	53	33.7^{d}	53	41.4^{b}	54	32.5^{e}
CA7	50	37.5^{a}	54	40.0^{c}	49	38.7^{c}
NA6	55	31.2^{f}	60	33.3^{f}	50	37.5^{d}
SCAN1	51	36.2^{b}	52	42.2^{a}	48	40.0^{a}
NA4	50	37.5^{a}	55	38.8^{d}	50	37.5^{d}
GA7	52	35.2^{c}	55	38.8^{d}	47	39.0^{b}
KA2	55	31.2^{f}	60	33.3^{f}	49	38.7^{c}
C.D (0.05)		0.26		0.29		0.27

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

Kataria (2012) investigated the antifungal activity of actinomycetes isolated from rhizosphere of medicinal plants against eight indicator test fungi *viz*., *Fusarium oxysporum, Aspergillus flavus, Penicillium pinophilum, Chaetomium globosum, Helminthosporium oryzae, Phytophthora drescleaoryzae and Aspergillus niger* and observed that all actinomycetes exhibited the potential to inhibit the growth of tested fungi.

According to Crawford *et al.* (1993), actinomycete strains isolated from the rhizosphere of *Taraxicum officinale* were active against *Pythium ultimum*. Ouhdouch and Barakate (2001) recovered 10 actinomycete isolates from medicinal plant rhizosphere soils, most of which belonged to *Streptomyces* sp. and possessed good antifungal activity against different phytopathogens. *Streptomyces* strain exhibited antipathogenic activity was isolated by Thangapandian *et al.* (2007) from rhizosphere soils of different medicinal plants.

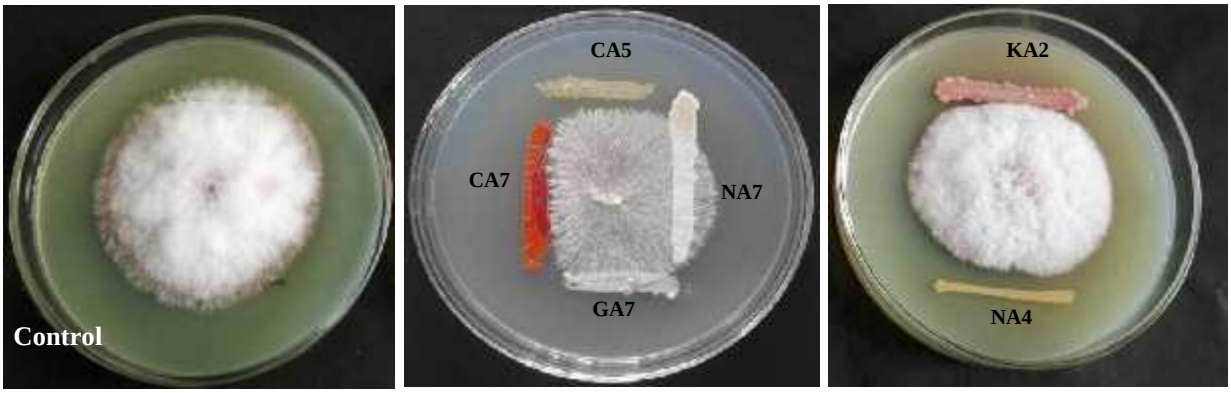
4.6.1 Effect of volatile metabolites secreted by actinomycetes in the growth inhibition of fungal phytopathogens

Volatile compounds may possess antimicrobial activity and play a significant role in microbial interactions where there is no direct contact between them. In the present study, the gaseous metabolites released by the actinomycete isolates inhibited the growth of fungal plant pathogens inoculated in the inverted face of the petri plate (Plate 4.8).

The persual of data depicted in Table 4.23 and Fig. 4.6 reveals a statistical difference in the per cent growth inhibition of all fungal phytopathogens. Among all, the isolate NA4, showed significantly higher inhibition in the growth of *F. oxysporum* (36.25%) while, lowest (21.87%) by SCAN1 over control. The significantly higher inhibition (44.44%) in the growth of *Sclerotium rolfsii* was shown by isolate GA7 followed by NA8 (41.67%) and NA6 (36.33%). Isolate NA8 showed statistically highest per cent inhibition (38.12%) in the growth of *Rhizoctonia solani.* Whereas, four isolates *viz*., CA7, NA6, NA4 and GA7 were found to be statistically at par.

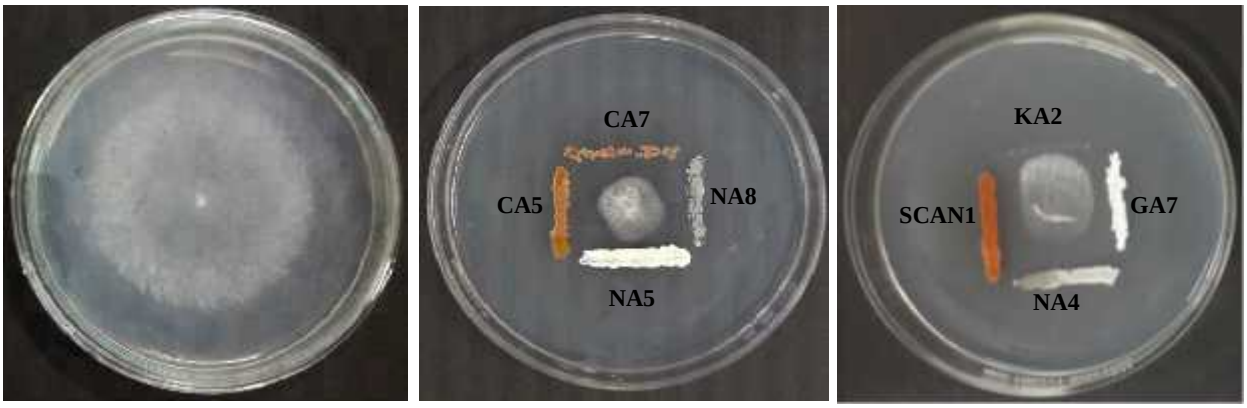
This inhibition could be due to the production of diverse kinds of volatile compounds including HCN and ammonia which are responsible for limiting fungal growth thereby contributing towards antagonism (Rezzonico *et al.*, 2007).

Our results are in accordance with those of Cordovez *et al.* (2015) who found that volatile compounds produced by *Streptomyces* strains W47 and W214 were able to retard the



(a) ***Fusarium oxysporum***

(b)***Sclerotium rolfsii***



(c) ***Rhizoctonia solani***

Plate 4.7 Antagonistic activity of actinomycete isolates obtained from *Arnebia euchroma* (Ratanjot) against fungal phytopathogens (a) *Fusarium oxysporium* (b) *Sclerotium rolfsii* and (c) *Rhizoctonia solani*

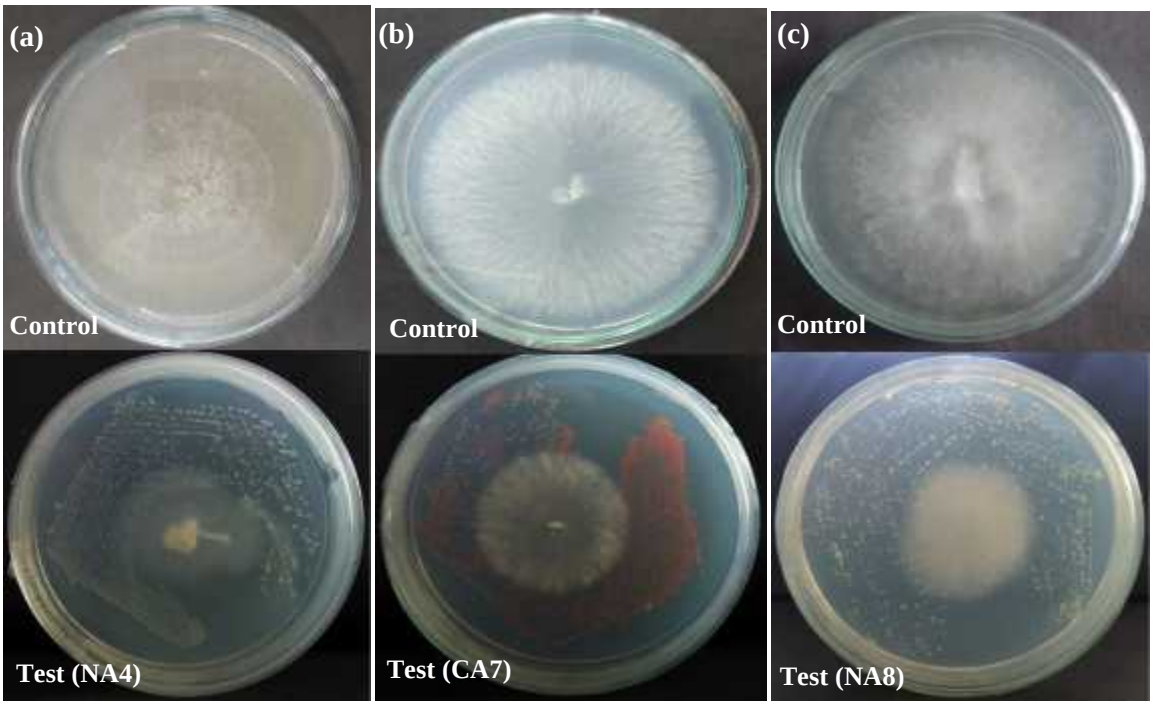


Plate 4.8 Effect of volatile compounds secreted by actinomycetes on growth inhibition of soil borne fungal phytopathogens (a) *Rhizoctonia solani* (b) *Sclerotium rolfsii* (c) *Fusarium oxysporum*

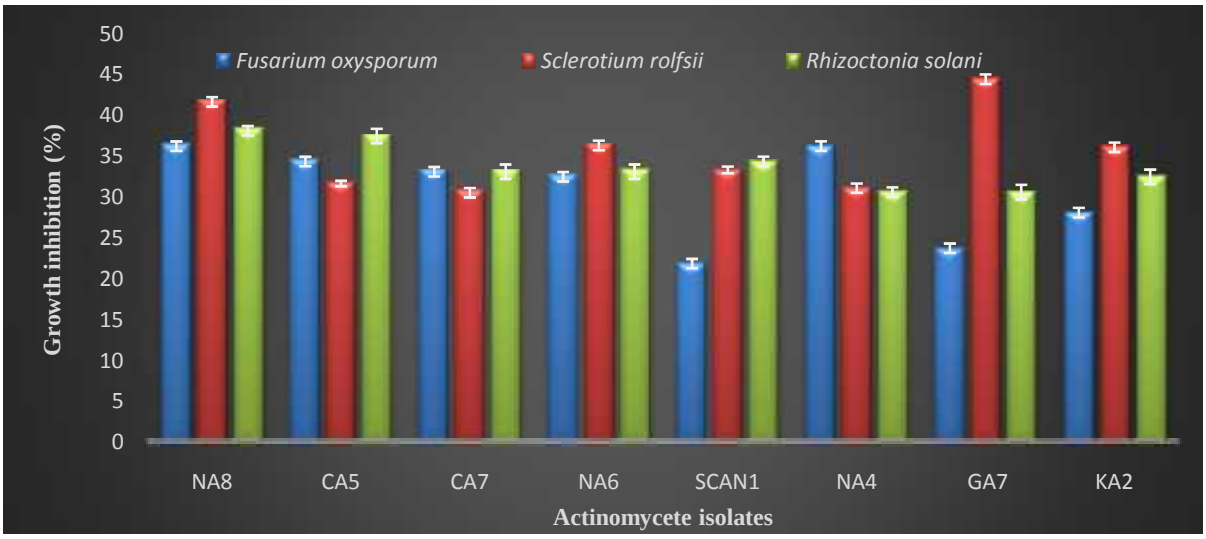


Fig. 4.6 Percent inhibition in the growth of fungal phytopathogens due to the secretion of volatile metabolites by actinomycetes obtained from *Arnebia euchroma* (Ratanjot)

growth of *Rhizoctonia solani*. Wu *et al*. (2015) reported 100 and 56.30 percent inhibition in the growth of *Sclerotinia sclerotiorum* and *Fusarium oxysporum*, respectively due to the volatile metabolites secreted by the actinomycete isolates.

Table 4.23. Effect of volatile compounds secreted by actinomycetes on growth inhibition of soil-borne fungal phytopathogens

Actinomycetes isolate	Percent growth inhibition of fungal pathogens					
	***Fusarium oxysporum* (C=80mm)**		***Sclerotium rolfsii* (C=90mm)**		***Rhizoctonia solani* (C=80mm)**	
	mm diameter	**% Inhibition**	**mm diameter**	**% Inhibition**	**mm diameter**	**% Inhibition**
NA8	21	35.25[b]	15	41.67[b]	19	38.12[a]
CA5	25	34.37[c]	32	31.67[f]	20	37.50[b]
CA7	27	33.12[d]	35	30.55[h]	27	33.12[d]
NA6	28	32.50[e]	24	36.33[c]	27	33.12[d]
SCAN1	45	21.87[h]	30	33.33[e]	25	34.37[c]
NA4	22	36.25[a]	34	31.11[g]	31	30.62[f]
GA7	42	23.75[g]	10	44.44[a]	31	30.62[f]
KA2	35	28.12[f]	25	36.11[d]	28	32.50[e]
C.D(0.05)		0.15		0.13		0.20

Each value represents mean of three replicates. According to one way ANOVA, significant differences are indicated by different letters. Same letters represent that their values are statistically at par.

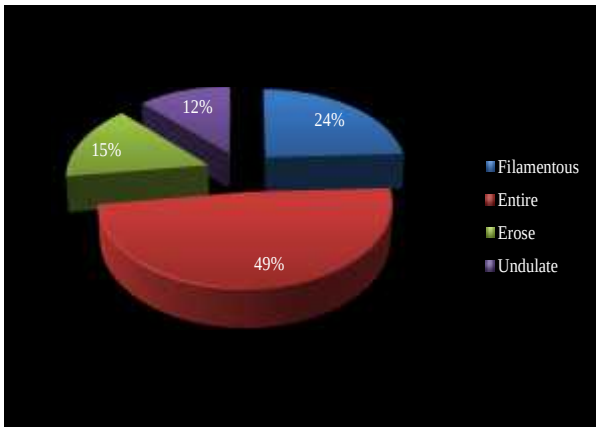
4.7 IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL ISOLATES

4.7.1 Morphological characterization

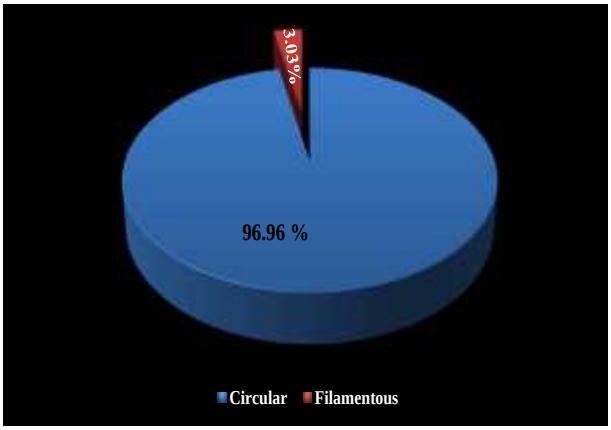
Actinomycetes, being the most economical and biotechnologically valuable prokaryotes, responsible for the production of about half of the discovered antibiotics and bioactive secondary metabolites (Kumari *et al*., 2012). Around 7,600 compounds are reported to be produced by *Streptomyces* species amongst actinomycetes (Olano *et al*., 2009). Different morphological characteristics like color, form, elevation and margin of actinomycete isolates were noted down and depicted in Table 4.24. Microscopic studies revealed that all the actinomycetes isolates were gram positive filamentous bacteria (Plate 4.9). Majority of the colonies were flat, umbonate and raised with mainly entire margin (Fig. 4.7). The color of the colony varied from yellow to cream.

Table 4.24 Morphological characteristics of actinomycetes isolates

Sr. No.	Isolates	Colony morphology				Gram's reaction	Pigmentation
		Form	Elevation	Margin	Colour		
1	GA1	Circular	Undulate	Filamentous	White	+	-
2	GA2	Circular	Umbonate	Entire	White	+	-
3	GA4	Circular	Flat	Erose	White	+	-
4	GA5	Circular	Convex	Entire	Orange	+	-
5	GA6	Circular	Raised	Undulate	White	+	-
6	GA7	Circular	Flat	Erose	White	+	-
7	NA1	Circular	Flat	Filamentous	Cream	+	-
8	NA2	Circular	Flat	Filamentous	Yellow	+	-
9	NA3	Circular	Umbonate	Undulate	Yellow	+	-
10	NA4	Circular	Umbonate	Entire	Brown	+	-
11	NA5	Circular	Convex	Filamentous	Grey	+	-
12	CA1	Circular	Convex	Filamentous	White	+	-
13	CA3	Circular	Convex	Filamentous	White	+	-
14	CA4	Circular	Convex	Entire	Brown	+	Brown
15	KA1	Circular	Raised	Erose	Yellow	+	Brown
16	KA2	Circular	Umbonate	Curled	Yellow	+	-
17	SCANI	Circular	Umbonate	Undulate	Orange	+	-
18	SCAKI	Circular	Raised	Entire	Grey	+	-
19	KMN2	Circular	Convex	Entire	Yellow	+	-
20	SCAN4	Filamentous	Raised	Filamentous	Brown	+	-
21	SCAK5	Circular	Flat	Entire	Cream	+	Brown
22	SCAC3	Circular	Flat	Entire	Brown	+	-
23	SCAK3	Circular	Flat	Undulate	Cream	+	Brown
24	KMN1	Circular	Raised	Erose	Cream	+	-
25	NAN2	Circular	Convex	Entire	Cream	+	Brown
26	NA8	Circular	Convex	Entire	Cream	+	-
27	CA5	Circular	Convex	Entire	Red	+	-
28	CA6	Circular	Convex	Entire	Cream	+	Brown
29	CA7	Circular	Raised	Entire	Orange	+	Brown
30	NA9	Circular	Raised	Entire	White	+	-
31	NA7	Circular	Convex	Filamentous	White	+	-
32	NA10	Circular	Raised	Entire	Cream	+	Brown
33	NA6	Circular	Undulate	Erose	Cream	+	Brown



Margin



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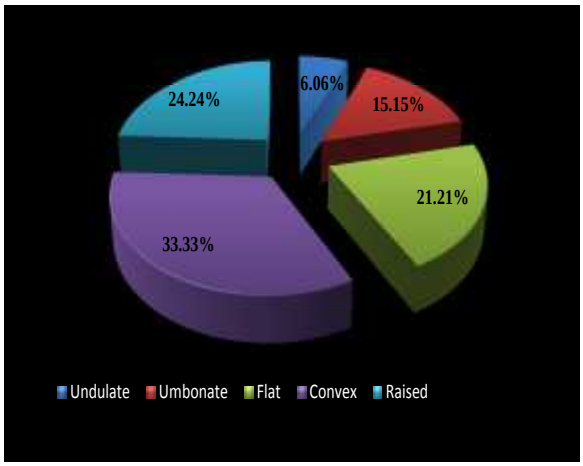
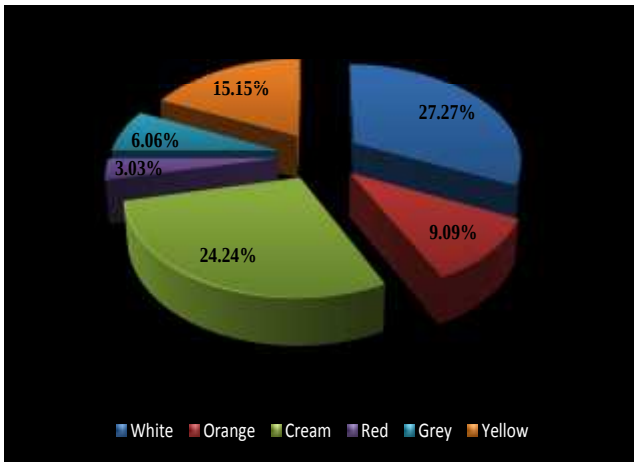


Elevation



Colour

Fig. 4.7 Morphological characterization of actinomycetes isolated from the rhizosphere of *Arnebia euchroma* (Ratanjot)

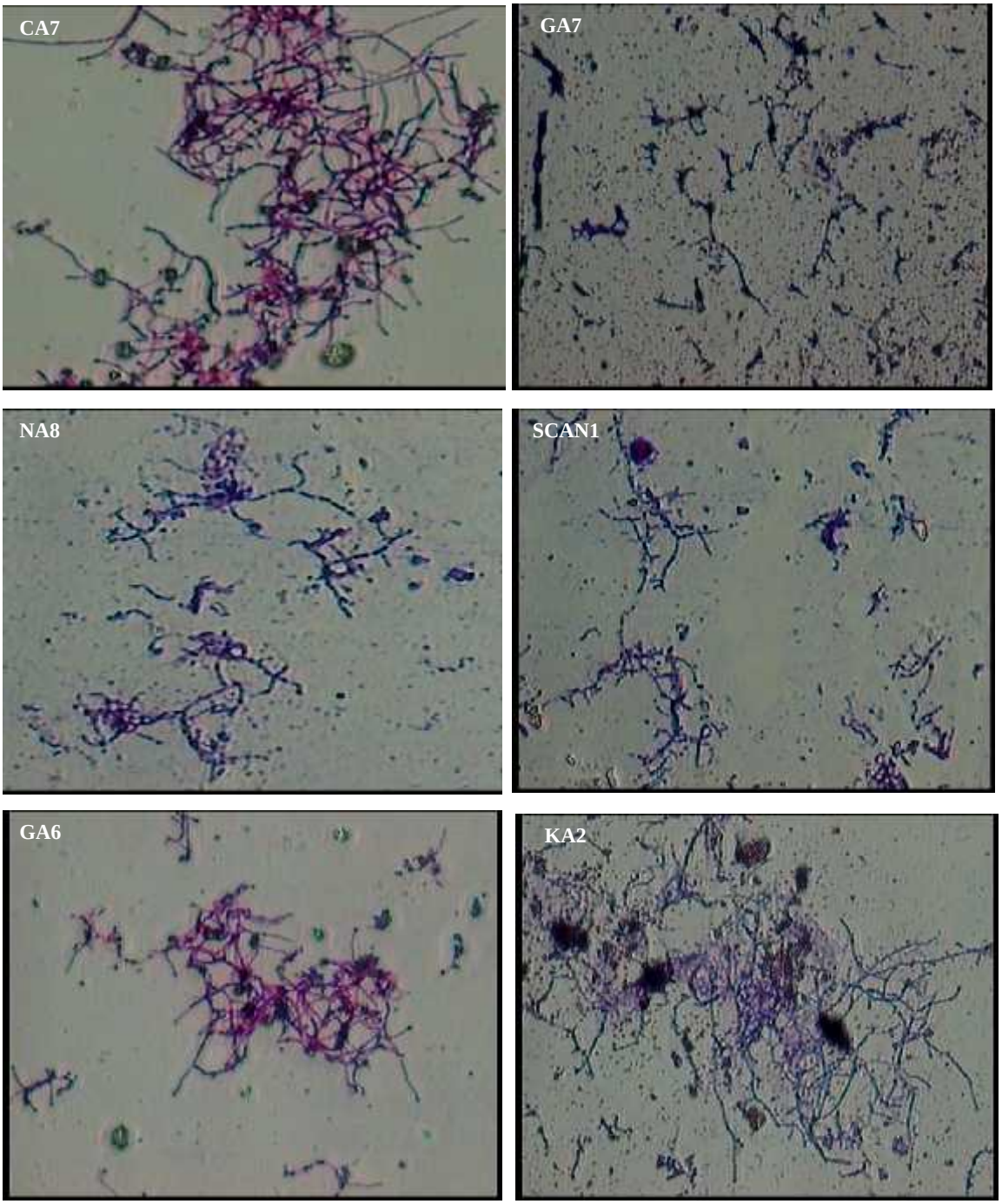


Plate 4.9 Microscopic view of actinomycetes obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot)

4.7.2 Physiological and biochemical characterization

Physiological characterization revealed that all actinomycete isolates showed optimum growth at temperature of 28±2ºC (Table 4.25). Out of nine isolates, three isolates namely GA6, GA7 and CA7 showed optimum growth at alkaline pH (9.0) while for rest of the isolates *viz*., NA4, KA2, SCAN1, NA8, CA5, NA6 neutral pH (7.0) was found to be optimum. Isolates GA6, GA7, NA4, CA7 and NA6 showed pellicle growth in liquid media while, isolates KA2, SCAN1, NA8 and CA5 showed sedimentary growth. Similar physiological characteristics were used by Akond *et al*. (2016) for the characterization of actinomycetes.

Biochemical characterization of actinomycetes revealed that isolates KA2 and NA8 fermented mannose with acid but no gas production while, isolate GA7 fermented inulin with gas production but no acid production. On the other hand, isolate NA6 showed gas but no acid production while used arabinose as carbon source (Table 4.25). All isolates except NA6 were found positive for MR test. Isolate NA4, among all, was found to be negative for TSI test. Two isolates GA6 and SCAN1 were observed to be negative for citrate utilization test while, rest showed positive test (Plate 4.10). Positive oxidase test was shown by majority of the isolates and only isolate NA6 was found to be negative for this test. Only six isolates i.e. GA7, SCAN1, NA8, CA5, CA7, NA6 were found catalase positive among all isolates. None of the isolates was found to be positive for indole test while, only one isolate i.e. GA6 showed gelatin liquefaction. On analogy, biochemical characterization of actinomycetes has been reported by other workers (Dhananjeyan *et al*., 2010 and Chaudhary *et al*., 2013). Shrivastava *et al*. (2012) identified and characterized actinomycetes based on their biochemical, physiological traits and 16S rRNA gene sequencing as *Streptomycetes* spp. Gautham *et al.* (2012) characterized *Streptomyces* species, the major antibiotic producing filamentous bacteria recovered from Western Ghats soils of Karnataka, India on the basis of their morphological characteristics.

According to Bergey's manual of Systematic Bacteriology, out of 9, 5 actinomycete isolates were tentatively identified as *Streptomyces* sp. while 2 were belonged to *Nocardia* sp. Rest of two isolates remained unidentified. Bergey's manual of Systematic Bacteriology a major taxonomic compilation of prokaryotes, gives information about all the recognized species of prokaryotes and contains number of keys to evaluate their characteristics (Madigan *et al*., 1997).

Table 4.25 Biochemical and physiological characterization of potential actinomycetes isolates

Sr. No.	Isolates	Biochemical characterization																	Physiological Characterization									Tentative identification
		TSI	Citrate	Oxidase	Carbohydrate fermentation									Catalase	Gelatin liquefaction	MR	VP	Indole	Optimum Temperature ˚C				Optimum pH				Growth in Liquid media	
					La	Ma	Mo	Ar	In	Rh	De	Sb	Su						28	37	45	55	4.0	7.0	9.0	11.0		
1	GA6	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+	-	Pellicle	*Nocardia* sp.
2	GA7	-	+	+	-	-	-	-	G	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	Pellicle	*Nocardia* sp.
3	NA4	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	Pellicle	Unidentified
4	KA2	-	+	-	-	-	A	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	Sedimentary	Unidentified
5	SCAN1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	Sedimentary	*Streptomyces* sp.
6	NA8	-	+	+	-	-	A	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-	Sedimentary	*Streptomyces* sp.
7	CA5	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-	Sedimentary	*Streptomyces* sp.
8	CA7	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-	Pellicle	*Streptomyces* sp.
9	NA6	-	+	-	-	-	-	G	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	+	-	Pellicle	*Streptomyces* sp.

+ = Positive reaction, - = No reaction, A = Acid production, G = Gas production
TSI = Triple Sugar Iron, MR= Methyl Red, VP =Voges–Proskauer
La-Lactose, Ma-Mannitol, Mo-Mannose, Ar-Arbinose, In-Inulin, Rh-Rhamnose, De- Dextrose, Sb-Sorbitol, Su-Sucrose

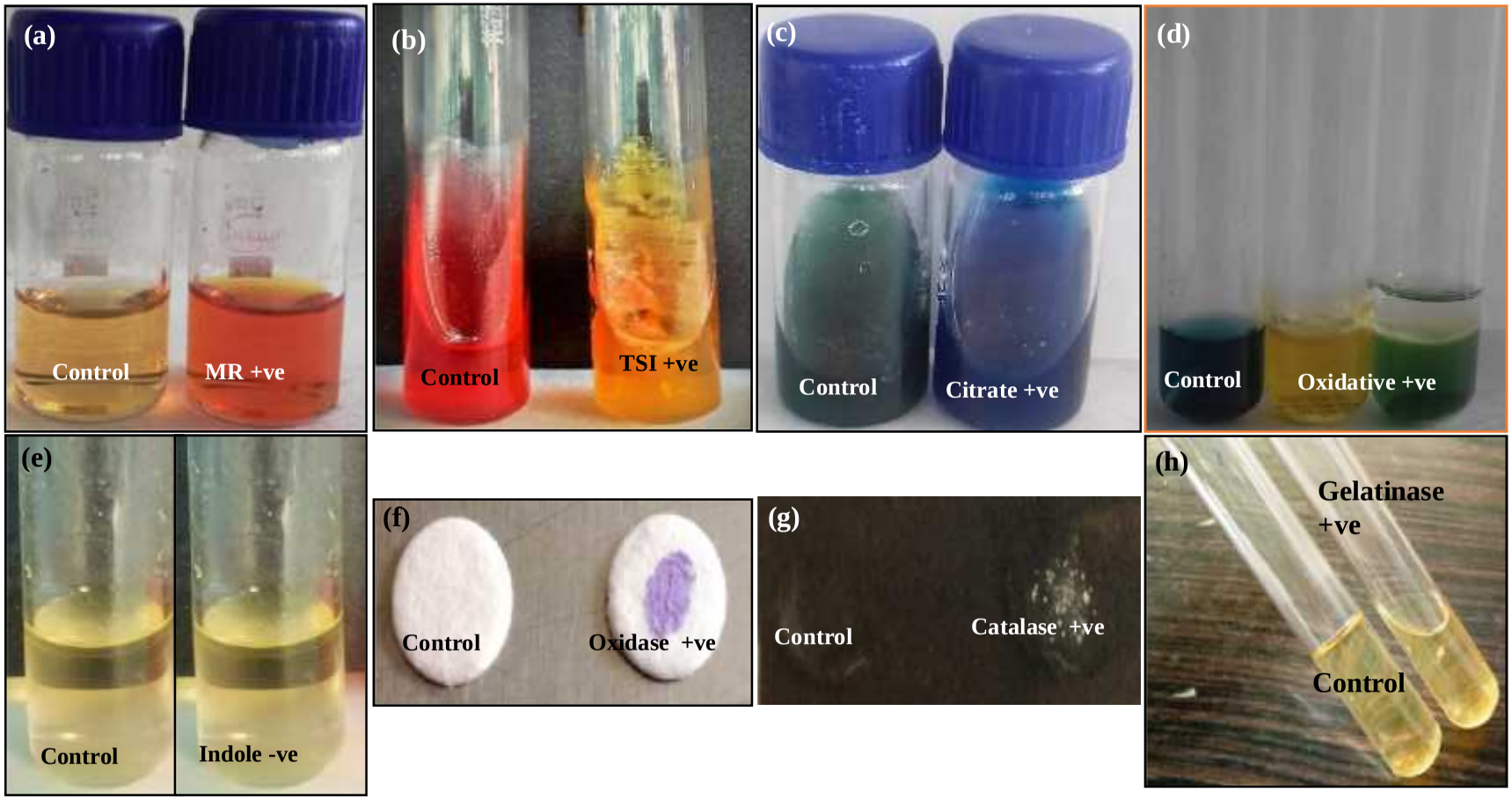


Plate 4.10 Biochemical characterization of actinomycete isolates (a) MR Test (b) TSI Test (c) Citrate Utilization Test (d) Oxidative Fermentation Test (e) Indole Test (f) Oxidase Test (g) Catalase Test (h) Gelatin liquefaction test

4.7.3 Molecular characterization of potential actinomycetes isolates

The field of microbial systematic has been revolutionized by the use of most frequently used marker (16S rRNA gene) (Woese *et al*., 1985; Amann *et al*., 1995; Mora and Amann, 2001). 16S rRNA gene has extensive distribution, highly conserved nature, and fundamental role of ribosome in protein synthesis, no horizontal gene transfer and its slow rate of evolution, which represents an appropriate level of variation between organisms. Hence, this gene is used for determining the phylogenetic relationship among bacteria (Stackebrandt and Goebel 1994; Mora and Amann, 2001; Clarridge, 2004)**.**

As sequencing of rRNA genes has become a common tool in microbial investigations, a considerable volume of sequence data of rRNA genes from diverse bacteria has been deposited in public data bank (NCBI) enabling the phylogenetic affiliation of bacteria.

Based on PGP potential, two potential actinomycete isolates (NA8 and CA7) were identified at genomic level using 16S rRNA gene sequencing technique. Genomic DNA was isolated using Quick-DNA^{TM} Fungal/Bacterial Microprep kit (Zymo) following manufacturer's instructions (Plate 4.11). Isolated DNA samples were sent for sequencing at National Centre for Cell Sciences (NCCS), National Centre for Microbial Resource (NCMR) Pune, Maharashtra (India).

16 S rRNA gene sequences of these actinomycetes (NA8 and CA7) were analyzed to determine the similarity (homology) with library sequences available in the NCBI, GenBank, USA. Sequences were BLASTed using on-line Blastin program (Altschul *et al*. 1997). These were then edited manually and analyzed using NCBI BLASTN program (http://www.ncbi.nih.gov/blast). The phylogenetic analysis was carried out in MEGA 7.0 software program. The sequences of NA8 and CA7 were submitted to NCBI, GenBank, USA (Appendix VI) and were assigned with the accession numbers MK836018 and MK 836019, respectively.

As depicted in Table 4.26, sequence similarity search for isolate NA8 showed 99.92 per cent similarity with the *Streptomyces rectiviolaceus* having accession no. NRRL B 16374 (T). Whereas, CA7 showed 99.12 per cent similarity with *S. Silaceus* with accession no. B24166 (T) Phylogenetic tree of isolate NA8 and CA7 with respect to other actinomycetes has been presented in Fig. 4.8.

Yilmaz *et al.* (2007) identified actinomycetes isolated from the rhizosphere of local endemic plants of Turkey as *Streptomyces lydicus, S. rochei, S. microflavus, S. griseoflavus, S. albidoflavus and S. violaceusniger* using 16S rRNA gene sequencing. Based on 16S rRNA gene sequencing, Zhang *et al*. (2011) and Ramakrishnan *et al*. (2009) identified actinomycetes recovered from the rhizospheres of medicinal plants as *Geodermatophilus ruber* and *Streptomycetes* sp. respectively.

Table 4.26 Molecular characterization of potential actinomycetes

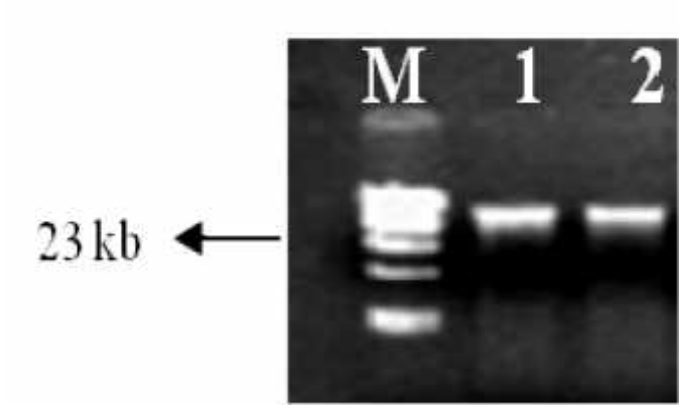
Isolate	Identified as	Closest homology with	Sequence length (bp)	Identity (%)	GenBank Accession no.
NA8	*Streptomyces rectiviolaceus*	*Streptomyces rectiviolaceus* NRRL B 16374 (T)	1226	99.92	MK836018
CA7	*Streptomyces silaceus*	*Streptomyces silaceus* NRRL B 24166 (T)	1377	99.12	MK836019

4.8 SEED GERMINATION BIOASSAY

The effect of growth regulators produced by two efficient actinomycetes isolates *viz., Streptomyces rectiviolaceus* (NA8) and *Streptomyces silaceus* (CA7) on root elongation of maize and pea seeds were evaluated. As shown in Table 4.27, Fig. 4.9 and Plate 4.12, the pre-germinated seeds of maize and pea showed significant increase in root length over the control. The highest root length of 2.6 cm was recorded for isolate NA8 over control (1.3 cm) in case of maize. Whereas, in case of pea, highest root length of 2.02 cm was recorded for isolate CA7 over control (1.46 cm). Our results are in agreement with Anwar *et al.* (2015) who evaluated the effect of *Streptomyces nobilis* WA-3 on the seeds of *Triticum aestivum* (Wheat) and found significant increase in shoot and root lengths of seeds over control.

Table 4.27 Effect of growth regulators produced by potential actinomycetes, *Streptomyces rectiviolaceus* (NA8) and *Streptomyces silaceus* (CA7) on root elongation in uniformly pre-germinated seeds of maize and pea

Treatment	Root length (cm)	
	Maize	Pea
Control	1.3	1.46
Streptomyces rectiviolaceus (NA8)	2.6	2.00
Streptomyces silaceus (CA7)	2.4	2.02
CD(0.05)	0.26	0.23



Lane M: Marker λ DNA/HindIII marker; Lane 1: DNA of NA8; Lane 2: DNA of CA7

Plate 4.11 Genotypic characterization of potential actinomycetes isolated from *Arnebia euchroma* (Ratanjot)

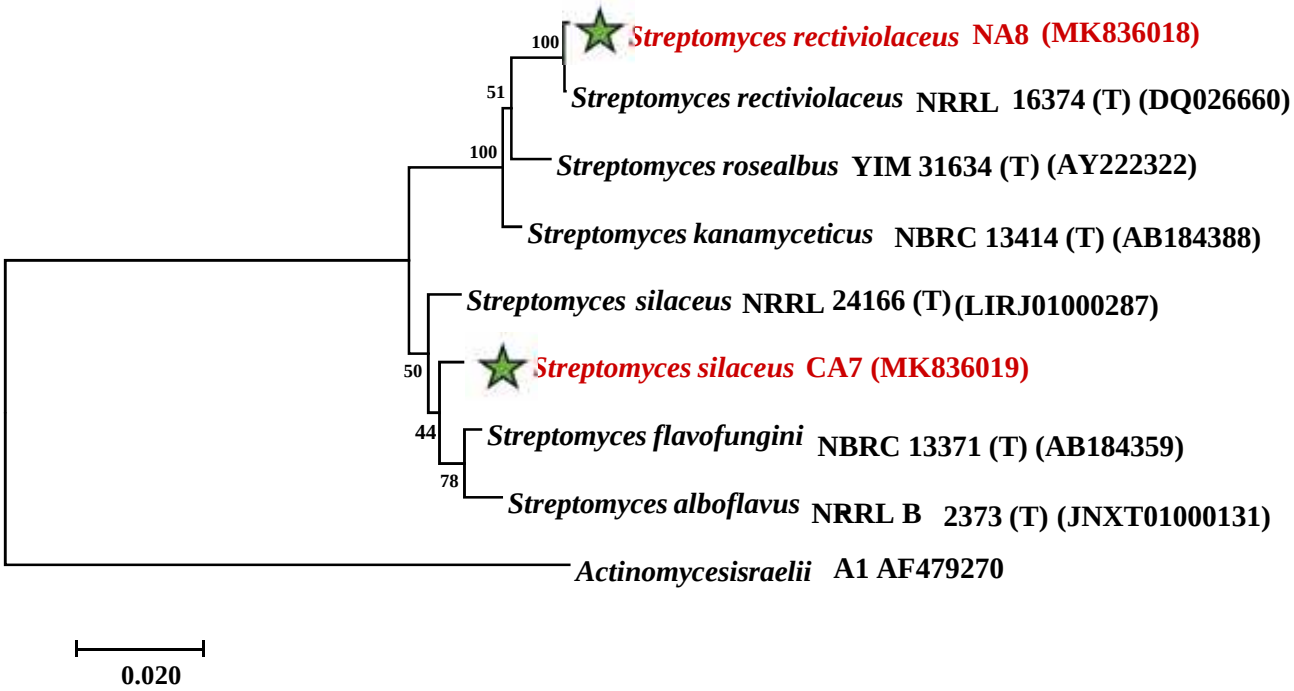
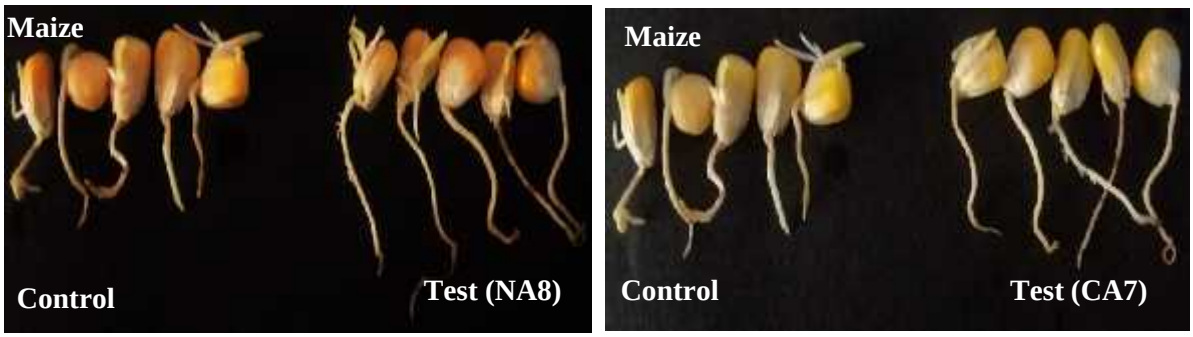
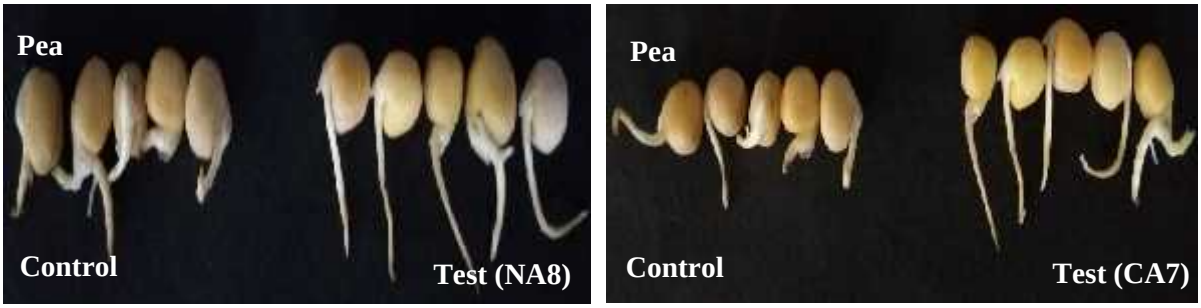


Fig 4.8 Phylogenetic tree showing evolutionary relationship of ★ potential PGP actinomycetes isolated from rhizosphere of *Arnebia euchroma* and its related taxa constructed using MEGA 7.0 software by neighbour-joining method

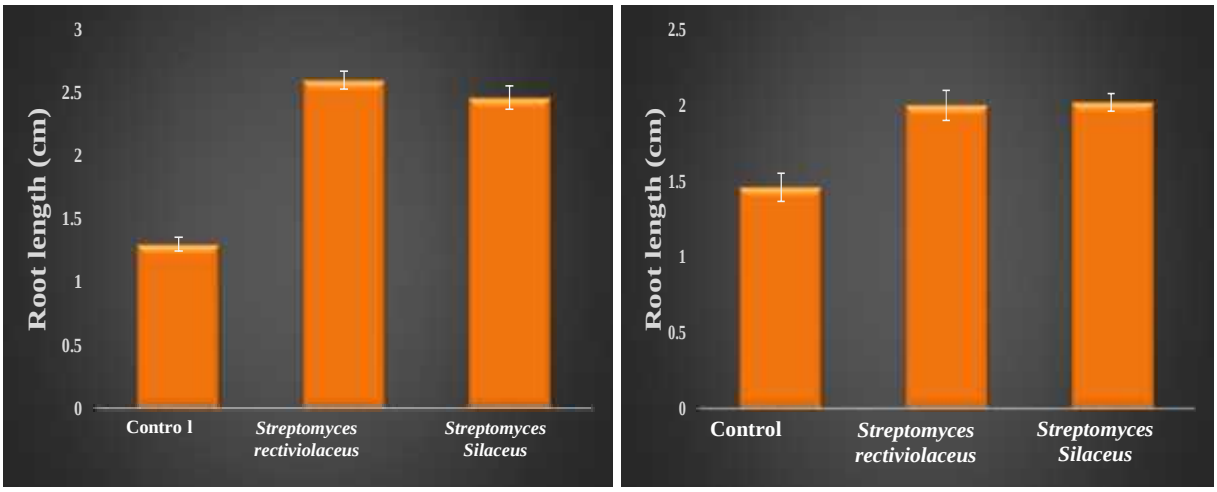


(a)



(b)

Plate 4.12 Effect of growth regulators produced by potential actinomycetes-*Streptomyces rectiviolaceus* NA8 and *Streptomyces silaceus* CA7 on root elongation in uniformly pre-germinated seeds of (a) maize (b) pea after 7 days of incubation at 28 ± 2 °C



(a) **(b)**

Fig 4.9 Effect of growth regulators produced by potential actinomycetes-*Streptomyces rectiviolaceus*NA8 and *Streptomyces silaceus* CA7 on root elongation in uniformly pre-germinated seeds of (a) maize (b) pea after 7 days of incubation at 28 ± 2 °C

The application of PGPR in agriculture has been reported to enhance plant growth, biomass and yield (Ahemad *et al*., 2014; Kundan *et al*., 2015). A significant improvement in root length, in maize and pea demonstrated plant growth promotion potential of the actinomycetes isolates *viz., Streptomyces rectiviolaceus* (NA8) and *Streptomyces silaceus* (CA7). This could be ascribed to production of IAA-like auxin which is one of the important mechanisms of plant growth promotion (Dimpka *et al.*, 2008).

Sreevidya *et al.* (2015) evaluated the effect of three actinomycetes on root and shoot lengths of chickpea. Out of these, one isolate significantly improved both root and shoot lengths. While, rest of two isolates significantly enhanced only root lengths in comparison to control.

4.9 PIGMENTS PRESENT IN THE ROOTS OF *Arnebia euchroma* (RATANJOT)

The pigments present in the roots of *Arnebia euchroma* were detected using Ultra Performance Liquid Chromatography (UPLC) technique. Results revealed that the major pigments present in the roots of *Arnebia euchroma* are ββ-dimethylacrylshikonin (0.145 per cent) followed by Deoxy-shikonin (0.098 per cent) and Shikonin (0.011 per cent) as depicted in Table 4.28 and Fig. 4.10 a & b). On contrary, acetyl shikonin was found to be the major pigment in *A. euchroma* through HPLC analysis (Malik *et al.*, 2008). Shikonin, alkannin and their derivatives were reported to be the main ingredients of *A. euchroma* (Royle) Johnst native to Northwest of China by Zhang *et al.* (2018).

Table 4.28 Percentage of pigments present in the roots of *Arnebia euchroma* (Ratanjot)

Compound	Content (%)
Shikonin	0.011
Deoxy-shikonin	0.098
ββ-dimethylacrylshikonin	0.145

Xiao *et al.* (2011) quantified shikonin and its derivatives in *Arnebia euchroma* using various chromatographic techniques and observed higher concentration of β,β′-dimethylacrylshikonin (2.15–1360 µg/mL) than shikonin(1.75–140 µg/mL). Shan-Lin and his coworkers (1984) identified six naphthaquinone pigments *viz.*, deoxyshikonin, β, β-dimethylacrylalkannin, acetylshikonin, β-acetoxyisovalerylalkannin, shikonin and β-hydroxyisovalerylalkannin in the roots of *A. euchroma.*

So, it can be inferred from these results that *A. euchroma* roots are rich in β,β-dimethylacryl shikonin, Deoxy-shikonin and Shikonin pigments which are responsible for imparting purple colour to the roots. Besides their use as pigments, shikonin and its derivatives have been used as a medicine for antimicrobial, anti-inflammatory and anti-tumor purposes as they have shown the ability to heal wounds, burns and hemorrhoids via proliferation of granulation tissue (Kamei *et al*., 2002 and Ordoudi *et al*., 2011).

4.10 CONSERVATION OF POTENTIAL ACTINOMYCETE ISOLATES

In order to conserve this valuable actinomycetes diversity, two potential actinomycete isolates *viz*., *Streptomyces rectiviolaceus* NA8 and *Streptomyces silaceus* CA7 were deposited in National Culture Collection Centre, National Centre for Cell Sciences (NCCS), National Centre for Microbial Resource (NCMR) Pune, Maharashtra, India and were assigned with accession number MCC 4170 and MCC 4262 (Appendix-VII), respectively.

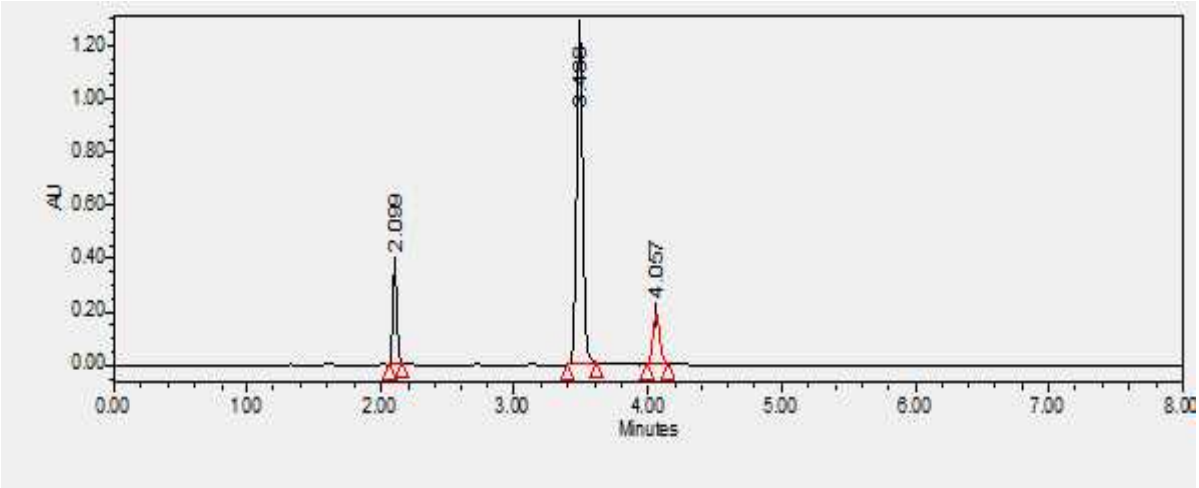
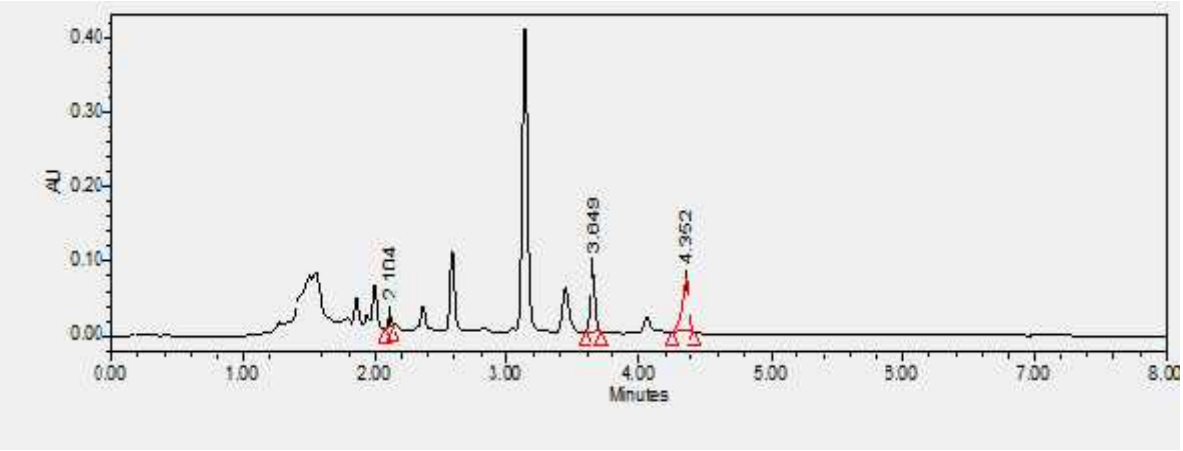


Fig. 4.10 (a) Chromatogram of standard mixture of 1) Shikonin 2) Deoxyshikonin and 3) ββ-dimethylacrylshikonin



Chromatogram of *Arnebia euchroma* sample

Fig. 4.10 (b)UPLC of Shikonin and its derivatives present in the roots of *Arnebia euchroma* (Ratanjot)

Chapter-5

SUMMARY AND CONCLUSIONS

Owing to low cost, easy accessibility, longer shelf life and probability of discovering some new molecules, use of herbal drugs is growing enormously. This has placed a profound pressure on medicinal plants ' biodiversity, especially on trans-Himalayan native plants such as *Arnebia euchroma*, commonly known as 'Ratanjot'. Therefore, the present study entitled **"Diversity of actinomycetes in the rhizosphere of *Arnebia euchroma* (Ratanjot): A critically endangered plant of Himachal Pradesh"** was proposed to evaluate plant growth promoting potential of actinomycetes diversity exists in the rhizosphere of *Arnebia euchroma* (Ratanjot). The results obtained in the present study are summarized as below.

The rhizospheric soil samples of *Arnebia euchroma* (Ratanjot) were collected from four sites namely Nako and Chango in Kinnaur and Gue and Kibber in Lahaul & Spiti districts of Himachal Pradesh. The collected samples were processed for elucidating the diversity of actinomycetes using Standard Plate Count technique. A total of 33 isolates (15 from Nako, 7 from Chango, 16 from Gue and 5 from Kibber) were obtained. A statistically significant difference was observed in the actinomycetes load obtained at selected sites and on different isolation media. The actinomycetes load was found to be significantly higher at Chango (Log CFU/mL=1.44) followed by Nako (Log CFU/mL=1.40) and Gue (Log CFU/mL=1.38) while, minimum at Kibber (Log CFU/mL=1.33). Four different media *viz.*, Starch Casein Agar (SCA), Actinomycetes Isolation agar (AIA), Nutrient Agar (NA) and Kenknight and Munaier's Medium (KM) were used for the isolation of actinomycetes. Amongst these, a significantly higher actinomycetes load was observed on SCA (Log CFU/mL=1.48) followed by AIA (Log CFU/mL=1.41), NA (Log CFU/mL=1.29) and KM (Log CFU/mL=0.88) thereby indicated, SCA as best medium for the isolation of actinomycetes.

All the actinomycetes isolates were evaluated for their PGP potential and only 45.45 per cent of the total isolates showed positive phytase activity and siderophore production while, 33.33 per cent showed phosphate solubilization activity qualitatively. On quantitative basis, significantly higher P-solubilization (53.66 µg mL^{-1}) was shown by NA8 while, lowest (6.44 µg mL^{-1}) by SCAN1. However, the phytase activity was observed to be significantly

higher (134.44 U/ml) for CA7 and minimum (15.33 U/ml) for NA10. Among all isolates, SCAK5 and SCAN1 showed significantly higher (17.24% SU) and lower siderophore (5.46% SU)) production, respectively whereas, three isolates *viz*., NA5, GA5 and NA9 were found to be statistically at par. All the isolates were found to be positive for nitrogen fixing activity. However, only 66.66 and 3.03 percent of total isolates showed ammonia and HCN production, respectively.

All the isolates were also screened for extracellular production of different lytic enzymes *viz*., pectinase, chitinase, cellulase, protease and lipase. However, 84.84, 90.90, 96.96, 72.72 and 42.42 percent of the total isolates exhibited pectinase, chitinase, cellulase, lipase and protease activities, respectively. Qualitatively, majority of the isolates (90.92%) exhibited excellent chitinase activity with maximum chitinase production was shown by isolate NA6 (33 mm) and minimum (4mm) by CA1. On quantitative basis, isolates NA6 and CA1 showed significantly higher (6.81 U/mL) and lower (0.42U/mL) chitinase activities, respectively while, isolates NA7, SCAN1, CA6, KMN2, KMN1 and CA5 were found to be statistically at par. On qualitative basis the highest protease activity (33 mm) was shown by isolate NA7 while, minimum (9 mm) by SCAK3 among all isolates. Quantitatively, isolates NA7 and NA10 showed significantly higher (28.13 U/mL) and lower (1.22 U/mL) protease activities, respectively, while, NA9 and GA4 were found to be statistically at par. Maximum cellulase activity (29mm) was shown by KMN1 while, minimum (6 mm) by GA6 qualitatively. On quantitative basis, the values of cellulase activity ranged between 24.18 - 31.21 U/mL being significantly highest (31.21 U/mL) for NA5 and lowest (24.18 U/mL) for GA6 whereas, isolates NA2, NA3, GA5, SCAC3, KMN1 and CA5 were found to be statistically at par. Qualitatively, highest pectinase activity was shown by isolate NA6 (29 mm) and lowest by isolate GA6. Quantitative estimation of pectinase activity revealed that its values varied from 1.60- 48.33 U/mL, being significantly highest (48.33 U/mL) for NA6 and lowest (1.60 U/mL) for GA6, respectively while, isolates NA3, GA1, NA1 and GA4 were observed to be statistically at par. Qualitatively, maximum lipase activity (28 mm) was shown by isolate NA6 while minimum by isolate NA5 (6 mm). Quantitatively, values of lipase activity ranged between 1000-2040 IU/mL. The isolate NA5 showed significantly highest (2040 IU/mL) lipase activity among all isolates while, six isolates *viz*., GA1, NA10, NA6, NA8, NAN2 and SCAK5, were found to be statistically at par.

All isolates showed good production of growth regulators i.e Auxins, Gibberellins and cytokinins. The values of auxins production ranged from 11.55 to 44.52 µg/ml and maximum production was shown by NA10 (44.52 µg/ml) while, minimum (11.55 µg/ml) by NA3 among all isolates. However, gibberellins production varied from 4.52 to 40.60 µg/ml, being highest for NA7 (40.60 µg/ml) and lowest for KMN2 (4.52 µg/ml). On analogy, cytokinins production ranged between 15.26 to 34.24 µg/ml, being highest for CA5 (34.0 µg/ml) and lowest for GA2 (15.0 µg/ml).

The potential actinomycete isolates were evaluated for their antagonistic activity against three soil borne fungal phytopathogens *viz*., *Fusarium oxysporum, Sclerotium rolfsii and Rhizoctonia solani* using dual culture technique. Among all, the isolate NA4 showed significantly higher inhibition against *F. oxysporum* (37.5 %) while, minimum (31.2%) by GA7 over control. A significantly higher inhibition (42.2 %) in the growth of *Sclerotium rolfsii* was shown by isolate SCAN1 followed by KA2 and NA6 (33.3%). Isolates SCAN1 and NA8 showed significantly highest (40.0 %) and lowest (31.2%) per cent inhibitions in the growth of *Rhizoctonia solani,* respectively. The gaseous metabolites released by the actinomycete isolates inhibited the growth of fungal plant pathogens and the isolate NA4 showed significantly higher inhibition against *F. oxysporum* (36.25 %) while, minimum (21.87%) by SCAN1 over control. The significantly higher inhibition (44.44 %) in the growth of *Sclerotium rolfsii* was shown by isolate GA7 followed by NA8 (41.67 %) and NA6 (36.33%). Isolate NA8 showed significantly higher (38.12 %) per cent inhibition in the growth of *Rhizoctonia solani.* Whereas, four isolates *viz*., CA7, NA6, NA4 and GA7 were found to be statistically at par.

Based on PGP potential, out of 33, only 9 potential isolates *viz*., GA6, GA7, NA4, KA2, SCANI, NA8, CA5, CA7, NA6 were selected for their morphological, physiological and biochemical characterization. Morphological and microscopic studies revealed that all the actinomycetes isolates were gram positive filamentous bacteria and majority of the colonies were flat, umbonate and raised with mainly entire margin. The color of the colony varied from yellow to cream. Physiological characterization revealed that all the isolates showed optimum growth at a temperature 28±2ºC. Out of nine, three isolates namely GA6, GA7 and CA7 showed optimum growth at alkaline pH (9.0) while, for the rest of the isolates neutral pH (7.0) was observed to be optimum. According to Bergey's manual of Systematic

Bacteriology out of 9, 5 actinomycete isolates were tentatively identified as *Streptomyces* spp. while 2 were belonged to *Nocardia* spp. Rest of the two isolates, remained unidentified.

Based on PGP potential, out of 9 only two actinomycetes isolates *viz*., CA7 and NA8 were subjected for molecular characterization using 16S rRNA gene sequencing technique and were identified as *Streptomyces silaceus* and *Streptomyces rectiviolaceus*, respectively. 16 S rRNA gene sequences of *Streptomyces silaceus* CA7 and *Streptomyces rectiviolaceus* NA8 were submitted to NCBI, GenBank, USA and were assigned with accession numbers MK836019 and MK836018, respectively.

The potential actinomycete isolates *viz*., *Streptomyces rectiviolaceus* NA8 and *Streptomyces silaceus* CA7 were deposited in National Culture Collection Centre, National Centre for Cell Sciences (NCCS), National Centre for Microbial Resource (NCMR) Pune, Maharashtra, India and were assigned with accession number MCC 4170 and MCC 4262 (Appendix-VII), respectively.

The effect of growth regulators produced by *Streptomyces silaceus* (CA7) and *Streptomyces rectiviolaceus* (NA8) on root elongation of maize and pea seeds was evaluated. The pre-germinated seeds of maize and pea showed significant increase in root length over the control. The highest root length of 2.6 cm was recorded for isolate NA8 over control (1.3cm) in case of maize. Whereas, in case of pea, highest root length of 2.02 cm was recorded for isolate CA7 over control (1.46cm).

The pigments present in the roots of *Arnebia euchroma* were detected using Ultra Performance Liquid Chromatography (UPLC) technique which revealed that the major pigment (s) present in the roots of *Arnebia euchroma* are ββ-dimethylacrylshikonin (0.145 %) followed by Deoxy-shikonin (0.098 %) and Shikonin (0.011%).

To conclude, the present study clearly identified multifarious plant growth promoting traits in actinomycetes that make them good candidates as plant growth promoters and biocontrol agents. Moreover, these traits might contribute towards the adaption of the plant under study in prevailing harsh environmental conditions. Therefore, application of such strains in agricultural fields may improve and enhance plant growth along with disease suppression in them.

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APPENDIX-I

Culture Media

Chrome AzurolSulphonate agar

King's medium B base

Proteose peptone	:	18 g
Dipotassium hydrogen phosphate	:	1.3 g
Magnesium sulphate heptahydrate	:	1.3 g
Distilled Water	:	900 ml

CAS-HDTMA solution

Chrome Azurol	:	60.5 mg dissolved in 50 ml distilled water
1 mM ferric chloride	:	10.0 ml
Hexadecyltrimethyl-ammonium bromide (HDTMA)	:	72.9 mg dissolved in 40 ml distilled water

Ferric chloride (1 mM)

Ferric chloride anhydrous	:	1.62 g
Hydrochloric acid	:	36.5 ml
Distilled Water	:	up to 100 ml

Chrome Azurol, $FeCl_3.6H_2O$ and HDTMA solutions were mixed slowly and CAS-HDTMAsolution was slowly added to King's medium B base with constant shaking. The pH was adjusted to 6.8 ± 0.2, added 18 g agar-agar, heated to boiling and sterilized by autoclaving at 121 °C for 15 min.

Pikovskaya's agar (PVK) (g L^{-1})

Glucose	:	10.0
Tricalcium phosphate	:	5.0
Ammonium sulphate	:	0.5
Potassium chloride	:	0.2
Magnesium sulphate	:	0.1
Ferrous sulphate	:	0.01

Manganous sulphate	:	0.01
Yeast extract	:	0.5
pH	:	7.0 ± 0.2
Bromo phenol blue (0.4%)	:	3.0 ml
Distilled Water	:	1000 ml

Added 18 g agar-agar, heated to boiling and sterilized by autoclaving at 121 °C for 15 min.

Pikovskaya's broth (PVK) (g L^{-1})

Glucose	:	10.0
Tricalcium phosphate	:	5.0
Ammonium sulphate	:	0.5
Potassium chloride	:	0.2
Magnesium sulphate	:	0.1
Ferrous sulphate	:	0.01
Manganous sulphate	:	0.01
Yeast extract	:	0.5
pH	:	7.0 ± 0.2
Bromo phenol blue (0.4%)	:	3.0 ml
Distilled Water	:	1000 ml

Nutrient agar (g L^{-1})

Peptic digest of animal tissue	:	5.0
Sodium chloride	:	5.0
Beef extract	:	1.5
Yeast extract	:	1.5
Agar-agar	:	18.0
pH	:	7.4 ± 0.2
Distilled Water	:	1000 ml

Nutrient broth (g L^{-1})

Peptic digest of animal tissue	**:**	5.0
Sodium chloride	**:**	5.0
Beef extract	**:**	1.5
Yeast extract	**:**	1.5
pH	**:**	7.4 ± 0.2
Distilled Water	**:**	1000 ml

Potato Dextrose Agar (g L^{-1})

Potatoes, infusion form	:	200.0
Dextrose	:	20.0
Agar	:	15.0
pH	:	5.6 ± 0.2
Distilled Water	:	1000 ml

Succinate broth (g L^{-1})

Potassium dihydrogen phosphate	**:**	3.0
Ammonium sulphate	**:**	1.0
Magnesium sulphate	**:**	0.2
Succinic acid	**:**	4.0
pH	**:**	6.8 ± 0.2
Distilled water	**:**	1000 ml

Actinomycetes Isolation Agar (g L^{-1})

Sodium caseinate	:	2.0
L-Asparagine	:	0.1
Sodium Propionate	:	4.0
Dipotassiumphosphate	:	0.50

Magnesium sulphate	:	0.001
Ferrous sulphate	:	0.1
Agar	:	15.0
pH	:	8.1 ± 0.2
Distilled Water	:	1000 ml

Starch Casein Agar (g L^{-1})

Soluble starch	:	10.0
Casein	:	0.3
Potassium nitrate	:	2.0
Magnesium sulphate	:	0.05
Sodium chloride	:	2.0
di-potassium hydrogen phosphate	:	2.0
Calcium carbonate	:	0.02
Ferrous sulphate	:	0.01
Agar	:	15.0
Distilled Water	:	1000 ml
pH	:	7.0 ± 0.1

Kenknight and &Munaier's medium (g L^{-1})

di-Potassium hydrogen phosphate	:	1.0
Sodium nitrate	:	0.1
Potassium chloride	:	0.1
Magnesium sulphate	:	0.1
Glucose	:	0.1
pH	:	7 ± 0.2
Distilled water	:	1000 ml

Phytase screening medium (g L^{-1})

Glucose	:	15.0
Ammonium nitrate	:	5.0

Magnesium sulphate	:	0.5
Potassium chloride	:	0.5
Ferrous sulphate	:	0.01
Manganese sulphate	:	0.01
Calcium phytate	:	5.0
Agar	:	15.0
pH	:	5.5 ± 0.2
Distilled water	:	1000 ml

Peptone broth (g L^{-1})

Peptone	:	10.0
Sodium chloride	:	5.0
Distilled water	:	1000 ml

Jensen's medium (g L^{-1})

Sucrose	:	20.0
Dipotassium phosphate	:	1.0
Magnesium sulphate	:	0.5
Sodium chloride	:	0.05
Ferrous sulphate	:	0.01
Sodium molybdate	:	0.005
Calcium carbonate	:	2.0
Agar	:	15.0
Distilled Water	:	1000 ml

Chitinase screening medium (g L^{-1})

Colloidal chitin	:	5.0
Magnesium sulphate	:	0.5
Dipotassium hydrogen phosphate	:	0.7
Dihydrogen potassium phosphate	:	0.3
Ferrous sulphate	:	0.01

Mangnese chloride	:	0.001
Sodium chloride	:	0.3
Yeast extract	:	0.2
Distilled Water	:	1000 ml

Skim milk agar medium (g L^{-1})

Skim milk	:	10.0
Peptone	:	5.0
Beef extract	:	3.0
Sodium chloride	:	5.0
Agar	:	15.0
Distilled Water	:	1000 ml

Carboxy Methyl Cellulose (CMC) agar (g L^{-1})

Peptone	:	5.0
NaCl	:	5.0
Yeast	:	3.0
Carboxy Methyl Cellulose	:	5.0
Agar	:	20.0
Distilled Water	:	1000 ml

Pectinase Screening media (g L^{-1})

Yeast extract	:	5.0
Peptone	:	5.0
Tryptone	:	10.0
Pectin	:	5.0
Agar	:	20.0
Distilled Water	:	1000 ml

Tributyrin agar medium (g L^{-1})

Peptic digest of animal tissue	:	5.0
Yeast extract	:	3.0

Agar	:	15.0
pH	:	7 ± 0.2
Tributyrin	:	10 ml
Distilled Water	:	1000 ml

Nutrient Gelatin (g L^{-1})

Gelatin	:	120.0
Beef extract	:	3.0
Peptone	:	5.0
Distilled Water	:	1000 ml
pH	:	6.8 ± 0.2

MR-VP broth (g L^{-1})

Glucose	:	5.0
Peptone	:	5.0
Dipotassium hydrogen phosphate	:	5.0
Distiiled water	:	1000 ml
pH	:	7.5

Simmon's citrate agar (g L^{-1})

Ammonium dihydrogen phosphate	:	1.0
dipotassium phosphate	:	1.0
Sodium chloride	:	5.0
Sodium citrate	:	2.0
Magnesium sulphate	:	0.2
Agar	:	15.0
Distilled Water	:	1000 ml

Oxidative-fermentation basal medium (g L^{-1})

Peptone	:	2.0
NaCl	:	5.0
K_2HPO_4	:	0.3
Glucose	:	10.0
Bromothymol blue	:	0.03
Agar	:	2.0
Distilled water	:	1000 ml

pH : 7.1

Triple Sugar Iron (TSI) Agar (g L^{-1})

Meat	:	3.0
Yeast extract	:	3.0
Peptone	:	20.0
Glucose	:	1.0
Lactose	:	10.0
Sucrose	:	10.0
Ferrous ammonium sulphate	:	0.2
Sodium chloride	:	5.0
Sodium thiosulphate	:	0.3
Agar	:	15.0
0.2% phenol red solution	:	12 ml
Distilled water	:	1000 ml
pH	:	7.3

Tryptone Broth (g L^{-1})

Tryptone	:	10.0
Sodium chloride	:	5.0
Final pH	:	7.5 ± 0.2

APPENDIX-II

Solutions and Reagents

Barton's reagent

A. Dissolved 25 g ammonium molybdate in 400 ml distilled water.

B. Dissolved 1.25 g ammonium metavanadate in 300 ml of boiling distilled water, cooled and then added 250 ml concentrated HNO_3.

The solution A and B were mixed and volume made 1 litre with distilled water.

Chrome Azurol Sulphonate (CAS) reagent

CAS stock solution (2 mM):

CAS	:	0.121 g
Distilled water	:	100 ml

Fe stock solution (1 mM)

Ferric chloride anhydrous	:	0.0162 g
Hydrochloric acid (10 mM)	:	10.0 ml

HDTMA solution:

HDTMA	:	0.0219 g
Distilled water	:	50 ml

Piperazine solution

Salt solution (30 ml)

K_2HPO_4-.0011g

NaCl-0.018g

NH_4Cl-0.037g

These salts were firstly mixed in 25ml distilled water& then mixed properly after that added remaining 5ml. To 25ml of salt solution added 1g piperazine and 6.8 pH was adjusted and final volume made 30 ml with D.W.

Mixed 1 ml iron solution with 8 ml CAS solution and to this added 40ml HDTMA solution in a 100 ml measuring cylinder. Added 50 ml piperazine solution and the volume made to 100 ml with distilled water. Stored in amber coloured bottle at 4 °C.

DNSA Reagent

2 g dinitrosalicyclic acid, 0.4 g phenol, 0.1 g sodium sulphite and 40 g Rochelle salt were dissolved in 100 mL of 2 % (w/v) NaOH solution and diluted to 200 ml with distilled water.

Salkowski reagent

Perchloric acid (35%)	**:**	49 ml
Ferric chloride (0.5 M)	**:**	1.0 ml

Ferric chloride (0.5 M):

Ferric chloride anhydrous	**:**	8.11 g
De-ionized water (15 Ω)	**:**	100.0 ml

Prepared fresh when required.

Kovac'sIndole Reagent

10g of p-dimethylaminobenaldehyde dissolved in 150 ml of isoamyl alcohol and 50 ml of concentrated HCl was added slowly.

Methyl Red indicator

Methyl red	: 0.20
Ethyl alcohol	: 60.0
Distilled water	: 40.0 ml

Voges-Proskauer (VP) Reagent

3ml of 5% solution of α-Naphthol in absolute ethanol and 1ml of 40% KOH

Nessler's Reagent

Mercuric chloride	:	10.0
Potassium iodide	:	7.0
Sodium hydroxide	:	16.0
Water	:	100.0 ml
pH	:	12 ± 0.05

0.1 M NaOH stock solution

4g NaOH pellets were dissolved in 1000 mL of Distilled water

Zinc acetate reagent

Zinc acetate	:	21.9 g
Glacial acetic acid	:	1 ml
Distilled Water	:	100 ml

APPENDIX-III

Buffers

Tris-acetate (TAE) buffer stock (50×)

Trisbase	**:**	242 g
Glacial acetic acid	**:**	57.1 ml
0.5 M EDTA (pH 8.0)	**:**	100 ml
Distilled water	**:**	up to 1000 ml

Prepared 1× TAE by diluting 50 times with distilled water

Ethylenediaminetetra acetic acid (EDTA, 0.5 M)

Dissolved 18.6 g EDTA in 50 ml distilled water, adjusted pH to 8.0 using sodium hydroxide pellets and volume made to 100 ml with distilled water.

EDTA will be soluble when pH reaches 8.0.

Sodium Acetate buffer (0.25 M)

Stock solutions

Sodium acetate	:	0.410g in 20 ml distilled water
Acetic acid	:	1.861g in 20 ml distilled water

Sodium phosphate buffer (0.05 M)

Stock solutions

Monobasic sodium phosphate	:	7.098 g in 1000 ml distilled water
Dibasic sodium phosphate	:	5.999 g in 1000 ml distilled water

Tris HCL buffer (0.1M)

Stock solution

Tris HCl: 15.760 g in 1000 ml distilled water (pH 8) 0.1 M Tris HCl (12.1g of Tris HCl/liter of distilled water) and 0.1M HCl were mixed together to get desired pH)

Appendix-IV

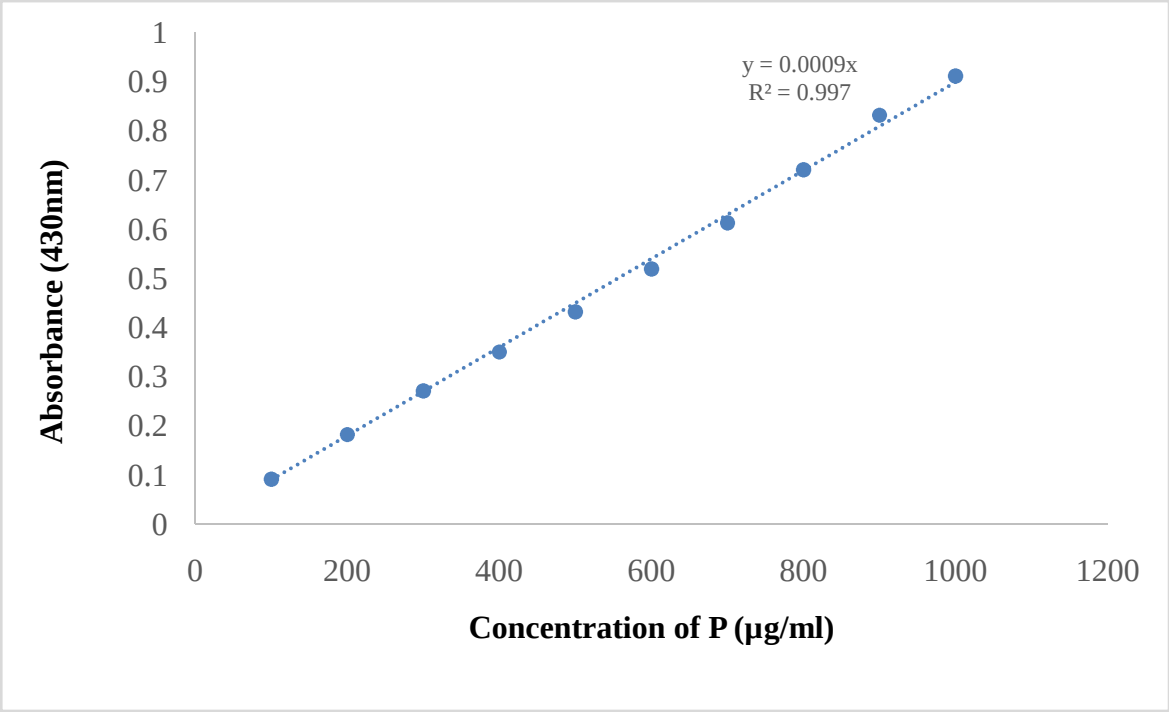

Fig. 1 Standard curve for KH_2PO_4

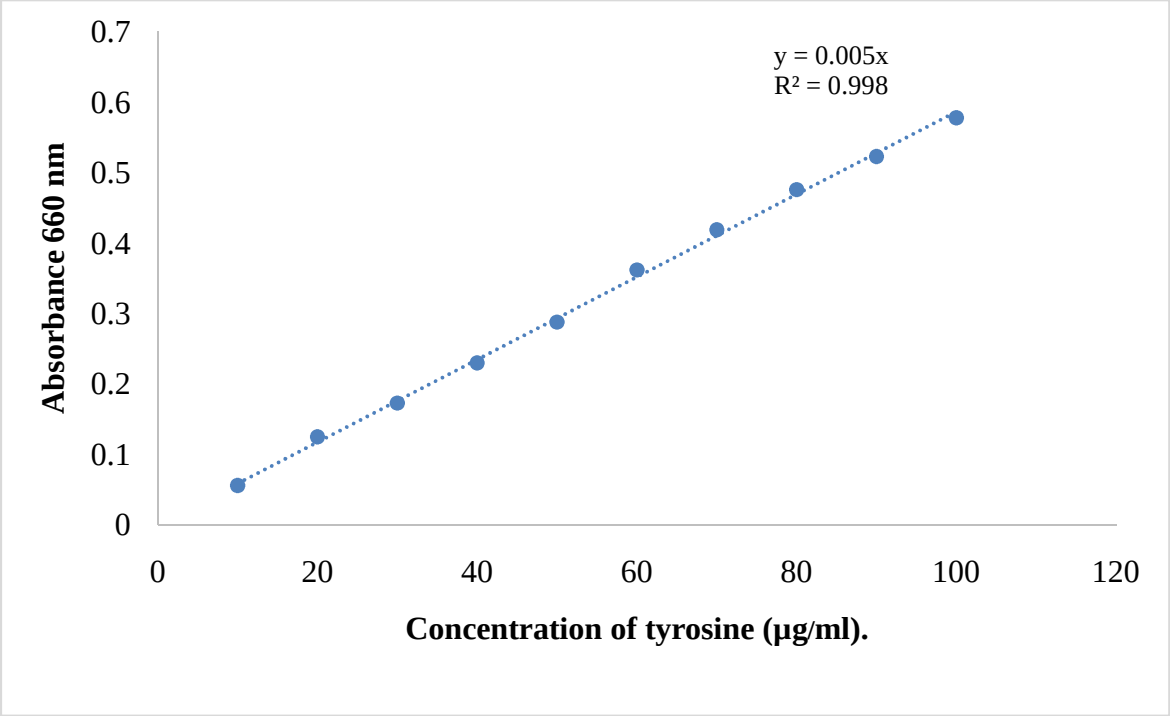

Fig. 2 Standard curve for tyrosin

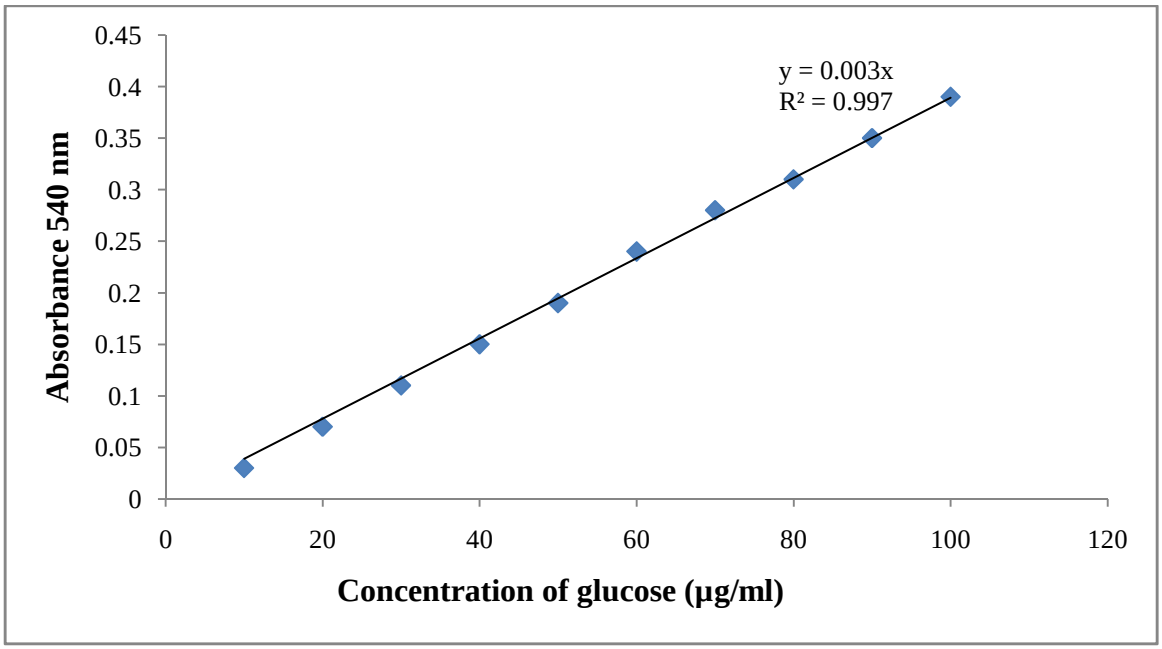

Fig. 3 Standard curve for glucose

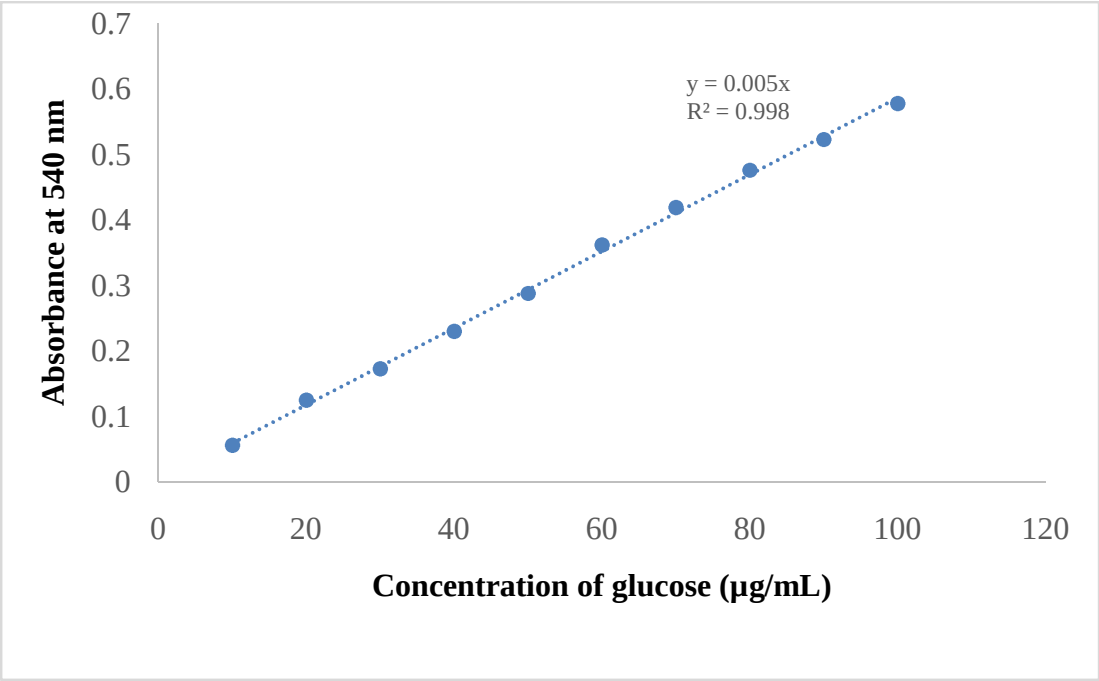

Fig. 4 Standard curve for glucose

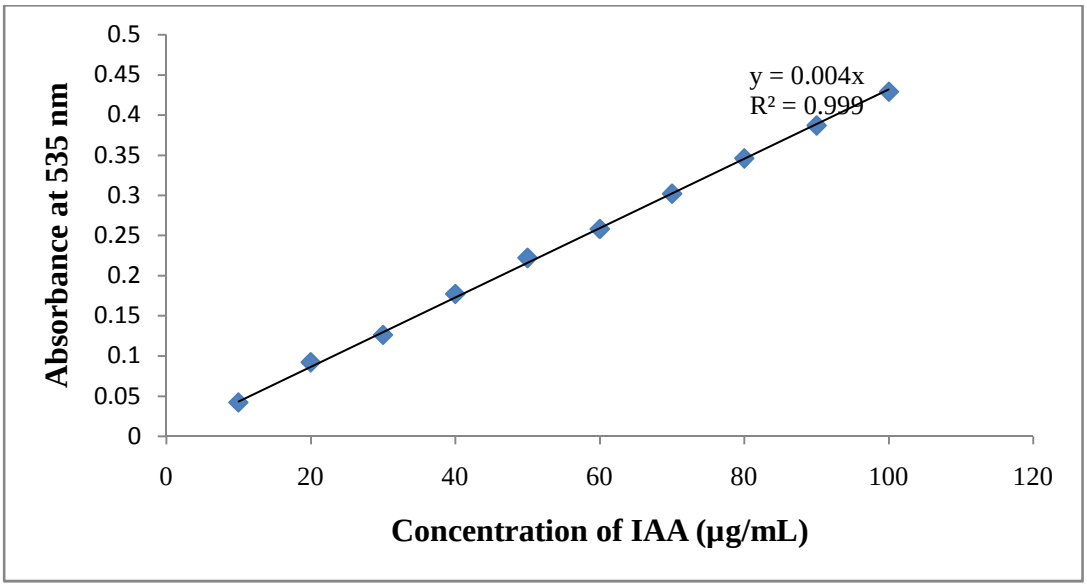

Fig. 5 Standard curve for IAA

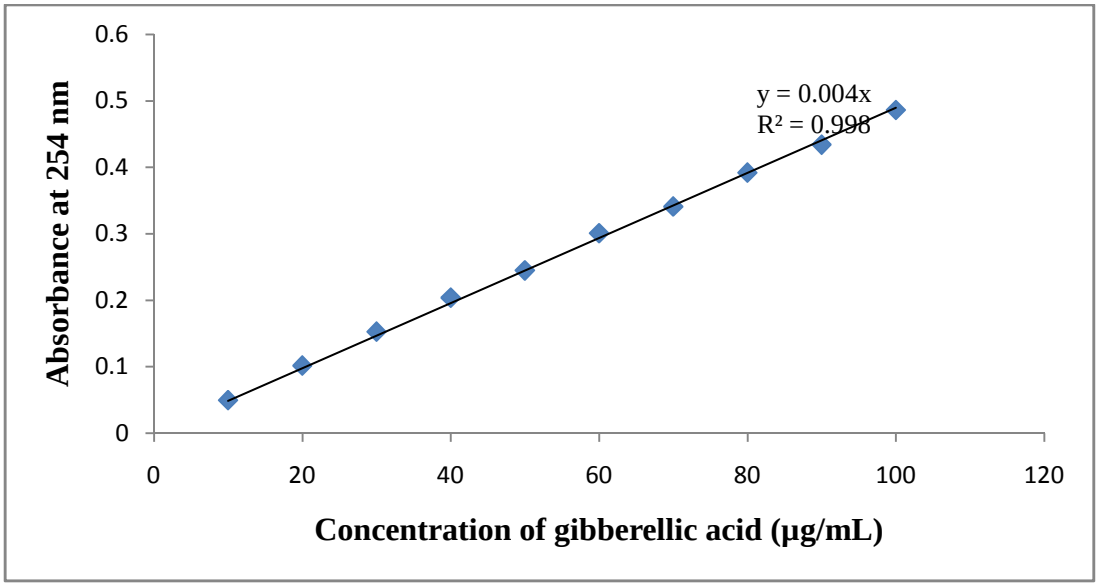

Fig. 6 Standard curve for gibberellic acid

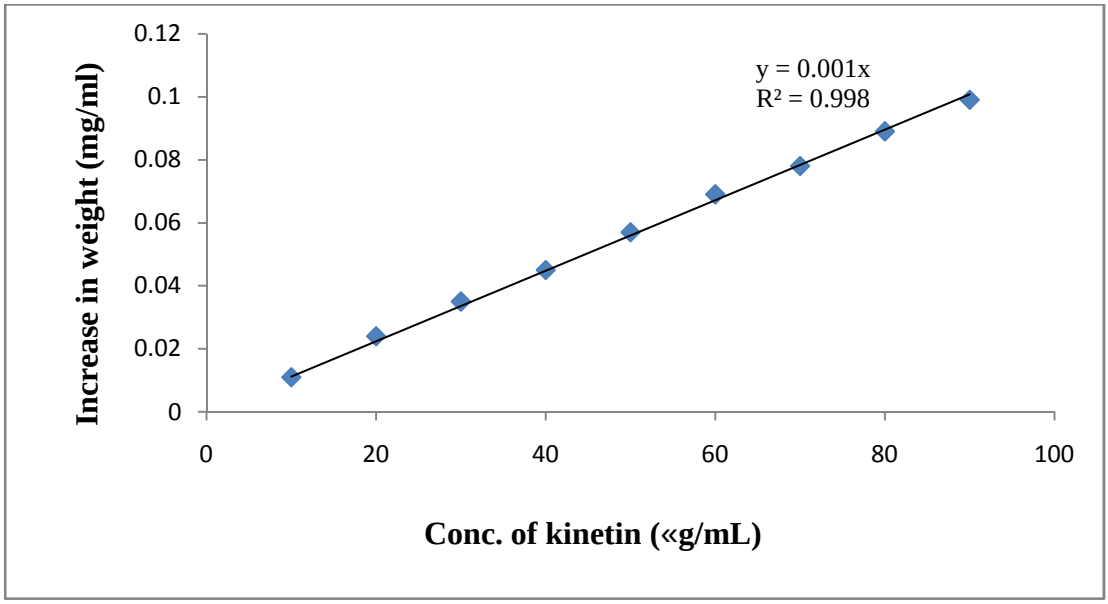

Fig. 7 Standard curve for kinetin

APPENDIX-VI

***Streptomyces rectiviolaceus* NA8 (MK836018)**

CGGTGTGTGCAAGGCCCGGGAAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCA
ACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAGACAGGCTTTTTGAGAT
TGCTCCGCCTCGCGGCTTCGCAGCTCATTGTACCTGCCATTGTAGCACGTGTGCAGCCCAAG
ACATAAGGGGCATGATGACTTGACGTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCT
CCTGTGAGTCCCCATCACCCCGAAGGGCATGCTGGCAACACAGAACAAGGGTTGCGCTCGTT
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GACCACAAGGGGGGCCGTATCTCTACGGCTTTCCGGGCGATGTCAAGCCTTGGTAAGGTTCT
TCGCGTTGCGTCGAATTAAGCCACATGCTCCGCTGCTTGTGCGGGCCCCCGTCAATTCCTTT
GAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGAACTTAATGCGTTAGCTGCGGCACCG
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TAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAATGGCCCAGAGATCCGCCT
TCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCGATCTC
CCCTACCACACTCTAGCCTGCCCGTATCGACTGCAGACCCGGGGTTAAGCCCCGGGCTTTCA
CAACCGACGTGACAAGCCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACAACGCTTGCG
CCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTC
ACTTTCGCTTCTTCCCTGCTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGC
GTCGCTGCATCAGGCTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC
TGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCGCCCTCTCAGGCCGGCTACCCGTCGTCGCC
TTGGTGAGCTTCTACCTCACCAACTAGCTGATAGGCCGCGGGCTCATCCTGCACCGCCGGAG
CTTTCAACCTTCTCCCATGCGAGAGAAAGTGTCATCCGGGTATTAGA

***Streptomyces silaceus* CA7 (MK 836019)**

TGACGGGCGGTGTGTGCAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTA
CTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAGACCGGCTTTT
TGAGATTCGCTCCACCTCGCGGTATCGCAGCTCATTGTACCGGCCATTGTAGCACGTGTGCA
GCCCAAGACATAAGGGGCATGATGACTTGACGTCGTCCCCACCTTCCTCCGAGTTGACCCCG
GCGGTCTCCCGTGAGTCCCCAACACCCCGAAGGGCTTGCTGGCAACACGGGACAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCACCT
GTACACCGACCACAAGGGGGGCACCATCTCTGATGCTTTCCGGTGTATGTCAAGCCTTGGTA
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TTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCACTTAATGCGTTAGCTGC

GGCACGGACAACGTGGAATGTTGCCCACACCTAGTGCCCACCGTTTACGGCGTGGACTACCA
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TCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTC
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CGTCGCCTTGGTGAGCCACTACCTCACCAACAAGCTGATAGGCCGCGGGCTCATCCTGCACC
GCCGGAGCTTTGAACCCCCTCCCATGCGAGAGAAGGTGTTATCCGGTATTAGACCCCGTTTC
CAGGGCTTGTCCCAGAGTGCAGGGCAGATTGCCCACGTGTTACTCACCCGTTCGCCACTAAT
CCACCCCGAAGGGCTTCATCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCGTCCT
GAGCCAGGATCAA

APPENDIX-V

ANOVA 1: Analysis of variance for total viable count of actinomycetes in the rhizosphere of *Arnebia euchroma* (Ratanjot) obtained from different sites on different media (Table 4.1)

Source of Variation	DF	Sum of Squares	MeanSquares	F-Calculated	Signficance
Factor A	3	0.129	0.043	106.757	0.00000
Factor B	3	0.199	0.066	164.970	0.00000
Intraction A X B	9	0.682	0.076	188.446	0.00000
Error	32	0.013	0.000		
Total	47	1.023			

ANOVA 2: Analysis of variance for quantitative estimation of phosphate solubilizing activity exhibited by actinomycetes obtained from *Arnebia euchroma* (Ratanjot) (Table 4.5)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	7	5,712.102	816.015	30,824.099	0.00000
Error	16	0.424	0.026		
Total	23	5,712.525			

ANOVA 3: Analysis of variance for quantitative estimation of phytase activity exhibited by actinomycetes obtained the rhizosphere of *Arnebia euchroma* (Ratanjot) (Table 4.7)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	10	53,890.815	5,389.082	86,699.108	0.00000
Error	22	1.367	0.062		
Total	32	53,892.183			

ANOVA 4: Analysis of variance for quantitative estimation of siderophore production by actinomycetes obtained from the rhizosphere of *Arnebia euchroma* (Table 4.9)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	14	559.335	39.952	4,690.255	0.00000
Error	30	0.256	0.009		
Total	44	559.590			

ANOVA 5: Analysis of variance quantitative estimation of chitinase activity exhibited by actinomycetes obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot) (Table 4.12)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	29	333.608	10.425	11.395	0.00000
Error	69	60.382	0.915		
Total	98	393.989			

ANOVA 6: Analysis of variance for quantitative estimation of protease activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot) (Table 4.14)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	13	6,061.275	189.415	513.482	0.00000
Error	85	24.346	0.369		
Total	98	6,085.622			

ANOVA 7: Analysis of variance for quantitative estimation of cellulase activity exhibited by actinomycete isolates obtained from the rhizosphere of *A. euchroma* (Ratanjot) (Table 4.16)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	31	2,318.733	72.460	71.128	0.00000
Error	67	67.236	1.019		
Total	98	2,385.970			

ANOVA 8: Analysis of variance for quantitative estimation of pectinase activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot) (Table 4.18)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	28	8,485.001	265.156	350.521	0.00000
Error	70	49.927	0.756		
Total	98	8,534.928			

ANOVA 9: Analysis of variance for quantitative estimation of lipase activity exhibited by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot) (Table 4.20)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	23	4,068,765.913	176,902.866	15.725	0.00000
Error	48	539,982.683	11,249.639		
Total	71	4,608,748.596			

ANOVA 10: Analysis of variance for production of plant growth regulators (auxins) by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot) (Table 4.21)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	32	4,933.497	154.172	146.594	1.618425
Error	66	69.412	1.052		
Total	98	5,002.909			

ANOVA 11: Analysis of variance for production of plant growth regulators (gibberellins) by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot) (Table 4.21)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	32	11,610.592	362.831	407.631	0.00000
Error	66	58.746	0.890		
Total	98	11,669.339			

ANOVA 12: Analysis of variance for production of plant growth regulators (cytokinins) by actinomycete isolates obtained from the rhizosphere of *Arnebia euchroma* (Ratanjot) (Table 4.21)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	32	2,272.542	71.017	69.064	0.00000
Error	66	67.866	1.028		
Total	98	2,340.408			

ANOVA 13: Analysis of variance for antagonistic activity of efficient actinomycetes against soil-borne fungal phytopathogens (*Fusarium oxysporum*) (Table 4.22)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	7	1.073	0.153	140.891	0.00000
Error	16	0.017	0.001		
Total	23	1.091			

ANOVA 14: Analysis of variance for antagonistic activity of efficient actinomycetes against soil-borne fungal phytopathogens (*Sclerotium rolfsii*) (Table 4.22)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	7	1.578	0.225	2,561.112	0.00000
Error	16	0.001	0.000		
Total	23	1.579			

ANOVA 15: Analysis of variance for antagonistic activity of efficient actinomycetes against soil-borne fungal phytopathogens (*Rhizoctonia solani*) by actinomycetes (Table 4.22)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	7	3.497	0.500	3,320.364	0.00000
Error	16	0.002	0.000		
Total	23	3.500			

ANOVA 16: Analysis of variance for effect of volatile compounds secreted by actinomycetes on growth inhibition of soil-borne fungal phytopathogens (*Fusarium oxysporum*) (Table 4.23)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	7	5.462	0.780	95.088	0.00000
Error	16	0.131	0.008		
Total	23	5.593			

ANOVA 17: Analysis of variance for effect of volatile compounds secreted by actinomycetes on growth inhibition of soil-borne fungal phytopathogens (*Sclerotium rolfsii*) (Table 4.23)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	7	3.537	0.505	79.975	0.00000
Error	16	0.101	0.006		
Total	23	3.638			

ANOVA 18: Analysis of variance for effect of volatile compounds secreted by actinomycetes on growth inhibition of soil-borne fungal phytopathogens (*Rhizoctonia solani*) (Table 4.23)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	7	1.205	0.172	12.776	0.00000
Error	16	0.216	0.013		
Total	23	1.420			

ANOVA 19: Analysis of variance for effect of growth regulators produced by potential actinomycetes, *Streptomyces rectiviolaceus* (NA8) and *Streptomyces silaceus* (CA7) on root elongation in uniformly pre-germinated seeds of maize (Table 4.27)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	2	0.606	0.303	22.430	0.00164
Error	6	0.081	0.014		
Total	8	0.687			

ANOVA 20: Analysis of variance for effect of growth regulators produced by potential actinomycetes, *Streptomyces rectiviolaceus* (NA8) and *Streptomyces silaceus* (CA7) on root elongation in uniformly pre-germinated seeds of pea (Table 4.27)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	2	3.055	1.528	90.037	0.00003
Error	6	0.102	0.017		
Total	8	3.157			

APPENDIX–VII

NCMR
राष्ट्रीय सूक्ष्मजीव संपदा केंद्र

National Centre for Microbial Resource
Member of WFCC, An IDA under Budapest Treaty, DNR of MoEF
राष्ट्रीय कोशिका विज्ञान केंद्र | National Centre for Cell Science

❖ CERTIFICATE OF DEPOSIT ❖

***Streptomyces sp.* CA7**

was received for public access deposit at NCMR from

Dr. Sunita Devi
Dept. of Basic Sciences,
College of Forestry,
Dr. Y. S. Parmar University of Horticulture & Forestry,
Solan, HP-173230

05 March 2019

and was allocated the accession number

MCC 4170

After confirming the viability, purity and authenticity of the strain in the NCMR facilities, it has been preserved using standard methods.

The strain is now available to the public.

Dr. Abhay Bajaj

Scientist, National Centre for Microbial Resource, Pune

16 July 2019

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एनसीसीएस भारत सरकार के जैवप्रौद्योगिकी विभाग का स्वायत्त संस्थान है.
NCCS is an Autonomous Institute of Department of Biotechnology, Govt. of India

NCMR
राष्ट्रीय सूक्ष्मजीव संपदा केंद्र

National Centre for Microbial Resource
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राष्ट्रीय कोशिका विज्ञान केंद्र | National Centre for Cell Science

❖CERTIFICATE OF DEPOSIT❖

***Streptomyces rectiviolaceus* Strain NAB**

was received for public access deposit at NCMR from

Dr. Sunita Devi

Department of basic sciences, College of Forestry, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, 173230
India

on *12 July 2019*

and was allocated the accession number

MCC 4262

After confirming the viability, purity and authenticity of the strain in the NCMR facilities, it has been preserved using standard methods.

The strain is now available to the public.

For

Dr. Shrikant Pawar

Scientist, National Centre for Microbial Resource, Pune

22 October 2019

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NCCS is an Autonomous Institute of Department of Biotechnology, Govt. of India

Department of Basic Sciences
Dr. Y.S. Parmar University of Horticulture and Forestry
Nauni, Solan–173 230, H.P.

Title of thesis	:	**Diversity of actinomycetes in the rhizosphere of *Arnebia euchroma* (Ratanjot): A critically endangered plant of Himachal Pradesh**
Name of student	:	Ms. Pooja Sharma
Admission No.	:	F-2017-33-M
Name of Major Advisor	:	Dr. Sunita Devi
Major field	:	Microbiology
Minor field(s)	:	Forest Products
Degree awarded	:	M.Sc.
Year of award of degree	:	2019
No. of pages in thesis	:	134+xxv
No. of words in abstract	:	494

ABSTRACT

Rhizospheric soil samples were collected from Nako and Chango in Kinnaur and Gue and Kibber in Lahaul & Spiti districts of Himachal Pradesh to decipher actinomycetes diversity in the rhizosphere of *Arnebia euchroma* (Ratanjot). Standard Plate Count technique was employed for the isolation of actinomycetes. In total, 33 isolates (15 from Nako, 7 from Chango, 16 from Gue and 5 from Kibber) were obtained. The actinomycetes load was found to be significantly higher at Chango (Log CFU/mL=1.44) followed by Nako (Log CFU/mL=1.40) and Gue (Log CFU/mL=1.38) while the minimum was recorded for Kibber (Log CFU/mL=1.33). Likewise, their significantly higher load was observed on SCA (Log CFU/mL=1.48) followed by AIA (Log CFU/mL=1.41), NA (Log CFU/mL=1.29) and KM (Log CFU/mL=0.88) proving SCA to be the best medium. With respect to their PGP potential, only 45.45 per cent of the total isolates showed phytase and siderophore production while 33.33 per cent showed P- solubilization qualitatively. Significantly higher P-solubilization (53.66 µg mL^{-1}) was shown by NA8 while the minimum (6.44 µg mL^{-1}) was recorded for SCAN1. However, the phytase activity was observed to be significantly higher (134.44 U/ml) for CA7 and minimum (15.33 U/ml) for NA10. Isolates SCAK5 and SCAN1 showed significantly higher (17.24% SU) and lower siderophore (5.46% SU)) production. All isolates exhibited nitrogen fixing ability while 66.66 and 3.03 percent of total isolates showed ammonia and HCN production, respectively. 84.84, 90.90, 96.96, 72.72 and 42.42 percent of the isolates exhibited pectinase, chitinase, cellulase, lipase and protease activities, respectively. Isolates NA6 and CA1 showed significantly higher (6.81 U/mL) and lower (0.42U/mL) chitinase activities, respectively. Maximum cellulase activity (31.21 U/mL) was shown by NA5 while the minimum (24.18 U/mL) was observed for GA6. Isolates NA7 and NA10 showed significantly higher (28.13 U/mL) and lower (1.22 U/mL) protease activities, respectively. Isolates NA6 and GA6 showed significantly higher (48.33U/mL) and lower (1.60 U/mL) pectinase activities, respectively. Only NA5 exhibited maximum lipase activity (2040 IU/mL). Maximum IAA production was shown by NA10 (44.52 µg/ml) while minimum (11.55 µg/ml) by NA3. However, maximum gibberellins production (40.60 µg/ml) was shown by NA7 while the minimum (4.52 µg/ml) was recorded for KMN2. Isolates CA5 and GA2 showed maximum (34.0 µg/ml) and minimum (15.0 µg/ml) cytokinins production, respectively. Eight potential isolates exhibited reasonable antagonism towards *Fusarium oxysporum, Sclerotium rolfsii* and *Rhizoctonia solani* ranging between 26.20 - 41.4 per cent. Likewise, volatile metabolites released by actinomycetes inhibited their growth ranging between 21.87 - 44.44 per cent. Based on morphological, biochemical and molecular characteristics, two potential isolates i.e. CA7 and NA8 out of 9 were identified as *Streptomyces silaceus* and *Streptomyces rectiviolaceus* and were assigned GenBank accession numbers MK836019 and MK836018, respectively. Evaluation of growth regulator's effect of CA7 and NA8 on root elongation revealed a significant increase in root length of pre-germinated seeds of maize and pea over the control. Ultra Performance Liquid Chromatography analysis showed the presence of ββ-dimethylacrylshikonin (0.145 %), Deoxy-shikonin (0.098 %) and Shikonin (0.011 %) pigments in the roots of *A. euchroma*.

Signature of Major Advisor **Signature of Student**

Countersigned

Professor and Head
Department of Basic Sciences
Dr. Y.S. Parmar University of Horticulture and Forestry
Nauni, Solan – 173 230 (H.P.)

BRIEF BIO-DATA

Name : **Pooja Sharma**

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Date of Birth : 18th April, 1996

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Certificate/Degree	Board/University	Year of passing	Division	Marks (%)
Matriculation	Central Board of Secondary Education (CBSE), New Delhi	2012	First	88.0
10+2	HP Board of School Education, Dharamshala	2015	First	71.3
B.Sc. (Hons), Microbiology	Himachal Pradesh University, Shimla	2017	First	76.2

Whether sponsored by some state/ Central Govt./Univ./SAARC : No

Scholarship/Stipend/Fellowship, any Other financial assistance receive during the study period : Yes

(Pooja Sharma)