Search for Novel Actinomycetes from soil as potential Biocontrol agent against Fungal root pathogens of *Phaseolus vulgaris* (L.) and *Vigna radiata* (L.)

Thesis submitted to the
University of North Bengal

For the Award of
Doctor of Philosophy

in

BOTANY

BY
Pushpanjali Ray

Supervisor
Professor B.N. Chakraborty

Immuno-Phytopathology Labrtoratory

Department of Botany

University of North Bengal

Raja Rammohanpur, Darjeeling

June, 2017

UNIVERSITY OF NORTH BENGAL
Department of Botany

Professor B. N. Chakraborty
Ph.D, FPSI, FNRS, FISMPP, FISPC, FMSI, FIMS
FAScT, FRSC (Lond.), FNAAS

ENLIGHTENMENT TO PERFECTION

IMMUNO-PHYTOPATHOLOGY LABORATORY
PO. N.B.U 734013, Siliguri, West Bengal, India
Phone : 0353-2776337 (O), 09800266298 (M)
Fax : 0353-2699001
E-mail : bncnbu@gmail.com
Website: www.iplnbu.ac.in

CERTIFICATE

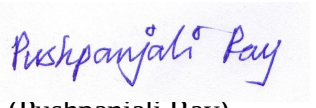
This is to certify that Ms. Puspanjali Ray has prepared the thesis entitled "**Search for Novel Actinomycetes from soil as potential Biocontrol agent against Fungal root pathogens of *Phaseolus vulgaris* (L.) and *Vigna radiata* (L.)**", for the award of PhD degree of the University of North Bengal under my guidance. She has carried out the work at the Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

(Professor B.N. Chakraborty)

(Professor B. N. Chakraborty)
Immuno-Phytopathology Laboratory
Department of Botany
University of North Bengal

DECLARATION

I declare that the thesis entitled **"Search for Novel Actinomycetes from soil as potential Biocontrol agent against Fungal root pathogens of *Phaseolus vulgaris* (L.) and *Vigna radiata* (L.)"** has been prepared by me under the guidance of Professor B.N. Chakraborty, Department of Botany, University of North Bengal**.** No part of this thesis has formed the basis for the award of any degree or fellowship previously.



(Pushpanjali Ray)

Department of Botany
University of North Bengal
Raja Rammohanpur, P.O.-NBU
Dist.-Darjeeling. Pin. 734013.

Date. 28/06/2017

ABSTRACT

The study undertaken was aimed for search of novel Actinomycetes from soil as potential biocontrol agent against fungal root pathogens of *Phaseolus vulgaris* (L.) and *Vigna radiata* (L.) from the terai areas of North Bengal with focus on Jalpaiguri district of North Bengal. Owing to the unique geographical location of the area under investigation, there exists a unique agro-climatic zone in the area of study and as is the fact that the area is a hot spot for agricultural and horticulture crop cultivation, with existence of tea gardens and areca nut plantations. The search for actinomycetes from these locations is indicative of the prerequisite for study, of area specific agriculturally important beneficial microorganisms for overall crop heath, cultivation and production respectively, with avenues for exploitation as potential bio control and bio growth enhancers.

The inventory for undertaking the study at hand was set as a module for each experimental set up and the road map was outlined, as per the aims and objectives delineated. Although the study pertains to isolation of actinomycetes at the initial phase, screening of the same was undertaken to assess and evaluate the same at the later part, so as, to ascertain their attributes in controlling known pathogens and growth promotion towards biological plant vigour of selected crops. The area under investigation was mainly agricultural field where crops and vegetables ranging from brinjal, tomato, okra, jute, potato etc grow. There is evident use of pesticides and fertilizers at these agriculture fields at the time the investigation was undertaken and so is the general practice. Recently there has been inclination into looking for organic modes of cultivation, irrespective of package of practices followed and crops considered. The selection of sites was also done to evidently see as to the type of microorganism that dwells in the soil along with chemical fertilizers. However, the present study would not look into the same ie., relation of isolates and their dwelling ecosystem in the soil with respect to the host.

With prelude to the assumption based on theory, the main objectives of the present study were to isolate actinomycetes from soil rhizosphere of agricultural field, their identification using conventional and molecular tools as per set protocol, their screening for plant growth promotion in relation to phosphate solubilization, plant

hormone efficacy, effect of plant primary defence compounds and secondary enhancers of plant physiology for overall shoot and root system development.

Moreover, the role of selected isolates as antagonist against target pathogens of *Fusarium solani* and *Sclerotium rolfsii* was also to be carried out as per set protocols. The objective of assessment was against two important pulses of Indian food industry, *Phaseolus vulgaris* and *Vigna radiate.*

A generalised review of literature of work ranging from experimental, comments, reviews etc has been catalogued during the course of the investigation and presented under subjects ranging from actinomycetes distribution throughout the world , with focus of their availability at inhospitable terrains, the lineage of actinomycetes, keys and theories of classification, Characteristics features, role in soil ecosystem, role in vertical and horizontal resistance, importance in agriculture and other anthropogenic activities, documentary of prevalent diseases of the target crops, their role with other pathogens and control measures etc.

The experimental as outlined in the materials and method were standardized protocols, that was embarked on to achieve set objectives of the study. The cultivars of crops selected were collected from various local markets of Darjeeling and Kalimpong district of West Bengal as well as the North Eastern States of Sikkim and Nagaland. The cultivars were grown and maintained, crop period after crop period, in test sites of Department of Botany, University of North Bengal. The test pathogens *Sclerotium rolfsii* and *Fusarium solani* were collected from National Fungal Collection Centre of India (NFCCI).

During the course of work, many viable strains of microorganism were isolated in actinomycetes growth media and maintained as stock and sub culture purposes. After, due screening using morphological and biochemical setups, wherein 17 isolates were identified as those belonging to actinomycetes. Upon screening for plant pathogen antagonism three isolates coded namely as ARHS/PO/15, ARHS/PO/26, ARHS/PO/27 were marked for future experimental for role in host pathogen interaction.

The Blast query in NCBI, GenBank database of 16S rDNA sequence of selected isolates on basis of set attributes confirmed the identity of ARSHO/PO/15 as *Streptomyces griseus* (KX 894282), ARHS/PO/26 as *S. tricolor* (KX894280) and ARHS/PO/27 as *S. flavogriseus* (KX894281).

All the selected isolates were able to enhance growth of target crop plants *Phaseolus vulgaris* and *Vigna radiata* when treatment of the plant were carried out with the isolates. The increased growth was in terms of shoot length, root length, leaf number, leaf area etc.

When the plants were challenge inoculated with plant root pathogen *Fusarium solani* and *Sclerotium rolfsii* the treated plant showed evident resistance against the pathogens in comparison to the untreated control plants. Biochemical analysis of the defence enzymes also showed marked correlation with the result. There was an increase in the level of the defence enzymes in the treated plants. Enzyme level was highest in the treated inoculated plants followed by treated plants. Lowest level was found in untreated control plants.

Phenol content and total protein content of the plant root and leaf samples also varied between different variety and different treatment. It was found that defence enzyme activity, phenol and protein content was higher in the leaf samples than root.

The tool of indirect immunofluorescence was used to observe the location of Chitinase and Glucanase in the tissues of root and leaf of *P. vulgaris* and *V. radiata*. The strong fluorescent apple green colour was indicative of the accumulation of these enzymes in the root and leaf tissue in the treated plants which was negligible in comparison in the control.

From the result of the present study it can be said that Actinomycetes isolates *Streptomyces griseus* (KX 312687), *S. tricolor* (KX894280) and *S. flavogriseus* (KX894281) can be used as bio protector and plant growth enhancer.

Preface

Microorganisms have always intrigued me as an individual, yet this endeavour was fascinating in the true terms of an adventure to look into one group with so much attention and deliverance. From an ounce of soil to the genes of the organism, from fascination to realisation, the work undertaken was a task that tried and tested many aspects of my individuality yet the path of my search to research has amalgamated into this compilation of facts and figures, discussions and results. But the same would not have been possible had it not been the leading lights in this path that was previously unknown to me.

I put forth my gratitude to Professor B.N. Chakraborty Immuno Phytopathology laboratory, Department of Botany, University of North Bengal for being the guiding light during the tenure leading to the submission of the work at hand. I would also like to thank Professor Usha Chakraborty for doing what she does best, providing constant support and silver lining of comments and inputs at rough edges from time to time where I had issues with my work.

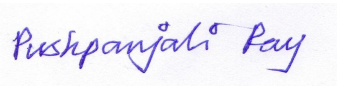
I would also like to take this opportunity to show gratitude to Professor A. Saha, preceding Head, Department of Botany, as well as present Head Professor A. Sen, for providing support and allowing myself to use the facility of the department for experimental setup and execution. Moreover, I would like to thank the faculty members Professor P.K. Sarkar, Professor A.P Das, Professor R. Chakraborty, Dr. P Mandal, Dr. S.C Roy, Dr. M. Chowdhury, for imparting knowledge and support time and again.,

It would not be fair, if I do not acknowledge the unrelenting support of fellow research scholars at the laboratory Dr. Amrita Acharya, Dr. Somnath Roy, Mr. Shibu Barman, Ms. Priyanka Bhattacharya, Ms. Jayanwita Sarkar, and Ms. Sweta Khati. I also thank Shri Shambu Banik for assisting in my field work. Last but not the least I would like to thank Dr. A. P. Chakraborty for his support in every sphere of my work.

I also take the privilege at this juncture to acknowledge the unrelenting support provided to me by my colleagues at work Dr. Rita Som Paul, and Shri Suman Sen faculty members, Department of Botany, A.C college and at the same time our non-teaching duo of Shri Sumanta Modak and Smt. Aliva Ghosh.

Not to forget the immense support by the Ex-Principal Dr. D.K. Basak and presently Dr. M.A Razzaque including all the College authority .And the silver lining was the support from my students in their own way, at times when thesis work load was at its highest.

I would like to extend my heartfelt gratitude to my family and friends. I sincerely thank my parents, my sister Dr. Madhurima Ray, my husband Dr. Kuldeep Rai and my especially to my one year old son Himakar, who knowingly and unknowingly was there at my side through frantic cries or loving smiles, during the course of completion of my work. I acknowledge that without their support and help it may not have been possible for me to complete this endeavour. Especially I am indebted to Kuldeep for all his support in completing this journey of mine. I am also thankful to my in laws for their support and encouragement.



(Pushpanjali Ray)
Immuno-Phytopathology Laboratory
Department of Botany
University of North Bengal
Date. 28/06/2017

Contents

List of Tables

List of Figures

Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.* CV2=Jwala (Cultiver 2), CV3=Kholar (Cultiver 3)

Figure 40. β 1-3 Glucanase activity in (A) leaf & (B) roots of varieties of *P. vulgaris* treated with actinomycetes formulation before and after challenge inoculation with pathogen *Fusarium solani*; UH= Untreated healthy, UI=Untreated Inoculated,T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.* CV2=Jwala (Cultiver2), CV3= Kholar (Cultiver 3)

Figure 41. Chitinase activity in (A) leaf & (B) roots of varieties of *P vulgaris* treated with actinomycetes formulation before and after challenge inoculation with*Fusarium solani*;UH= Untreated healthy, UI=Untreated Inoculated,T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.* CV2=Jwala (Cultivar 2), CV3=Kholar (Cultivar 3)

Figure 42. (A-D) *Phaseolus vulgaris* (Cultivar 2, Jwala), in pot condition, A-Control, Untreated Healthy (UH). B- Control, Untreated Inoculated (UI). C- T1, Treated Healthy (T1H). D- T1 Treated Inoculated (T1I), E-T2 Treated Healthy (T2H), F-T2, Treated Inoculated (T2I), G- T3, Treated Healthy (T3H). H-T3, Treated Inoculated (T3I) T1= treated with *Streptomyces griseus*, *T2=Streptomyces tricolor,* T3= *Streptomyces flavogriseus,* Inoculated with *F. solani*

Figure 43. (E-H) *Phaseolus vulgaris* (Cultiver 3, Kholar), in pot condition, A-Control, Untreated Healthy (UH). B- Control, Untreated Inoculated (UI). C- T1, Treated Healthy (T1H). D- T1 Treated Inoculated (T1I), E-T2 Treated Healthy (T2H), F-T2, Treated Inoculated (T2I), G- T3, Treated Healthy (T3H). H-T3, Treated Inoculated (T3I) T1= treated with *Streptomyces griseus*, *T2=Streptomyces tricolor,* T3= *Streptomyces flavogriseus,* Inoculated with *F. solani*

Figure 44. Disease index (Evident symptom of Disease) after challenge inculcation with target pathogen (*Sclerotium rolfsii*) and host response upon respective isolate treatments in *Vigna radiata.*T1= treated with *Streptomyces griseus*, *T2=Streptomyces tricolor,* T3= *Streptomyces flavogriseus*

Figure 45. PAL activity in (A) leaf & (B) roots of varieties of *V. radiata* treated with actinomycetes formulation before and after challenge inoculation with *Sclerotium rolfsii*; UH= Untreated healthy, UI=Untreated Inoculated,T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.*

Figure 46. Total phenol content in (A) leaf & (B) roots of varieties of *V. radiata* treated with actinomycetes formulation before and after challenge inoculation with *Sclerotium rolfsii*; UH= Untreated healthy, UI=Untreated Inoculated,T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.*

Figure 47. Peroxidase activity in (A) leaf & (B) roots of varieties of *V radiata* treated with actinomycetes formulation before and after challenge

Figure 55. (A-F) FITC labeling of root & leaf tissue of *Vigna radiata* with Pab of glucanase enzyme & Pab of chitinase enzyme after treatment with *S.tricolor* (KX894280) and pathogen challenge. A-TS of root tissue of control; (B-C & D)-TS of cortical & vascular tissue in roots ; (E & F) TS of leaf & localization of glucanase enzyme after pathogen challenge.

List of Appendices

Chapter 1
INTRODUCTION

North Bengal, as the name implies is Located in the Northern part of the State of West Bengal in India. It is located at 89°7'30''E and 26°59'34'' N. The area comprises of Steep mountainous regions and vast plains. Hence it is rich in Flora and fauna. Likewise, extensive research has been undertaken to study the diversity with respect to flora and fauna of the region. However, the study on microbial diversity is limited in comparison .This provides a window of an opportunity to undertake research and study the microbial diversity of the region. Moreover, the cultivation of horticultural and agricultural crops is also indispensable source of livelihood for a large section of marginalized stakeholders belonging to the area. Thus to an extent it can be inferred that agriculture is a mainstay of rural economy in this region. In agriculture management two of the most important goals are to ensure the crop of enough nutrients and to prevent it from diseases.

Traditionally, these goals have been achieved by using pesticides and a high input of fertilizer. However, these management practices evidently contributed to a high loss of bio diversity all over the landscape. Therefore, other approaches should be investigated (Kaushik, 2007). Comparatively the North Bengal plains experience a tropical to subtropical type of climate with high relative humidity and temperature. The climatic factors favour growth and multiplication of microorganisms which may be either beneficial or pathogenic in nature. Farmers use chemicals as fertilizer, insecticide or pesticide to combat these and attain balance for productivity and profit. But as is known, this may not be as holistic an approach for sustainable farming and livelihood respectively. Hence an alternative mode without apprehension in the form of bio-control agents as growth enhancers, control, and antagonist is needed.

However as put forth by Chakraborty (2016) Most of the farmers in the country do not have sufficient and clear knowledge on the use of bio pesticide. Ministry of Agriculture as well as Ministry of Science and Technology, Government of India has taken initiative to establish Rural Bio resource centres in different states. Key drivers in the market are increasing consumer demand for organic farming practices that encourage organic farming practices and thus promoting bio-control products.

Moreover, parallel study of the soil ecosystem and its inhabitants is also necessary. Preliminary investigation to isolate microorganisms from the soil rhizosphere has been undertaken by some workers in the Darjeeling and Jalpaiguri district of west Bengal. However it is opined that mere isolation may not be the solution, to assist the soil system and the resulting crop cultivation. Efforts are being made throughout the world, to not just isolate but to identify certain microorganism from the selection that may be exploited in term of its attributes as antagonist and plant health growth promoter. Works in *Frankia*, PGPRs *like Bacillus, Pseudomonas* etc, Vesicular Arbascular Mycorrhiza (Kamal *et al*., 2014), isolates from fermented foods have been carried out in the recent past for the biota in and around areas in North Bengal. However there is minimal evidence of works on the same lines for Actinomycetes.

Any living being prevalent in the ecosystem is unique either in terms of physiology, morphology, habitat or mode of survival. Yet in terms of prospecting for beneficial microorganism, that protects crop system from pathogens, and at the same time boosts or maintains the plant vigour, there should be certain threshold criteria that has to be there so that the organism upon its application at field may provide the same results as was laboratory based, on which respective selection was made among many isolates.

Actinomycetes and its related species just upon superficial observance, may fit into the role mainly due to fact that they have been isolated from all around the world and under extremes of ecological condition, ranging from the depths of the marine trench, to deserts, hot water geysers, extreme high altitude, freezing temperatures, humid to extreme heat. Hence if a certain group of organism with its diversity does inhabit mostly every type of habitat, then it does need attention.

The relevance of actinomycetes as a biological medium for agricultural purposes can also be linked to the fact that they may be able to survive the drastic changes that has undertaken in the agricultural fields of the farmer owing to indiscriminate use of chemicals and also anthropogenic activities, and at the same time assist the cultivation of crops by providing protection and integrated nutrient to the soil rhizosphere. According to Crawford *et al.* (1993) they are quantitatively as well as qualitatively vital part of the rhizosphere. Recently Doolotkeldieva *et al.* (2015) have put forth that use of

a formulation *Streptomyces fumanus* as colonizer of rhizosphere of plants after competition with existing microflora.

The antibiotic aspects of *Streptomyces* species a type of actinomycetes cannot be ruled out either, however clinical study of the same has many results which have everyday human use and the number is increasing day by day. Behal (2000) reported secondary metabolites have antifungal, antitumor and immunosuppressive activities.. However, focus on their role for plant health and not just human health should also be considered, as good food from plants is the mainstay of healthy human health.

The role of actinomycetes as antagonist against many pathogens either fungal, bacterial, nematodes, pests etc. has been proved by many workers (Sowndhararajan and Kang,2012, Ningthoujam *et al*.,2009,Srividya *et al*.,2012, Heng *et al.*,2015, Hastuti *et al.*, 2012, Intra *et al*., 2011, Ara *et al*.,2012b). Shrivastava *et al.,* (2017) have also clearly concluded that *Streptomyces aureofaciens* K20 under saline stress condition can be used for bio control of *M. Phaseolina*. However it is also to be believed that microorganism isolates from respective agroclimatic zone will perform better in terms of bio-control and growth enhancement rather than a foreign enitity. Hence isolation assay and evaluation of target isolates from respective ecosystem may be the key to sustainable agriculture. . Janaki *et al*., (2016) have added that search should be on for newer compounds from mangrove habitat as well. The same has been supported by Basha and Rao (2017) stating exploration of mangrove inhabiting actinomycetes for active secondary metabolite should be done. The host range of crops for which detailed and positive investigations in relation to actinomycetes, varies from fruits, grasses, vegetables, plantation crops.

Pulses are also an important crop as far as balanced diet is considered. There has been works where actinomycetes have assisted in bio control and growth enhancement of crop plants (Couillerot *et al*., 2013). Cultivation of pulses even though is undertaken at other states of India as well, there is emerging scope for agribusiness of the same in the North East states of India including North Bengal, as evidenced by the fact that about 21 varieties of *Phaseolus* are cultivated in a single district of Tuensang in Nagaland state of India.

There is always evidence that many bio control agents like *Trichoderma viride, Ttrichoderma harzianum, Bacillus, Pseudomonas,* VAM formulation provide similar

results to an extent. However, the unique character of actinomycetes is its ability to survive in any type of soil system and even in chemical infested environment. As actinomycetes are known to be durable organism they are appropriate for soil application (Sharma 2014), as *Streptomyces* gn-2 strains improve the composition of microflora in the rhizosphere, mostly by attracting saprophytic microorganisms: ammonificators and oligotrophs (Doolotkeldieva *et.al.*2015). Jog *et al.*, (2014) have even proposed three *Streptomyces* strain namely mhrcr0816, mhcr0817 and mhce 0811 as potential bio inoculants, which were isolated from wheat roots and rhizosphere.

Though it may be early to put forth, but these can be taken as pointers for earmarking this group as potential agent for phyto- remediation of the soil system in area in-and around North Bengal, where extensive use of chemicals as insecticide spray, or fertilizers has taken place mostly owing to the fact that there are about 178 tea gardens in the Jalpaiguri and adjoining areas.

The tools and techniques for any experimental undertaking are as important as the aims and objectives outlined relevant to the hypothesis and review prepared for attaining certain results. Conventional to modern techniques were followed to assay the potential of the isolates under various experimental designs. Conventional anatomical studies to molecular characterization were carried forward with the single aim to provide a holistic evaluation of the objective at hand.

As put forth by Sunar (2014) there has been few evidence of beneficial microorganism being isolated from North Bengal and other North Eastern state of India. Mere isolation and identification can be one aspect of the study but identification and assay of the same as beneficial has to run parallel, so that the there is an existing repository of area specific bio-control and bio growth enhancers from the region and not just samples for identification purpose. As put forth an area specific repository of data on bio agents will ensure organic cultivation minimizing side effect on health issues is looked into, more so when most of North East till date is organic by default.

In view of the above situation a study was undertaken to found a biological method of improving the overall plant health and disease resistance capacity with the following

- Isolation of actinomycetes from soil.
- Morphological and biochemical characterization of isolates.
- Screening of isolates for phosphate solubilizing activity.
- *In vitro* antagonistic activity of selective actinomycetes isolates against fungal pathogens (*Fusarium solani*, *Sclerotium rolfsii*).
- Molecular detection of potential isolates of actinomycetes.
- Evaluation of potential actinomycetes as bio protector against fungal root pathogens of pulses (*Phaseolus valgaris* and *Vigna radiata).*

Chapter 2
LITERATURE REVIEW

There have been many definitions of actinomycetes, though in generalization all are same yet many researchers have put forward respective definitions and interpretations for better understanding. Actinomycetes are in most cases reported as gram positive bacteria that grow in form of mycelia. They are mainly aerobic; inhabit the soil in most of the cases, with very few exceptions. The name for the bacterium is derived from the anaerobic species, *Actinomyces bovis*. (Schlegel 1992).

As put forth by Oskay *et al., (*2004*)* actinomycetes are soil inhabiting microorganisms which are globally distributed. Many actinomycetes have been isolated and screened from the soil system, in recent decades, indicating upto 70-80% of relevant secondary metabolites available commercially (Khanna *et al.,* 2011). Historically, most actinomycetes isolated from the soil have been those belonging to genus *Streptomyces* and *Micromonospora* (Basilio *et al.,* 2003). Out of 22500 biologically active compounds derived in some form or other from microorganisms, 45% are from actinomycetes, 38% from fungi and those from bacteria account 17% (Berdy 2005). 70% of the total antibiotic production is related to species of *Streptomyces*, also those from *Micromonospora* was less than one-tenth as many as *Streptomyces* (Lam 2006)

Abundance of Actinomycetes localization and distribution

Among the abundant isolates that have been isolated by researchers are members of *Actinoplanes*, *Streptomyces*, *Nocardia*, *Actinomadura*, *Micromonospora*, *Nonomuria*, and *Streptosporangium* (Wang *et al*., 1999). Evidently, actinomycetes denote a high share of soil microbial biomass, and seem to be of importance among the microbial flora of the rhizosphere. The interaction found in plant and the Actinomycete, could be both deleterious and beneficial for the host (Couillerot *et al*.,2013). A large number of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. The population of actinomycetes has been time and again marked as one of the major group of soil population which may vary with the soil types. Among actinomycetes group, streptomycetes is suggested as the most dominant. The non streptomycetes are also called rare actinomycetes.The genus

streptomyces is one of the largest genus of Actinobacteria and also the type genus of the family Streptomycetaceae. Over 500 species of *Streptomyces* bacteria have been described so far. The genus *Streptomyces* belong to the Domain Bacteria, Phylum Actinobacteria, Order Actinomycetales and the Family Streptomycetaceae.

Isolations within India

Screening of actinomycetes have been undertaken for many attributes like four different strains from laterite soil in Guntur region of Andhra in India (Kavitha *et al.,* 2010).15 strains of actinomycetes were isolated from Lucknow in Uttar Pradesh (Pandey *et al.,* 2011) Five actinomycetes strains of *Isoptericola variabilis was* isolated from 25 samples of Cauvery river basin. (Muthu *et al.,* 2013). six strains (in total) of actinobacteria were isolated from the soil samples collected from various arid and semi regions around Jaipur, Jhunjhunu, Sikar of Rajasthan (Masand *et al.,* 2015).10 isolates with distinct respective morphology were isolated and purified on starch casein agar from forest soils of Mahabubnagar district, Andhra Pradesh by Balakrishna *et al*., (2012)

Throughout the world

Similarly, other investigations carried through-out the world shows evidence of the ability of actinomycetes to inhabit many parts of the world. Heng *et al*., (2015) have isolated 110 *Streptomyces* isolates from samples of peat soil of Malaysia.A thermophilic actinomycetes *Thermasporomyces composti* gen. nov., sp. nov. was isolated from compost (Yabe *et al*., 2011). 31 strains of potential antibiotic producing actinomycetes from sediments as well as water of Tana Lake, Ethiopia were isolated by Gebreyohannes *et al*., (2013). 60 actinomycetes isolates were isolated from soil samples that were collected from different selected locations of Saudi Arabia. (Ababutain *et al.,* 2012). A total of foryt four strains of actinomycetes were isolated from Caspian Sea sediments at a depth of 5-10 m (Mohseni *et.al*. 2013).

Understanding Actinomyctes

Evidence indicates that actinomycetes are quantitatively vital within the rhizosphere. (Barakate *et al* 2002, Crawford *et al*.1993, Doumbou *et al*.2001,). Couillerot *et al*.,(2013) during their work on biocontrol and biofertilizer activities evaluation of *Streptomyces anulatus* S37 have stated that they provide protection to various plants, from soil-borne fungal pathogens and the antagonistic property

against pathogenic fungi, which have allowed these bacteria to be used as a bio control agent, with attributes such as fungus-anatagonistic root colonizer. Like the other Actinobacteria, streptomycetes are gram-positive, and have genomes with high GC-content, grow in soil and decaying vegetation, with permanent substrate and aerial mycelia which are mostly branching. Aerial mycelia have characteristic long chains of arthrospores during mature stage in their life cycle, also called as sporophore, which serve to enhance the spread of the organism, through budding of conidia. The morphological structure of the sporophore, colonial morphology, colour, size and odour are diagnostic features, used to differentiate many species and strains. .Streptomycetes can be easily distinguished by their distinct "earthy" odour which is due to production of a volatile metabolite, geosmin which was isolated from *Streptomyces griseus* (Schlegel 1992).

Others too have noted that they are responsible for the earthy smell of freshly upturned healthy soil (Sprusansky *et al.,* 2005). And as outlined by Chaudhary *et al*.,(2013) actinomycetes decomposes complex mixtures of dead plant, animal fungal materials which are conductive for crop production as the by-products are extra cellular enzymes.Actinomycetes also produce auxin and gibberlin like compounds which is realted to plant growth. (Persello-Cartieaux *et al*.2003, Bloemberg *et al.* 2001). Studies of Cummins and Harris (1956) established that actinomycetes have a cytomembrane composition comparable to that of gram-positive bacterium, and conjointly indicated that the chemical composition of the cytomembrane may furnish sensible strategies of differentiating numerous varieties of actinomycetes. Fuentesa *et al.* (2010) reported that growth of 12 out of 18 actinomycetes isolates was closely realted to the presence of other microorganism and the pesticide namely (chlordane, lindane or methoxychlor) and highest growth and pesticide removal were observed with chlordane. Sharma (2014) has said that spores of most actinomycetes endure desiccation and show slightly higher resistance to dry or wet heat than vegetative cells hence are appropriate for soil applications.

Growth after germination during the life cycle of the gram positive , *Streptomyces coelicolor* a soil dwelling bacterium starts when spores come in contact with a suitable source of nutrient . The filamentous vegetative cells known as “substrate hyphae” grow following apical tip extension along with branching, ultimately resulting in a tangled filamentous network. The second filamentous cell types emerge with the

gradual aging of the vegetative colonies, taking to the air as aerial hyphae which is at a distance from the substrate hyphae, by undergoing septation and compartalization into 40 to 60 units of equal size. Known as “prespore” these structures are the precursors for metamphosis into spores. There are many steps to the maturation, ultimately cumulating in the deposition of a grey polyketide pigment, upon the surface of the respective spores and evtentually turning the colour of aerial mycelium to grey from white. (Davis and Chater, 1990). The switch from substrate hyphal growth to aerial growth is understood to coincide with the sensing of environmental stress and nutrient deficiencies. Nitrate depletion, for instance, is known to coincide with initiation of formation of aerial hyphae (Karandikar *et al*. 1997), and while the presence of glucose is understood to inhibit development of the aerial hyphae (Redshaw *et al*., 1976). The emergence of aerial hyphae in turn results in production of various secondary metabolites, and those have subsequent and significant application in varous fields like medical science as antibiotics, antifungal drugs and also varous important chemotherapeutic agents. Further there has been suggestions that physiological parameters through tests can be used as indispensable tools for classification and identification of actinomycetes. (Kampfer *et al*.,1991)

Actinomycetes as source of antibiotic.

As is known antibiotic producing ability is the best known ability of actinomycetes. As reported by other workers as well approximately 70% of all antibiotics known to mankind has been isolated from actinomycetes, (Ayari *et al*. 2012) It is also stated that in comparison to other microbes, novel therapeutic antibiotics are being discovered from this group at a frequent rate in various chemotypic and biologically active forms namely daptomycin, thiednamycin and echinocandins. (Newman and Cragg, 2007). It has been put on record by Marinelli (2009) that of the total marketed microbial drugs two-thirds are produced by streptomycetes.

Table 1. List of antibiotic compound produced by actinomycetes

Type	Active component	Microorganism
Antifungal compound	Nystatin	*Streptomyces noursei*
	Amphotericin B	*Streptomyces nodosus*
	Natamycin	*Streptomyces natalensis*
Antibacterial compound	Erythromycin	*Saccharopolyspora erythrea*
	Neomycin	*Streptomyces fradiae*
	Streptomycin	*Streptomyces griseus*
	Vancomycin	*Streptomyces orientalis*
	Daptomycin	*Streptomyces roseosporus*
	Rifamycin	*Streptomyces mediterranei*
	Chloramphenocol	*Streptomyces venezuelae*
	Puromycin	*Streptomyces alboniger*
	Lincomycin	*Streptomyces lincolnensis*
Other bioactive compounds	Brasilinolide A	*Nocardia brasiliensis* IFM0406
	Tetrodotoxin	Marine actinomycetes
	Niromycin A	*Streptomyces endus* N40
	Salinosporamide A	*Salinispora tropica*

Relevance of Actinomycetes and economic importance

Bignell *et al*. (2010) have reported a new biosynthetic gene cluster found in *Streptomyces scabies* that produce coronafacic acid, which is part of the plant toxin, coronatine which mimics the plant hormone jasmonate, thus playing a major role in contributing to virulence.

Means of Bio-remediation

Due to fact that actinomycetes have been isolated from mostly any type of habitat or agroclimatic zone , it can be inferred that there must be some ability in these organism to be able to auto remedy various type of soil or biological structure. Lin *et al* (2011) demonstrated that a strain of *Streptomyces parvulus* , new in its occurence, isolated from waste-water sludge could even degrade a pyrethroid based insecticide named cypermethrin. Polti *et al*., (2007) put up, that if bioremediation of heavy metals and other organic compounds is to be considered then actinomycetes are relatively well suited owing to the relevance that as they constitute a prevalent microbial component in many soil biota, attributed due to their metabolic diversity and growth characteristics, mycelial form and relatively rapid colonization of selective substrates.

Isolation technique for Actinomycetes

There are reports of many types of techniques, which have been followed as such or with certain modification for isolation of actinomycetes by reserachers from respective area of location and climatic zone. Sahin and co-workers followed simple soil dilution method with starch casein agar as the media for isolation of thermophilic *Streptomyces*. In brief about 1g of soil samples was aspetically transferred to 9ml of sterile Ringer's solution (oxiod) ¼ strength, which was manually shaken for a span of half an hour, with intent to disperse the bacteria.Preheated in water bath at 55^0C for a duration of 6 min was opitimised to heat the suspension at tenfold dilution. Aliquots (0.2 ml) of 10^{-2} to 10^{-5}dilutions were evenly spreed on the dried starch casein plate surfaces at (pH 7.2; 20) with supplements of cycloheximide (only 50mg ml^{-1}) and filter sterilized rifampicrin (0.5 mg ml^{-1}).7 days incubation of inoculated plates in replicas of four was done at 55^0C.Colony forming units (c.f.u) per gm per dry wgt of sample was used in counting the expression of isolates in each plate (Sahin *et al*.2002). Similar method was applied by Lo *et al*. (2002) but by using HV agar medium. Same was done earlier in past too by El-Nakeeb and Lechevakier (1963) followed dilution technique for aerobic actinomycetes but used many types of media like Gaueze's agar medium, benedicts medium modified, chitin medium, soyabean meal glucose medium, Czapek's agar medium and gave a conclusive report that AGS medium or Arginine-glycerol-salt medium supports isolation of many types of actinomycetes. Hsu and Lockwood (1975) on the other hand pointed out that chitin agar was superior for isolation and at the same time enumeration. Moncheva further said that starch-casein-nitrate agar can be used for isolation, cultivation as well as maintenance of isolated soil actinomycetes (Moncheva *et al*., 2002). Other related works include isolation of a moderate halophilic actinomycets, strain called as HA-9 or *Nocardiopsis kunsanensis* from salt urns in Kunsan in Republic of Korea, by simply amending seawater in the Bennet medium. Through their work, another research group Hayakawa *et al*.,(2000) formulated an experimental, termed rehydration and centrifugation (RC) method, wherein supplementation of media by yet another simple enrichment technique allowed direct isolation from soil and litter of selective and rapid isolation of many types of zoosporic actinomycetes.

Similarly,Takahashi and Omura (2003) described selective isolation of novobiocin or Actinoplanes strains of *Kitasatospora* using chemotactic (KCl)

substituted by gellan gum as a solidifying agent and appraised nine novel compounds and proposed two new genera, five new species and one new subspecies. Couillerot *et al.,* (2013) in their work have used supplements in Olsons media and nutrient agar, namely actidione to inhibit fungus and nalidixic acid to inhibit bacteria capable of swarming. Soil dilution method was used by Srividya *et al*., (2012) to isolate Streptomycetes strain from solanaceae rhizospheric soils of brinjal, capsicum and chilli grown in Bangalore and Assam. Serial dilution method diluted upto 10^6 dilutions with plating on starch casein agar following spread plate technique was used by Gopalakrishnan *et al*., (2011) to isolate 137 cultures from herbal compost. Similarly Heng *et al*., (2015) have isolated 110 actinomycetes isolates from peat soil samples of areas in Malaysia, by agitating soil sample with orbital shaker and plating on starch casein agar at 28^0C. Modified standard dilution technique was followed by Ara *et al*.,*(*2012a), for collection of 105 actinomycetes strains from the soils of Riyadh in Saudi Arabia.

Study of morphology of actinomyctes

Studies have been made to understand each aspect of actinomycetes growth and survival under various condition. In the past, Shirling and Gottlieb (1966) examined isolates for pigmentation, colour of aerial mycelium and related morphological features. Abbas using the same method, did grow cultures for 4 weeks and observation were made at weekly intervals for morphological properties of colony, cells and spores (Abbas 2006).

Even variation of the media for growth was done for morphological study, like Sahin *et al.,*(2002) chose oatmeal agar as the medium of growth and visual examination on the basis of aerial spore mass colour, substrate mycelia pigmentation and colouration of media by diffusible pigments was done, and at the same time peptone-yeast extract in iron agar plates were used to observe production of any dark colored melanin pigments. Also there is confinement of study and investigation of fine structure of germinating spores to *Streptomyces* genera as put forth by Kalakoutswl and Agre (1973). Hence, there has been suggestions for need of modification of colour grouping method, and objective color determination method (Pridham 1965).

Production of melanine

Actinomycetes have another feature of being able to synthesize as well as excrete, melanin and melanoid, which are uaully dark colored and forms the basic criteria of taxonomical study, tests have been conducted by growing actinomyctes in peptone-yeast extract agar and synthetic tyrosine agar, to study the melanine production. This was done by dispensing 10 µl of selected media in liquid form in test tubes and then inoculating loop full of *Streptomyce*s spore, sunjecting the same to a stationary stage, facilitating better observance at 27^0 c for seven days (Dastager 2006)

Enzyme activity

Enzyme activity of actinomycetes, mainly fungal cell wall degrading enzymes such as cellusase, chitinase, β 1-3-glucanase have been extensively studied by different scientists. Production of chitinase from endophytic *Streptomyces aureofaciens* CMUAC130 and likewise effect on phytppathogenic fungi has been reported Taechowisan *et al*., (2003). Srividya *et al* (2012) reported chitinase, Glucanase, cellulose, protease production by *Streptomyces* sp. 9p.

IdentificationTechniques

Various techniques are adapted by the researchers for identification of different groups of actinomycetes. Identification can be done by the conventional classical method where the morphological attributes are noted for the identification and classification. The identification key as described in Bergey's Manual of Determinative Bacteriology(Buchanan and Gibbon 1974) is very much useful for identification of Streptomycetes group.Colour of the aerial spore mass, production of melanoid pigment, spore chain morphology, spore structure,sporulation types all these characters are routinely followed(Li *et al*., 2016) for the morphological identification procedure. Chemotaxonomy is another method of identification of organisms where the chemical variation in the organisms are considered for classification. Being Gram positive bacteria the presence of Diaminopimelic Acid (DAP) isomers in the cell wall is the most important chemical characteristic of Actinomycetes as described by Schon and Groth (2006). Molecular identification of the actinomycetes is done by the nucleic acid sequencing method. The sequence of 16S ribosomal DNA is being used by various researchers for accurate identification of the actinomycetes up to genus level. With help of the 16S rDNA sequences phylogenetic relationship of different actinomycetes can be

confirmed. Yu *et al*., (2015) isolated actinomycetes from wetland and successfuly performed their molecular profiling following the method. Other workers like Jami *et al*.,(2015), Labeda *et al.,* (2014), Muthu *et al.*,(2013) also reported molecular identification of the actinomycetes based on the 16S rDNA technique.

Plant protection by biocontrol agents.

Plant diseases and pathogenic microorganisms are a major and chronic threat to crop production as well as crop loss affecting food production. Evidently, owing to dependancy on agrochemicals and proven protection against pathogens agriclutral production has increased manyfold in the decades gone by. However, the flip side of the same has been deterioting health related conditions to humans upon comsumption or contact and resulting environmental pollution. Also increase of resistance against pathogens to wide range of fungicides is there. (Prapagdee *et al*., 2008). Furthermore, the growing effective cost of pesticides, predominantly in developing countries of the world, and increase in awareness of people against harmful effect of these chemicals has led to a search for substitutes for chemical properties.On similar lines, various microrganism to microorganism interaction for growth enhancement , antagomisn and overall soil system uogradation has been underaken as experimental set ups against pathogens and pest using either bacteria, fungi, actinomycetes etc. For instance Xiao *et al*. (2002) observed that application of isolated strains of actinomycetes rather significantly reduced severity of root rot in host alfalfa and soybean caused by *Phytophthora medicaginis* and *Phytophthora sojae.* in sterilized vermiculate as well as naturally infested field soil.

Similarly, *Streptoverticillium albireticuli* upon isolation and cross with fungal pathogens *Rhizoctonia solani*, *Phytophthora cinnamomi* and *Fusarium oxysporum* showed remarkabile ability towards antifungal activity(Park *et al*., 2002).Phytophthora blight pathogen of red pepper, *Phytophthora capsici* growth was inhibited by thermostable low molecular weight substance of *Streptomyces halstedii* (Joo, 2005)

Mechanism of biocontrol

Biological control for direct protection of plants from pathogens involve the use of antagonistic microorganisms.The machanism by which these microorganisms carry out their function are their abillity to parasitize the pathogen directly, production of antibiotic against the pathogen, their ability to compete for space and nutrients and to

survive in the presence of other microorganisms. These mocroorganisms produce enzymes which attack the cell component of pathogen, induce defence response in plants they surround. Although thousands of microorganisms have been shown to interfare with the growth of plant pathogens in the laboratory , only a few are effective in the field condition. The most important of them are *Trichoderma harzianum, Pseudomonas fluorescens* and *Bacillus subtilis* (Agrios 2005).The genus *Streptomyces* is gaining reputation as a suitable alternative for much needed organic method of desease control in plants, as there is an unprecendented awareness in the global market aimed at not only crop protection but a general need for use or consumption of natural products, having genuinely low or nil toxic trail, upon application for the same (Behal 2000). Actinomycetes isolates as biocontrol agents exert a direct inhibitory effect on hyphal growth and structure of fungal pathogens like *Botrytis cinerea* to reduce disease incidence, even though the exact mechanisms by which actinomycetes isolates operate is not elucidated. (Couillerot *et al*., 2013)

Table 2. Actinomycetes as bio control agent

Actinomycetes	**Host Plant**	**Disease**	**Pathagen**	**Reference**
Streptomyces Sp. *S. thermotolerans* and *Streptomyces sp.* N0035	Yam	Spot diseases in Yam	*Curvularia eragrostides* (Henn.) *Meyer Colletotrichum gloeosporioides* (Penz.)	Soares *et al* 2006
Streptomyces GS 93-23	Alfa alfa	Phytophthora root rot on alfalfa	*P. medicaginis*	Xiao 2002
Streptomyces griseus	Tomato	Fusarium disease of tomato	*Fusarium oxysporium f. sp.* Lycopersici	Anitha and Rabeeth. 2009
Strepromyces griseus, S. *hygroscopicus* var. *geldanus* and *S. noursei* *S.cellulosae* *S. herbaricolor* *S. coeruleofuscus*	Pea	Rhizoctonia root rot on pea	*Rhizoctonia solani* *Phytophthora megasperma* var.*sojae*	Rothrock and Gottlieb., 1981
Strepromyces	Cucumber	Damping off	*Pythium aphanidermatum*	Costa *et al.* 2013
Streptomyces	Sunflower	Sunflower Head and	*Sclerotium sclerotiorum*	Baniasadi *et al.*, 2009

		Stem rot disease		
Actinomycetes isolate 19	Radish	Rhizoctonia root rots	*Rhizoctonia solani*	Sahaya *et al*.,2012
Streptomyces sp.	Sugar beet	Root rot	*Sclerotium rolfsii*	Errakhi *et al.* 2009
Nocardia sp. AzL025 Streptosporangium sp. AzR 021 *and* 048	Lettuce	Root rot	*Pythium and phytophthora sp.*	Verma *et al.*, 2009
Actinomycetes isolate A5005 *and* A 5314	Rice	Rice Blast	*Magnaporthe grisea*	Hong-Sik and Yong-Hwan , 2000
Streptomyces sp.	Chilli pepper	Chilli anthracnose	*Colletotrichum gloeosporioide*	Suwam *et al.*, 2012
Streptomyces Sp.	Tomato	Bacterial wilt in tomato	*Ralstonia solanacearum*	Sreeja and Surendra., 2013
Streptomyces hydroscopicua	Chilli	Stem rot disease of Chilli	*Sclerotium rolfsii*	Pattanapipitpaisa Kamlandharn., 2012
*Streptomyces sindeneusis*isolate *263*	Rice	Rice Blast	*Magnaporthe oryzae*	Zarandi *et al.*, 2009
Actinomycetes	Soybean	Damping off	*Sclerotium rolfsii*	Sastrahidayat *et al.*, 2011
Strepromyces sp.	Sweet pea	Powdery mildew disease	*Oidium sp.*	Sangmanee *et al.*, 2009
Streptomyces nigellus NRC 10	Tomato	Dumping off	*Pythium ultimum*	Helmy *et al*. 2010
Streptomyces lydicus WYEC108	Pea	Pythium seed rot and root rot.	*P. ultimum*	Yuan and Crawford., 1995
Actinomycetes	Lettuce	Damping - off	*Pythium lutimum*	Crawford *et al.* 1993
Streptomyces Viridodiasticus and *Micromonospora carbonacea*	Lettuce	Basal drop disease of lettuce	*Sclerotinia minor*	El-Tarabily *et al.*, 2000

Antimicrobial activity.

Sahin and co-workers (2003) studied *Streptomyces* isolates under, *in vitro* condition for microbial activity against gram positive and gram-negative bacteria and

yeast. The result of the study inferred that 5 isolates, 3 identified as *Streptomyces antibioticus* (MU106, MU107), *S. rimosus* (MU114) showed prominent level of activity against chosen microbe of coagulase-negative *Staphylococcus* (CoNS) as well as yeast. Similar reports have been on effective antimicrobial activity of *Streptomyces* isolates have been put forth by reserachers during various time Ilic (2005), Laidi *et.al*., (2006) and Charoensopharat *et al*.,(2008).

Lytic enzyme production

The ability of any organism to act as antagonist against other microbes or pathogen is due to various ability, and production of lytic enzyme capable of destroying fungal cell wall resulting in hyperparasitic activity is one such character, supported by results and accounts from many sources, mostly or coveniently found among actinomycetes.Spore germination and at the same time germ tube elongation in *Botrytis cinerea* was inhibited by chitinase produced by *Serratia plymuthia* (Frankowski *et al*., 2001).The ability of *Serratia marcescens* to produce extracellular chitinase is what helps it to act as antagonist against *Sclerotium rolfsii* (Ordentlich *et al*.,1988). *Pseudomonas stutzeri* synthesize extracellular chitinase as well as laminarinase that digest and lyse *Fusarium solani* mycelia (Lim *et al.*, 1991). Evidence has been reported that *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 synthesize β-1,3-glucanase, which undertakes cell wall lyses of *Fusarium oxysporum* f. sp. *cucumerinum* (Singh *et al*., 1999). Similarly cell walls of *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Pythium ultimum* are destroyed by the same β-1,3-glucanase but synthesized by *B. cepacia* (Fridlender *et al*., 1993). Endophytic actinomycetes also produce lytic enzymes which inhibit fungal growth. For example, Castillo *et al.* (2002) demonstrated that an endophytic bacterium *Streptomyces* sp. strain NRRL 30562 isolated from *Kennedia nigriscans* produced munumbicins, an antibiotic which can inhibit *in vitro* growth of phytopathogenic fungi, *P. ultimum*, and *F. oxysporum* (Compant *et al.*, 2005).*Streptomyces griseus* upon interactive setups showed evidence for production of a metabolite which inhibited soil-borne plant pathogens (*Alternaria alternate*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Fusarium solani*) and two isolates of *Aspergillus flavus. In vitro* lytic activity presdisposition provides setup of appropriate condition and the effect of biocontrol organism in field level treatment. (Anitha and Rabeeth, 2010)

In-built resistance in plants.

Systemically activated resistance (SAR) is a phenomenan which comes, after primary infection takes place upon invasion by a necrotizing pathogen resulting in increased level of salicylic acid and pathogen related protiens and subsequent hypersentitive reaction (Agrios 2005). Whereas Induced sustemic resistance (ISR) is triggered by non-pathogenic strains of root colonizers. ISR does not cause visible sympotoms on the host plants and first PGPB-elicited ISR was observed on *Dianthus caryophillus* with reduced susceptibility to wilt desease caused by *Fusarium sp* (Van Peer 1991). Similarly, in *Cucumis sativa* with reduced susceptibility to wilt caused by *Fusarium* sp. (Van Peer1991) and on cucumber (*Cucumis sativus*) with reduced susceptibility to foliar disease caused by *Colletotrichum orbiculare* (Wei *et al*.,1991). Traditionally, reports supported that only rhizobacterial strains of PGPB, brought about ISR through physiological mediation, but evidence of same by *Pseudomonas fluorescens* an endophytic bacteria against red rot caused by *Colletotrichum falcatum* on sugarcane has also been reported (Viswanathan and Samiyappan.,1999), as has *Burkholderia phytofirmans* PsJN against *Botrytis cinerea* on grapevine (Barka *et al*.,2000).

Defence mechanisms of ISR- mediated by PGPB

The chronology or sequential networking of various reactions within the plant tissue system when a pathogen attackes is dynamic both interanlly and externally too. For optimum defence PGPB triggered ISR leads to enhancement of plant defence enzyme systhesis which in turn strengthens plant cell wall and host response to metabolic responses and physiology. Duffy *et al.,* (2003). Indicated in responses to pathogen colonization of epidermal and hypodermal cells, endophytic *P. fluorescens* WCS417r induce outer peripheral and outer radial end of the first cortical cell wall thickening. Similarly, phenolic compound accumulation along with exodermal or cortical cell wall stregthening was reported in *Burkholderia phytofirmans* PsJN-grapevine interaction upon colonization. (Compant *et al*.,2005).

***Streptomyces* as bio controlling agent**

Evidently *Streptomyces* sysnthesize a variety of fungal cell wall-degrading enzymes, such as chitinase, cellulases, hemicellulases, amylases, glucanases, etc and other antifungal compunds. Fungal inhibition can be related to chitinase production

(Gupte *et al.,* 2002; Dahiya *et al.,* 2006), *Streptomyces viridodiasticus* syntesized chitinase extract has been found to suppress basal drop disease causing fungal pathogen *Sclerotinia minor* in lettuce (El-Tarabily *et al.* 2000).It is further supported that fungal growth inhibition is related to chitinase production in various plants (Gupte *et al*., 2002; Dahiya *et al.,* 2006). Evidence is also there that *Aspergillus* sp. and *Fusarium subglutinans* growth is inhibited by *Streptomyces* sp of maize rhizosphere (Bressan 2003). Control of foliage diseases by culture filtrates of streptomycetes has also be worked out with satisfactory results wherein nine out of ten samples controlled or suppressed one or other disease under green house condition (Pridham *et al.,* 1956). Even under artificial condition *Streptomyces ambofaciens* controlled *Fusarium* wilt in cotton and *Pythium* damping-off in tomato plants (Reddi and Rao.,1971).

***Streptomyces* as growth enhancer**

There is a wide array of compound that assist and contribute towards the vitality and vigour of plants. Indole-3-acetic acid (IAA) is one such hormone, it is the principal form of auxin in regulating cellular processes like cell division, elongation, differentiation. At the same time plays a key role in shortening of root length and root hair formation. Hence IAA assists in increasing the nutrient absorption ability of the palnt. Other role in developmental activity includes embryo and fruit development, vascular tissue differentiation, tropism of plant, apex formation and apical dominance (Shrivastava *et al.,*2008). There has been observation regarding induction of IAA systhesis by *Streptomyces* species, six in number when tryptophan is present, viz *S. violaceus* and *S. exfolitus* through catabolization of IAM, ILA, IET, IAAld into IAA, with other possible pathways into IAA biosynthesis (Manulis *et al.,* 1994). Igarashi *et al*.(2002) reported secretion of indole-3-acetic acid (IAA) by *S.violaceus, S.scabies, S.griseus, S. exfoliates, S. coelicolor* and *S. lividans* when L-trytophan was induced. So are reports that strain of *Streptomyces* sp MBR52 augmented elongation and emergence of plant adventitious roots (Meguro *et al*., 2006)

***Streptomyces* as Plant growth inhibitors or herbicidal agents**

There have been many reviews and works on the many beneficial aspects of plant actinomycetes interaction. There is support to the fact that *Streptomyce*s also inhibit the growth of certain plants and in doing so can be exploited as potential herbicide. Certain metabolite systhetesized by *Streptomyces* sp strain SANK 63997

produced herbicidal antibiotics called Herbicidin H, the strain was isolated from leaves of *Setaria virdis* var *pachystachys* (Hasegawa *et al.*, 2006). It is not only *Streptomyces* but other actinomycetes which happen to have herbicidal properties, there has been reports of strain SANK 61299 of *Dactylosporangium sp.* from *Cucubalus* sp. producing two growth inhibitors along with streptol acting on adverse germination of *Brassica rapa* (Okazaki 2003).

Endophytic actinomycetes as biocontrol agents

Actinomycetes are found at various types of habitat and in different roles in the ecosystem, most of the time in soil rhizosphere, in lakes or pond, in sediments or in riverine soil as free living, sprophytes etc. Moreover there are groups which are in direct interaction with the living tissue of plants, these are known as endophytic actinomycetes, and may be beneficial like *Frankia* which has suggestive role in nitrogen fixation and *Rhizobium* which is believed to do the same in legume plants. Endophytic actinomycetes have been demonstrated to improve and promote growth of host plants, as well as to reduce disease symptoms. Management of beneficial potentials of endophytic actinomycetes to favour plant growth could be realized by a better understanding of the physiological and molecular interactions between these microbes and plants. (Simizu ,2011).Thirty-eight strains belonging to *Streptomyces, Microbispora, Micromonospora* and *Nocardia* were isolated from surface sterilized healthy wheat tissues (Coombs and Franco, 2003)Similarly, 59 endophytic isolates isolated from root tissues of *Zingiber Officinale* and *Alpinia galangal* showed maximum antifungal activity against *Candida albicans*, mostly *Streptomyces aureofaciens* (Taechowisan *et al*, 2005)

Actinomycetes metabolites that affect plant's life

Endophytic colonizers do get shielded from external factors and get nutrition from the host, and in turn different kinds and forms of bioactive metabolites are synthesized by them which aids in the plant vitality and vigour. The control point of such attributes may be the ability to fix nitrogen, produce phytohormones, inhibit phytopathogen growth or incidence through related phenomemen like antibiotic secretion, siderophore production, competition for nutrient and most importantly directly or indirectly brining about systemic disease resistance.

Use of *Streptomyces* as biocontrol agent for plant disease management

Okazaki *et al.,* (1995) and Matsumoto *et al.* (1998) reported that a variety of actinomycetes inhabit a wide range of plants as symbionts, parasites or saprophytes, and most of them belonging to the genera, *Streptomyces* and *Microbispora.*

Actinomycetes and their role in various plant pathogen interaction
***Pythium* seed and root rot of pea.**

Extracellular metabolites produced by *Streptomyces lydicus* WYEC108 was instrumental in combating fungal pathogens of pea, which was evident by inhibition of *Pythium ultimum* and *Rhizoctonia solani* together with *Streptomyces lydicus* WYEC108 grown in liquid medium. Even spore coating of pea seeds by the respective actinomyetes showed maximum inhibition of infection or invasion by test pathogen *P ultimum,* even under conditions of high oospore count in the soil of growth. Even seed and root was indicatively mimimized or suppresed under controlled condition. Also formulations of the strain as spore and peat moss-sand media in sterile and non sterile soil under pathogen infestation, was able to positively affect pea plant growth and vigour. With proven ability to lyse fungal cell wall and distort fumgal hyphae the strain *S. Lydicus* WYEC108 can be said to be a potential biocontrol agent when controlling Pythium seed and root rot (Yuan and Crawrford., 1995).

Lettuce damping-off caused by *Pythium ultimum*

Seed germination of lettuce under open and glass house condition in pathogen infested soil was optimal in works carried out in England inferreing that damping off by *Pythium ultimum* was controlled when formulations of actinomycetes was applied, isolated from both rhizospheric and non rhizospheric soil. Same isolates inhibited growth of other root pathogens as well (Crawford *et al*., 1993).

Basal drop disease of lettuce.

Isolation and screening of microbes from lettuce growing fields of Al-Ain, United Arab Emirates resulted in availalibity of countless number of bacterial, *Streptomyces* and non-Streptomyces isolates, which in turn showed ability to sysnthesize higher level of Chitinase. Further *in vitro* assay of these isolates against a known pathogen *Sclerotina minor* casual organism of basal drop disease resulted in marking three isolates namely *Serratia marcescens, Streptomyces viridodiasticus* and

Microsperma carbonacea as most effective in disease supression. The trio, upon further assay for β-1,3-glucanase showed prominent results, of not only for enzyme synthesis but also *in vitro* pathogen incidence and infection reduction under glass house condition (EL-Tarabily *et al*., 2000)

Leaf spot diseases of yam

Reports indicate that *Streptomyces* strain AC26 was effective in halting the growth of both spore and mycelium of *Curvularia eragrostides* (Henn.) causing leaf spot disease of yam. At the same time *Streptomyces thermotolerance* and *Streptomyces* sp N0035 did the same towards *Colletotrichum gloeosporioides*. The result was in tune with the chitinolytic activity of the strains and sysnthesis of secondary metabolites (Soares *et al*., 2006)

***Phytophthora* root rot on alfalfa**

Xiao et al., (2002) put forward results stating post emergence damping-off caused by *Phytophthora* on alfalfa was inhibited by application of formulations of *Streptomyces* isolates with the added advantage of increase in plant vigor and forage yield. Similarly, supportive was findings that a *Streptomyces* strain GS 93-23 acted as prominent bio control agent against *Phytophora medicaginis* in alfalfa plants even in infested conditions. The same strain under *in vitro* conditions inhibited growth of alfalfa and soybean pathogens *Pythium ultimum, Phoma medicaginis Aphanomyces euteiches.* Further it also showed ability against diverse soil borne pathogens for integrated control.

Fusarium disease of tomato

Experiments with *Streptomyces griseus* in testing inhibition of *Fusarium oxysporum f.sp. lycopersici* under *in vitro* condition showed prominent sign of pathogen growth inhibition supported by indiciation of presence of inhibitory substance, antibiotic, and enzymes such as protease, Glucanase etc. Even cell wall lysis was observed (Anitha and Rabeeth 2009).

Rhizoctonia root rot on pea

Experimental set ups with known antibiotic producing *Stretomyces* namely *Streptomyces griseus, Streptomyces hygroscopicus.var.geldanus,Streptomyces noursei* showed prominent zones of inhibition against pathogens *Rhizoctonia solani* and

Phytophthora megasperma var sojae. *Streptomyces hygroscopicus* provided complete control over disease development upon pre application of strain seven days before pathogen infestation of planting soil and *Streptomyces herbaricolor* and *Streptomyces coeruleofuscus* was found to provide consistent control over different conditions against the pathogens (Rothrock and Gttleieb, 1981)

Disease of interest as per present investigation.

There are several records of fungi belonging to ascomycetes, deuteromycetes, basidiomycetes as being causal agent of root rot and wilt disease, prominant among them are *Sclerotium rolfsii, Rhizoctonia solani, Thielaviopsis, Acermonium, Fusarium solani* as well as its other species which even cause stem rot in various crop plants.

Fusarium root rot

Fusarium solani and *Fusarium oxysporium* are prominent pathogens of root rot in non grain crops. *Fusarium solani f.sp.phaseoli* causes *Fusarium* root rot and it is a prominent disease effecting common bean (*Phaseolus vulgaris* L.) and also other crops like soybean, peanut, asparagus etc. The symptomology is evidently initial reddish colour of young tap roots, which eventually attain dark tinge over some time and the affect is enhanced with the gradual cracks along main root and ultimate killing of secondary roots.

Further disease development marks yellowing of leaves, growth retardation, leaf fall or simple death of the plant even without visible wilting.

Generally production of asexual spores is there in *Fusarium solani* , which are either micro conidia, macro conidia, chlamydospores which are thick walled. Chlamydospores are profusely found in killed plant tissues or colonized organic stock in soil. Chlamydospore helps the fungi pass on unfavourable condition of low temperatures and drought as inactive stage and attains active state with proximity to seedling root system. This happens during an early phase of the growing season when warm conditions prevail.

Sclerotial rot

Sclerotium rolfsii is very harmful soil – borne fungus which is pathogenic to an array of host, it over winters in its mycelial form within the infected host tissue or the plant debri. A round structure called sclerotia, either in free condition or association

is the main over wintering structure and is the primary inoculum bringing about disease by persistingly dwelling near the soil surface. (Aycock 1966; Punja 1985). Sclerotial dissemination is either by the cultural practices, infested transplanted seedling, wind, or water itself, and like other sclerotium producing fungi has capability of overwintering as sclerotia or sterile mycelium.(Akram *et al.,* 2008). The form genus *Sclerotium* is characterized by dark brownish, black to tanned sclerotia mostly spherical in shape and on internal examination has a rind, cortex and medulla.(Punja and Rahe 1992). It also has a teleomorphic state (Punja 1988) and is prevalent in occurrence where weather is warm. Infection initiates as a lesion in the soil line area of the stem and gradually moves upward in a cottony at times fluppy appearance of mycelium, at times parallel leaf wilting, yellowing and die back is also there. Of the diseases that it causes collar rot is the most frequent as well as prominent in terms of occurrence and loss (Singh and Pavgi 1965).

Table 3: List of different Diseases of *Vigna radiata* and bio control agent

Bio-control agent	Disease	Pathogens	References
Trichoderma spp.	Root rot of *Vigna mungo*	*Macrophomina phaseolina*	Leo *et al.* 2010
Pseudomonas spp.	Seedlings damping off and stem rot of cowpea (*Vigna unguiculata* L. Walp)	*Pythium aphanidermaturm*	Dieudonne *et al* 2007
Trichoderma viride	Root rot diseases of *Vigna radiata*	*Rhizoctonia solani Sclerotium rolfsii Macrophomina Phaseolina, Alternaria alternate, Furarium solani and Colletrichum capsicii*	Mishra *et al* 2011
T. harzianum	Root rot diseases of *Vigna radiata*	*Macrophomina phaseolina*	Kumari *et al.* 2012
Burkholderia sp. Strain TNAU-1	Root rot diseases of *Vigna radiata*	*Macrophomina phaseolina*	Satya *et al*, 2011
Fluorescent *Pseudomonas* (MRFP)	Root rot diseases of *Vigna radiata*	*Fusarium solani Rhizoctonia solani Macrophomina phaseolina*	Ara *et al*, 2012a

Table 4: List of different Diseases of *Phaseolus vulgaris* and bio control agent

Bio control agent	Disease	Pathogen	References
Trichoderma harzianum	Root rot of *Phaseolus vulgaris*	*Fusarium solani,Rhizoctonia solani,Fusarium oxysporium,Sclerotium rolfsii* and *Pythium* spp.	El-Mohamedy *et al.* 2013
*Glomus*spp	Root rot of *Phaseolus vulgaris*	*Rhizoctonia solani*	Hathout *et al.* 2010
Trichoderma harzianium, Glomus intraradices, Azotobacter chroococcum	Root rot of *Phaseolus vulgaris*	*Rhizoctonia solani, Fusarium solani* f.sp. *phaseoli*	Matloob *et al*. 2013 Kilicoglu and Ozkoc, 2013 Bilgi *et al.* 2008

Disease reporting

Fusarium* and *Sclerotium* root rot disease in *Phaseolus vulgaris

Commonly known as either bean, dry bean, French bean, *Phaseolus vulgaris* is from the family Fabaceae. The main desirable part of the crop is its pod which has value in terms of nutrient consumption as well as commercial importance. Root rot is the most prominent and loss-making soil borne disease of *Phaseolus vulgaris* L ,with a broad spectrum of causal agents or pathogens , which includes *Fusarium* sp. *Pythium* sp. *Rhizoctonia solani*.

The occurrence is global in disposition and distributed over all bean growing locations. Abawi and Pastor-Corales (1990), Mukankusi (2011), Abeysinghe (2007) reported Fusarium root rot of *Phaseolus vulgaris* from different parts of the world characterized by reddish to brown lesions evidently at the lower hypocotyls and along tap root of the plant. Infected portions enlarge with gradual aging of the host and its pathogen, attaining a brown colour, at the same time longitudinal cracks form within the older lesions , with discolouration and decay of the ortical cells. The outlined fungus as pathogen is very persistant into disposition for survival and distribution in the soil, with remarkable tools to dwell in the soil for a long duration of time, hence has been deemed difficult to control and contain (Abeysinghe 2007). In similar line of work Abawi and Pastor-Corrales (1990) reported Sclerotium root rot in *Phaseolus.*

Sclerotium* root rot and collar rot disease of *Vigna.

Mungbean or *Vigna radiata* is an annual legume crop belonging to the Fabaceae family, which is an important food supplement in many parts of the world and has high economic value. *Sclerotium rolfsii* root rot and collar rot disease of *Vigna* reported by Yaqub and Shahazad (2005) and Sharma *et al*.,(2002) is the prominent and important disease as per crop loss and disease development.

Formulation

Studies in Kyrgyzstan reported a laboraty made biofertilizer (Patent # 1703, registered by10/12/2012 in the State Register of Kyrgyz Republic) on the basis of *Streptomyces fumanus gn*-2 for the treatment of wheat and bean seeds, before planting them in soil with low fertility in order to determine the effect of this biological agent on germination rate, growth of seedlings and shoots, maturation phase of plants. And resistance of these plants to pathogens confirmed that the introduction of *Streptomyces fumanus* as a biological agent in soil together with the seeds stimulated the growth and reproduction of useful and important microorganisms in the soil environment.(Doolotkeldieva *et al* ,2015).

Yuan and Crawford designed and undertook a process of mass production of *Streptomyces lydicus* WYEC108 as a peat moss-sand formulation. Spores were collected from SPA plates where heavy sporulation was there, aided with a sterile spatula for transfer to sterile sand , which in turn was mixed into sterile sand-peat moss carrier and was maintained at room temperature for future use and storage.(Yuan and Crawford 2005)

Another mode of formulation preparation involves autoclaving 500 ml conical flasks with 50g moist wheat bran for 20 minutes at 121^0C, on three simultaneous occasions (Roiger and Jeffers 1991). Yet another of formulations involved inoculation of substrate with 25ml of spore suspension in 10% glycerol, under aseptic condition with incubation at 28^0C under dark condition for a period of three weeks and routine shaking for uniform growth and colonization. (EL-Tarabily *et al.,* 2000). Simple yet seemingly relevant formulations involve direct seed coating with *Streptomyces* spore, tried on sterilized tomato seeds by soaking in *Streptomyces* spore suspension for half an hour (Dhanasekharan *et al*., 2005)

Scenario at national level

India being a country with a huge population which is increasing exponentially over the years needs tons of food grain each year to feed its people. As the cultivable land is decreasing day by day and the cost of manure and other materials for agricultural use are increasing at the same time the use of biocontrol agents is the only way out. By using the indigenous micro organisms present in soil this aim can be fulfilled. Indian scientists who are working in this field are Dhanasekharan *et.al* (2005) working on biological control *Rhizoctonia* dumping off of tomato by *Streptomyces*. Shrivastava and co workers working on Production of indole-3-acetic acid by immobilized actinomycete (Shrivastava *et al.,* 2008). Anitha and Rabeeth (2010) working on *Streptomyces griseus*.Patel *et al* (2014) working at Gujrat with the Actinomycetes having antibacterial and anti fungal properties. Masand and Menghani (2015) are working at Rajasthan with the same aim. Srividya *et al*.(2012) working with *Streptomyces* sp. 9p having biocontrol ability against chilli fungal phytopathogens. Goplakrishnan *et al*.(2011)evaluated Actinomycetes isolates for biological control of *Fusarium* wilt of chickpea. Janaki *et al* (2016) isolated actinomycetes from mangrove plant rhizosphere and tested their antifungal properties. Shrivastava *et al*., (2017) isolated *Streptomyces aureofaciens* K20 and found its biocontrol ability against *Macrophomina phaseolina.*

Chapter 3
METHODOLOGY

3.1 Identification of Study area:

Random survey was conducted among agricultural field and forests in various locations in sub Himalayan area of Jalpaiguri District ($26^{o}15'47''$ & $26^{o}59'34''$ N Latitude and $88^{o}23'2''$ & $89^{o}7'30''$E Longitude) of North Bengal in the State of West Bengal. The average rainfall is from 2500mm-3000mm*, 80% of which falls during monsoon and about 110 rain days. Soil is acidic to neutral in nature and low in fertility. The criteria of location selection were based on prior study of areas where target crops (pulses and beans) are being cultivated. (* source IMD)

3.2 Soil sampling and collection

The soil sample was collected from different locations, after considering a minimal depth of 20cm to 25cm using sterile spatula. Initial soil layer was removed manually by hand hoe. The samples were then transferred under sterile condition in Ziplock plastic bags and stored in airtight plastic containers (Cello) with ice packs in polypropylene ice box at (4^{0}C to 5^{0}C) and transported to Laboratory (2 hrs).The samples were then pre-treated and stored in incubator for future usage.

3.3 Determination of pH

The soil sample from various locations was tested to determine the pH of the soil. pH is important for the occurrence and inhabitation by respective microflora. The determination was done by help of digital pH meter.(Systronics)

3.4 Sterilization & cleaning of Glassware

The glassware was cleaned with chromic acid solution by soaking them in the solution overnight. Then 3% commercially available Lysol solution was used to treat the glassware. Finally washing was done by running tap water repeatedly. After drying the glassware were kept in a hot air oven for 3-6 hours at 140-180°C. Sterilizations of the glassware were done by autoclaving them.

3.5 Isolation of Microorganism from soil rhizosphere

The soil samples were pre-treated to facilitate isolation of actinomycetes and avoid contamination by other microorganism. The samples about 100 gm in each slot were air dried, heated aseptically in laminar air flow by layering the soil sample to 2cm and incubated in chamber at 28^0C for two days (Gebreyohannes *et al.*,2013). The treated samples were crushed in already baked mortar,sieved and stored for immediate use.

3.5.1 Soil dilution technique

Warcup's soil dilution method (1955) was followed for isolating microorganisms from the rhizosphere with certain standarized modifications. The soil suspension was prepared by dissolving 10g of sample in 30 ml sterile saline water and blending with a magnetic stirrer for 1hr. The suspension was allowed to settle, until distinct layers were visible, one of the sediment and other of the supernatant.

Then, following method of Masayuki *et al*., (1988), with some modification, Stock solution was prepared by diluting 1gm of sediment in 9ml of sterile saline water and shaken again in vortex mixer. 1 ml was taken to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} by serial dilution method.

3.6. Screening of Actinomycetes from rhizosphere soil

0.1 ml of suspension from each dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} was taken with the help of sterile micro pipette and were evenly spread on previously prepared Starch casein nitrate agar media and Oatmeal Agar (ISP Medium 3) under aseptic conditions. Replicates, three in number, were used for plating each sample. The setting was incubated at 28^0C for 7 to 21 days (Masand *et al*.,2015), under constant observation and as per growth of the microorganism under sterile conditions.

Media composition

SCN Media (Starch- Casein-Nitrate)

Soluble Starch.....10gm
Casein.....0.3gm
Potassium nitrate.....2gm
Sodium chloride.....2gm
Di Potassium hydrogen phosphate.....2gm
Magnesium sulphate.....0.05gm
Calcium carbonate.....0.02gm
Agar.....15gm
Dist.water.....1000ml
P^H 7

Oatmeal Agar (ISP Medium 3)

Oatmeal.....20gm
Agar.....18gm
Trace salt soln.....1.0gm
Dist water.....1000ml

Trace salt solution

$Feso_4$.....0.1gm
$MnCl_2$.....0.1gm
$ZnSO_4$.....0.1gm
Dist. H_2O.....100ml

Water and oatmeal was boiled and simmered for 20 minute. The solution was filtered through cheese cloth and agar was added and volume was supplemented back to 999ml, brought to boil and autoclaved at 121^0C for 15 minute, and 1ml trace salt solution was added aseptically.

3.7 Maintenance and preservation of isolates

Pure colonies were screened visually, identified, and isolated under sterile conditions and was maintained in Starch casein nitrate agar slants at 4^0C for future studies.

3.8 Assessment of actinomycelial growth

17 isolates from pure colonies were obtained after screening 72 plates from different locations. The maintained slants were used to streak and stab isolates in both liquid and solid media:

3.8.1. Solid media: Isolates were streaked in Starch- Casein-Nitrate Agar media and was incubated at 28^0C for seven days with constant observation.

3.8.2. Liquid media: Isolates were streaked in Starch- Casein-Nitrate broth in Erlenmeyer flask and was incubated at 28^0C for seven days with constant observation, after shaking in rotary shaker for 30 minutes.

3.9 Classical Approach for characterization of isolates

Both macroscopic and microscopic methods as outlined within the identification key of Guide to the Classification and Identification of the Actinomycetes and Their Antibiotics (Selman and Hubert 1953) and Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons,1974) were followed for characterization of isolates.

3.9.1 Morphological method

To study the isolates, strains were transferred from 7 day old mother culture and was inoculated into 1.5% agar medium as stabs and incubated at 37^0C. Upon observation that the isolate had suspended and mixed in media (semi solid), 2 drops from medium was pipetted onto a sterile glass slide in laminar air flow. Subsequently, 1 to 3 drops of agar was spread well on the slide. When a thin film was formed, the setting was incubated at 27^0C under sterile conditions. Observation was made under microscope at timely interval.

3.9.1.1. Spore chain morphology (Microscopic morphology)

Spore chain was characterized morphologically after grouping into 'sections'. (Sivakumar *et.al*, 2005) The species belonging to genus *Streptomyces* was divided into three sections (Shirling and Gottlieb,1966), those being rectifexibiles (RF), retinaculiaperti (RA) and Spirales (S). When two types of chains were present for the same strain, both types were considered.

3.9.1.2.Study of Aerial mycelium and substrate mycelium (Macroscopic morphology)

The colour of the aerial mycelium bearing spore was observed by the naked eye and the colour of the spore mass was noted .If the spore mass have two different colour mixed up then both the colour were noted. For these isolates were grown in Oatmeal Agar media (ISP 3) for 7 days at 28ºC and the colour of the isolates were recorded.

Reverse side pigments: Isolates were streaked on Oatmeal agar media and incubated at 28ºC for 7 days. Pigmentation on the reverse side of the colony was noted as distinctive (+) and non distinctive (-) and the isolates were divided in these two groups.

3.9.1.3. Study of Diffusible pigments

For the study of diffusible pigment production the isolates were grown in Liquid media for a duration of 7 days. The isolates were divided into two groups on the basis of their ability to produce soluble pigment.

3.9.1.4. SEM studies of isolates

For scanning electron microscopy, pellet collection was done after centrifugation (3000 rpm) of isolate culture grown in nutrient broth medium and simultaneous washing in 0.1M phosphate buffer saline. Prefixing of the sample, under vacuum, was carried in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 6.8). Passing through different alcohol grades with initiation from the lower grade was done for dehydrolysis of the samples. Critical point drying in CO_2 (CPD 030; BAL TEC Vaduz, Liechtenstein), was undertaken after mounting the sample on slab and coating with 20 nm silver palladium alloy. JEOL JSM 5200 Scanning Electron Microscope (Tokyo, Japan) was used for the examination.

3.10. Biochemical characterization of isolates

3.10.1. Gram reaction

Gram staining was done by the method as described by Buchanan and Gibbson(1974) with some modifications. Actinomycetes were grown in SCN plates. After incubation period of 3 days one sterile coverslip was placed on the actinomycetes colony aseptically. The impression on the coverslip was air-dried and heat-fixed. Then the coverslip was flooded with crystal violate solution(crystal violate 2.0gm, 95%alcohol 20ml, ammonium oxalate 1%W/V, aqueous solution-80ml) and kept for 1 minute. Then the coverslip was flooded with Burke's iodine solution (iodine 1.0gm, KI 2.0gm, distilled water 100ml) and kept for 1minute. Then slides were washed with distilled water for 5seconds and 95% alcohol was poured drop by drop keeping the coverslip in 45º angle until the excess stain comes out. Again washed with distilled water and finally counter stained with safranine (2.5 W/V safranine in 95% alcohol- 10ml, distilled water 100ml.) for 2 minutes washed with water and dried. The gram reaction and morphological characters were observed under oil immersion objective.

3.10.2. Starch degradation

The bacteria were streaked on sterilized starch agar plate (NA + 0.1% soluble starch) and incubated for five days at 37º C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

3.10.3. Catalase production

24 hour old bacterial culture was flooded with 0.5 ml 10% H_2O_2 solution and formation of gas bubbles indicated the positive reaction.

3.10.4. Indole production.

Inoculation of isolate was done in 10ml of Davis Mingoli's broth which was supplemented by 0.1% tryptophan. Incubation was done at 37^0C for duration of 7 days. Careful layering of the culture was done by 2 ml of Ehrlich bobme reagent (P- dimethlyaminobenzaldehyde 10g in 100 ml concentrated HCL.) on the surface. This was allowed to stand for a few minutes and was observed. The formation of a ring at the interface of medium and reagent if any indicated the production of Indole.

3.10.5. H_2 S production

Streaking of isolates was done on SIM agar slants and incubation undertaken for 48hrs at an optimum of 37^0C. Visible darkening (affect) along the inner lines of the slant walls was taken as indicative positive result for H_2S production.

3.10.6. Urea digestion

Colour change of the medium was taken as indicator for the presence of urease. Streaking of isolates about half a loop unto medium was done and the set with paraflim cover was incubated for a duration of 7 days at controlled condition of 37^0C.

3.10.7. HCN production

Procedure described by Wei *et al.,* (1991) with standardized modifications was followed for determination of hydrocyanic acid (HCN) production.

During the follow up of procedure, NA medium with glycine (4.4g L^{-1}) amendment in petri plate was used to grow bacteria. Whatmann filter paper was cut in thin strips which were in turn soaked in picric acid solution. The papers were attached on to the lid of the plates and was sealed with parafilm. The setup of the same in replicates was incubated for 2 to 4 days. Change in colour of the filter paper strip, from brown to red was taken as indicator for ability of the isolate to produce HCN.

3.11. Physiological characterization of isolates

3.11.1. Growth of isolates at different salt concentration

Growth of the isolates at different NaCl concentration was determined by growing the isolates in SCN media supplemented with 1%, 2%,5%,8% and 10% (w/v) NaCl. Isolates were streaked onto petriplates having SCN media supplemented with 1%, 5% and 10% (w/v) NaCl and incubated at 28ºC for 5days. (Sahin *et al*.,2002)

3.11.2. Resistance of isolates to different antibiotics: Screening of the isolates towards resistance to different antibiotics was performed by Agar diffusion method as described by Sahin *et al.* (2002) with some modifications. Antibiotic discs were placed on the inoculated SCN plates. Plates were incubated for 5 days at 28ºC and the zone of growth inhibition was measured.

3.12. *In vitro* Screening and evaluation of phosphate solubilizing activity of isolated microorganism.

3.12.1. Qualitative analysis for phosphate solubilizing activity (screening)

Actinomycetes isolates were screened in Pikovskaya medium for phosphate solubilization activity (Pikovskaya,1948). Isolates were streaked in Pikovskaya media and incubated at room temperature for 7 days. A clear halo zone around the growth indicates phosphate solubilization activity. The plates were observed for clear zone around the colony and diameter of the halo zone was measured.

3.12.2.Quantitative analysis for phosphate solubilizing activity (Evaluation)

Phosphate solubilising activity of respective isolates and its evaluation was undertaken by culturing the isolates in Pikovskaya's liquid medium, which in turn was supplemented by 0.5% tri Calcium phosphate and 0.5% rock phosphate separately.The flasks were constantly shaken in rotary incubator at 100rpm and kept for 10 days at 28^0C.Kundsen and Beegle's (1988)Amoniummolybdate ascorbic acid method was followed for estimation of phosphate. Amount of the phosphate utilized or solubilized by the isolates were expressed as mg/L phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified Pikovskaya liquid medium (yeast extract- 0.5 gm/L, dextrose- 10.0gm/L, Calcium phosphate/ Rock phosphate- 5.0gm/L, ammonium phosphate 0.5gm /L, Potasium chloride- 0.2gm/L, Magnesium sulphate- 0.10gm/L, Manganese sulphate - 0.0001gm/L, ferrous sulphate- 0.0001gm/L, pH- 6.6). 7days old culture grown on SCN

slants was taken as 5%v/v of actinobacterial suspension and inoculated in 50/250 ml (v/v) broth. The broth upon centrifugation at 10000 rpm was allowed to settle and the supernatant was collected. Mixing of 2 ml of aliquot and 8ml colorimetric solution (60gm/L Ammonium paramolibdate, 1.455gm Antimony potassium tartarate, 700ml/L conc.H_2SO_4 and 132ml/L of ascorbic acid) was done in a thorough basis and incubated for 20 min till colour development took place. Percent transmittance of the solution was taken on a colorimeter with wavelength set at 882nm.

3.13. IAA production.

3.13.1. Quantitative test for IAA production

To enhance acetic acid (IAA) production Tryptophane (0.1mM) was used in a high C/N ratio medium, wherein isolate were grown for 24 to 48 hrs (Prinsen *et al.*, 1993).Assay in culture supernatant was done for IAA by Pillet- Chollet method described by (Dobbelaere *et al*., 1999). Reagent preparation and composition was done by adding 12g $FeCl_3$ per litre in 7.9 M H_2S0_4. 1ml of the recently pepared reagent was added to 1ml sample supernatant and was mixed well, kept in the dark for 30 minutes at room temperature. Absorbance was measured at 530nm.

3.14. *In vitro* Screening and evaluation for antibacterial activity

Cross streak method was applied to assess antibacterial activity. Single streak at one end of the on SCN plate was done. Incubation was done for 7 days and seeding allowed by a single streak in perpendicular to the plane of the previous one. Due observance of size of inhibition zone upon interaction of microbial strains, upon further incubation was done and recorded (Lertcanawanichakul and Sawangnop, 2008).

3.15. *In vitro* Screening and evaluation for antifungal activity

3.15.1. Inhibition of fungal mycelial growth in solid media

The efficacy of individual isolates was tested *in vitro* for inhibiting growth of the pathogen in dual culture using PDA and SCN media. Each actinomycetes isolate was placed at one side of the agar plate about 1cm away from the edge and 7mm diameter block of the pathogen taken from growing edge of the fungal culture was inoculated at the other half of the Petri plate. In another experiment actinomycetes were placed circling the pathogen. For each test three replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28ºC and inhibition zone towards the fungus colony in individual plate was quantified.

Results were expressed as mean % of inhibition in presence of the actinomycetes isolate (Gamliel *et al*.1989) The percent inhibition in the radial colony growth was calculated by the following formula:

$$\% \text{ inhibition} = 100 - (R^2 \times 100 / r^2)$$

Where r= Radius of growth of pathogen in control: R=Radius of growth of pathogen in treated set

3.15.2. Inhibition of fungal growth by metabolites

Extraction of antifungal compounds from Actinomycetes

Extraction of the metabolites from the actinomycetes grown in solid media was carried out with some modification of the method described by Soares *et al*., (2006). The actinomycetes were grown in solid argentine glycerol agar (AGS) medium at 28°C for 7 days. After that 10 ml sterile distilled water was added aseptically to each petriplate containing the actinomycetes and the plates were incubated for 24hours at room temperatute. The metabolite suspension obtained was centrifuged at 12000rpm for 15 min. After centrifugation the supernatant was collected and cold sterilized by passing through sterilized micro filter (0.22 µm pore size). The metabolites suspensions were transferred to sterile glass vials and kept at 4°C until further use.

3.15.2.1. Effect on Fungal mycelial growth

The method of Soares (2006) was followed with modification. Autoclaved PDA medium (20ml) was mixed with 0.5ml of test compound solution and plated into petriplates (70 mm dia.). After solidification, agar block (6mm dia.) containing growing mycelium of the test fungus (4 day old) was placed in the centre of each petriplate. Control plates were mixed with solvent alone. The plates were incubated at 30ºC ± 1 ºC and radial growth of mycelium was measured after every 2, 4, 6 and 8 days interval.

3.15.2.2. Effect on fungal spore germination

Fungal spores of test fungi were bio assayed against Secondary metabolitesobtained from selected isolates on glass slides following the method of Soares *et al.,* (2006). 40µl of the metabolites suspension was taken in a grooved glass slide to which 40 µl of fungal pore suspension was added. The control treatment was prepared with sterile distilled water instead of metabolites suspension. The slide was incubated in a moist Petri plate for 24 h at 30ºC ± 1 ºC. Finally one drop of lacto phenol - cotton blue was

added to each spot to fix the germination of spore or conidia. The slides were observed under microscope and the percentage of germination was determined.

3.15.2.3. Effect on Sclerotia germination

For assessing the effect of the cell free culture filtrate on the sclerotial germination of *S. rolfsii* the sclerotia were scrapped off from the culture growing in 7 days old PDA plates. The sclerotia were then soaked 1 hour in cell free culture filtrate solution. The sclerotia soaked in sterile distilled water and in uninoculated sterile PDB served as the control. After soaking the sclerotia were transferred asceptically to the petri plate containing sterile Black paper. These sterile black papers were also soaked in culture filtrate for at least 30minutes and incubated at room temperature. Percent germination as well as the radial growth of the germinating sclerotia was measured.

3.16.Qualitative test for Chitinase production

Conventional plate method in chitinase detection agar (CDA) was followed to determine the secretion of chitinase. Composition included 1% (w/v) colloidal chitin with 15g of agar in medium (Na_2HPO_4 -6.0g, KH_2PO_4 -3.0g, NaCl -0.5g, NH_4Cl 1.0g, Yeast extract- 0.05g and distilled water 1L; pH 6.5). Incubation of the inoculated organism was done on CDA plate at 28^0C for duration of 7 to 10 days. Evidently the occurrence of clear zone around the growing zone is indicative of chitinase activity (Kamil *et al.,* 2007). The colloidal chitin was prepared by following the method described by Mathivanan *et. al.,*(1997). Slow addition of chitin powder (5gm) was done to 60 ml of concentrated Hydrochloric acid at 4^0C ; kept under vigorous stirring overnight. The mixture was added to 2L of ice cold 95% ethanol with rapid stirring and kept overnight at 25 ºC. The precipitation formed was collected by centrifugation at 7000rpm for 20 minutes at 4 ºC and washed with sterile distilled water until the colloidal solution become neutral (pH 7). The prepared colloidal solution (5%) was stored at 4 ºC until further use.

3.17.Isolation of genomic DNA

Isolation of actinomycetes genomic DNA was done by growing the isolates in Nutrient broth(NB) for 3-4 days. Liquid nitrogen was used for crushing the cell mass. Isolation of genomic DNA was done following the method of Muthu et al.,(2013) with some modifications.

3.17.1. Preparation of genomic DNA extraction buffer.

At desired pH the following buffers for DNA extraction was prepared from appropriate composition of desired chemicals.

Lysis Buffer
50 mM Tris, pH 8.0
100 mM EDTA
100mM NaCl
1% SDS
Genomic DNA Buffer
10 mMTris, pH 8.0
0.1 mM EDTA

3.17.2. Extraction of actinomycetes genomic DNA

The target isolates were grown in nutrient broth for 3-4 days and other isolation procedures were followed. This involved initial centrifugation of culture in broth for 5 minutes at 28^0C at 10000 rpm. The pellet in the tube after separation was collected for future use and supernatant discarded. The pellets so collected was washed carefully with distilled water about three to four times and then re-suspended in 0.5 ml of lysis solution of 100mMTris-Hcl (pH 7.5), 20 mM EDTA, 250mM NaCl and 2% SDS and 1mg/ml lysozyme.To it 5µl of RNAse(50mg/ml) was added and incubated at 37ºC for 3 hours.10µl of Proteinase K solution (20mg/ml) was added to it and further incubated at 65ºC for 3 min. Extraction of the lysate was done with equal volume of tris water saturated phenol:chloroform:isoamyl alchohol (25:24:1) and centrifugation carried out at 10,000 rpm for 5 minutes, the resulting aqueous phase was then transferred into a fresh tube and DNA precipitation was done with double volume of chilled ethanol (100%). Then DNA was pelleted by centrifuging at 10000 rpm for 5 min at 4^oC and subsequent air drying was carried out, the resulting DNA was dissolved in 40 µl of TE buffer and stored at 4^oC.

3.17.3. Purification of genomic DNA

Total genomic DNA extraction of the isolated microorganism as per the above procedure was followed by RNAase treatment. In doing so resuspension of the genomic DNA was done in 100 µl1 X TE buffer and incubated at at 37^oC for 30 min with RNAse (60µg). After due incubation, re-extraction of the sample was done with PCL solution (Phenol: Chloroform: Isoamylalcohol 25:24:1), and RNA free DNA was precipitated with chilled ethanol as was done and described earlier.

3.17.4. Spectrophotometric quantification of genomic DNA

Measurement of DNA Concentration using Spectrophotometry

The pure sample (without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), was subjected to spectrophotometry to measure amount of UV irradiation absorbed by the bases in the DNA . For quantitating DNA or RNA, readings was to be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample.

1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

1 O.D. at 260 nm for RNA molecules = 40 ng/ul of RNA

The reading at 280 nm gives the amount of protein in the sample.

Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantification of the amount of nucleic acid will not be possible.

3.17.5. Agarose gel eletrophoresis to check DNA quality.

Gel electrophoresis is an important molecular biology tool which allows us to study DNA. At the same time it is instrumental in determining the sequence of nitrogen bases, size of any insertion or deletion, finding presence of a point mutation if any, and also has usage in distinguishing variable sized alleles at a single locus as well as assesses the quality and quantity of DNA present in a sample.

3.17.6. Preparation of DNA samples for electrophoresis

Preparation of agarose gel for DNA gel electrophoresis, involved melting a specific amount (0.8%) of agarose in 1X TBE buffer, cooling the solution and casting the same in gel tray with ethidium bromide and solidifying of the Gel for 15-20 minutes.

3.17.7. Run gel electrophoresis for DNA fraction

Electric supply was applied at a constant current of 90mA and voltage 75 volt (BioRAD Power Pac 3000) by firmly attaching the electrical lead of the gel tank, for 90 minutes.

The DNA migrated from cathode to anode and run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then electric current was turned off and gel was removed from the tank and examined on UV transilluminator and photographed for analysis.

3.18. Sequencing of r DNA

The sequencing was done using r DNA. Bi-directional mode using ITS primer pairs by CROMAS was followed for DNA sequencing, resulting in chromatogram generation. This provided detailed and relevant information about the sequence.

Sequence analysis

Bio-informatic algorithms tool was used for analysis of DNA sequence information was using MEGA 4.

Chromatogram of sequence

The chromatogram of the DNA sequence was analysed by the software Chromus.

Editing and alignment of sequence data

Editing of the DNA sequences was performed using Bio Edit software and alignment was done by using ClustralW algorithms.

3.19. BLAST of Sequence

DNA sequence analysis based on different characteristic for microorganism identification was carried out using alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997). The homology of sequence was used as basis for identification of microorganism. (http://ncbi.nlm.nih.gov/blast).

3.20. Submission of rDNA Sequence to NCBI GenBank

The DNA sequences were deposited to NCBI GenBank through BankIt procedure, and approved as per the ITS sequence, after complete annotation and provision of collection of accession numbers.

3.21. Multiple sequence alignment and Phylogenetic analysis

The sequenced PCR product was aligned with extype isolates' sequences from NCBI GenBank for identification as well as for studying phylogenetic relationship. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal., 1973).

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and these are represented in the units of number of base substitutions per site. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA-4 (Tamura *et al*., 2007). 16S rDNA of all the three isolates were aligned to study the range of homology present in the conserved regions following the ClustalW algorithm (Thompson *et al.,* 1994) using the Bioinformatic tool BioEdit.

3.22. Plant Material

Seed samples of *Vigna radiate* and *Phaseolus vulgaris* were collected from local markets, and other sources. Preliminary seed germination test, viability test under new agro-climatic zone at Immune Phyto-Pathology laboratory in North Bengal University were conducted at random . One seed variety of *Vigna radiata,*and two seed variety of *Phaseolus vulgaris* was selected for proper identification and further experimental set up.Set up was made for seed propagation and plant growth both in field and pot at open and glass house condition.

Plant materials

***Vigna radiata*:** Cultivar 1 (CV1):Mung/ Paheli Dal (common name)

***Phaseolus vulgaris*:** Cultivar 2 (CV2):Rajmash/ Jwala (common name)

Cultivar 3 (CV3): Rajmash/Kholar (common name)

Plant materials were maintained in the open field (Front Line Demonstration) premises of Immuno-Phytopathology laboratory, Department of Botany, N.B.U. and Glass house for pot experiments for selected field treatment of inoculants.

3.23. Selection of potent isolates

On the basis of their different attributes like PSA, IAA production, biocontrol activity potent isolates will be selected for *in vivo* study

3.24. *In Vivo* study of efficiency of selected isolates on plant growth promotion

3.24.1. Application of actinomycetes

3.24.1.1. Seed coating

For seed coating or seed bacterization the isolates were grown in NB for 48 h at 28ºC and centrifuged at 12,000rpm for 15 minute. The cell pellet was suspended in sterile

distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer to obtain a final density of 3 $X10^6$cfu ml^{-1}. Surface sterilized seeds of *Phaseolus vulgaris* and *Vigna radiata* dried under sterile air steam were soaked in the cell suspension using 0.2% sterilized carboxymethylcellulose as an adhesive. Seeds were soaked overnight and next day seeds were sown following method of Errakhi *et al.*,(2007) with some modification.

3.24.1.2. Soil drench

For soil drench the method of Karimi *et al*.,(2012) was followed. The isolates were grown in NB for 48 h at 28ºC and centrifuged at 12,000rpm for 15 minute. The cell pellet obtained, was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer to obtain a final density of 3 $X10^6$cfu ml^{-1} .

The cell suspension was applied to the plants in both pots and field. Soil was autoclaved prior to sowing of seeds. Applications were made by distributing 100 ml per pot at regular interval of 15 days. In the field the rhizosphere of plants were flooded with the suspension.

3.24.1.3. Foliar spray

The bacterial pellet obtained by the method described above was suspended in sterile distilled water at a concentration of $3x10^6$ cfu ml^{-1} after the addition of a few drops of Tween -20. The solution was sprayed until run off on the foliar part of the plants. The spraying was done every fifteen days till the new shoots started appearing. The growth parameters such as number of leaves, branches and height of plants were observed.

Growth promotions in seedlings were evaluated after 15day in terms of increase in height, shoot and root length, number of leaf, leaf area of the treated as well as untreated control plants under the same environmental and physical conditions (temperature 20±5ºC, RH 60-80%)

As both the plants belong to the Family Fabaceae they have the ability to produce root nodule to symbiotically fix atmospheric nitrogen with help of nodule forming bacteria *Rhizobium* sp. The effect of isolates on formation of root nodule expressed in terms of Nodulation index was also observed according to the formula suggested by Ben Rebah *et al.*,(2002).

Nodulation index=A x B x C ≤ 18

Nodule size = A (small=1, medium=2, large=3)

Nodule colour = B (white=1, pink=2)

Nodule number= C (few=1, several=2, many=3)

3.24.2. Pathogen inoculum preparation and application

3.24.2.1. Preparation of inoculum

The inoculum of pathogen *Fusarium solani* and *Sclerotium rolfsii* was prepared in sand maize meal media which increases the survival capacity and viability of the inoculum in soil, and inoculation of healthy plants was done. The following preparations were used for experimental purposes:

Sand maize meal medium

Marked quantity of washed and sterilized sand: water: maize meal ratio of (9: 1.5: 1, w:w:v) was taken in either autoclavable plastic bag or conical flasks (150g), and was sterilized at 20lb for 20 minutes for future use upon inoculation and mass culture of fungal pathogen (Biswas and Sen, 2000).Care was taken to select mycelia bits from margins and transition zones of actively growing culture, and the whole mass was incubated at 28ºC$\pm$2º C for 15-20 days. Inoculum was found viable for pathogenicity when it was two weeks olds under optimum condition, soil application involved 100gm inocula at the rhizosphere of each of the potted plants. Proper irrigation and aeration was maintained for optimal condition for pathogen growth.

3.24.2.2. Disease assessment

Determination of the effects of selected bacterial isolates on disease reduction was done by categorisation of the various treatment under the following types: i. Untreated control, ii. Inoculated with pathogen, iii. Inoculation with actinomycetes isolates, iv.Inoculation with both actinomycetes and fungal pathogen.

The rhizosphere of plants pre- treated with the antagonists or without treatment was inoculated with pathogen. In pre- treated plants, pathogen inoculation was done 3 days after application of antagonist. Disease assessment was performed after 7, 14, 21, 28 days of inoculation.

Above ground and underground symptoms was used for assessment of the disease intensity and its recording (Roots, colour, rotting, leaves withering, shoot tip withering, defoliation etc.) Disease intensity was calculated by using 0-6 scale as adopted by Mathew and Gupta (1996). The disease infections observation were recorded in a continuous 0-6 scale, where

0 = no symptoms:

1 = Small roots turn rotten lesion appeared at the collar region;

2 = Middle leaves start wilting and 10-20% of root turn brown;

3 = Leaves wilted and 20-40% roots become dry with browning of shoot ;

4 = Extensive rotting at the collar region of roots, 60-70% of roots and leaves wilted, browning of shoot over 60%;

5 = 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80%

6 = Whole plants die, since 100% roots were

Open Field condition

Table 5.The arrangement (dummy) of the plants in Field condition.

Vigna radiata/ Phaseolus vulgaris	Control	Isolate treatment		
Healthy (H)	UH	T_1H	T2H	T3H
Inoculated (I)	UI	T_1I	T2I	T3I

T1= Treated with *Streptomyces griseus*

T2= Treated with *Streptomyces tricolor*

T3= Treated with *Streptomyces flavogriseus*

Six blocks in replicates of three each was made.

Disease Incidence (DI%)

For calculating the Disease Incidence, the disease index was recorded based on the score 0-6, depending on above ground and underground symptoms. Disease Incidence was calculated as per methodology described by Xue *et al*., (2013) using the following formula:

Disease Incidence (DI%)=[∑(the number of plants on this index x Disease index)/(Total number of plant investigated x highest disease index)] x 100%

3.25. Extraction and assay of defence enzyme activity in treated plants.

3.25.1. Associated changes in Phenylalanine ammonia lyase (PAL) activity extraction and estimation

Extraction of PAL (EC.4.3.1.5) was done by following the method described by Chakraborty *et al.,* (1993) with modifications. Using 0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of ß mercaptoethanol under ice cold conditions in a mortar and pastel 1gm sample was crushed, traces of sea sand was used to make available a fine slurry, which in turn was centrifuged in 15000 rpm for 20 minutes at 4^{o}C. The supernatant so collected after due recording of its volume was used for assay else stored at 20^{0}C.

Assay of Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300µM sodium borate (pH 8.8), 0.3 ml of 30 µM L-phenylalanine and 0.5ml of supernatant along with 1.9 ml distilled water making a total volume of 3ml. Following incubation for 1 h at 40 ºC the absorbance at 290nm was taken against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced in 1 min g^{-1} fresh weight of tissue.

3.25.2. Associated changes in β-1,3 glucanase activity, extraction and estimation

Extraction of ß -1,3 glucanase (EC.3.2.1.39) was done following the method described by Pan *et al.* (1991). Crushing was done in liquid nitrogen using 1g of plant leaf sample at 4 ºC using mortar and pastel, using 5ml of chilled 0,05 M sodium acetate buffer (pH 5.0) as the extraction buffer. Centrifugation was then carried out at 10000 rpm for 15 minutes at 4 ºC then supernatant was collected and used as crude enzyme extract. Estimation of the ß-1,3- glucanase was done by following the Laminarin-di-nitro-salicylate method (Pan *et. al.,* 1991). Equal volume of crude enzyme extract at 62.5µl was added to 62.5 µl of laminarin (4%) and incubation was done at 40ºC for 10 minutes and reaction was allowed to take place, which was stopped by adding 375µl dinitrosalicylic reagent and simultaneous heating was carried out for a duration of about 5 minutes over conventional boiling water bath.

The treated solution mixture was in turn diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500nm. The blank taken was a mixture of laminarin and

crude enzyme with zero time incubation. The enzyme activity was expressed as µg glucose released per min per gm fresh tissues.

3.25.3. Associated changes in Chitinase activity.

Extrection of chitinase (EC. 3.2.39) was done by following the method described by Boller and Mauch (1988) with modifications. Leaf sample of 1gm weight from identified plants was crushed in liquid nitrogen, using 5ml of chilled 0.1M Sodium Citrate (pH5) as extraction buffer. The resulting homogenate was centrifuged for 10 min at 12,000rpm and the supernatant so collected was used as enzyme source.

Chitinase activity was measured according to the method described by (Boller and Mauch,1988). 10µl Na-acetate buffer (1M) pH 4, 0.4ml of enzyme solution, 0.1ml of colloidal chitin (1mg) in respective composition was used as assay mixture. Colloidal chitin was prepared as per the method of (Roberts and Selitrennikoff, 1988). After 2hour of incubation at 37 ºC the reaction was stopped by centrifugation at 10,000g for 3minutes. An aliquot of supernatant (0.3ml) so obtained was pipetted into a glass reagent tube containing 30µl of potassium phosphate buffer (1M) pH7.1 and incubated with 20µl of (3%w/v) desalted snail gut enzyme Helicase (Sigma) for 1hour, upon which the pH of the reaction mixture was levelled to 8.9 by addition of 70µl of sodium borate buffer (1M) pH 9.8. The reaction mixture was then incubated in a boiling water bath for 3 minutes and then rapidly cooled in an ice water bath. Again incubation was carried out for 20 minutes at 37^{o}C on addition of 2ml of DMAB (ρ-di-methyl-amino-benzaldehyde). Thereof with immediate effect the absorbance value at 585nm was measured using a UV-VIS spectrophotometer. For the protocol N-acetyl glucosamine (GlcNAc) was used as standard (Reissig *et al.*,1959). The enzyme activity was expressed as µg GLcNAc min $^{-1}$ mg $^{-1}$ fresh tissues.

3.25.4. Associated changes in Peroxidase activity.

For the extraction of peroxidase (EC.1.11.1.7) the plant tissue was macerated to powdered form in liquid nitrogen and extracted in 0.1 M Sodium borate buffer (pH 8.8) under cold conditions using ice cubes and flakes with addition of 2 mM ß mercaptoethanol, the homogenate so obtained was centrifuged immediately at 15000 rpm for 20 minutes at 4 ºC. The volume of the supernatant was recorded after carefull collection in a vial, and the same was used for assay or stored at -20^{o}C (Chakraborty *et al*., 1993).

For determination of peroxidase activity, 100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 5.4), 100µl of 4mM H_2O_2, 100 µl O-dianisidine (5mg ml-1 methanol) and 1.7ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O- dianisidine in presence of H_2 O_2 (Chakraborty *et al*., 1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/ min^{-1}.

3.26. Extraction of total phenol contents from leaves and roots

Phenol was extracted from the fresh leaves and roots following the method of Mahadeven and Sridar, (1982). Initially 1gm of freshly washed and weighed root or leaf samples were cut into small pieces and put in boiling alcohol within a hot water bath for 5-10 minutes with fixed proportion of 4ml alcohol for a gm of tissue. The tubes with the reaction mixture were allowed to boil for 15 minutes after which they were gradually cooled. The samples were the decanted and crushed in mortar and pestle at room temperature. The extract so obtained was passed on through two layers of muslin cloth and filtered through Whatmann filter paper No.1, and the final volume was adjusted with 80% ethanol, which was then subjected to boiling for 15 minutes and then gradual cooling was done. Crushing in mortar and pestle was then done thoroughly at room temperature.

The extract so obtained, was passed through two layers of cheese cloth and then filtered through Whatmann No.1 filter paper. Final volume was adjusted with 80% ethanol, under dark condition to prevent light induced degradation of phenol.

Estimation of total phenols contents

Total phenol content was estimated by Folin Ciocalteau‘s reagent, following the method of Mahadevan and Sridhar (1982). 1ml of test solution in dilutions of 10^{-2} and 10^{-1} (leaf and root) with added 1ml of 1N folin –ciocalteau reagent (1:1) along with 2ml of 20% sodium carbonate solution (Na_2CO_3) was taken in a test tube mixed well and boiled in water bath for exactly 1minute. With gradual cooling dilution of the reaction mixture was done upto 25ml by ddition of distilled water. Absorbance of the solution was measured in a Systronics photometric colorimeter Model 101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.27. Extraction and estimation of total protein contents from leaves and roots

Soluble protein was extracted from the leaves and roots of treated and control plants following the method of Chakraborty *et al*., (1995). The methodology involved freezing the plant tissues in liquid nitrogen and simultaneous grinding in 0.05 mM sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2 S_2 O_5$, 0.5 mM $MgCl_2$ with addition of 2mM PMSF with crushing and centrifugation at 4ºC for 20 min at 12000rpm.The estimation from the supernatant was done following method of Lowry et al. (1951).Bovine serum albumin (BSA) was taken as the standard. The process involved taking supernatant with protein sample 1ml, along with addition of 5ml of alkaline reagent (1ml of 1% $CuSO_4$ and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% $Na_2 CO_3$ in 0.1 NaOH). The reaction mixture was incubated for 15 minutes at room temperature and 0.5 ml of 1N Folin Ciocalteau reagent was added with further incubation for a duration of 15 minutes and then optical density of the solution was measured at 720nm.

3.28. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of cross-section of plant roots and leaves samples were done using FITC labelled goat antirabbit IgG following the method of (Chakraborty and Saha, 1994)

3.28.1. Immunolocalization of Chitinase and Glucanase enzymes by indirect immunofluorescence staining of leaf and root

The steps involved making fine cross section of healthy and treated roots and leaves and subsequent immersion in PBS,ph 7.2.These sections were further treated with normal serum or antiserum in ratio(1:50) in PBS and was allowed to incubate for 1hr at room temperature with cover. The sections were then thoroughly and carefully washed with PBS-Tween pH 7.2 for 15 minutes for about three times. Then sections were subjected under dark conditions to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescence (FITC) and incubation was done for half an hour, under same condition. Sections were again washed thrice with PBS- Tween and were carefully mounted on a grease free slide with 10% glycerol. Fluorescence's of the target sections under treatment were observed using using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and plate photographs were taken.

Chapter 4
EXPERIMENTAL

4.1. **Isolation and identification of actinomycetes isolates from sample area.**

Soil samples were collected from agricultural fields of different parts of Jalpaiguri district of West Bengal. The area falls between 26°15'47'' & 26°59'34'' N Latitude and 88°23'2'' & 89°7'30''E Longitude of North Bengal in the State of West Bengal. The average rainfall is from 2500mm-3000mm, 80% of which falls during monsoon and have about 110 rain days. Soil is acidic to neutral in nature and low in fertility. Agricultural fields were chosen because of the fact that they have a diversity of soil microflora present in the root rhizosphere of the crop plants. These natural microflora boost the growth and disease resistance activity of the crop plants . Jalpaiguri being a prominent agriculture based district it is very important that microorganism based bioformulations and biofertilizers are developed for this region because of the fact that chemical formulations kills and changes not only the soil microflora but also changes the character of the soil and greatly affect the biodiversity. So a potent microorganism from the natural system will be of great importance which not only have positive effect on plant growth and health but also will maintain the balance of the ecosystem. The soil sample was collected from agricultural field from a minimal depth of 20cm to 25cm using sterile spatula. Initial soil layer was removed manually by hand and the samples were then transferred under sterile condition in Ziploc plastic bags and stored in airtight plastic containers (Cello) and transported to Laboratory. Samples were air dried and then heated in a incubator at 28ºC for two days to facilitate actinomycetes isolation. Warcup's serial dilution method was modified to some extent for the isolation process. A total number of 17actinomycetes were isolates from various sources, mainly from the potato growing fields.

Table 6. GIS position, code and colony characteristics of actinomycetes isolates

Soil sample code	GIS location of sampling	Colony colour
ARHS/PO/11	N26º44'54.08" E 88º48'14.53"	Reddish white
ARHS/PO/12	N26º44'54.88" E 88º48'14.04"	reddish
ARHS/PO/13	N26º44'53.18" E 88º48'13.24"	Whitish
ARHS/PO/14	N26º44'54.89" E 88º48'14.34"	Pinkish white
ARHS/PO/15	N26º44'57.23" E 88º48'15.24"	Reddish white
ARHS/PO/16	N26º47'09.44" E 88º22'06.53"	Pinkish
ARHS/PO/17	N26º44'54.08" E 88º48'14.53"	Reddish
ARHS/PO/18	N26º44'54.08" E 88º48'14.53"	Grayish white
ARHS/PO/20	N26º44'54.08" E 88º48'14.53"	Grayish
ARHS/PO/22	N26º44'54.08" E 88º48'14.53"	Whitish
ARHS/PO/23	N26º44'54.08" E 88º48'14.53"	Pinkish
ARHS/PO/24	N26º44'54.08" E 88º48'14.53"	Grayish
ARHS/PO/25	N26º44'54.08" E 88º48'14.53"	Grayish red periphery
ARHS/PO/26	N26º44'54.08" E 88º48'14.53"	Pinkish
ARHS/PO/27	N26º44'54.08" E 88º48'14.53"	Grayish
ARHS/PO/28	N26º44'54.08" E 88º48'14.53"	Whitish
ARHS/PO/29	N26º44'54.08" E 88º48'14.53"	Whitish

ARHS= Actinomycetes of Root rhizosphere, PO= Potato

4.1.1. Morphological characterization of isolates

Isolates were streaked on SCN media and incubated at 28ºC for 7 days. Morphological characterization of the isolates were done by observing colour of both aerial and substrate mycelium, production of aerial spore mass and by production of melanine pigment. Colours of the substrate mycelium in most isolates were insignificant. In most cases it was pale yellow. It was found that most of the isolates produce white or grey

colour spore mass with some of the isolates having different tints. Some of the isolates produced dark brown or chocolate coloured melanin pigments. Detail results have been presented in table 7.

Table 7. Morphological characteristic of isolates

Isolate code	Media	OptimumGrowth temperature	Colour of Aerial mycelium	Colour of Substrate mycelium	Melanoid pigment Production	Diffusible Pigments
ARHS/PO/11	SCN	28ºC	Reddish white	Pale yellow	-	-
ARHS/PO/12	SCN	28ºC	Reddish	Pale yellow	-	-
ARHS/PO/13	SCN	28ºC	Whitish	Pale yellow	-	-
ARHS/PO/14	SCN	28ºC	Pinkish white	Pale yellow	+	+
ARHS/PO/15	SCN	28ºC	Reddish white	Pale yellow	-	_
ARHS/PO/16	SCN	28ºC	Pinkish	purple	+	+
ARHS/PO/17	SCN	28ºC	reddish	Pale yellow	-	-
ARHS/PO/18	SCN	28ºC	Grayish white	purple	+	+
ARHS/PO/20	SCN	28ºC	grayish	Pale yellow	-	-
ARHS/PO/22	SCN	28ºC	whitish	Pale yellow	-	-
ARHS/PO/23	SCN	28ºC	Pinkish	purple	+	+
ARHS/PO/24	SCN	28ºC	Grayish	Pale yellow	-	_
ARHS/PO/25	SCN	28ºC	Grayish red periphery	Pale yellow	-	+
ARHS/PO/26	SCN	28ºC	Pinkish	purple	+	+
ARHS/PO/27	SCN	28ºC	Grayish	Pale yellow	-	_
ARHS/PO/28	SCN	28ºC	whitish	Pale yellow	-	_
ARHS/PO/29	SCN	28ºC	Whitish	Pale yellow	-	_

ARHS= Actinomycetes of Root rhizosphere, PO= Potato, SCN= Starch Casein Nitrate agar media

Figure 1: (A-Q) Growth Pattern of different actinomycetes isolates

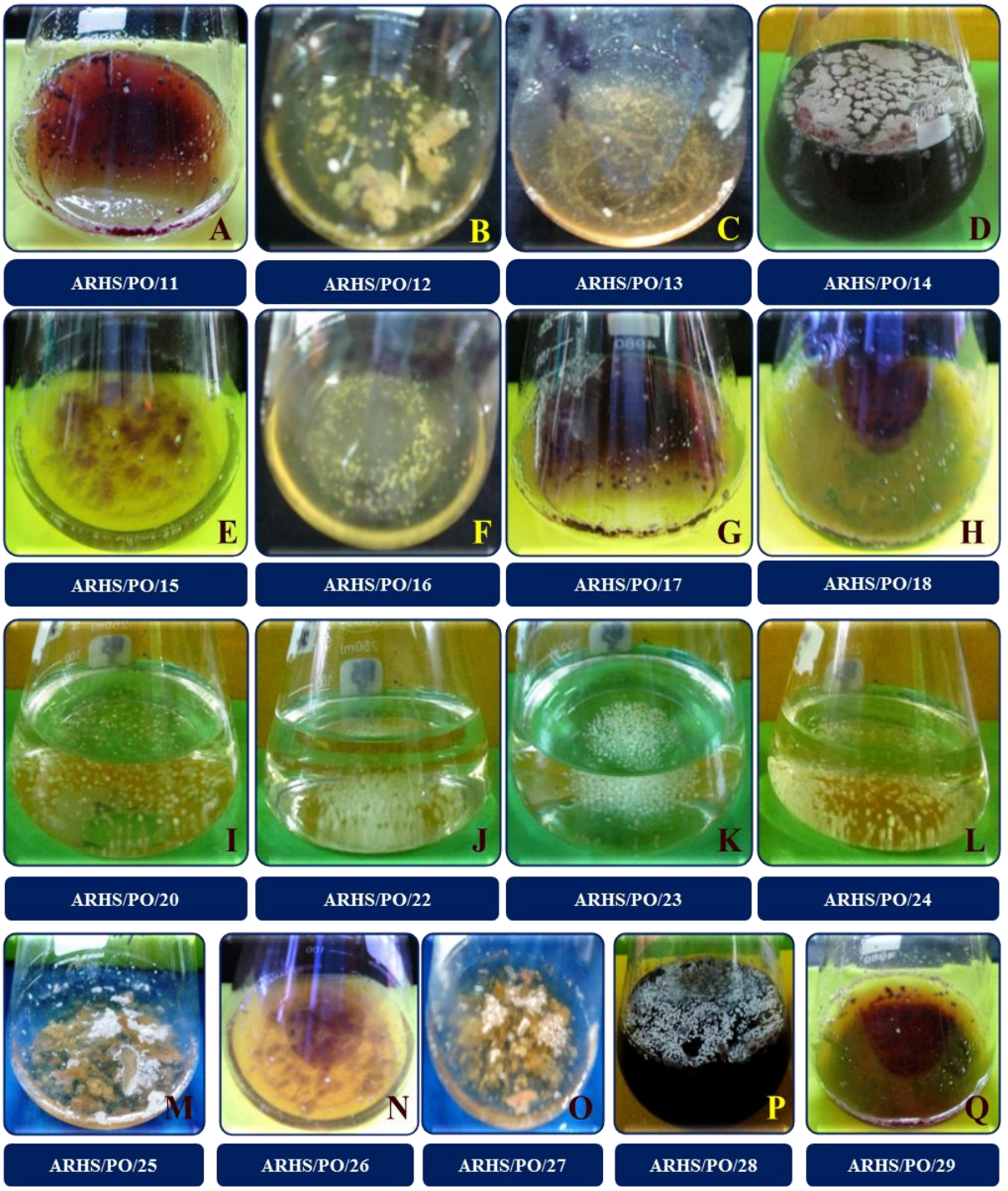


Figure 2: (A-Q) Screening for pigmentation by the Actinomycetes isolates.

4.1.2. Biochemical characterization of isolates.

All the isolatesobtained from different regions were first categorized as Gram positive organisms. Basic biochemical characterizations like Starch hydrolysis, H_2S production, catalase production, Indole production were characterized. The detail result is summarized in Table 8.All the isolates showed positive result for Gram staining, starch hydrolysis, catalase production (Fig. 3).

Table 8. Biochemical characterization of actinomycetes isolates

Isolates	Spore	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Urase production	Cellulase Production	Indole Production	Identification
ARHS/PO/11	+	+	-	+	+	+	+	+	+	-	+	+	*Streptomyces sp.*
ARHS/PO/12	+	+	-	+	+	+	+	+	+	-	+	+	*Streptomyces sp.*
ARHS/PO/13	+	+	-	-	+	+	-	-	+	-	-	+	*Streptomyces sp.*
ARHS/PO/14	+	+	-	+	+	+	-	-	+	-	-	+	*Streptomyces sp.*
ARHS/PO/15	+	+	-	-	+	+	-	-	+	-	-	+	*Streptomyces sp.*
ARHS/PO/16	+	+	-	+	+	+	+	+	+	-	+	+	*Streptomyces sp.*
ARHS/PO/17	+	+	-	+	+	+	-	-	+	-	-	+	*Streptomyces sp.*
ARHS/PO/18	+	+	-	+	+	+	+	+	+	-	+	+	*Streptomyces sp.*
ARHS/PO/20	+	+	-	+	+	+	-	-	+	-	-	+	*Streptomyces sp.*
ARHS/PO/22	+	+	-	+	+	+	-	-	+	-	-	+	*Streptomyces sp.*
ARHS/PO/23	+	+	-	+	+	+	-	-	+	-	-	+	*Streptomyces sp.*
ARHS/PO/24	+	+	-	+	+	+	+	+	+	-	+	+	*Streptomyces sp.*
ARHS/PO/25	+	+	-	+	+	+	+	+	+	-	+	+	*Streptomyces sp.*
ARHS/PO/26	+	+	-	+	+	+	-	-	+	-	-	+	*Streptomyces sp.*
ARHS/PO/27	+	+	-	-	+	+	+	+	+	-	+	+	*Streptomyces sp*
ARHS/PO/28	+	+	-	+	+	+	-	-	+	-	+	+	*Streptomyces sp.*
ARHS/PO/29	+	+	-	+	+	+	-	-	+	-	+	+	*Streptomyces sp*

+= present, -= absent

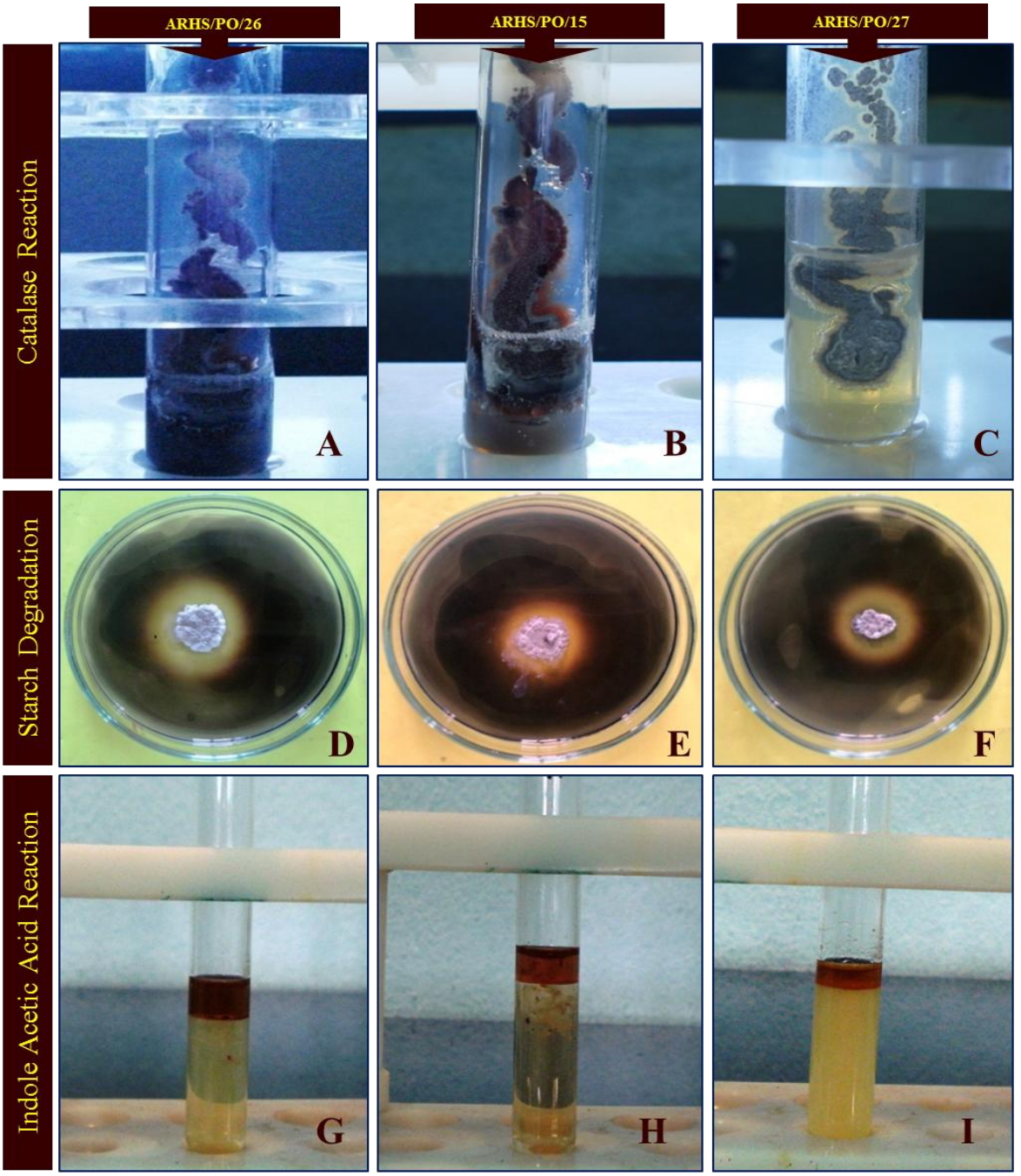


Fig. 3. (A-L) Biochemical characterization of actinomycetes isolates. (A-C) Catalase reaction; (D-F) Starch Degradation reaction;(G-I) Indole Acetic Acid (IAA) reaction.

4.1.3. Physiological characterization of isolates.

Physiological characterizations of the isolates were done by observing the growth of isolates in different media. Whereas the isolates grow vigorously in SCN media growth in NA is less. Sporulation in Oatmeal agar (ISP 3) was highest. Growth of the isolates in different temperature, different pH and salt concentration(1%, 2%, 5%, 8% and 10% salt concentration) were observed. The result showed that though in 1% salt concentration growth of the isolates were normal, growth was hampered in 5% salt concentration and in 10% salt concentration no growth was observed.(Fig. 5).Optimum temperature of the isolates for growth was in the range of 28ºC to35ºC. Resistance of isolates to different antibiotics were also characterized. Isolates were highly resistant to ampicillin followed by streptomycin and least resistant to kanamycin (Fig.4) Table 9.

Table 9 : Physiological characterization of actinomycetes isolates

Isolates	Growth on Media		Sporulation in ISP 3	NaCl Concentration			pH		Temperature			Resistance against		
	SCN	NA		1%	5%	10%	pH7	pH10	15ºC	28ºC	35ºC	Ampicillin (25µg/disc)	Streptomycin (25µg/disc)	Kanamycin (25µg/disc)
ARHS/PO/11	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/12	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/13	+++	+	+++	++	+	-	+++	+	+	+++	+++	+	+	-
ARHS/PO/14	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/15	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/16	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/17	+++	+	+++	++	+	-	+++	+	+	+++	+++	+	+	-
ARHS/PO/18	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/20	+++	+	+++	+	-	-	+++	+	+	+++	+++	+	+	-
ARHS/PO/22	+++	+	+++	++	+	-	+++	+	+	+++	+++	+	+	-
ARHS/PO/23	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/24	+++	+	+++	++	+	-	+++	+	+	+++	+++	+	+	-
ARHS/PO/25	+++	+	+++	+	-	-	+++	+	+	+++	+++	+	+	-
ARHS/PO/26	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	+
ARHS/PO/27	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/28	+++	+	+++	++	+	-	+++	++	+	+++	+++	+	+	-
ARHS/PO/29	+++	+	+++	+	-	-	+++	+	+	+++	+++	+	+	-

+++= maximum growth, ++=moderate growth, +=less growth, - = no growthFor antibiotic resistance += resistance, - = susceptible

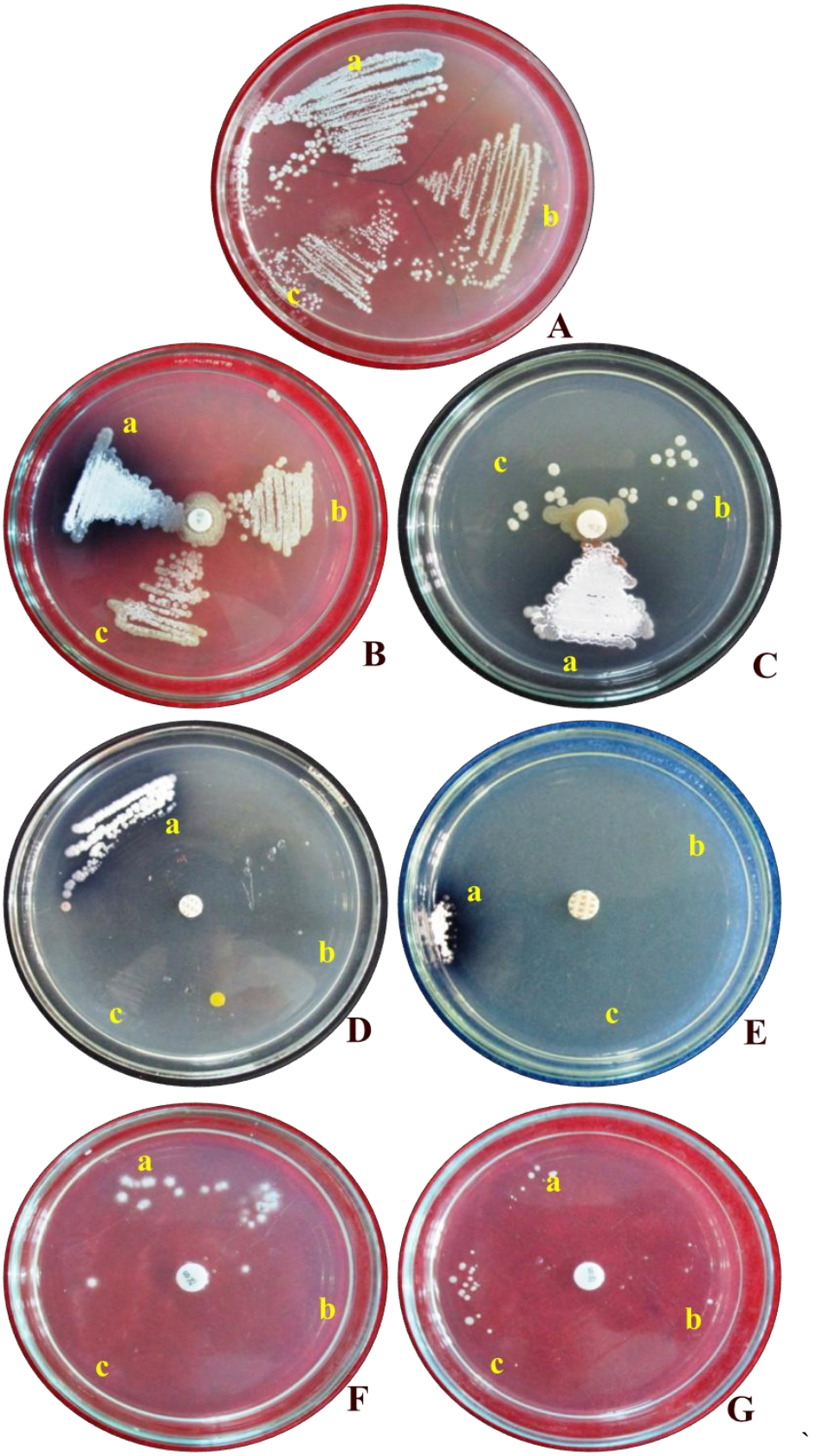


Fig. 4 . (A-G): Screening of Antibiotic Resistance of Selected Isolates; (a) ARHS/PO/26; (b) ARHS/PO/27; (c) ARHS/PO/15.A- Control; B-G Resistance to ; Amphicilin [B-C], Kanamycin [D-E], Streptomycin [F-G]

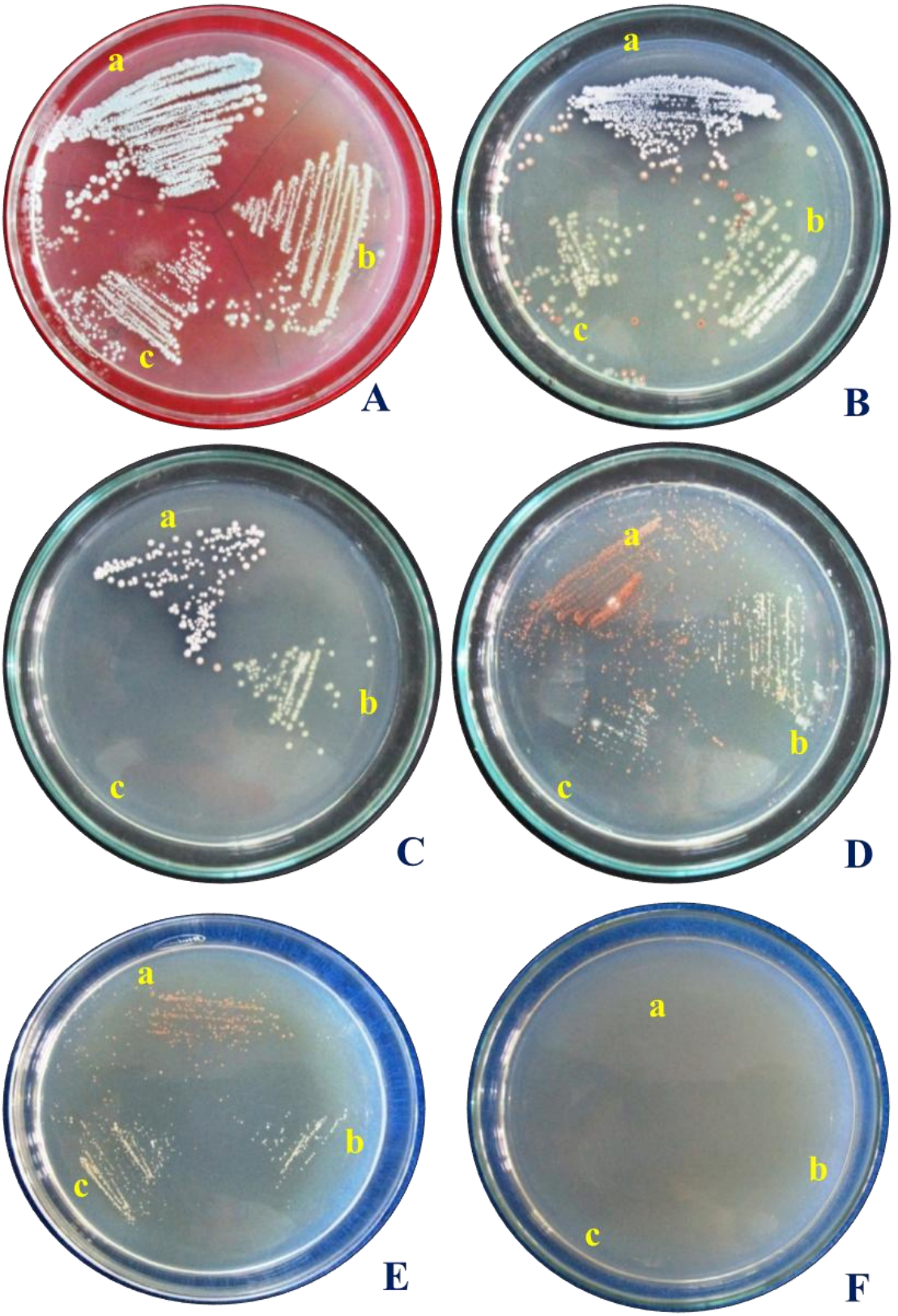


Figure 5: (A-F) Visual Assessment; Growth of Selected Isolates in different salt concentrations. . A- Control, B - 1% NaCl; C - 2% NaCl; D - 5% NaCl L; E -8 % NaCl ; F- 10% NaCl.

4.2. Assessment of growth of isolates in media

4.2.1. In solid media

Pure culture of the isolates obtained from different sources was preserved in SCN slants. Morphological study, growth pattern, time of growth was observed in SCN plates for 7 days at 28ºC. Colour of aerial and substrate mycelia, Spore formation, melanin pigment production were observed in the isolates (Fig.1)

4.2.2. In liquid media

Isolates were inoculated in liquid SCN media (50/250 v/v) and kept in a rotary shaker at 28ºC for 7 days. Colonies were visible to the naked eye and diffusible pigment formation was also observed (Fig.2)

4.2.3.Microscopic observation

Microscopic observations of the isolates were made and photographs were taken with help of normal bright field microscope and Ocular attached stereo digital camera (Fig.6). Actinomycetes cells were found to be rod shaped in structure and they bind end to end to form chain like structure. By observing the characteristics of the spore bearing hyphae and the particular structures of the spore chains the isolates can be devided into three sections belonging to the group streptomycetes, namely rectiflexibiles (RF), Retinaculiapetri (RA) and Spirales(S). (table.10)

Table 10 . Grouping of isolates on the basis of spore chain morphology

Isolate	Spore chain morphology
ARHS/PO/11	RF
ARHS/PO/12	S
ARHS/PO/13	S
ARHS/PO/14	S
ARHS/PO/15	RF
ARHS/PO/16	S
ARHS/PO/17	RA
ARHS/PO/18	RF
ARHS/PO/20	S
ARHS/PO/22	RA
ARHS/PO/23	S
ARHS/PO/24	S
ARHS/PO/25	S
ARHS/PO/26	SRA
ARHS/PO/27	S
ARHS/PO/28	S
ARHS/PO/29	RA

Rectiflexibiles =RF, Retinaculiapetri =RA,Spirales=S

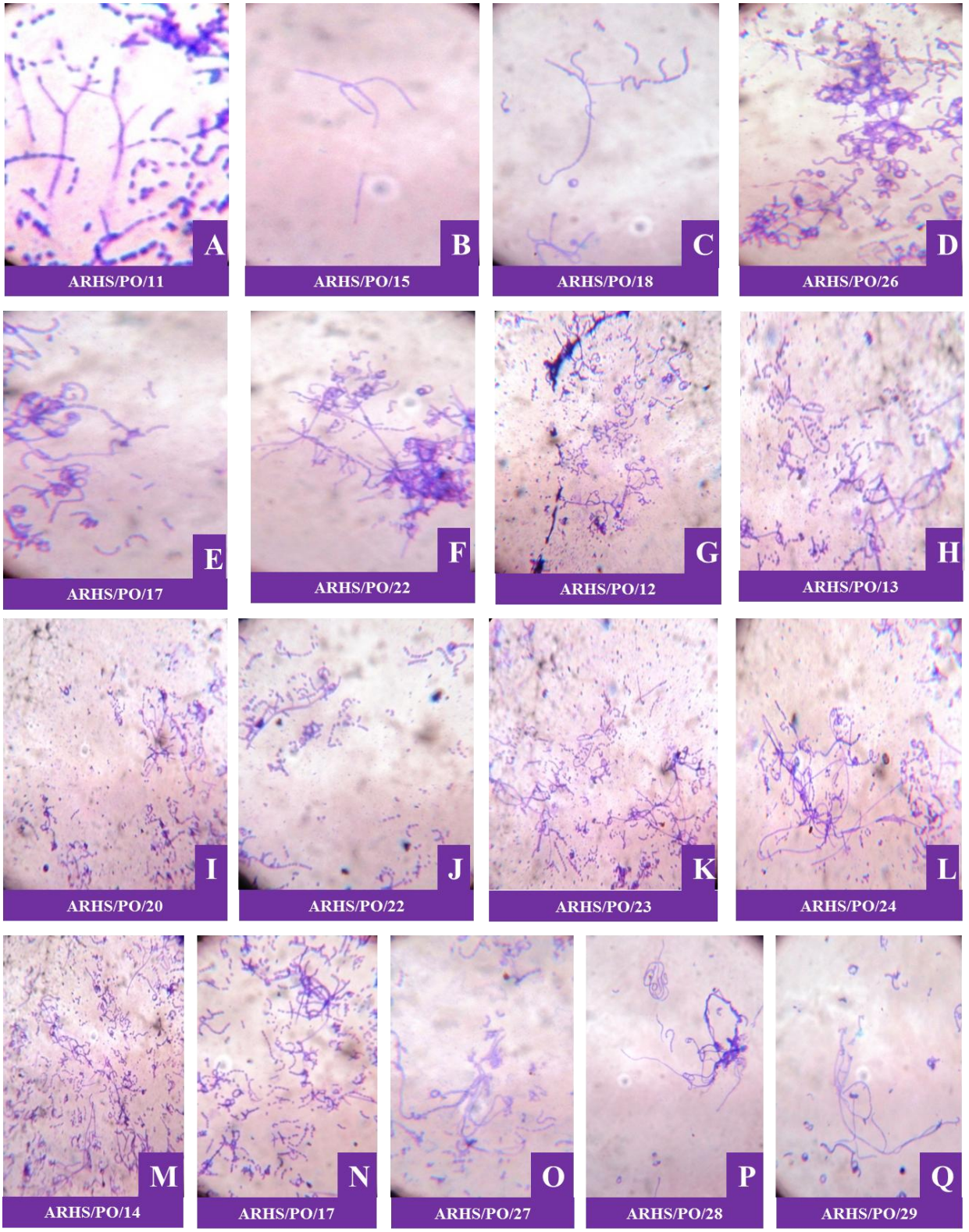


Figure 6: (A-Q) Microscopic identification of isolates based on morphological characters. (A-C) Rectiflexible, (D-E) Retinaculiapetri, (F-Q) Spirales.

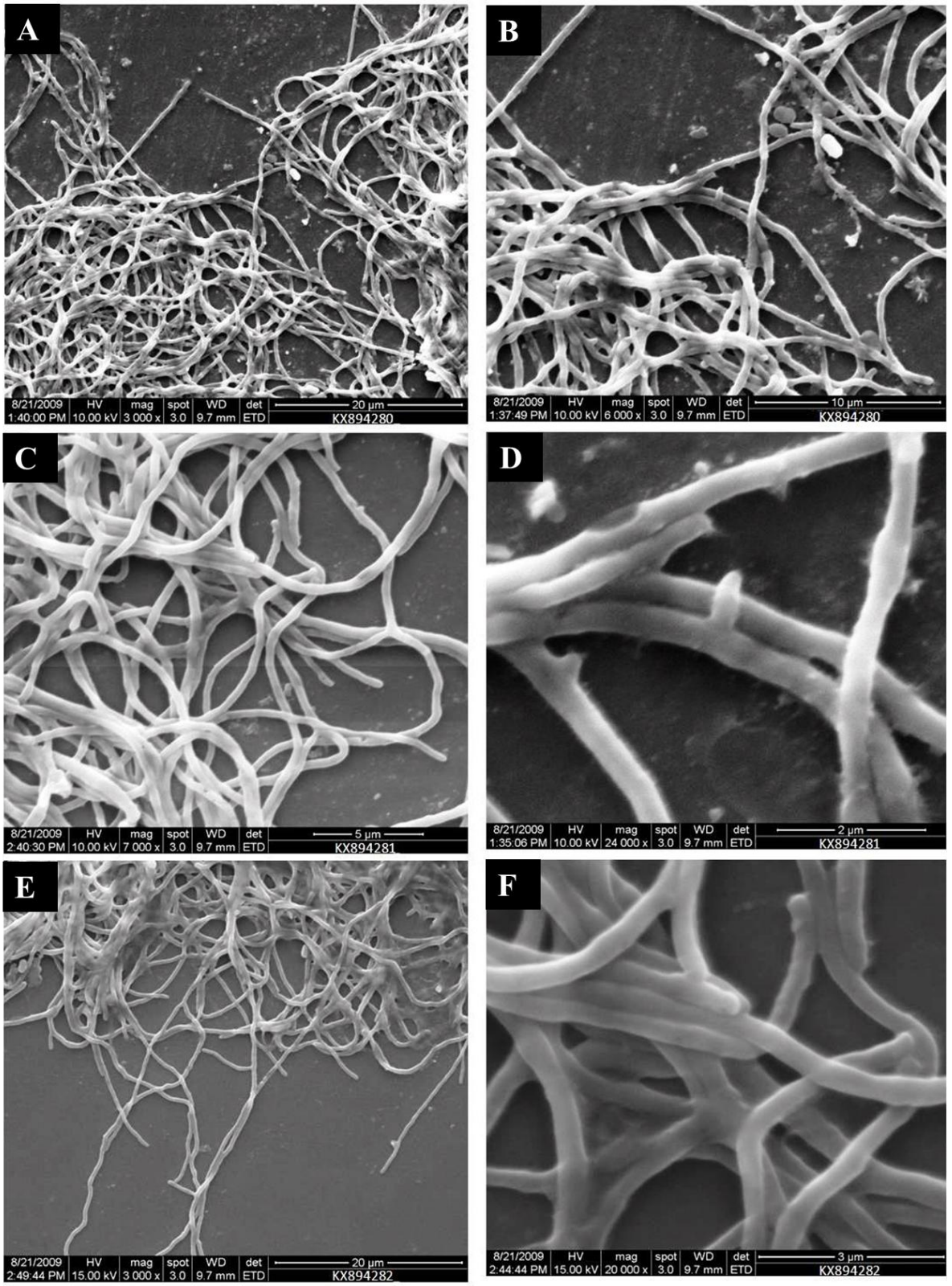


Figure7: (A-F) Scanning Electron Micrograph of different actinomycetes isolates. (A-B). *Streptomyces tricolor* (KX894280). (C-D). *Streptomyces flavogriseus* (KX894281). (E-F). *Streptomyces griseus*(KX894282).

4.2.4. Deposition of the Isolates to the National Agriculturally Important Culture Collection(NAIMCC)

Some of the isolates were initially identified on the basis of their morphological and biochemical properties By National Centre of Fungal Taxonomy, as *Streptomyces* spp. belonging to streptomycetes group of actinomycetes. These cultures were later submitted to the National Agriculturally Important Culture Collection (NAIMCC) of National bureau of Agriculturally Important Microorganisms, and their accession numbers are listed in Table 11.

Table 11.NAIMCC accession numbers of selected isolates

Isolate Code	NAIMCC acc no.
ARHS/PO/14	NAIMCC-B00913
ARHS/PO/15	NAIMCC-B00915
ARHS/PO/16	NAIMCC-B00917
ARHS/PO/17	NAIMCC-B00914
ARHS/PO/27	NAIMCC-B00916

4.3 *In vitro* Screening and evaluation of isolates for growth promoting attributes.

4.3.1. Phosphate solubilization

4.3.1.1. In solid medium

A total of 17 actinomycetes isolates were obtained from the agricultural fields. All the isolates were initially screened for their ability to solubilize phosphate *in vitro* by plating the isolates in Pikovskaya agar (PKV)medium. Formation of a clear halo zone around the colony indicate phosphate solubilizing property (Fig.8) The diameter of the halo zone formed was recorded to compare the efficacy of the isolates for phosphate solubilization (Table.12)

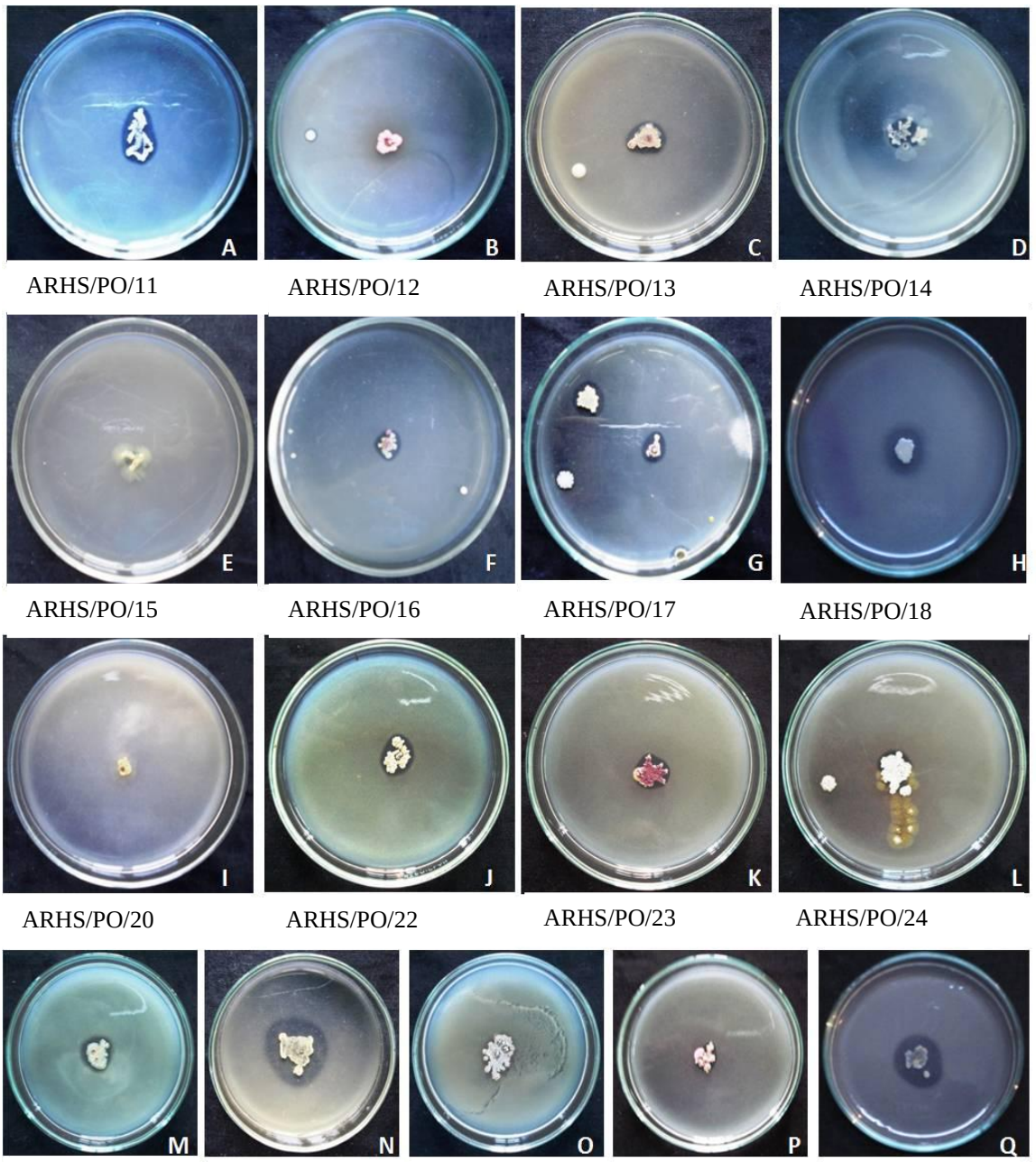


Figure 8: (A-Q) Screening for phosphate solubilizing activity by actinomycetes isolates in PVK solid media.

Table 12: Screening of Phosphate solubilization in the solid PKV medium

Isolates	Diameter of hallo zone(cm)	
	3 days	**7days**
ARHS/PO/11	0.10±0.091	0.25±0.008
ARHS/PO/12	0.16±0.02	0.34±0.025
ARHS/PO/14	0.21±0.014	0.45±0.06
ARHS/PO/16	0.20±0.035	0.43±0.012
ARHS/PO/17	0.13±0.071	0.31±0.032
ARHS/PO/18	0.17±0.050	0.35±0.061
ARHS/PO/20	0.11±0.001	0.27±0.055
ARHS/PO/22	0.18±0.062	0.38±0.023
ARHS/PO/23	0.24±0.021	0.51±0.01
ARHS/PO/24	0.12±0.011	0.29±0.06
ARHS/PO/25	0.18±0.006	0.39±0.1
ARHS/PO/26	0.28±0.071	0.57±0.21
ARHS/PO/28	0.22±0.023	0.49±0.
ARHS/PO/29	0.14±0.044	0.35±0.

Values are mean of three replicates, ± =standard error

4.3.1.2. In Liquid medium

The isolates which showed positive result for phosphate solubilization on PKV medium were further evaluated for phosphate solubilization capacity in liquid PKV media amended with Tricalcium phosphate and rock phosphate.The initial total phosphate content of Tricalcium phosphate was 920mg/L and of rock phosphate was 592mg/L. The amount of the total phosphate solubilized is presented in Table 13. The initial pH of the culture medium was 7. After 7 days of incubation pH in the control remained constant but dropped significantly in the broth culture containing samples.

Table13. *In vitro* quantification of phosphate solubilization by isolates in modified PKV broth culture

Isolates	TCP(µg/ml)	RP(µg/ml)
ARHS/PO/11	374.61 ± 1.21	244.86 ±0.53
ARHS/PO/12	478.23 ± 0.84	296.47 ±1.29
ARHS/PO/13	-	-
ARHS/PO/14	512.73 ± 0.56	392.39 ±0.68
ARHS/PO/15	-	-
ARHS/PO/16	536.99 ± 0.84	399.09 ±0.81
ARHS/PO/17	363.36 ± 1.23	279.79 ±1.05
ARHS/PO/18	417.52 ± 1.43	325.63 ±0.86
ARHS/PO/20	387.70 ± 0.95	211.59 ±0.72
ARHS/PO/22	476.38 ± 0.80	271.27 ±0.92
ARHS/PO/23	519.13 ± 0.38	281.58 ±0.45
ARHS/PO/24	347.53 ± 0.47	203.63 ±0.87
ARHS/PO/25	447.14 ±0.77	255.75 ±0.55
ARHS/PO/26	629.56 ±1.11	409.28 ±1.67
ARHS/PO/27	-	-
ARHS/PO/28	514.64 ±0.91	264.76 ±0.61
ARHS/PO/29	509.36 ±0.47	326.74 ±0.40

TCP=tricalcium phosphate RP= rock phosphate, Values are mean of three replicates, ± =standard error

4.3.2. IAA production

Isolates were grown for 48h in high C/N ratio medium. Trytophane (0.1mM) was added in order to enhance acetic acid (IAA) production by the isolates. Production of IAA in culture supernatant was assayed by Pillet- Chollet method. The detailed result is summarised in Table 14. The result shows that IAA production is highest in ARHS/PO/26, ARHS/PO/27 and ARHS/PO/15. ARHS/PO/26 produces highest amount of IAA.

Table14. Quantification of IAA production in actinomycetes isolates

Isolates	IAA(mg/L)
ARHS/PO/11	11.21 ±0.33
ARHS/PO/12	15.32 ±0.47
ARHS/PO/13	7.61 ±0.15
ARHS/PO/14	16.61 ±0.75
ARHS/PO/15	20.56 ±0.59
ARHS/PO/16	18.70 ±0.34
ARHS/PO/17	11.47 ±0.32
ARHS/PO/18	12.09 ±0.54
ARHS/PO/20	9.35 ±0.28
ARHS/PO/22	13.42 ±0.64
ARHS/PO/23	10.50 ±0.49
ARHS/PO/24	13.76 ±0.26
ARHS/PO/25	12.39 ±0.46
ARHS/PO/26	23.70 ±0.32
ARHS/PO/27	21.46 ±0.45
ARHS/PO/28	17.44 ±0.51
ARHS/PO/29	14.39 ±0.44

Values are mean of three replicates, ± =standard error

4.4. *In vitro* Screening and evaluation for antifungal activity

4.4.1.Inhibition in solid media

All the isolates were tested *in vitro* for inhibiting growth of the pathogen in dual culture using PDA and SCN media. Each actinomycetes isolate was placed at one side of the agar plate about 1cm away from the edge by streaking and 7mm diameter block of the pathogen(*Fusarium solani* and *Sclerotium rolfsii*) taken from growing edge of the fungal culture was inoculated at the other half of the Petri plate. In another experiment actinomycetes were placed circling the pathogen. After 7 days inhibition zone towards the fungus colony in individual plate was recorded.

Results of *in vitro* pairing experiments were recorded and enlisted in table (15, 16) Figs. (9.10) The result shows that maximum inhibition against *Fusarium solani* was exhibited by ARHS/PO/27, similarly ARHS/PO/26 showed maximum inhibition against *Sclerotium rolfsii.*

Table 15. *In vitro* pairing of the isolates with S*clerotium rolfsii* for evaluation of antifungal activities

Interacting microorganisms	Isolates	Radius of fungal colony after 7 days growth(cm)	% of Inhibition
Sclerotium rolfsii		2.6±0.09	-
S. rolfsii+Streptomyces sp.	ARHS/PO/11	1.99±0.08	41.66
S. rolfsii+Streptomyces sp.	ARHS/PO/12	1.72±0.11	56.18
S. rolfsii+Streptomyces sp.	ARHS/PO/13	1.91±0.09	46.66
S. rolfsii+Streptomyces sp.	ARHS/PO/14	1.77±0.04	53.72
S. rolfsii+Streptomyces sp.	ARHS/PO/15	1.81±0.33	51.81
S. rolfsii+Streptomyces sp.	ARHS/PO/16	2.00±0.67	40.95
S. rolfsii+Streptomyces sp.	ARHS/PO/17	1.93±0.86	44.90
S. rolfsii+Streptomyces sp.	ARHS/PO/18	1.93±0.41	40.90
S. rolfsii+Streptomyces sp.	ARHS/PO/20	1.9±0.01	46.66
S. rolfsii+Streptomyces sp.	ARHS/PO/22	1.68±0.32	58.22
S. rolfsii+Streptomyces sp.	ARHS/PO/23	1.92±0.04	45.45
S. rolfsii+Streptomyces sp.	ARHS/PO/24	1.92±0.78	45.77
S. rolfsii+Streptomyces sp.	ARHS/PO/25	1.87±0.41	48.25
S. rolfsii+Streptomyces sp.	ARHS/PO/26	1.41±0.04	70.26
S. rolfsii+Streptomyces sp.	ARHS/PO/27	1.55±0.56	64.86
S. rolfsii+Streptomyces sp.	ARHS/PO/28	1.76±0.07	54.05
S. rolfsii+Streptomyces sp.	ARHS/PO/29	1.72±0.02	56.45

Values are mean of three replicates, ± =standard error

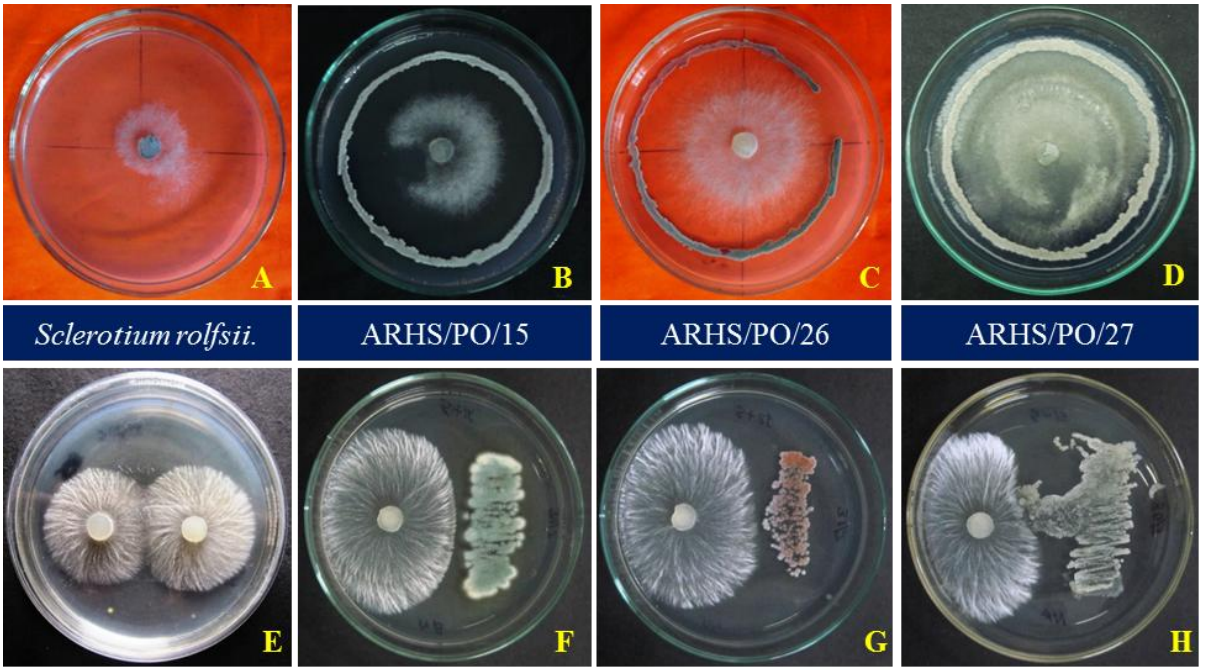


Figure 9: (A-H) *In vitro* antagonist activity assay of selected isolates (ARHS/PO/15, ARHS/PO/26 and ARHS/PO/27) with *Sclerotium rolfsii.;(*A &E) Control; (B-D) radial method ; (F-H) linear method

Table 16. *In vitro* pairing of the isolates with *Fusarium solani* for evaluation of antifungal activities

Interacting microorganisms	Isolates	Radius of fungal colony after 7 days growth(cm)	% of Inhibition
Fusarium solani		1.9±0.05	-
F.solani+Streptomyces sp.	ARHS/PO/11	1.07±0.01	67.8
F.solani+Streptomyces sp.	ARHS/PO/12	1.23±0.89	57.8
F.solani+Streptomyces sp.	ARHS/PO/13	1.07±0.63	67.8
F.solani+Streptomyces sp.	ARHS/PO/14	1.2±0.06	60.0
F.solani+Streptomyces sp.	ARHS/PO/15	1.17±0.07	62.2
F.solani+Streptomyces sp.	ARHS/PO/16	1.2±0.91	60.0
F.solani+Streptomyces sp.	ARHS/PO/17	1.2±0.51	60.0
F.solani+Streptomyces sp.	ARHS/PO/18	1.25±0.84	56.7
F.solani+Streptomyces sp.	ARHS/PO/20	1.09±0.07	66.7
F.solani+Streptomyces sp.	ARHS/PO/22	1.09±0.23	66.7
F.solani+Streptomyces sp.	ARHS/PO/23	1.2±0.44	60.0
F.solani+Streptomyces sp.	ARHS/PO/24	1.17±0.01	62.2
F.solani+Streptomyces sp.	ARHS/PO/25	1.07±0.03	67.8
F.solani+Streptomyces sp.	ARHS/PO/26	1.09±0.01	66.7
F.solani+Streptomyces sp.	ARHS/PO/27	0.64±0.03	88.5
F.solani+Streptomyces sp.	ARHS/PO/28	1.25±0.06	56.7
F.solani+Streptomyces sp.	ARHS/PO/29	1.15±0.31	63.4

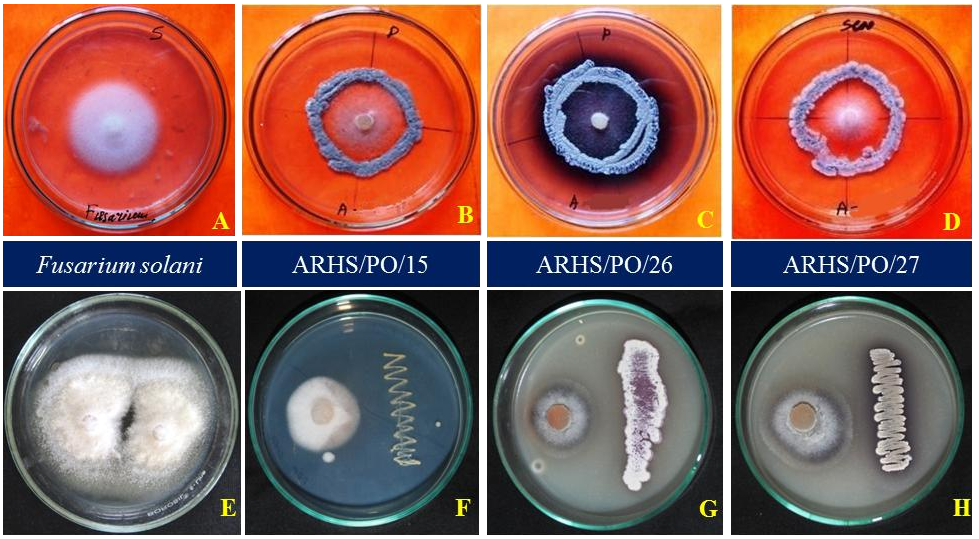


Figure 10: (A-H) *In vitro* antagonist activity assay of selected isolates (ARHS/PO/15, ARHS/PO/26, ARHS/PO/27) with *Fusarium solani*. (A &E) Control, (B-D) radial method ; F-H, linear method.

4.4.2. Inhibition of fungal growth by metabolites

4.4.2.1. Inhibition of spore germination

Fungal spores of *Fusarium solani* wereb ioassayed against metabolitesobtained from selected isolates . A drop of the test solution was placed on a clean, grease free grooved slide and following which a drop of spore suspension was placed over it. The slide was incubated in a moist Petri plate for 24 h at 30ºC ± 1 ºC. Finally one drop of lacto phenol - cotton blue solutionwas added to each spot to fix the germination of spore. The slides were observed under microscope and the percentage of germination was determined. The result showed that *Fusarium solani* spore germination was hampered in presence of metabolites suspension. ARHS/PO/27 had the highest germination inhibition rate with only 10.82% spore germination rate (Table 17, Fig 11).

Table 17. Effect of metabolites on spore germination of *Fusarium solani*

Treatment	% of spore germination
Control	89.7± 0.87
ARHS/PO/15	37.11± 0.65
ARHS/PO/26	32.6± 0.21
ARHS/PO/27	10.82± 0.86

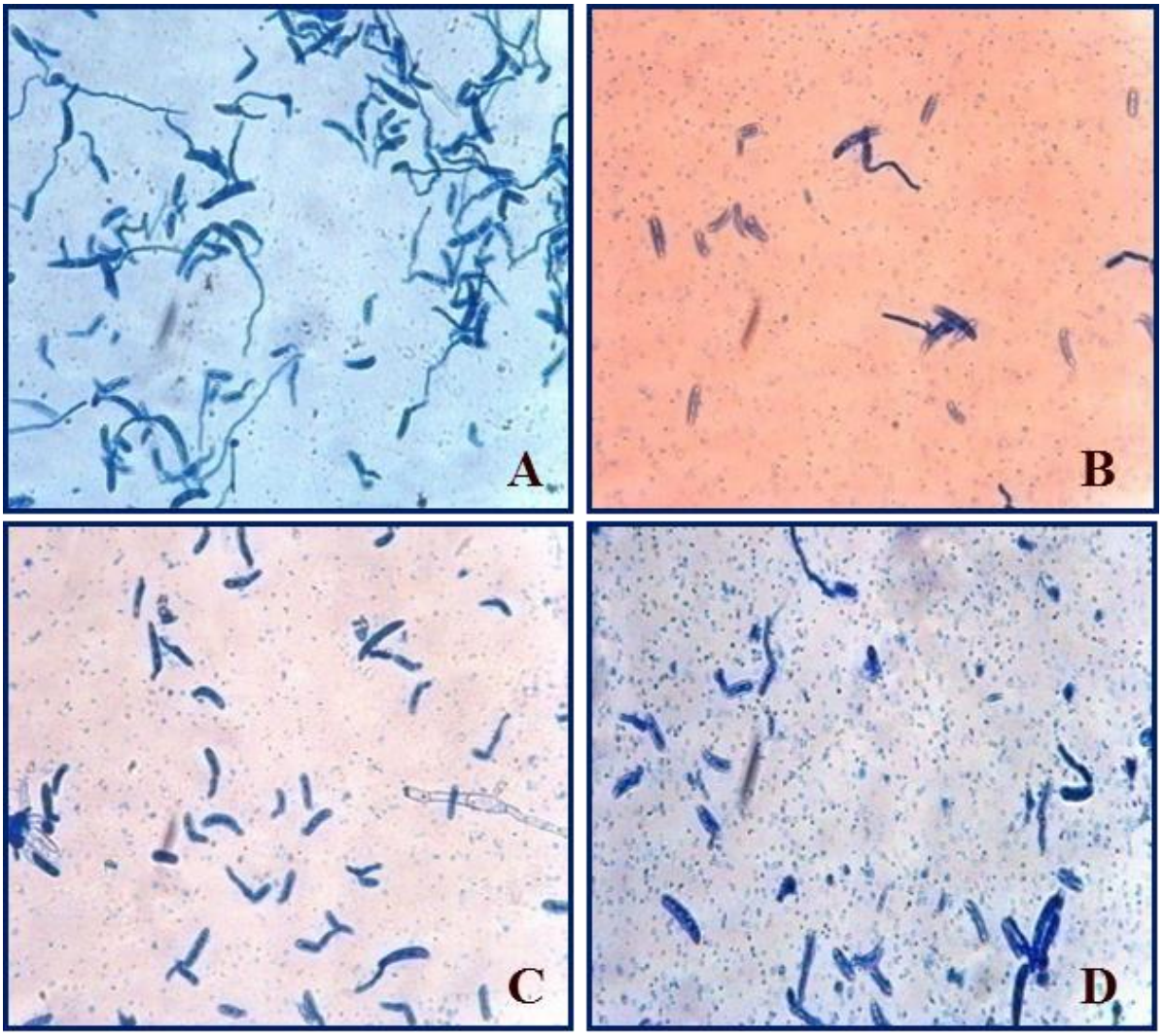


Figure 11: (A-D) Effect of metabolites of actinomycetes on conidial germination of *Fusarium solani.* A- Control ,B-ARHS/PO/15, C-ARHS/PO/26, D-ARHS/PO/27

4.4.2.2. Inhibition of mycelial growth

Autoclaved PDA medium (20ml) was mixed with 0.5ml of test compound solution and plated onto petriplates. After solidification, agar block from 4 days old cultures of *F.solani* and *Sclerotium rolfsii* was placed in the centre of each petriplate. The plates were incubated and radial growth of mycelium was measured.In case of *Fusarium solani* maximum inhibition was noted in metabolites of *Streptomyces flavogriseus* and in *Sclerotium rolfsii* maximum inhibition was noted in *S. tricolor* (Table 18,19)

Table 18. Effect of metabolites on radial growth of *Fusarium solani* after 48 hours incubation

Treatment	Diameter(mm)	% Inhibition
Control(*F. solani)*	8±0.98	-
F. solani+ S.griseus	6±0.70	43.74
F. solani+ S.tricolor	5±0.67	60.93
F. solani+ S.flavogriseus	2±0.03	93.75

Table 19. Effect of metabolites on radial growth of *Sclerotium rolfsii* after 72 hours incubation

Treatment	Diameter (mm)	% Inhibition
Control (*S.rolfsii)*	10±1.09	-
S. rolfsii+ S.griseus	3±0.07	91.0
S.rolfsii+ S.tricolor	2±0.08	96.0
S.rolfsii+ S.flavogriseus	7±0.99	51.0

4.4.2.3. Inhibition of sclerotia germination

For assessing the effect of the metabolites on the sclerotial germination of *S. rolfsii* the sclerotia were scrapped off from the culture growing in 7 days old PDA plates. The sclerotia were then soaked 1 hour in metabolites solution. After soaking the sclerotia were transferred asceptically to the petriplate containing sterile Black paper. These sterile filter papers were also soaked in metabolites solution for at least 30minutes and incubated at room temperature. Percent germination (Table 20) as well as the radial growth of the germinating sclerotia (Table 21), (Fig. 12) was measured.

Table 20. Effect of metaboliteson Sclerotia germination of phytopathogenic fungi

Treatment	**Germination % of Sclerotia**
Sclerotium rolfsii+ Streptomyces griseus	70.0
Sclerotium rolfsii + Streptomyces tricolor	40.0
Sclerotium rolfsii + Streptomyces flavogriseus	50.0

Table 21. Effect of metabolites on radial growth of Sclerotia after 72 hours incubation

Treatment	Diameter (mm)	% Inhibition
Control (*S rolfsii)*	15±1.8	-
S. rolfsii+ S.griseus	8±0.08	71.5
S.rolfsii+ S.tricolor	2±0.04	98.2
S.rolfsii+ S.flavogriseus	5±0.27	88.88

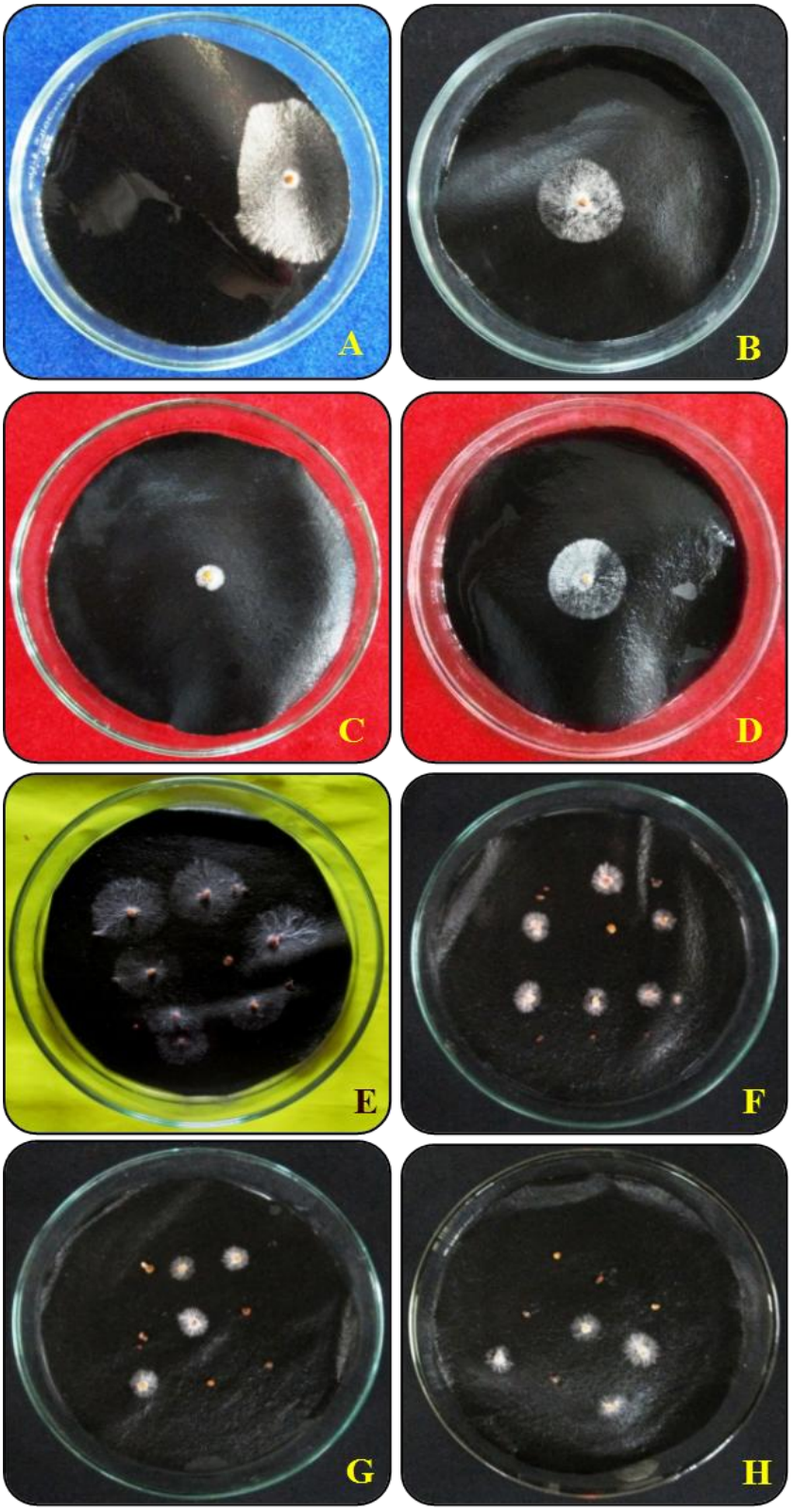


Figure12: (A-H): Effect of metabolites of actinomycetes on germination of sclerotia of *Sclerotium rolfsii*. (A–D) Single sclerotium; A- Control; B- ARHS/PO/15; C-ARHS/PO/26; D-ARHS/PO/27. (E-H) Germination of sclerotia; E-Control; F-ARHS/PO/15; G-ARHS/PO/26; H- ARHS/PO/27

4.5. *In vitro* Screening and evaluation for antibacterial activity.

In vitro screening and evaluation for antibacterial activities of the selected isolates were carried out with bacterial isolates of *Bacillus megaterium*, *Bacillus cereus* and *Escherichia coli*. *In vitro* pairing of the isolates with bacterial isolates shows that none of the isolates inhibit the growth of *Bacillus megaterium* which is a potent plant growth promoting rhizobacteria (Fig.13.)

Figure 13: *In vitro* screening for antibacterial activity.

4.6 Molecular detection

Genomic DNA of potent isolates ARHS PO 15, ARHS PO 26 and ARHS PO 27 were suspended in 100µl 1X TE buffer and stored in -20°C until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free. Purity of DNA evaluated in terms of the ratio between absorbance of A260 and A280 showed that genomic DNA was ~1.8. PCR amplification of ITS region of 16 S rDNA was carried out using primer pair, Forward primer:5'AGAGTTGATCMTYGCTWAC3' and reverse primer 5'CGYTAMCTTWTTACGRCT3'.

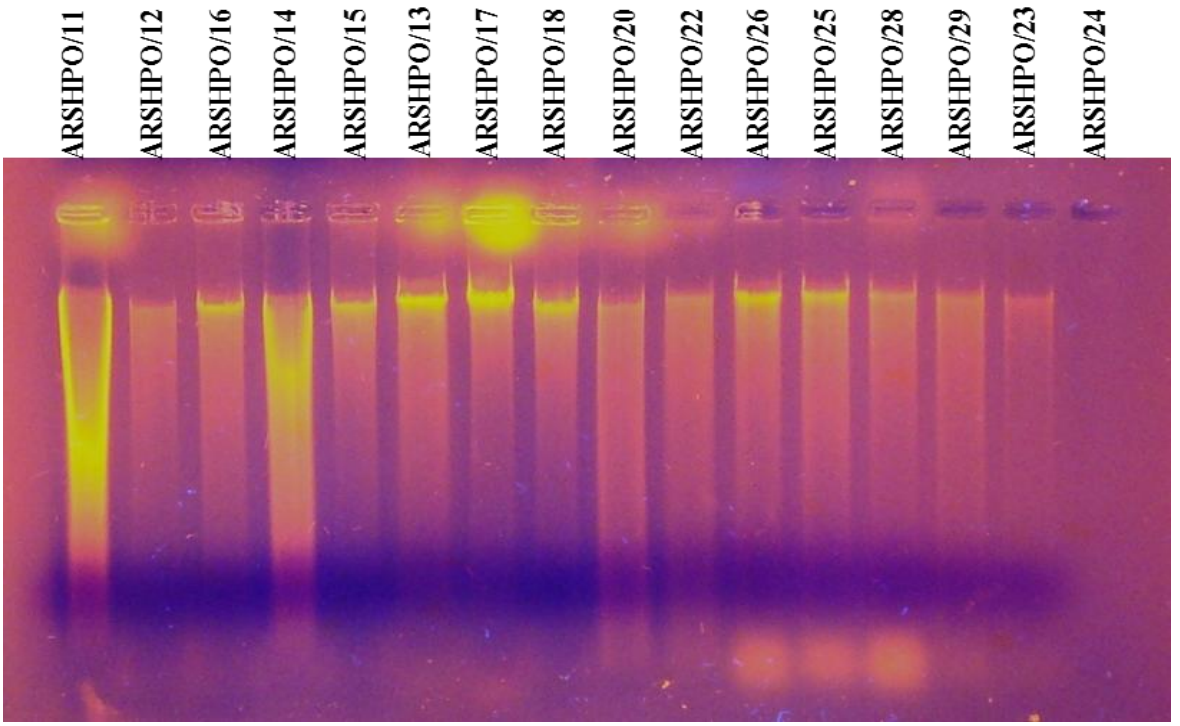


Figure14: Agarose Gel electrophoresis of genomic DNA of isolates from various sources.

4.6.1*Streptomyces griseus*

4.6.1.1. 16 S rDNA sequence analysis

The BLAST query of the 16S rDNA sequence of ARHS PO 15 against GenBank database confirmed that the isolate is *Streptomyces griseus.* The sequences have been deposited in NCBI, GenBank database under the accession no. KX894282.The sequence and chromatograms have been represented in Figure 15.

4.6.1.2 Multiple Sequence Alignment

A multiple sequence alignment of ITS gene sequences of *Streptomyces griseus* was conducted. Sequences of other strains obtained from NCBI GenBank database showing maximum homology with the strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Figure 16).

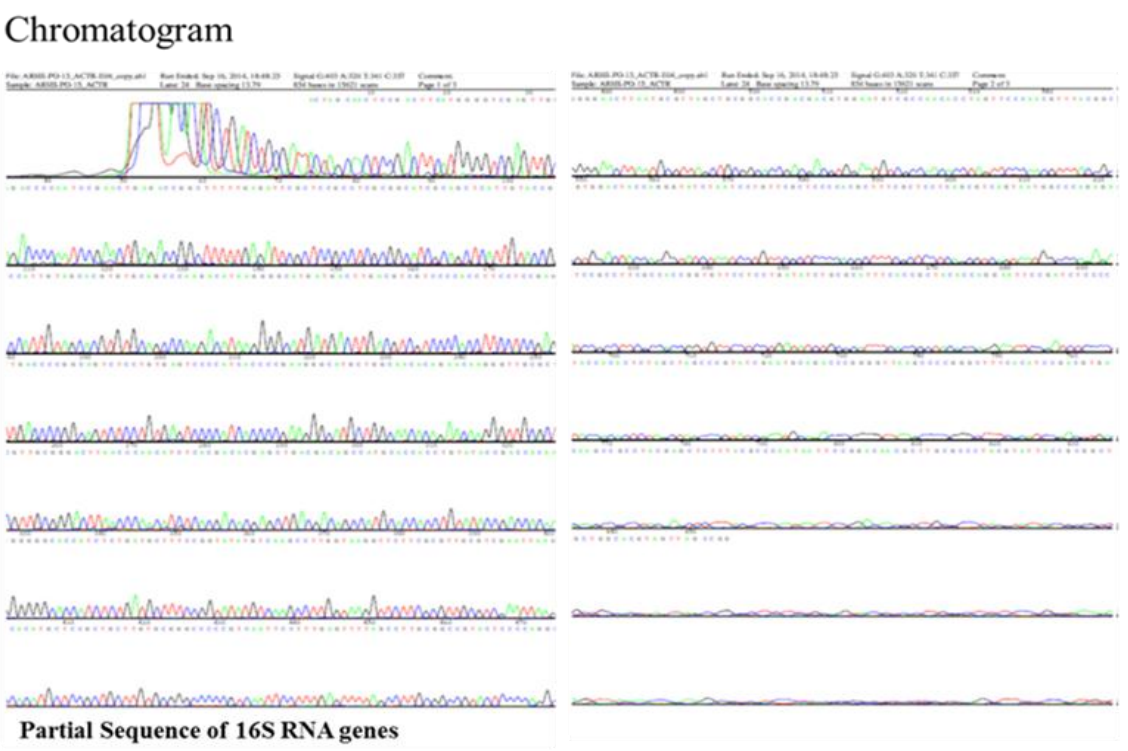


Fig.15 . Chromatogram and sequence deposit of 16S r RNA region *Streptomyces griseus* ARHS/PO/15 (NCBI-KX894282)

Figure16: 16S RNA gene sequence alignments of isolate *Streptomyces griseus* ARHS/PO/15 (NCBI-KX894282) with other extypes from NCBI GenBank Database.

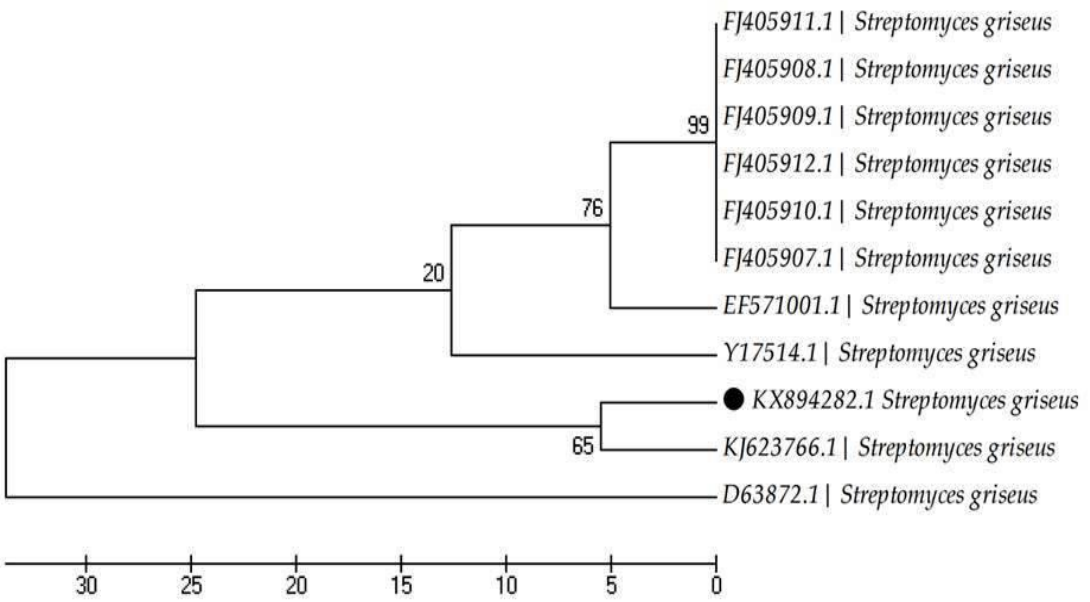


Figure17: Phylogenetic placement of *Streptomyces griseus*(KX894282) with other ex-type strain sequences obtained from NCBI GenBank Database.

4.6.1.3 Phylogenetic analysis

Phylogenetic analysis was carried out with extype strain sequences obtained from NCBI Genbank database which showed maximum homology with *Streptomyces griseus*(KJ623766) (Table 22).The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 435 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4(Fig. 17)

Table 22: GenBank accession numbers and geographic location of extype strains of *Streptomyces griseus* that showed homology with isolate ARHS/PO/15

Sl No	Accession No	Strain or Isolate	rDNA Sequence	Origin
1	FJ405911	D30	1365bp	China
2	FJ405908	FXJ124	1365bp	China
3	FJ405909	FXJ162	1365bp	China
4	FJ405912	E3	1365bp	China
5	FJ405910	FXJ175	1365bp	China
6	FJ405907	FXJ70	1365bp	China
7	EF571001	52-1	1487bp	Hungary
8	Y17514	10/ppi	435bp	USA
9	KJ623766	S131	1411bp	Egypt
10	D63872	ATCC25497	1532bp	Japan
11	**KX894282**	**ARSH/PO/15**	**852bp**	**India**

4.6.2. *Streptomyces tricolor*

4.6.2.1. 16 S rDNA sequence analysis

The BLAST query of the 16S rDNA sequence of ARHS PO 26against GenBank database confirmed that the isolate is *Streptomyces tricolor*. The sequences have been deposited in NCBI, GenBank database under the accession no. KX894280.The sequence and chromatograms have been represented in Figure 18.

4.6.2.2 Multiple Sequence Alignment

A multiple sequence alignment of ITS gene sequences of *Streptomyces tricolor* was conducted. Sequences of other strains obtained from NCBI GenBank database showing maximum homology with the strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Figure 19).

Chromatogram

Partial Sequence of 16S RNA genes

GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
CAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAA
GTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCT
AGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCT
CTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGC
ACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACAT
ACACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGG
TCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGA
GCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCTAACTCGACCCCATGAAGTCGGAGT
CGCTA GTAAT

Sequence Deposited: NCBI
Accession No: KX894280
Version : KX894280.1
DNA Linear: 1055bp

Title:
***Streptomyces tricolor* strain ARHS/PO/26 16S ribosomal RNA gene, partial sequence**

Origin

```
   1 gtagccggcc tgagagggcg accggccaca ctgggactga gacacggccc agactcctac
  61 gggaggcagc agtggggaat attgcacaat gggcgaaagc ctgatgcagc gacgccgcgt
 121 gagggatgac ggccttcggg ttgtaaacct ctttcagcag ggaagaagcg aaagtgacgg
 181 tacctgcaga agaagcgccg gctaactacg tgccagcagc cgcggtaata cgtagggcgc
 241 aagcgttgtc cggaattatt gggcgtaaag agctcgtagg cggcttgtca cgtcggttgt
 301 gaaagcccgg ggcttaaccc cgggtctgca gtcgatacgg gcaggctaga gttcggtagg
 361 ggagatcgga attcctggtg tagcggtgaa atgcgcagat atcaggagga acaccggtgg
 421 cgaaggcgga tctctgggcc gatactgacg ctgaggagcg aaagcgtggg gagcgaacag
 481 gattagatac cctggtagtc cacgccgtaa acggtgggca ctaggtgtgg gcaacattcc
 541 acgttgtccg tgccgcagct aacgcattaa gtgccccgcc tggggagtac ggccgcaagg
 601 ctaaaactca aaggaattga cgggggcccg cacaagcggc ggagcatgtg gcttaattcg
 661 acgcaacgcg aagaacctta ccaaggcttg acatacaccg gaaagcatca gagatggtgc
 721 cccccttgtg gtcggtgtac aggtggtgca tggctgtcgt cagctcgtgt cgtgagatgt
 781 tgggttaagt cccgcaacga gcgcaaccct tgtcccgtgt tgccagcaag cccttcgggg
 841 tgttggggac tcacgggaga ccgccggggt caactcggag gaaggtgggg acgacgtcaa
 901 gtcatcatgc cccttatgtc ttgggctgca cacgtgctac aatggccggt acaatgagct
 961 gcgataccgc aaggtggagc gaatctcaaa aagccggtct cagttcggat tggggtctgc
1021 taactcgacc ccatgaagtc ggagtcgcta gtaat
```

Figure 18: Chromatogram and sequence deposit of 16S r RNA region of *Streptomyces tricolor* ARHS/PO/26 (NCBI-KX894280)

Figure 19: 16S r RNA gene sequence alignments of isolate *Streptomyces tricolor* ARHS/PO/26 (NCBI-KX894280) with other extypes from NCBI GenBank Database.

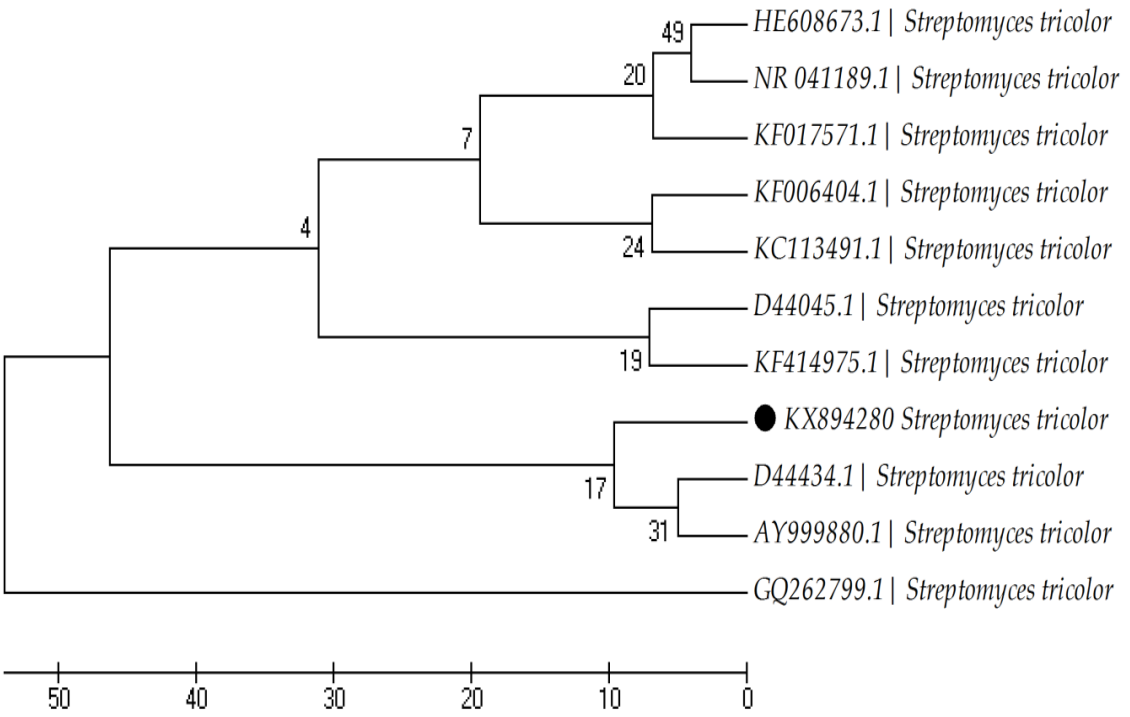


Figure20: Phylogenetic placement of *Streptomyces tricolor*(KX894280)with other ex-type strain sequences obtained from NCBI GenBank Database.

4.6.2.3 Phylogenetic analysis

Phylogenetic analysis was carried out with extype strain sequences obtained from NCBI GenBank database which showed maximum homology with *Streptomyces tricolor*(AY999880) (Fig.20) (Table 23).The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 119 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 .

Table 23: GenBank accession numbers and geographical location of ex-type strains of *Streptomyces* species that showed homology with isolate ARHS/PO/26

Sl No	Accession No	Strain or Isolate	rDNA Sequence	Origin
1	HE608673	D0710T2_2B_S3	784bp	Spain
2	NR041189	NBRC15461	1450bp	Japan
3	KF017571	ICN14	1314bp	India
4	KF006404	ERINLG	1030bp	India
5	KC113491	Vh85	1339bp	India
6	D44045	JCM4295	120bp	Japan
7	KF41975	Mhce0811	788bp	India
8	D44434	JCM5065	121bp	Japan
9	AY999880	AS4.1867/CSSP401	1406bp	USA
10	GQ262799	Vh85	1341bp	India
11	**KX894280**	**ARSH/PO/26**	**1055bp**	**India**

4.6.3 *Streptomyces flavogriseus*

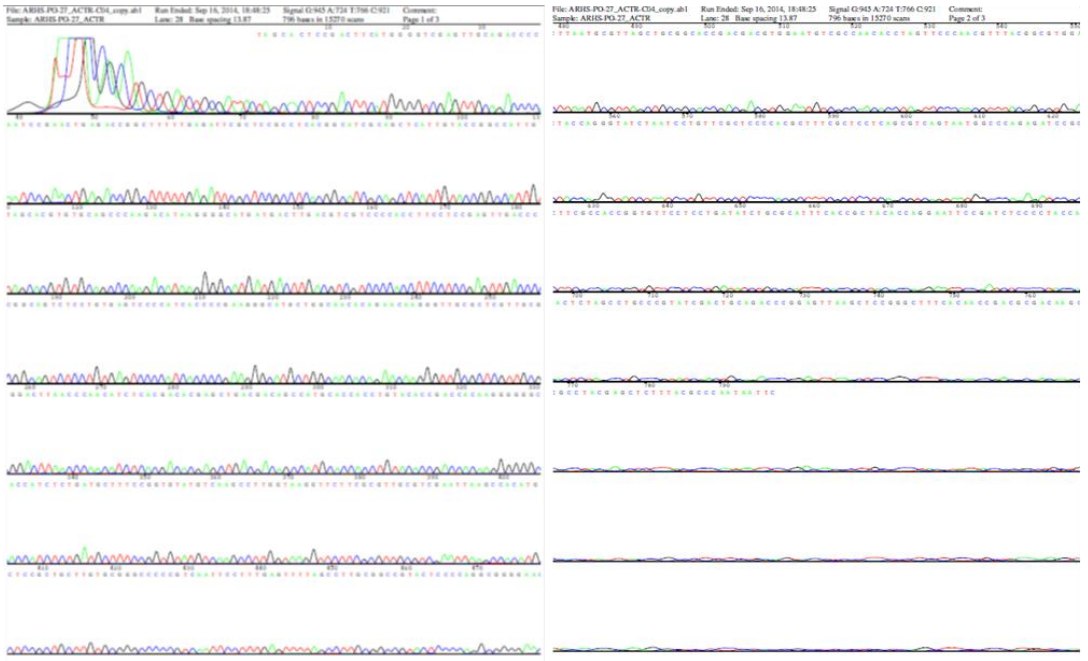
4.6.3.1. 16 S rDNA sequence analysis

The BLAST query of the 16S rDNA sequence of ARHS PO 27against GenBank database confirmed that the isolate is *Streptomyces flavogriseus*. The sequences have been deposited in NCBI, GenBank database under the accession no. KX894281.The sequence and chromatograms have been represented in Figure 21 .

4.6.3.2. Multiple Sequence Alignment

A multiple sequence alignment of ITS gene sequences of *Streptomyces flavogriseus* was conducted. Sequences of other strains obtained from NCBI GenBank database showing maximum homology with the strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Figure 22).

Chromatogram



Partial Sequence of 16S RNA genes

GGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAA
GCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCC
GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATA
CGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGC
GAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACG
GCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAA
CCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGG
GACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACA
CGTGCTACAATGGCCGGTACAATGAGCTGCGAAGTCGTGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTC
TGCAACTCGACCCCATGAAGTCGGAGG

Origin

Sequence Deposited: NCBI
Accession No: KX894281
Version : KX894281.1
DNA Linear: 1045bp

Title:
***Streptomyces flavogriseus* strain ARHS/PO/26**
16S ribosomal RNA gene, partial sequence

```
   1 ggtagccggc ctgagagggc gaccggccac actgggactg agacacggcc cagactccta
  61 cgggaggcag cagtggggaa tattgcacaa tgggcgaaag cctgatgcag cgacgccgcg
 121 tgagggatga cggccttcgg gttgtaaacc tctttcagca gggaagaagc gaaagtgacg
 181 gtacctgcag aagaagcgcc ggctaactac gtgccagcag ccgcggtaat acgtagggcg
 241 caagcgttgt ccggaattat tgggcgtaaa gagctcgtag gcggcttgtc acgtcggatg
 301 tgaaagcccg gggcttaacc ccgggtctgc attcgatacg ggctagctag agtgtggtag
 361 gggagatcgg aattcctggt gtagcggtga aatgcgcaga tatcaggagg aacaccggtg
 421 gcgaaggcgg atctctgggc cattactgac gctgaggagc gaaagcgtgg ggagcgaaca
 481 ggattagata ccctggtagt ccacgccgta aacgttggga actaggtgtt ggcgacattc
 541 cacgtcgtcg gtgccgcagc taacgcatta agttccccgc ctggggagta cggccgcaag
 601 gctaaaactc aaaggaattg acgggggccc gcacaagcag cggagcatgt ggcttaattc
 661 gacgcaacgc gaagaacctt accaaggctt gacatatacc ggaaagcatc agagatggtg
 721 ccccccttgt ggtcggtata caggtggtgc atggctgtcg tcagctcgtg tcgtgagatg
 781 ttgggttaag tcccgcaacg agcgcaaccc ttgttctgtg ttgccagcat gcccttcggg
 841 gtgatgggga ctcacaggag actgccgggg tcaactcgga ggaaggtggg gacgacgtca
 901 agtcatcatg ccccttatgt cttgggctgc acacgtgcta caatggccgg tacaatgagc
 961 tgcgaagtcg tgaggcggag cgaatctcaa aaagccggtc tcagttcgga ttggggtctg
1021 caactcgacc ccatgaagtc ggagg
```

Figure 21: Chromatogram and sequence deposit of 16S r RNA region *Streptomyces flavogriseus* ARHS/PO/27(NCBI-KX894281)

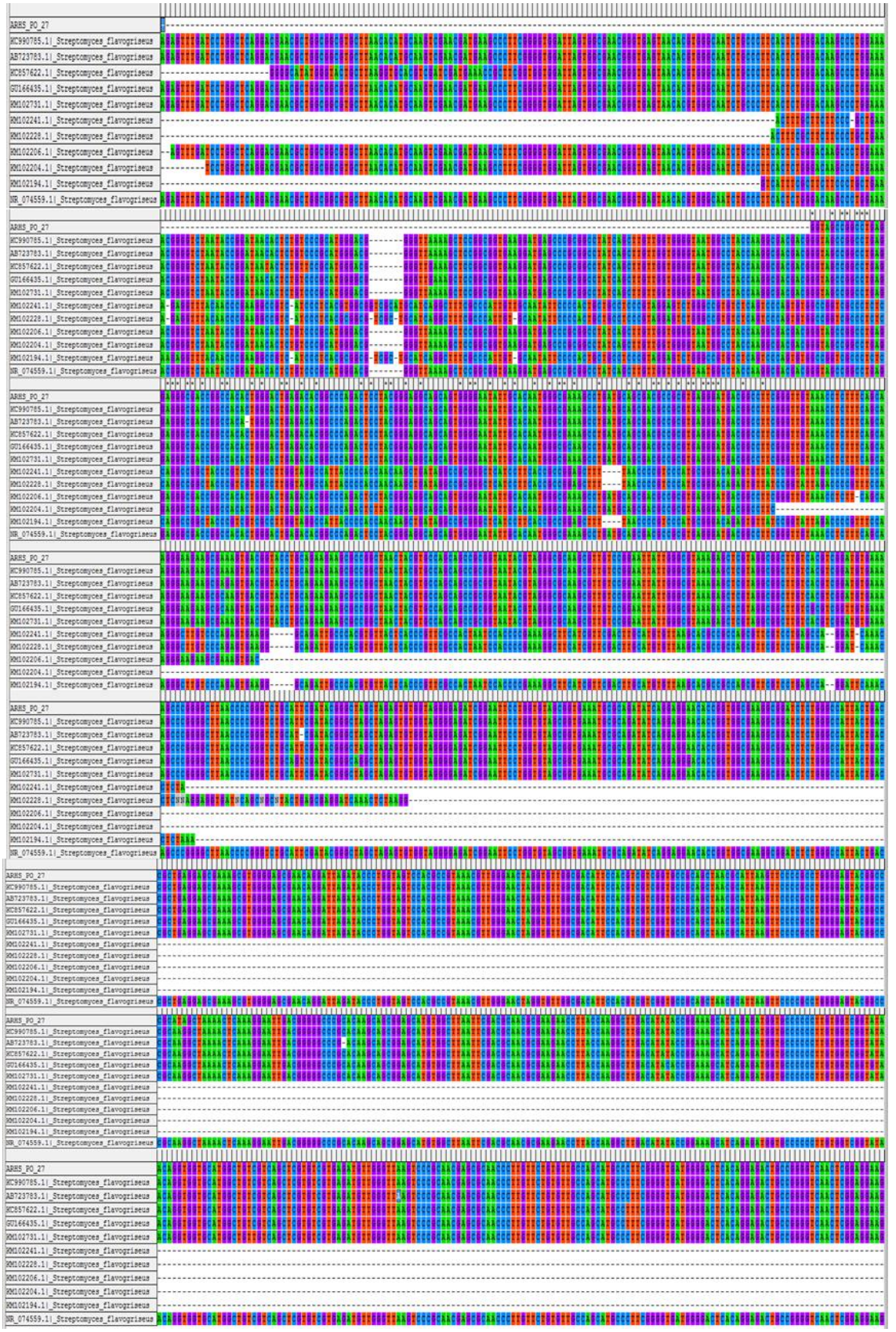


Figure22: 16S rRNA gene sequence alignments of isolate *Streptomyces flavogriseus* ARHS/PO/ 27 (NCBI-KX894281) with other extypes from NCBI GenBank Database.

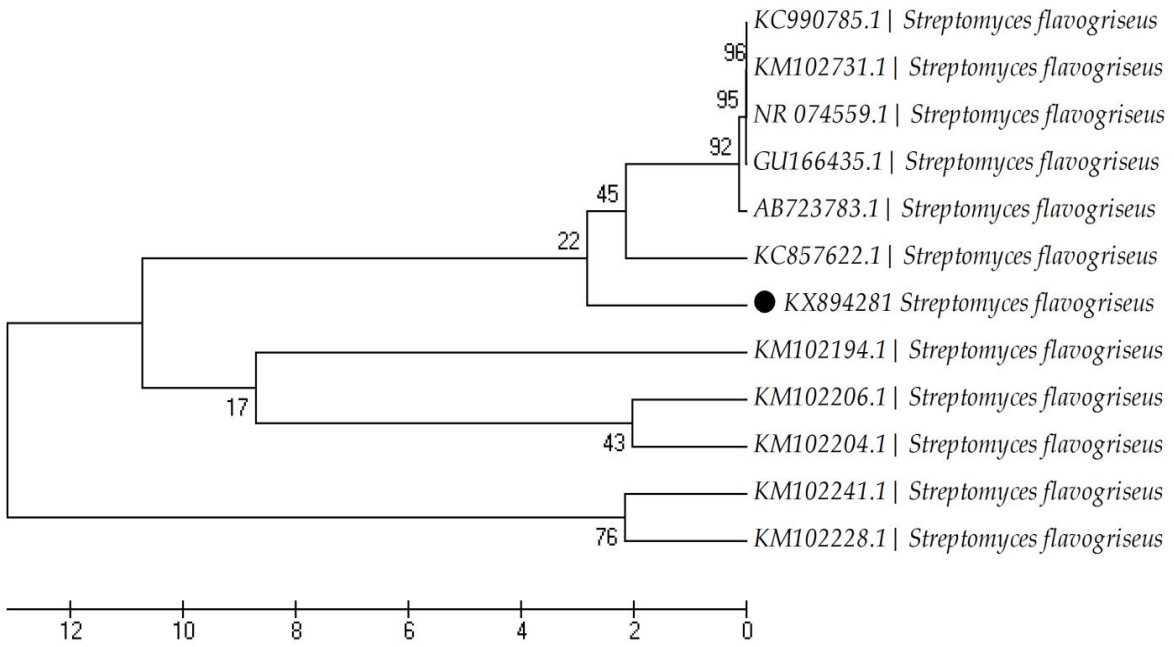


Figure 23: Phylogenetic placement of *Streptomyces flavogriseus*(KX894281)with other ex-type strain sequences obtained from NCBI GenBank Database

4.6.3.3 Phylogenetic analysis

Phylogenetic analysis was carried out with extype strain sequences obtained from NCBI GenBank database which showed maximum homology with *Streptomyces flavogriseus* (KC857622) (Fig.23).The evolutionary history was inferred using the UPGMA method . The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 398 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.(Fig 23)

Table 24: GenBank accession numbers and geographical location of extype strains of *Streptomyces* species that showed homology with isolate ARHS/PO/27

Sl No	Accession No	Strain or Isolate	rDNA Sequence	Country
1	KC990785	ACTK2	1483bp	India
2	KM102731	NJ-4	1484bp	China
3	NR074559	ATCC33331	1514bp	USA
4	GU166435	030	1489bp	Korea
5	AB723783	NRC2012	1197bp	Egypt
6	KC857622	NRC10	1350bp	Saudi Arabia
7	KM102194	2LA10	451bp	Canada
8	KM102206	3LD2	445bp	Canada
9	KM102204	5LA3	398bp	Canada
10	KM102241	8LC11	446bp	Canada
11	KM102228	6LE2	490bp	Canada
12	**KX894281**	**ARSH/PO/27**	**1045bp**	**India**

4.6.4. Multiple sequence alignment *of Streptomyces griseus, Streptomyces tricolor, Streptomyces flavogriseus*

A multiple sequence alignment of ITS gene sequences of *Streptomyces griseus Streptomyces tricolor, Streptomyces flavogriseus* was conducted. Sequences of other strains obtained from NCBI GenBank database showing maximum homology with the strains was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Figure 24).

Figure24: Multiple sequence alignment of *Streptomyces griseus* (KX894282*), Streptomyces tricolor* (KX894280) and *Streptomyces flavogriseus* (KX894281) with other extypes from NCBI

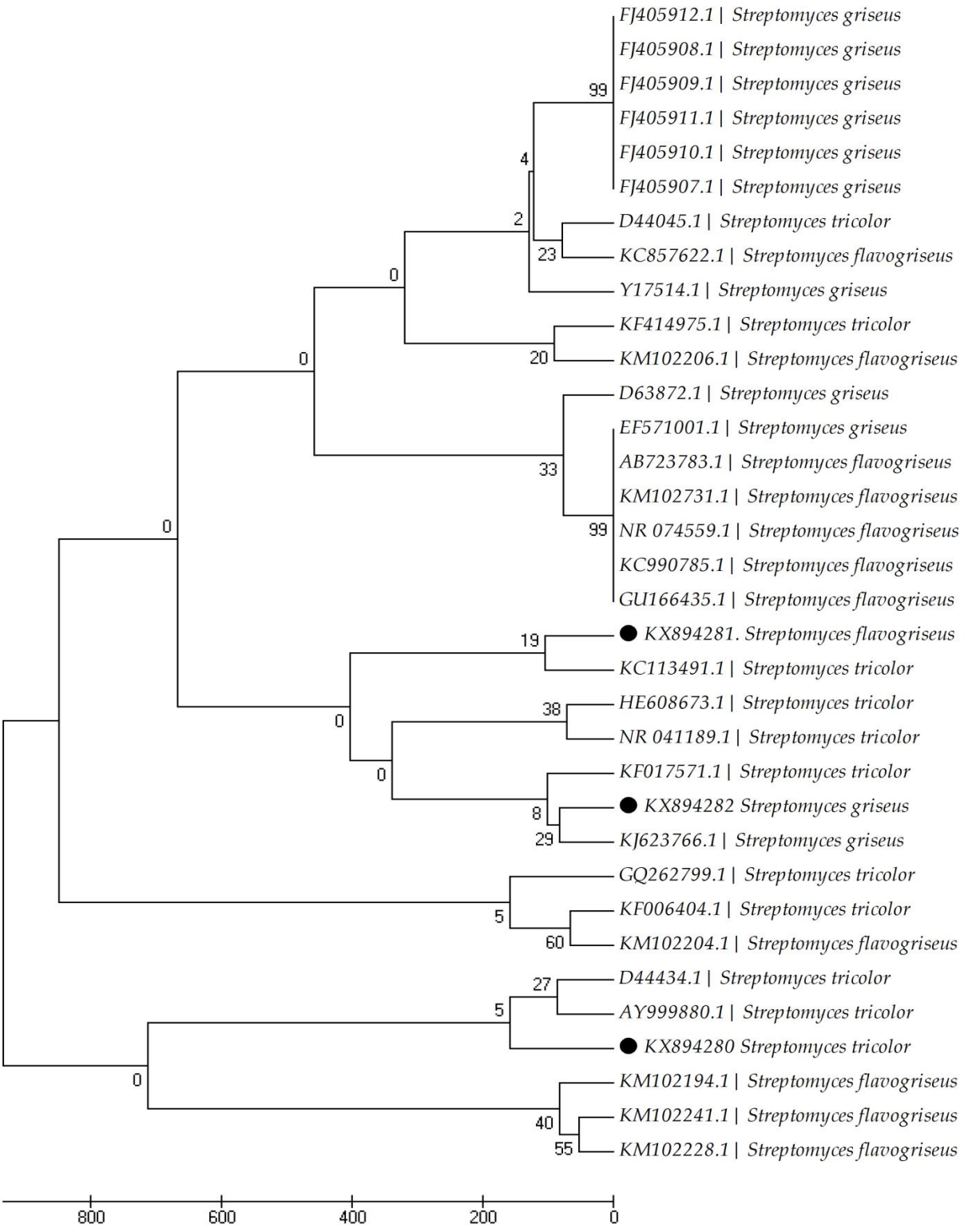


Figure25: UPGMA based Phylogenetic placement of *Streptomyces griseus* (KX894282), *Streptomyces tricolor* (KX894280) and *Streptomyces flavogriseus* (KX894281) with other extype strain sequences obtained from NCBI GenBank Database.

Table 24: GenBank accession numbers and geographic location of extype strains of *Streptomyces griseus, Streptomyces tricolor and Streptomyces flavogriseus* that showed homology with isolate ARHS/PO/15, ARHS/PO/26, ARHS/PO/27

Sl No	Accession No	Strain or Isolate	rDNA Sequence	Country
1	FJ405911	D30	1365bp	China
2	FJ405908	FXJ124	1365bp	China
3	FJ405909	FXJ162	1365bp	China
4	FJ405912	E3	1365bp	China
5	FJ405910	FXJ175	1365bp	China
6	FJ405907	FXJ70	1365bp	China
7	EF571001	52-1	1487bp	Hungary
8	Y17514	10/ppi	435bp	USA
9	KJ623766	S131	1411bp	Egypt
10	D63872	ATCC25497	1532bp	Japan
11	**KX894282**	**ARSH/PO/15**	**852bp**	**India**
12	HE608673	D0710T2_2B_S3	784bp	Spain
13	NR041189	NBRC15461	1450bp	Japan
14	KF017571	ICN14	1314bp	India
15	KF006404	ERINLG	1030bp	India
16	KC113491	Vh85	1339bp	India
17	D44045	JCM4295	120bp	Japan
18	KF41975	Mhce0811	788bp	India
19	D44434	JCM5065	121bp	Japan
20	AY999880	AS4.1867/CSSP401	1406bp	USA
21	GQ262799	Vh85	1341bp	India
22	**KX894280**	**ARSH/PO/26**	**1055bp**	**India**
23	KC990785	ACTK2	1483bp	India
24	KM102731	NJ-4	1484bp	China
25	NR074559	ATCC33331	1514bp	USA
26	GU166435	030	1489bp	Korea
27	AB723783	NRC2012	1197bp	Egypt
28	KC857622	NRC10	1350bp	Saudi Arabia
29	KM102194	2LA10	451bp	Canada
30	KM102206	3LD2	445bp	Canada
31	KM102204	5LA3	398bp	Canada
32	KM102241	8LC11	446bp	Canada
33	KM102228	6LE2	490bp	Canada
34	**KX894281**	**ARSH/PO/27**	**1045bp**	**India**

4.6.5. Phylogenetic analysis

Phylogenetic analysis was carried out with extype strain sequences obtained from NCBI GenBank database which showed maximum homology with *Streptomyces griseus* (KX894282), *Streptomyces tricolor* (KX894280) and *Streptomyces flavogriseus*(KX894281)(Table 25). The evolutionary history was inferred using the

UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 119 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Figure 25).

4.7. Evaluation of potent Actinomycetes isolates on plant growth promotion

On the basis of initial screening of the antagonism of the isolates against the plant pathogenic fungi, phosphate solubilizing activity, IAA production three isolates were selected for enhancement of growth of two crop plant, *Phaseolus vulgaris* and *Vigna radiata*. Growth promotions in seedlings were evaluated in terms of increase in height, shoot and root length, number of leaf, leaf area, root shoot fresh and dry weight of the treated as well as untreated control plants. The result shows that the isolates have significant positive effect on the growth of the two crop plants, *Phaseolus vulgaris* and *Vigna radiata* in pot condition. Enhancement of nodule formation was also taken into account and it was found that nodulation frequency increased in treated plants in comparison to the untreated plants though Nodulation Index did not vary that much.

4.7.1. Evaluation of potential Growth promotion of *Phaseolus vulgaris* upon treatment

The Isolates *Streptomyces griseus* (KX894282), *S. tricolor* (KX894280) and *S.flavogriseus* (KX894280) have positive effect in *Phaseolus vulgaris* growth promotion. Treatments were done in form of seed coating, foliar spray and soil drench in regular intervals. The final result showed that *S. tricolor* (KX894280) is the most effective in plant growth promotion.Growth promotion in *Phaseolus vulgaris* after treatment with three potent isolates is summarized in the Table (26 and 27) and Fig(26 and 27).

Table 26: Growth promotion in *Phaseolus vulgaris* following treatment with actinomycetes

Treatment		Shoot length(cm)			Root length(cm)		
		7 days	**15 days**	**30days**	**7 days**	**15 days**	**30days**
CV2	Untreated Healthy	9.5 ±1.02	10.00±0.12	20.00±1.82	2.1±0.02	3.1±0.02	8.80±0.42
	Treated with *Streptomyces griseus*	9.7±0.98	12.00±0.18	30.00±3.18	2.9±0.20	3.9±1.52	17.00±1.32
	Treated with *S. tricolor*	10.6±1.20	15.15±0.75	33.75±1.70	2.5±0.07	4.7±1.71	15.38±1.07
	Treated with *S. flavogriseus*	11.7±1.03	14.00±0.53	30.25±1.93	3.0±.08	4.1±0.87	20.25±4.87
CV3	Untreated Healthy	8.9±1.15	11.2±0.51	23.25±1.65	2.8±0.04	3.4±1.12	10.80±1.64
	Treated with *S. griseus*	9.2±1.21	13.00±0.27	28.50±2.21	2.3±0.05	3.2±0.92	14.75±3.52
	Treated with *S. tricolor*	10.3±1.10	14.9±0.10	35.50±2.10	3.1±.08	4.8±0.08	17.80±1.08
	Treated with *S. flavogriseus*	9.7±1.11	12.50±1.19	31.50±1.19	2.8±0.14	4.7±0.14	14.83±0.44
CD (P=0.05)	Treatments	1.10	2.81	4.46	1.41	1.25	8.38
	Varieties	0.78	1.99	3.15	1.00	0.88	5.92

Values are mean of 10 plants. ± denote standard errorCV2=Cultivar 2(Jwala), CV3=Cultivar3(Kholar). Treatment with *Streptomyces griseus, Streptomyces tricolor, Streptomyces flavogriseus*

Table 27: Growth promotion in *Phaseolus vulgaris* after treatment with actinomycetes isolates.

Treatment		Leaf number			Leaf area(cm^2)		
		7 days	**15 days**	**30days**	**7 days**	**15 days**	**30days**
C2	Untreated Healthy	2±1.02	3±0.41	11±0.12	15±0.04	23.5±0.1	28.40±4.04
	Treated with *Streptomyces griseus*	3±0.98	5±1.09	13±0.32	18±0.05	29.8±1.51	35.06±0.85
	Treated with *S. tricolor*	4±1.20	8±1.34	15±0.37	21±1.17	45.2±0.97	79.50±4.17
	Treated with *S. flavogriseus*	2±1.03	6±0.44	13±0.77	16.5±0.24	37.9±0.34	62.70±3.64
C3	Untreated Healthy	2±1.15	3±1.14	10±0.61	19.2±1.87	29.2±1.36	49.63±8.37
	Treated with *Streptomyces griseus*	3±1.21	4±0.52	14±1.22	20.5±0.36	35.6±0.16	72.48±4.66
	Treated with *S. tricolor*	2±1.10	4±1.78	17±0.18	23.7±2.32	46.8±2.22	87.18±9.32
	Treated with *S. flavogriseus*	3±1.11	5±1.04	14±0.74	22.01±1.64	41.6±0.64	78.90±5.84
CD (P=0.05)	Treatments	2.83	3.89	2.83	3.17	4.46	28.16
	Varieties	2.00	2.75	2.00	2.24	3.15	19.91

Values are mean of 10 plants. ± denote standard errorC2=Cultivar 2(Jwala), C3=Cultivar 3(Kholar), Treatment with *Streptomyces griseus, Streptomyces tricolor, Streptomyces flavogriseus*

4.7.2. Evaluation of potential Growth promotion of *Vigna radiata* upon treatment

The isolates *Streptomyces griseus* (ARHS/PO/15), *S. tricolor* (ARHS/PO/26) and *S. flavogriseus* (ARHS/PO/27) have positive effect in *Vigna radiata* growth promotion. Treatments were done in form of seed coating, foliar spray and soil drench in regular intervals. The final result showed that *S.tricolor* (ARHS/PO/26) is the most effective in plant growth promotion.Growth promotion in *Vigna radiata* after treatment with three potent isolates is summarized in the Table (28, 29) and Figure (28,29).

Table 28: Growth promotion in *Vigna radiata* following treatment with Actinomycetes

Treatment	**Shoot length(cm)**			**Root length(cm)**		
	7 days	**15 days**	**30days**	**7 days**	**15 days**	**30days**
Untreated Healthy	09.0±01.73	14.0±0.21	19±1.21	2.0±0.67	2.85±0.55	4.52±0.16
Treated with *Streptomyces griseus*	15.0±01.14	19.0±1.63	26±1.73	2.6±0.93	4.7±1.12	9.05±1.74
Treated with *S. tricolor*	13.0±01.05	18.0±1.75	27±1.04	2.5±0.46	4.5±1.22	9.72±0.36
Treated with *S. flavogriseus*	14.0±0.1.73	17.0±1.54	23±1.11	2.3±1.22	4.6±1.33	9.51±0.54

Values are mean of 10 plants. ± denote standard error. Treatment with *Streptomyces griseus, Streptomyces tricolor, Streptomyces flavogriseus*

Table 29: Growth promotion in *Vigna radiata* after treatment with actinomycetes

Treatment	**Leaf number**			**Leaf area(cm^2)**		
	7 days	**15 days**	**30days**	**7 days**	**15 days**	**30days**
Untreated Healthy	4±0.67	6±0.67	16±0.67	6.85±0.25	6.25±1.24	7.78±0.33
Treated with *Streptomyces griseus*	6±0.93	15±0.93	21±0.93	8.8± 1.15	6.3± 1.14	9.49±0.41
Treated with *S. tricolor*	6±0.46	10±0.46	23±0.46	8.55±1.24	6.45±1.22	9.77±0.33
Treated with *S. flavogriseus*	7±1.22	12±1.22	20±1.22	8.6±0.54	6.5±0.73	8.98±0.41

Values are mean of 10 plants. ± denote standard error . Treatment with *Streptomyces griseus, Streptomyces tricolor, Streptomyces flavogriseus*

4.7.3. Effect of treatment upon nodule formation

4.7.3.1. Effect on nodule formation in *Phaseolus vulgaris*

Effect of the potent isolates upon nodulation was evaluated by comparing the nodulation frequency of the isolates in pot condition. Though the nodulation frequency revealed positive effect of treatments on nodule formation, nodulation Index among the treatments do not have much deviation from the control (Figs. 30, 32) and Table 30.

Table 30: Nodulation frequency and Nodulation Index of pot grown one month old *Phaseolus* plants among different treatments

Cultivar	Treatments	Nodule frequency	Nodulation Index
Jwala(CV2)	Untreated Healthy	16.6±1.02	8.5±0.66
	Treated with *Streptomyces griseus*	18.2±0.98	9.7±0.49
	Treated with *S. tricolor*	23.3±1.23	11.5±0.42
	Treated with *S. flavogriseus*	20.9±2.05	10.31±0.01
Kholar (CV3)	Untreated Healthy	17.9±0.74	9.78±0.2
	Treated with *Streptomyces griseus*	19.5±0.23	9.93±1.06
	Treated with *S. tricolor*	19.7±0.02	12.04±1.22
	Treated with *S. flavogriseus*	21.3±0.03	10.55±1.09
CD(P=0.05)	Treatments	5.26	1.10
	Varieties	3.72	0.78

Values are mean of 10 plants. ± denote standard error CV2=Cultivar 2(Jwala), CV3=Cultivar 3(Kholar), Treatment with *Streptomyces griseus, Streptomyces tricolor, Streptomyces flavogriseus*

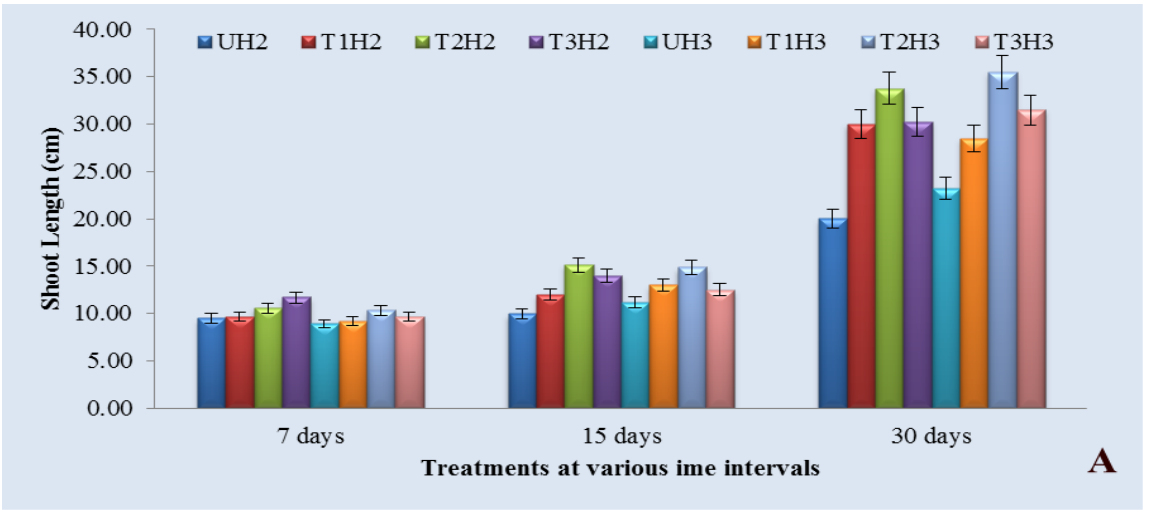
4.7.3.2. Effect on nodule formation in *Vigna radiata*

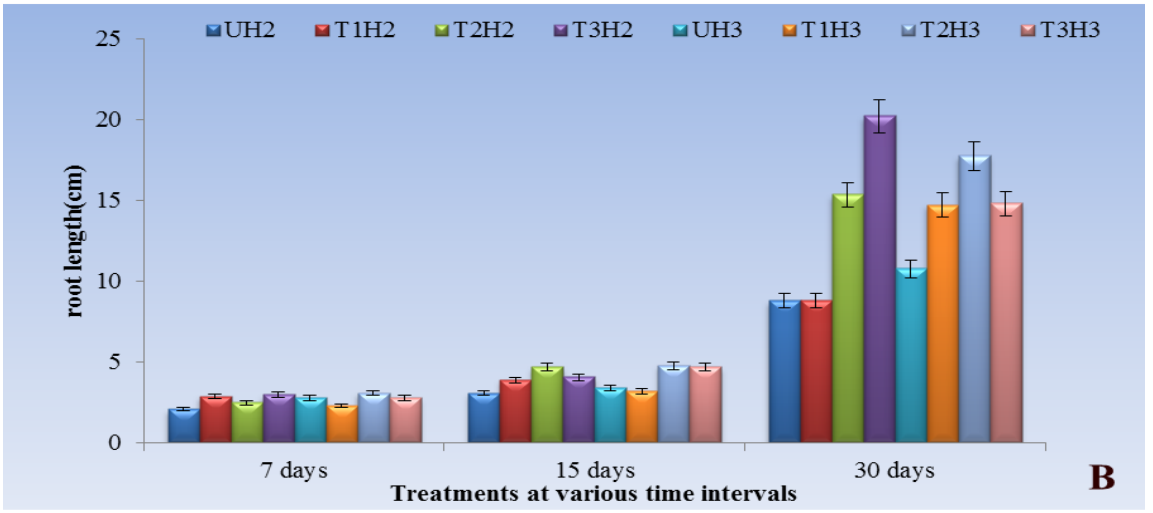
Effect of the potent isolates upon nodulation was evaluated by comparing the nodulation frequency of the isolates in pot condition. Though the nodulation frequency revealed positive effect of treatments on nodule formation, nodulation Index among the treatments do not have much deviation from the control (Fig 31, 33) table 31.

Table 31: Nodulation frequency and Nodulation Index of pot grown one month old *Vigna* plants among different treatments

Treatment	Nodule frequency	Nodulation Index
Untreated Healthy	4.57±0.67	6.0±0.03
Treated with *Streptomyces griseus*	5.87±0.25	7.0±0.05
Treated with *S. tricolor*	8.25±0.09	8.5±0.97
Treated with *S. flavogriseus*	7.25±0.11	6.25±1.05

Values are mean of 10 plants. ± denote standard error. Treatment with *Streptomyces griseus, Streptomyces tricolor, Streptomyces flavogriseus*





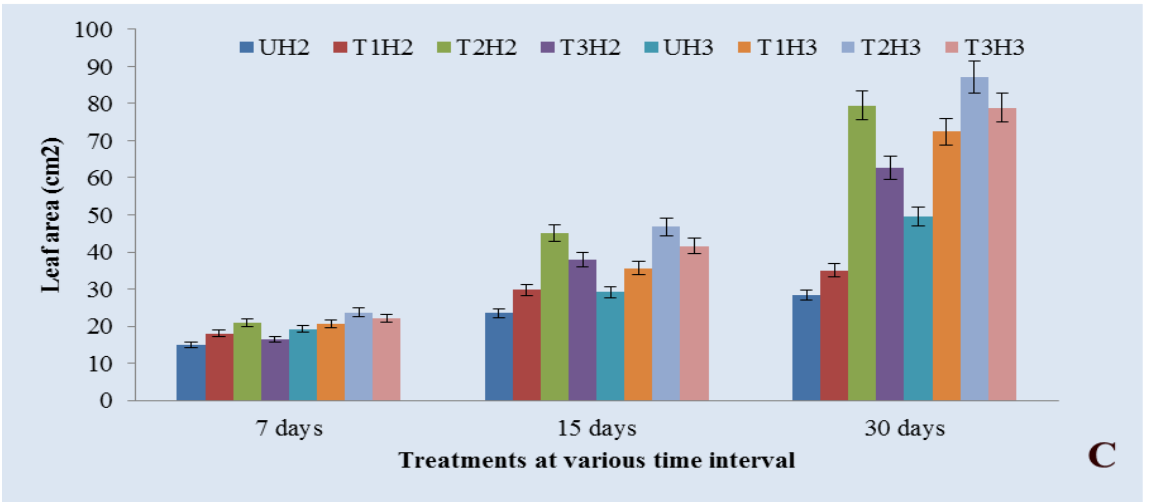


Figure 26: Evaluation of growth promotion (A) Shoot Length , (B) root Length & (C) Leaf Area of varieties of *P vulgaris* treated with actinomycetes formulation before and after challenge inoculation with *Fusarium solani***.** UH2= Untreated Healthy (Jwala/CV2), UH3= Untreated Healthy (Kholar/CV3) T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3= Treated with *Streptomyces flavogriseus.*

Figure 27: (A-L) *Phaseolus vulgaris* in open field and pot condition ; (A-D) *Phaseolus vulgaris* in open field condition ; (E-H) *Phaseolus vulgaris* Cultivar Jwala (CV2) in pot condition,(I-L) *Phaseolus vulgaris* Cultivar Kholar (CV3) in pot condition,(E & I) Control, (F&J) T1, (G&K) T2,(H&L) T3.T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor*,T3= Treated with *Streptomyces flavogriseus.*

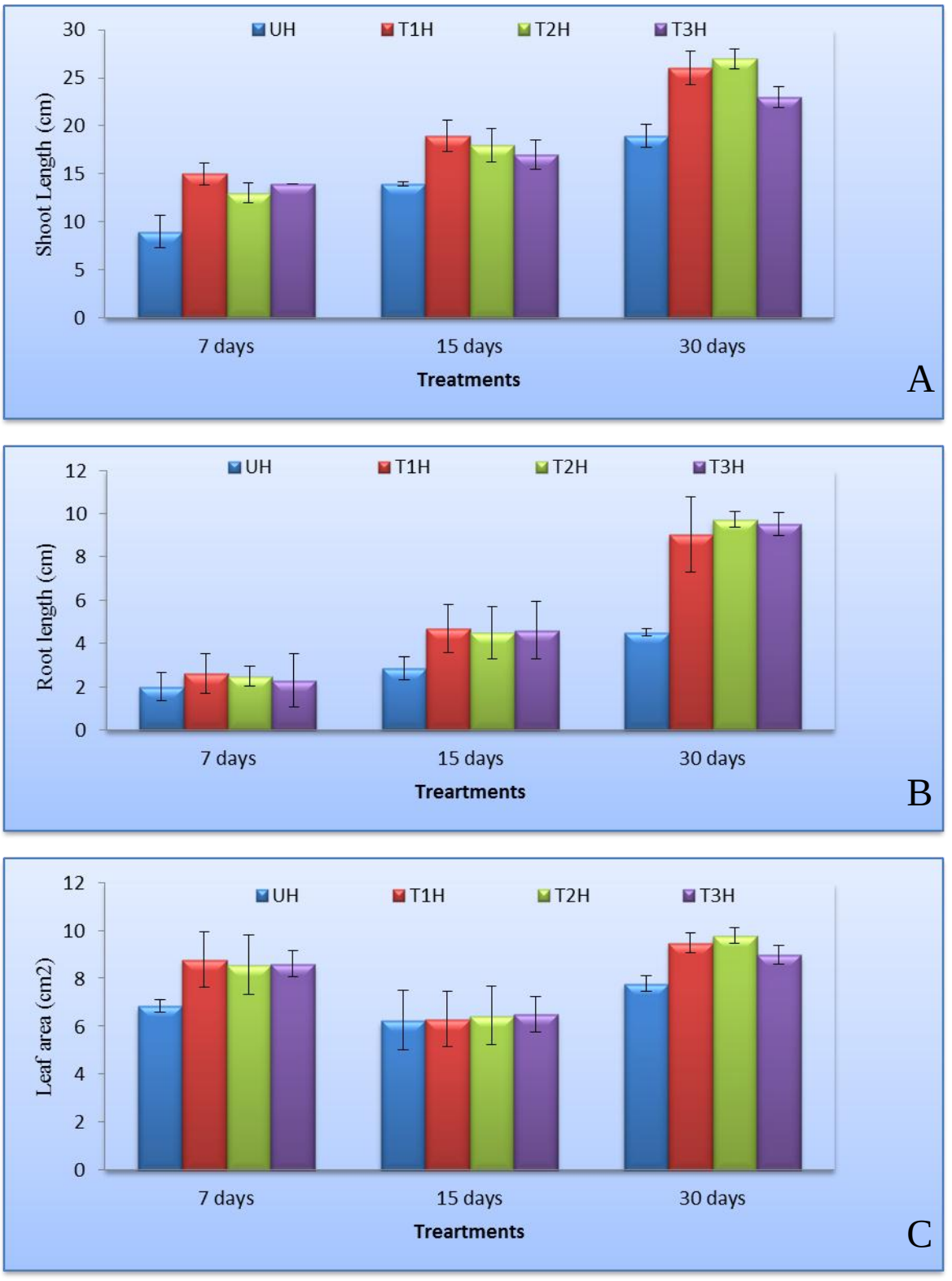


Figure 28: Evaluation of growth promotion (A) Shoot Length , (B) root Length & (C) Leaf Area of *Vigna radiata* treated with actinomycetes formulation before and after challenge inoculation with *Sclerotium rolfsii.* T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3= Treated with *Streptomyces flavogriseus.*

Figure 29: (A-D)*Vigna radiata* in open field condition; A- Control; B- T1; C- T 2; D-T3 (E-H) *Vigna radiata* in pot condition, E- Control; F- T1;G- T 2; H-T3. T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.*

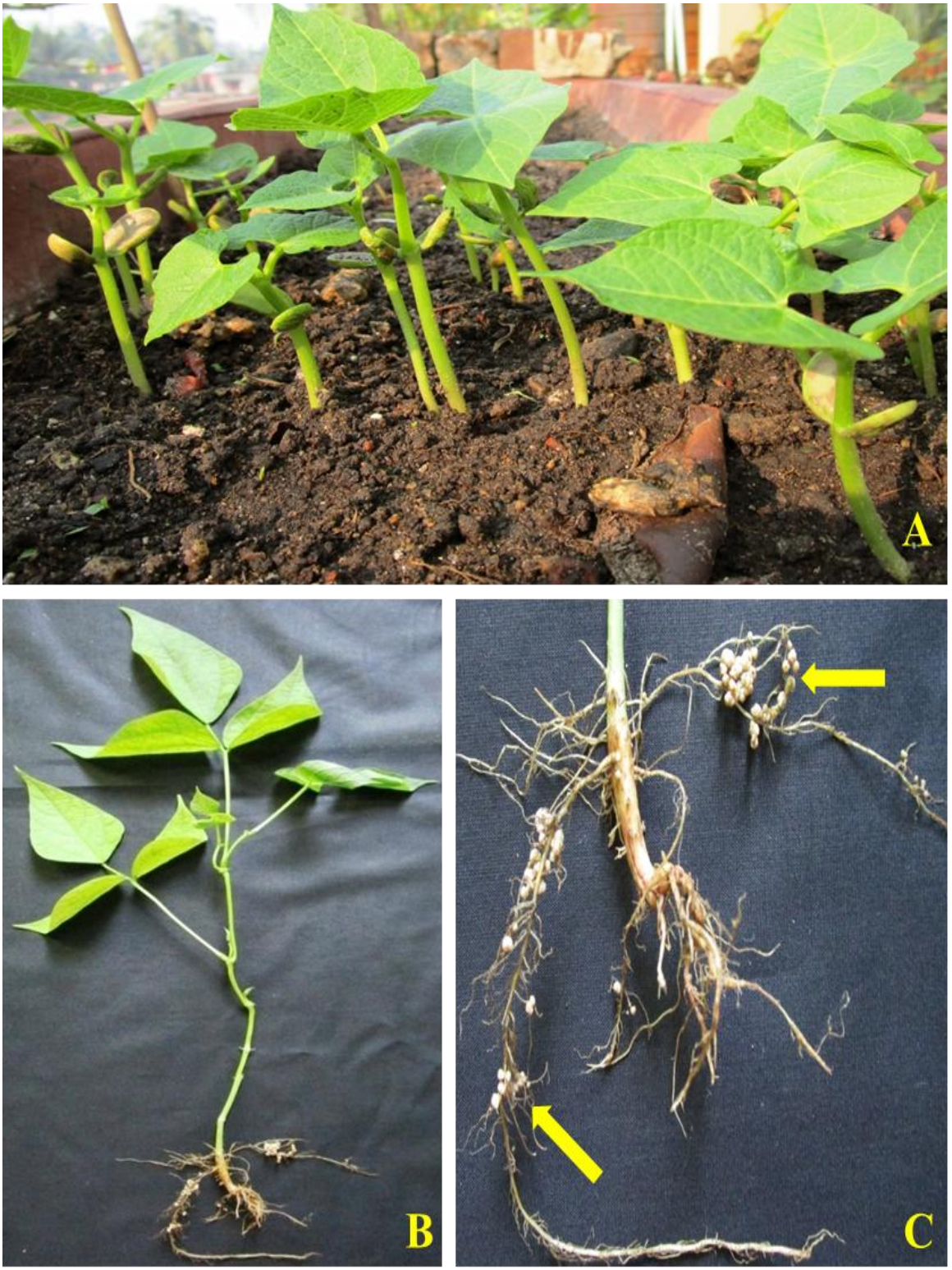


Figure 30: Nodulation of *Phaseolus vulgaris* and the effect following application with actinomycetes. A- Field grown plant ; B-Uprooted plant, C- Root nodulation

Figure 31: Nodulation of *Vigna radiata* and the effect following application with actinomycetes; A- Field grown plant ; B-Uprooted plant; C-Root nodulation

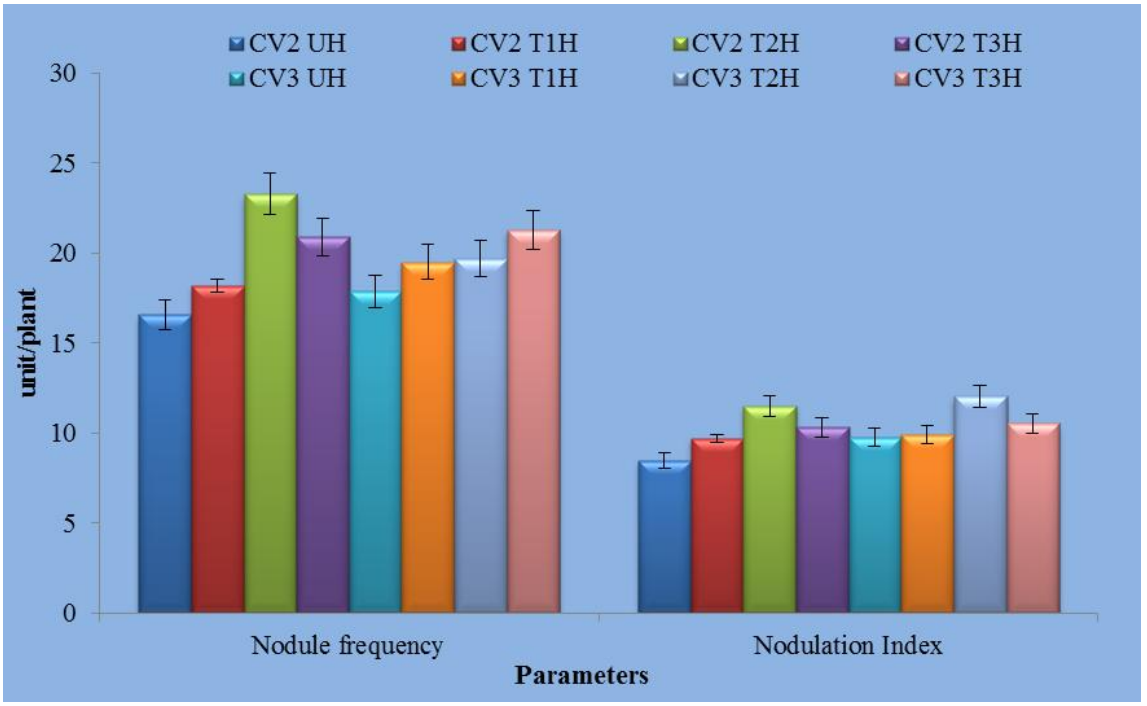


Figure 32: Nodulation frequency and Nodulation Index in *Phaseolus vulgaris* under various treatments in selected cultivars CV2(Jwala) and CV3(Kholar). UH= Untreated Healthy, T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3= Treated with *Streptomyces flavogriseus.*

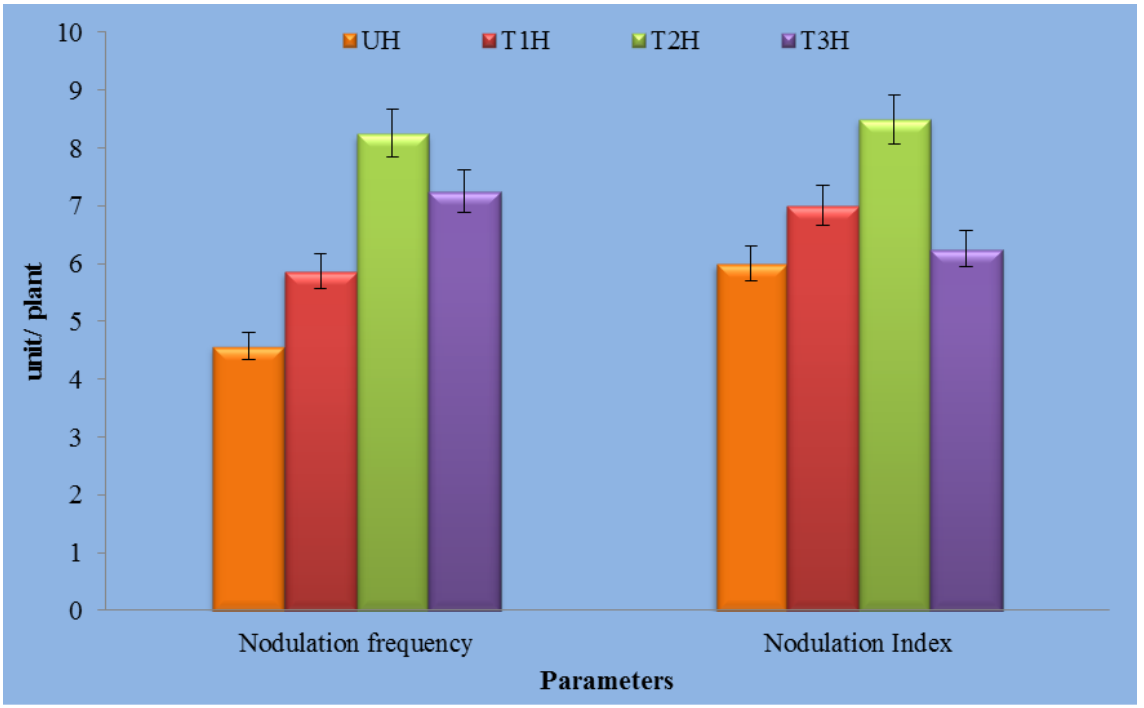


Figure 33: Nodulation frequency and Nodulation Index under various treatments in *Vigna radiata*. UH= Untreated Healthy, T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3= Treated with *Streptomyces flavogriseus.*

4.8. Fungal pathogen

Fungal pathogens were collected from Immuno-phytopathology laboratory, Department of Botany NBU, West Bengal and from National Fungal Collection Centre of India, (NFCCI) Pune ,Maharastra**.**

4.8.1. Fusarium solani

Virulent strain of *Fusarium solani* was initially collected from germplasm of Immuno-phytopathology lab, Dept of Botany, NBU. *In vitro* tests were conducted to prove the Koch's postulates. Another potent virulent strain with accession number NFCCI 606 was collected from NFCCI. Upon further investigation NFCCI Accession number 606 was found to be more active and further experiments were carried out with the same strain.

4.8.2. Sclerotium rolfsii

Virulent strain of *Sclerotium rolfsii* was initially collected from germplasm of Immuno-phytopathology lab, Dept of Botany ,NBU. *In vitro* tests were conducted to prove the Koch's postulates. Another potent virulent strain with accession number NFCCI 1002 was collected from NFCCI. Upon further investigation Accession number 1002 was found to be more active and further experiments were carried out with the same strain.

4.9. Histopathological study of roots of *Phaseolus vulgaris*, before and after pathogen inoculation

To understand and investigate the interaction of the pathogen with the host plant in different conditions, anatomical study with conventional fixations and staining was carried out under bright field microscopy. Root samples were collected prior to challenge inoculation by uprooting plants from the fields and light washing under Jet stream water and surface dried with tissue paper.

Dissection of root sample was done with a blade, various dimensions of transverse sections of samples were obtained and temporarily stored in watch glass. Visual parameters with a fine brush were used to select sections with optimum edge and visibility to open eyes. Differential staining with saffranine and light green and simple staining with lactophenol cotton blue both were applied for study of the healthy as well as the diseased plant tissue(Fig.34) Fungal hyphae penetrating the host tissue were seen in the sections.

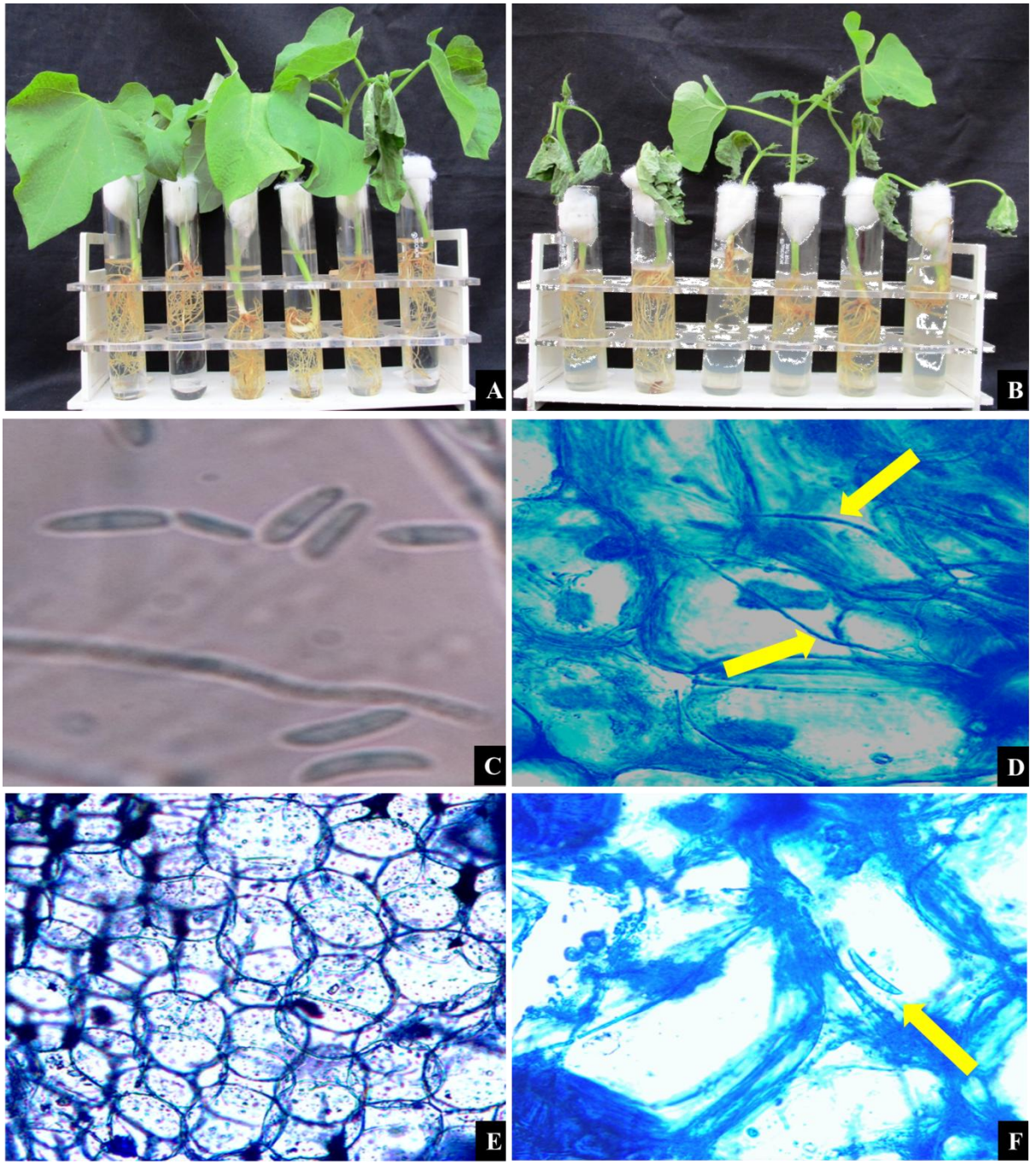


Figure34: Histopathological interaction of host and pathogen in *Phaseolus vulgaris.* A- Control in sterile water, B- Direct contact method with target pathogen, *Fusarium solani* C- Conidia of *F. solani* observed under microscopic field. (D & F)- Hyphal invasion of pathogen inside host tissue ;E-Control tissue section.

4.10. Histopathological study of roots of *Vigna radiata*, before and after pathogen inoculation

To understand and investigate the interaction of the pathogen with the host plant in different conditions, anatomical study with conventional fixations and staining was

carried out under light microscopy. Root samples were collected prior to challange inoculation by uprooting plants from the fields and light washing under steady stream water and surface dried with tissue paper.

Dissection of root sample was done with a blade; various dimensions of transverse sections of samples were obtained and temporarily stored in watch glass. Visual parameter with a fine brush was used to select sections with optimum edge and visibility to open eyes. Differential staining with safranin and light green and simple staining with lactophenol cotton blue both were applied for study of the healthy as well as the diseased plant tissue (Fig .35)

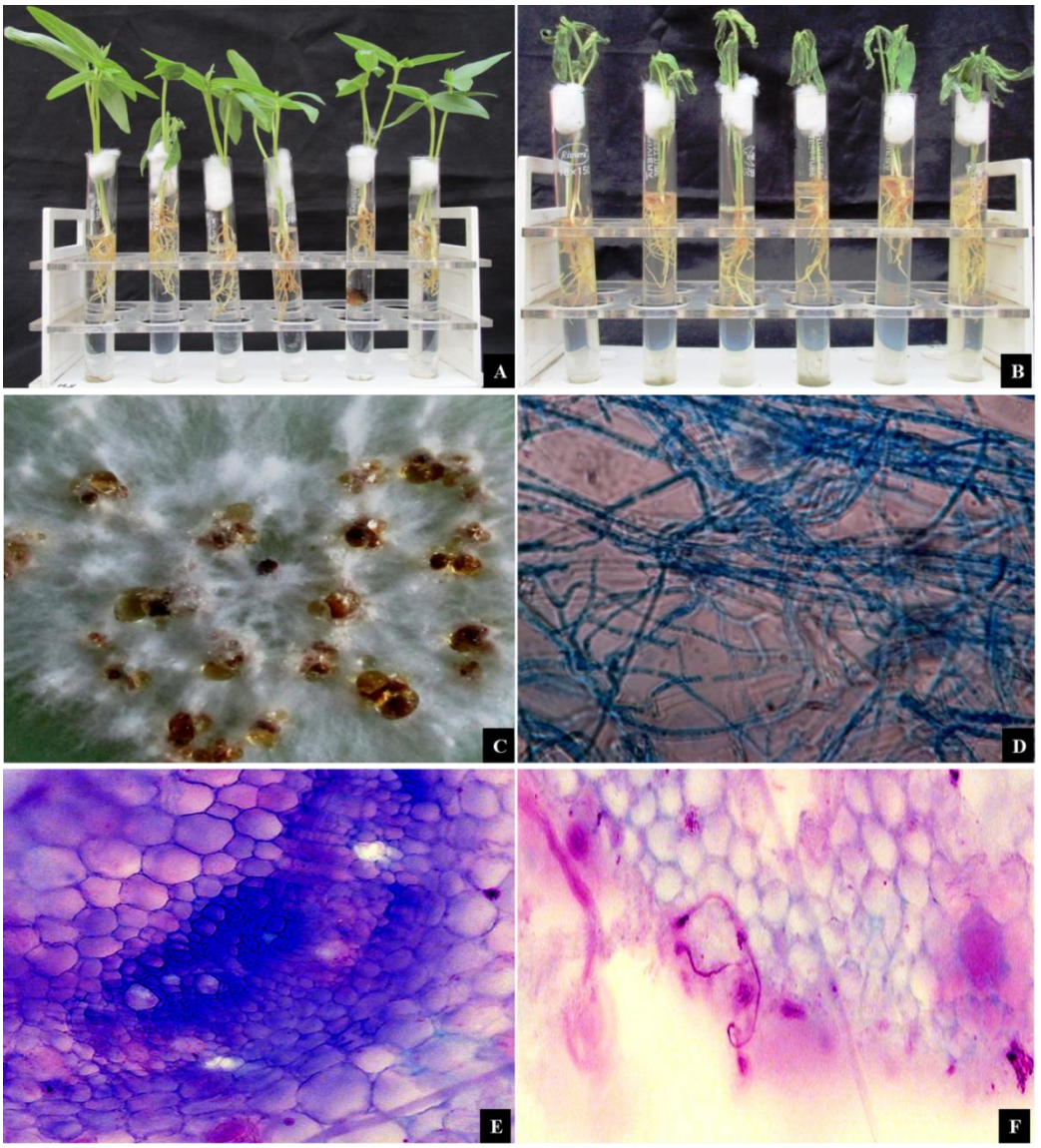


Figure 35: (A-F) . Histopathological interaction of Host & Pathogen in *Vigna radiata* .A- Control in sterile water, B- Direct contact method with target pathogen, *Sclerotium rolfsii* C- sclerotia of *S.rolfsii* observed under microscopic field. (D & F)-Hyphal invasion of pathogen inside host tissue ; E-Control tissue section.

4.11. Influence of Actinomycetes isolates on Fusarial root rot of *Phaseolus vulgaris*

4.11.1. Disease development

Effect of three actinomycetes isolates *Strptomyces griseus, S. tricolor, S. flavogriseus* in development of fusarial root rot of *Phaseolus vulgaris* caused by *Fusarium solani* was determined (Fig 36). 7 days old seedlings of *Phaseolus* was inoculated with *F. solani* and disease assessment was done after 7, 14, 21 and 28 days of inoculation. The disease index of the plants was recorded. The disease severity increased with time reaching the highest pick at the end of 28 days. It was observed that when the plants were pretreated with *Strptomyces griseus, S. tricolor,* and *S. flavogriseus* the disease severity was lower than the untreated control. *Strepomyces flavogriseus* was most effective in reducing the disease followed by *S. tricolor* and *S. griseus*.

4.11.2. Percent Disease Index (PDI%)

The disease severity in *Phaseolus vulgaris* inoculated with the pathogen *Fusarium solani* increased with time reaching a maximum value after 28 days (Table 32). But when the soil was pretreated with Actinomycetes isolates the maximum disease severity was reduced. *Streptonyces flavogriseus* (ARHS/PO/27) was the most effective to inhibit the root rot disease followed by *S. tricolor* (ARHS/PO/26) and *S. griseus* (ARHS/PO/15) (Fig 42, 43).

Table 32: Fusarial root rot development in the roots of *Phaseolus vulgaris* in presence and absence of actinomycetes isolates in pot condition

Treatments		**7 d**	**14d**	**21d**	**28d**
Jwala (CV2)	Control	20	35	53	90
	T1	8.3	23.33	41.66	66.66
	T2	6.6	15	33.33	53.33
	T3	5	10	20	30
Kholar (CV3)	Control	20	40	53.33	75
	T1	5	12	23.33	53.33
	T2	3.3	7.5	16.6	35
	T3	1.6	8.3	15	23.33

4.11.3. Associated Biochemical changes

Application of *Streptomyces griseus, S. tricolor, S. flavogriseus* to soil was found to affect the biochemical properties of plants. Disease establishment also affect the

biochemical characters. So the biochemical response of both the root and leaves of plants following application of actinomycetes isolates and challenge inoculated with the pathogen *Fusarium solani* was determined. The conference of resistance towards the pathogen was evaluated in terms of enhancement of key defence enzymes- PAL, POX,GLU and CHT in both the root and leaves of *Phaseolus vulgaris* (Figs. 37, 39, 40, 41). The results showed that the level of defence enzyme increases in plants treated with actinomycetes isolates and challenge inoculated with the pathogen followed by only treated plants. Amount of defence enzyme was higher in pathogen challenged plants than control plants. Total phenol content of the roots and leaves of plants were also evaluated which showed significant increase in treated plants (Fig 38).

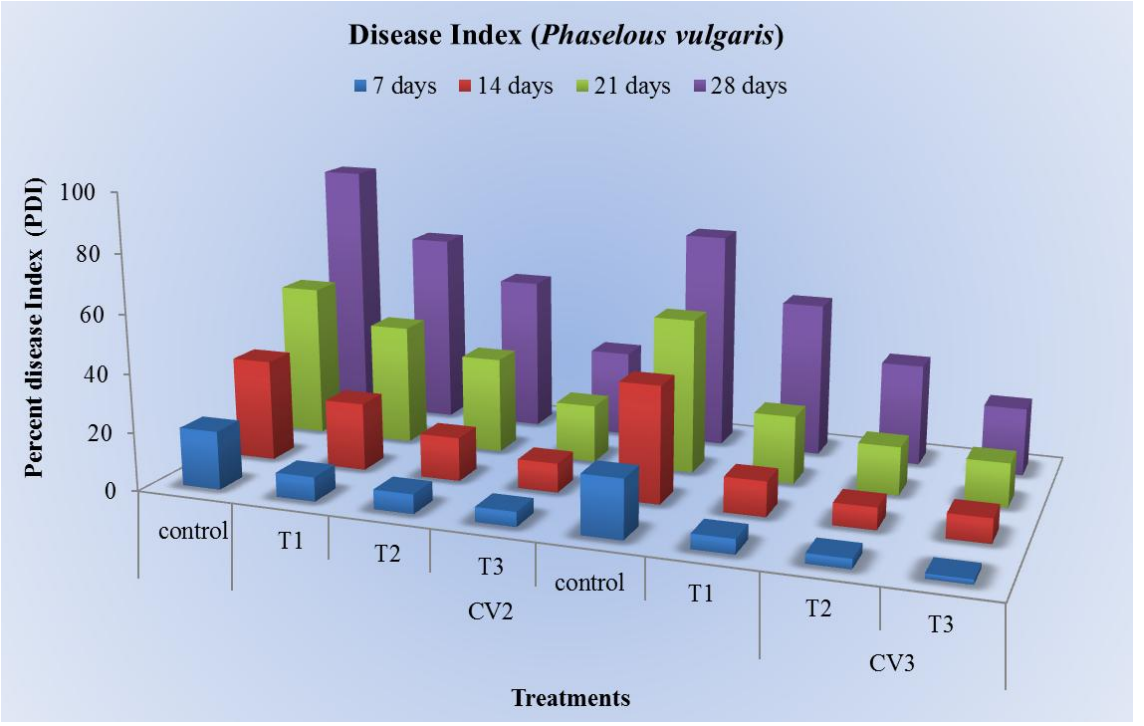


Figure36: Disease index (Evident symptom of Disease) after challenge inculcation with target pathogen (*Fusarium solani*) and host response upon respective isolate treatments in two cultivars of *Phaseolus vulgaris.* T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3=Treated with *Streptomyces flavogriseus*

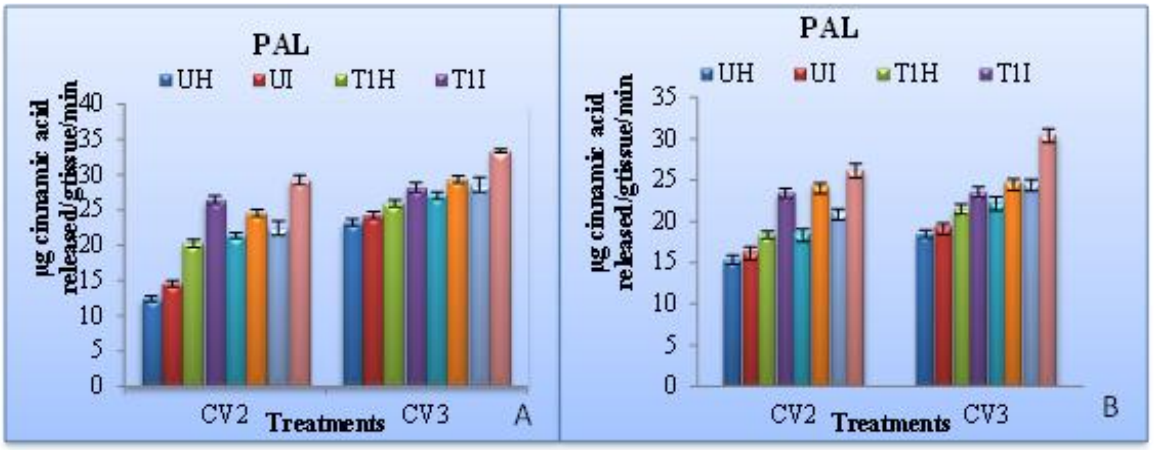


Figure 37: PAL activity in (A) leaf & (B) roots of varieties of *P. vulgaris* treated with actinomycetes formulation before and after challenge inoculation with *Fusarium solani;* UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.*CV2=Jwala, CV3=Kholar

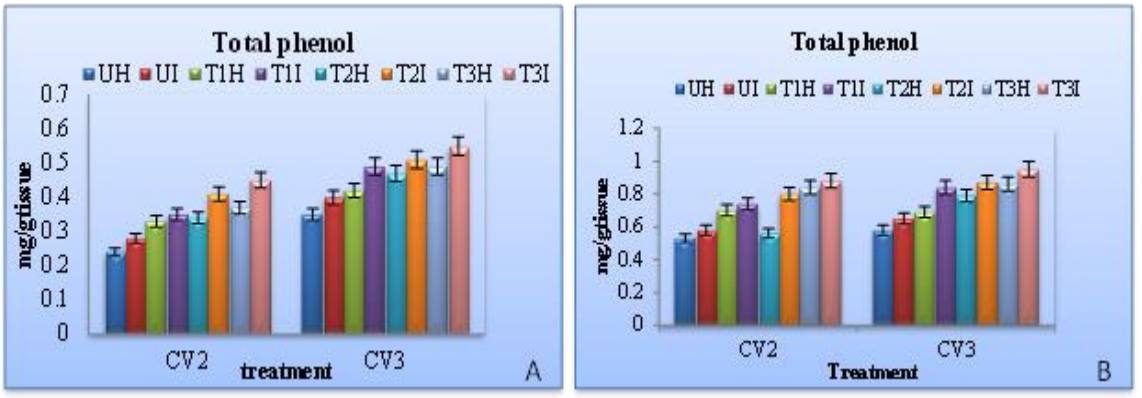


Figure 38: Total phenol content in (A) leaf & (B) roots of varieties of *P. vulgaris* treated with actinomycetes formulation before and after challenge inoculation with *Fusarium solani*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.* CV2=Jwala, CV3=Kholar

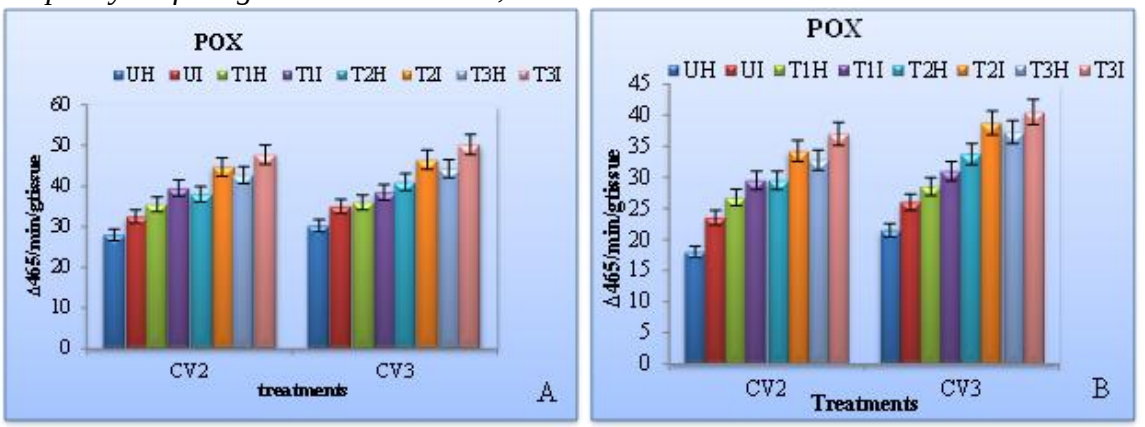


Figure 39: Peroxidase activity in (A) leaf & (B) roots of varieties of *P. vulgaris* treated with actinomycetes formulation before and after challenge inoculation with *Fusarium solani*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.* CV2=Jwala, CV3=Kholar

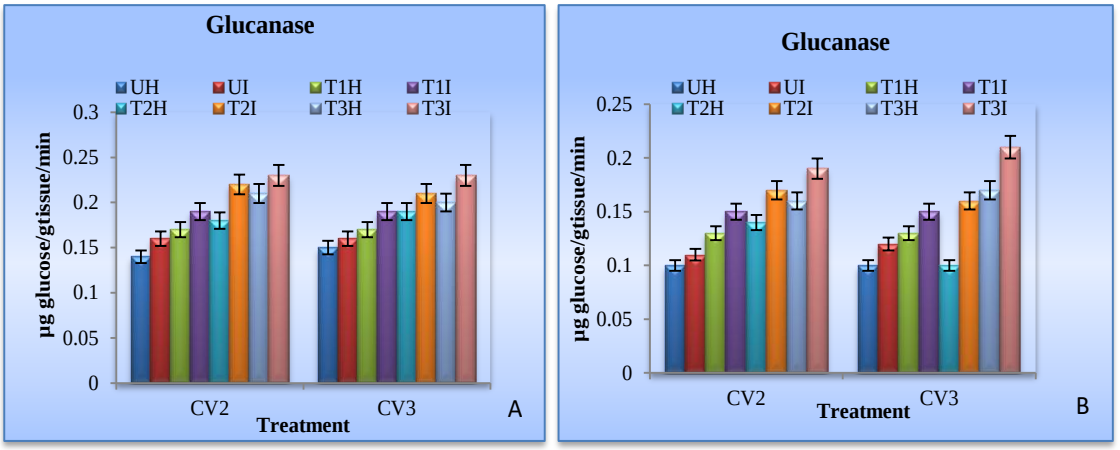


Figure 40: β-1,3 Glucanase activity in (A) leaf & (B) roots of varieties of *P. vulgaris* treated with actinomycetes formulation before and after challenge inoculation with pathogen *Fusarium solani*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3=Treated with *Streptomyces flavogriseus* CV2=Jwala, CV3=Kholar

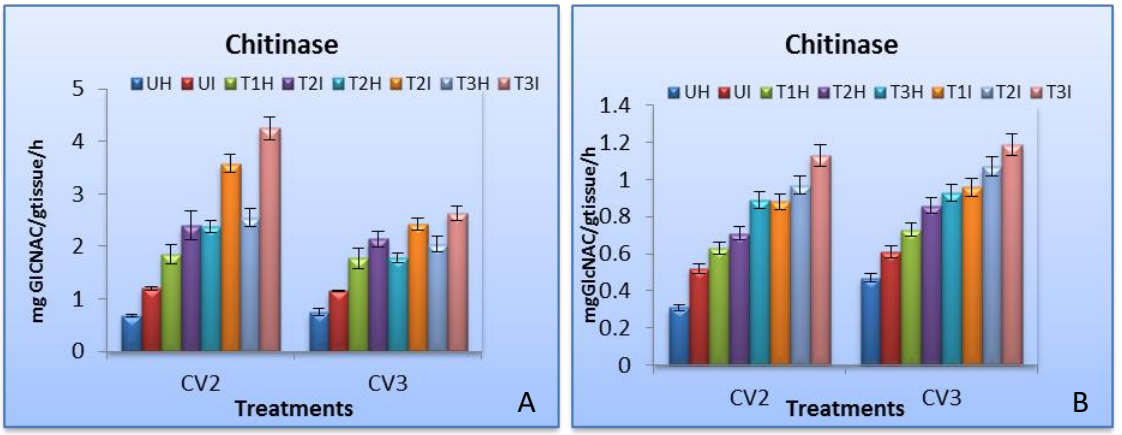


Figure41: Chitinase activity in (A) leaf & (B) roots of varieties of *P vulgaris* treated with actinomycetes formulation before and after challenge inoculation with *Fusarium solani*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3=Treated with *Streptomyces flavogriseus.* CV2=Jwala, CV3=Kholar

Figure 42: (A-D) *Phaseolus vulgaris* (Cultivar 2, Jwala), in pot condition, A- Control, Untreated Healthy (UH). B- Control, Untreated Inoculated (UI). C- T1, Treated Healthy (T1H). D- T1 Treated Inoculated (T1I), E- T2 Treated Healthy (T2H), F-T2, Treated Inoculated (T2I), G- T3, Treated Healthy (T3H). H-T3, Treated Inoculated (T3I)

T1= treated with *Streptomyces griseus*, *T2=Streptomyces tricolor,* T3= *Streptomyces flavogriseus,* Inoculated with *Fusarium solani*

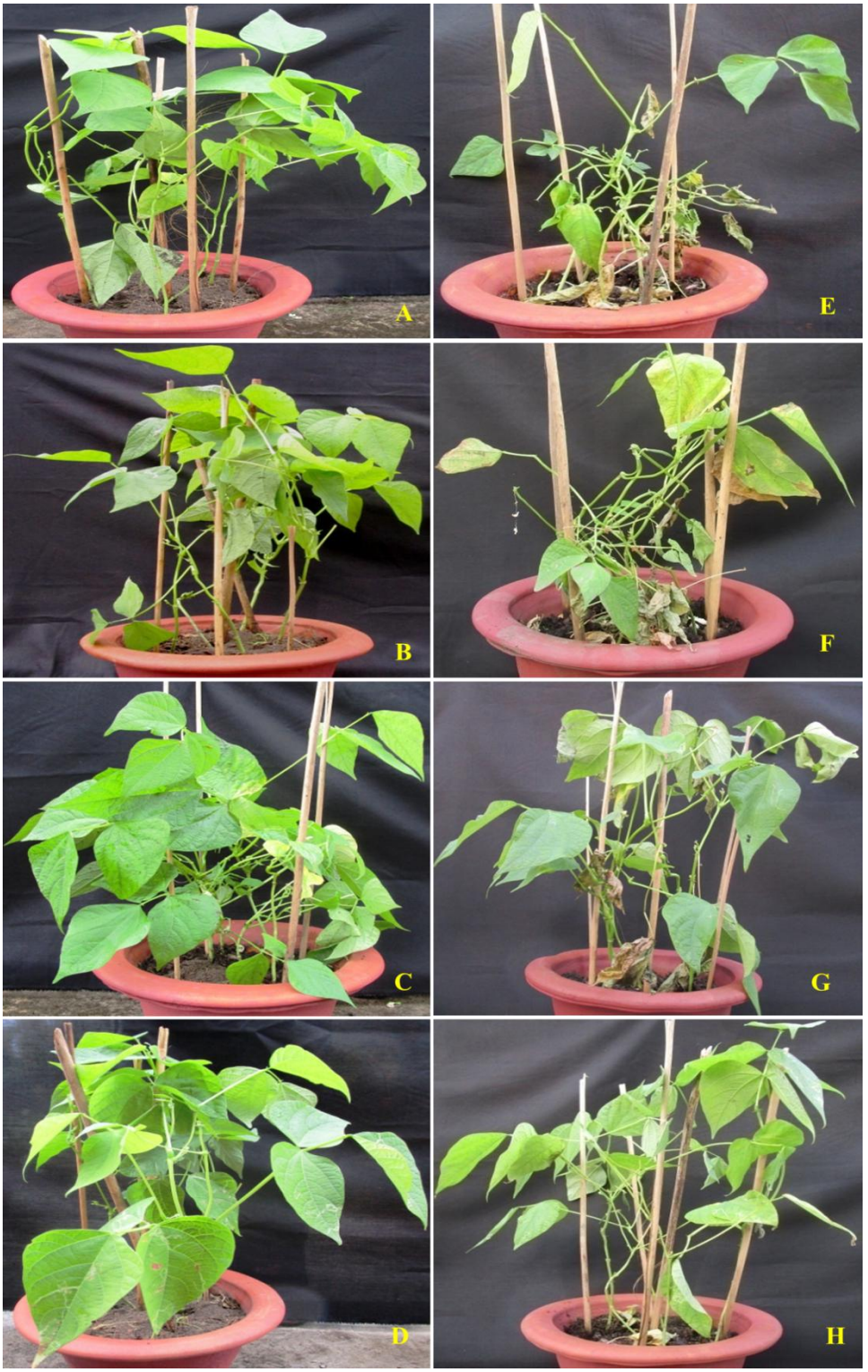


Figure 43: (E-H) *Phaseolus vulgaris* (Cultiver 3, Kholar), in pot condition, A- Control, Untreated Healthy (UH). B- Control, Untreated Inoculated (UI). C- T1, Treated Healthy (T1H). D- T1 Treated Inoculated (T1I), E- T2 Treated Healthy (T2H), F-T2, Treated Inoculated (T2I), G- T3, Treated Healthy (T3H). H-T3, Treated Inoculated (T3I)

T1= treated with *Streptomyces griseus*, *T2=Streptomyces tricolor*, T3= *Streptomyces flavogriseus*, Inoculated with *Fusarium solani.*

4.12. Influence of Actinomycetes isolates on sclerotial root rot of *Vigna radiata*

4.12.1. Disease development

Effect of three actinomycetes isolates *Streptomyces griseus, S. tricolor, S. flavogriseus* in development of sclerotial root rot of *Vigna radiata* caused by *Sclerotium rolfsii* was determined (Fig. 44).7 days old seedlings of *Vigna* was inoculated with *S. rolfsii* and disease assessment was done after 5, 10,15,21 and 28 days of inoculation. The disease index of the plants was recorded. The disease severity increased with time reaching the highest pick at the end of 30 days. It was observed that when the plants were pretreated with *Strptomyces griseus, S. tricolor,* and *S. flavogriseus* the disease severity was lower than the untreated control. *Strepomyces tricolor* was most effective in reducing the disease followed by *S. flavogriseus* and *S. griseus*.

4.12.2. Percent Disease Index (PDI%)

The disease severity in *Vigna radiata* inoculated with the pathogen *Sclerotium rolfsii* increased with time reaching a maximum value after 30 days. But when the soil was pre-treated with actinomycetes isolates the maximum disease severity was reduced to 53.3% in *Streptomyces tricolor* treated plants (Table 33) (Fig 50).

Table33: Sclerotal root rot development in the roots of *Vigna radiata* in presence and absence of actinomycetes isolates in pot condition

Treatments	7d	15d	21d	28d
Control	15	35	60	90
T1	8.3	20	46.66	66.66
T2	6.6	15	35	53.3
T3	7.8	16.6	40	60

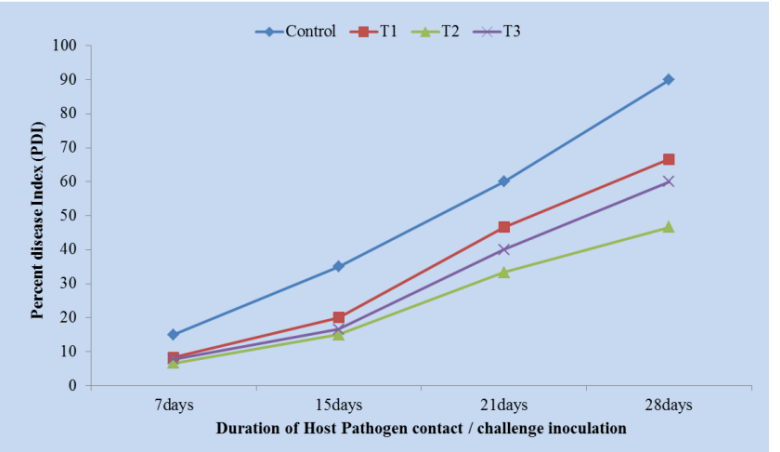


Figure 44: Disease index (Evident symptom of Disease) after challenge inculcation with target pathogen(*Sclerotium rolfsii*) and host response upon respective isolate treatments in *Vigna radiata.* T1= treated with *Streptomyces griseus, T2=Streptomyces tricolor,* T3= *Streptomyces flavogriseus*

4.12.3. Biochemical changes

Application of *S. griseus*, *S.tricolor*, *S. flavogriseus* to soil was found to affect the biochemical properties of plants. Disease establishment also affect the biochemical characters. So the biochemical response of both the root and leaves of plants following application of actinomycetes isolates and challenge inoculated with the pathogen *Sclerotium rolfsii* was determined. The conference of resistance towards the pathogen was evaluated in terms of enhancement of key defence enzymes- PAL, POX, GLU and CHT in both the root and leaves of *Vigna radiata*. The results showed that the level of defence enzyme increases in plants treated with actinomycetes isolates and challenge inoculated with the pathogen followed by only treated plants. Amount of defence enzyme was higher in pathogen challenged plants than control plants (Figs. 45, 47, 48, 49). Total phenol content of the roots and leaves of plants were also evaluated which showed significant increase in treated plants (Fig 46).

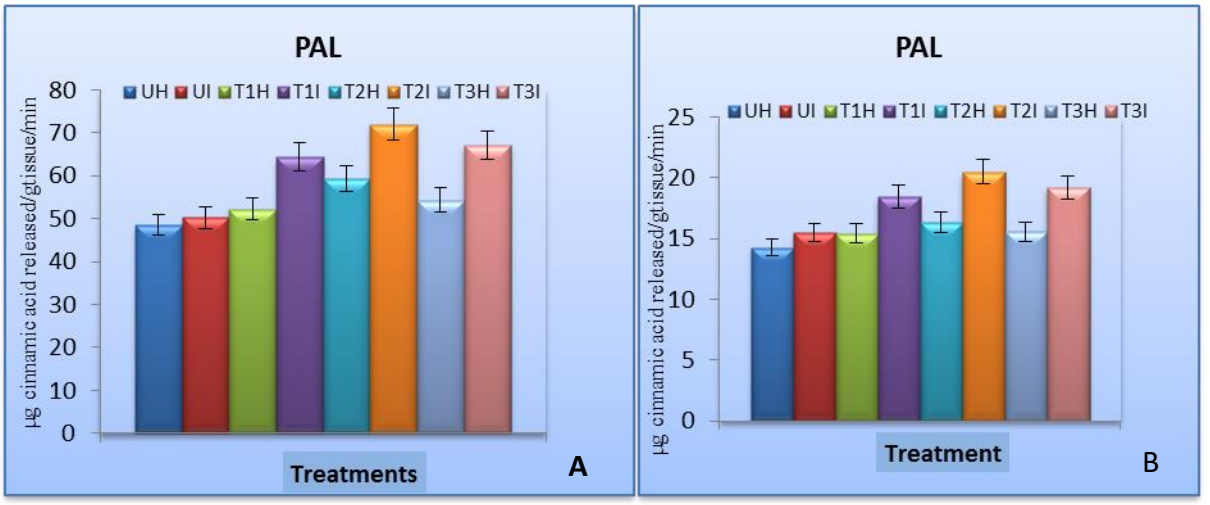


Figure 45: PAL activity in (A) leaf & (B) roots of varieties of *V radiata* treated with actinomycetes formulation before and after challenge inoculation with *Sclerotium rolfsii*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3=Treated with *Streptomyces flavogriseus.*

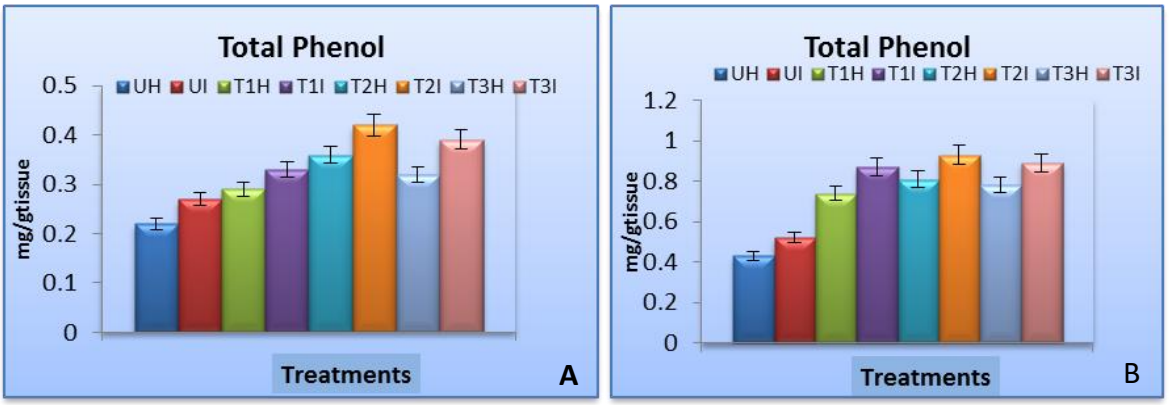


Figure 46: Total phenol content in (A) leaf & (B) roots of varieties of *V radiata* treated with actinomycetes formulation before and after challenge inoculation with *Sclerotium rolfsii*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3=Treated with *Streptomyces flavogriseus.*

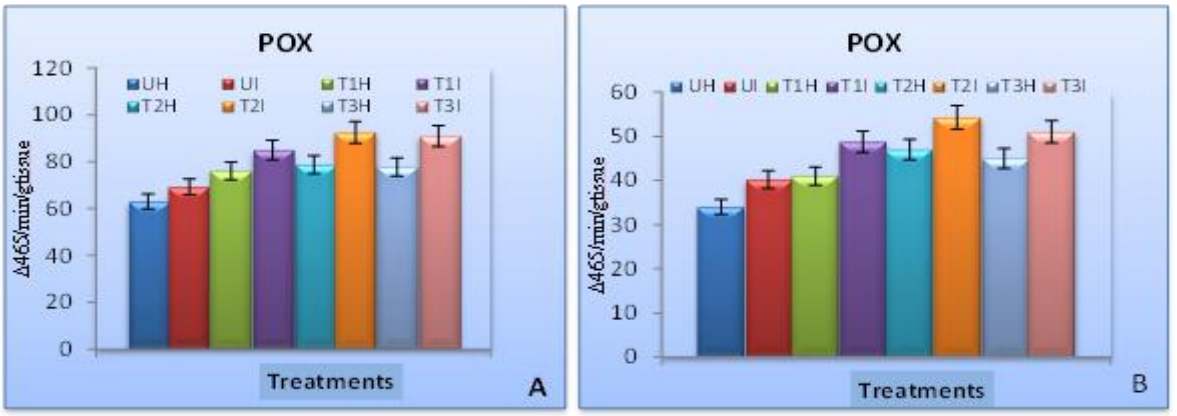


Figure 47: Peroxidase activity in (A) leaf & (B) roots of varieties of *V radiata* treated with actinomycetes formulation before and after challenge inoculation with *Sclerotium rolfsii*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3=Treated with *Streptomyces flavogriseus.*

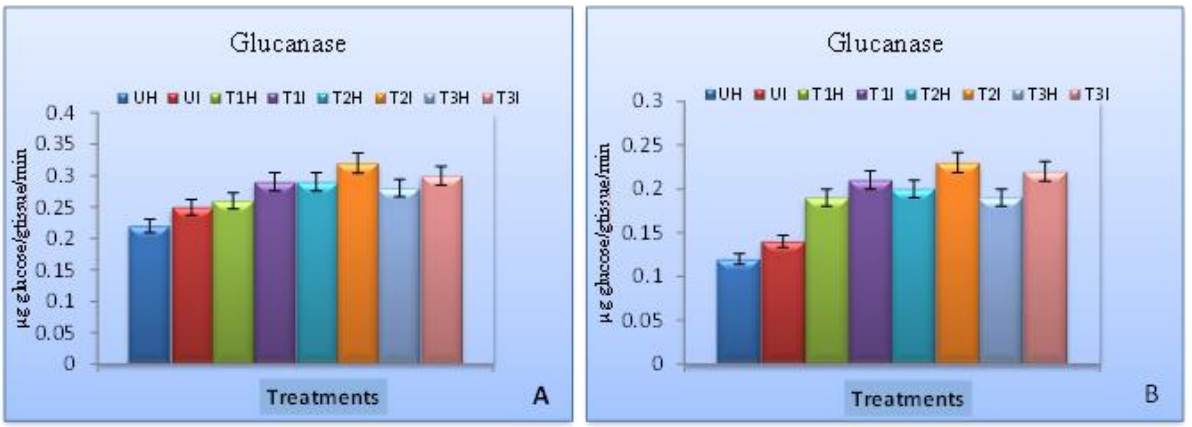


Figure 48: . β 1-3 Glucanase activity in (A) leaf & (B) roots of varieties of *V radiata* treated with actinomycetes formulation before and after challenge inoculation with pathogen *Sclerotium rolfsii*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus*, T2= Treated with *Streptomyces tricolor*, T3=Treated with *Streptomyces flavogriseus.*

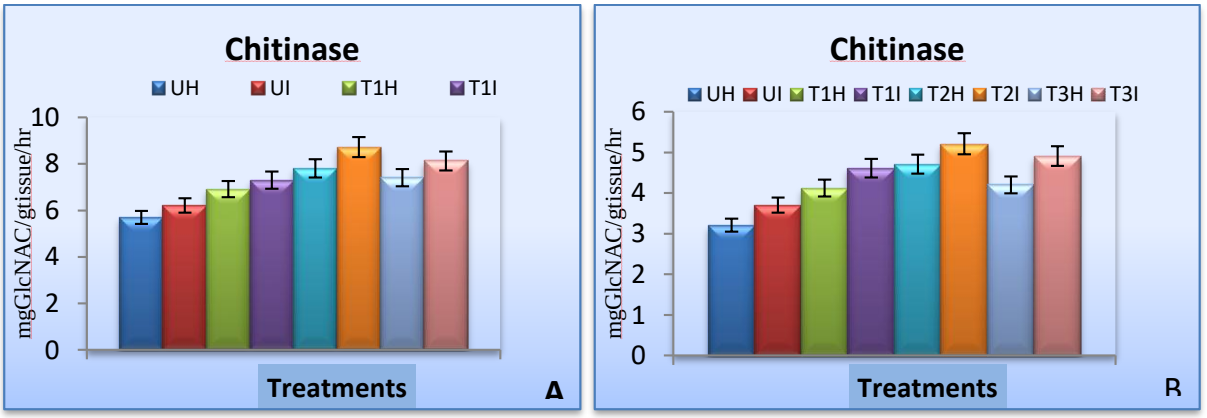


Figure 49: Chitinase activity in (A) leaf & (B) roots of varieties of *V radiata* treated with actinomycetes formulation before and after challenge inoculation with *Sclerotium rolfsii*; UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.*

4.13. Artificial inoculation of *Phaseolus vulgaris* and *Vigna radiata* with actinomycetes formulation and associated changes in protein content before and after fungal inoculation

4.13.1. Total protein in *Phaseolus vulgaris*

Total protein was extracted from root and leaf tissue of *Phaseolus vulgaris* plants. The plants were artificially inoculated with *Fusarium solani* and treated with actinomycetes formulation. Result showed that protein content was higher in treated plants in comparison to the untreated healthy plant. The increase was highest in case of plants challenge inoculated with the pathogen, *Fusarium solani*. Protein content in the leaves was higher than the root. Detailed information can be obtained from the Table 34 and Table 35.

Table34. Protein content in Leaf of varieties of *P. vulgaris* treated with actinomycetes formulation before and after inoculation with pathogen

Cultiver	Treatments	Activity (mg/gm tissue)
Jwala (CV2)	UH	22.04±0.02
	UI	31.11±0.09
	T1H	33.12±0.09
	T2H	37.23±0.13
	T3H	40.25±0.13
	T1I	49.28±0.17
	T2I	53.13±0.09
	T3I	61.31±0.18
Kholar(CV3)	UH	19.39±0.12
	UI	23.55±0.18
	T1H	26.46±0.18
	T2H	37.08±0.02
	T3H	36.63±0.20
	T1I	61.29±0.17
	T2I	69.06±0.02
	T3I	67.37±0.17
CD(P=0.05)	Treatments	14.60
	Varieties	7.30

UH=Untreated healthy, UI= Untreated Inoculated, T1=treated with *Streptomyces griseus*, T2= treated with *S. tricolor*, T3= treated with *S. flavogriseus*

Table 34 a. ANOVA of the data in table 34 (Total Protein content)

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	3786.5	7	540.9285	14.17963	0.0012	3.78704354
Columns	11.1556	1	11.1556	0.292427	0.60544	5.591447848
Error	267.038	7	38.14829			
Total	4064.693	15				

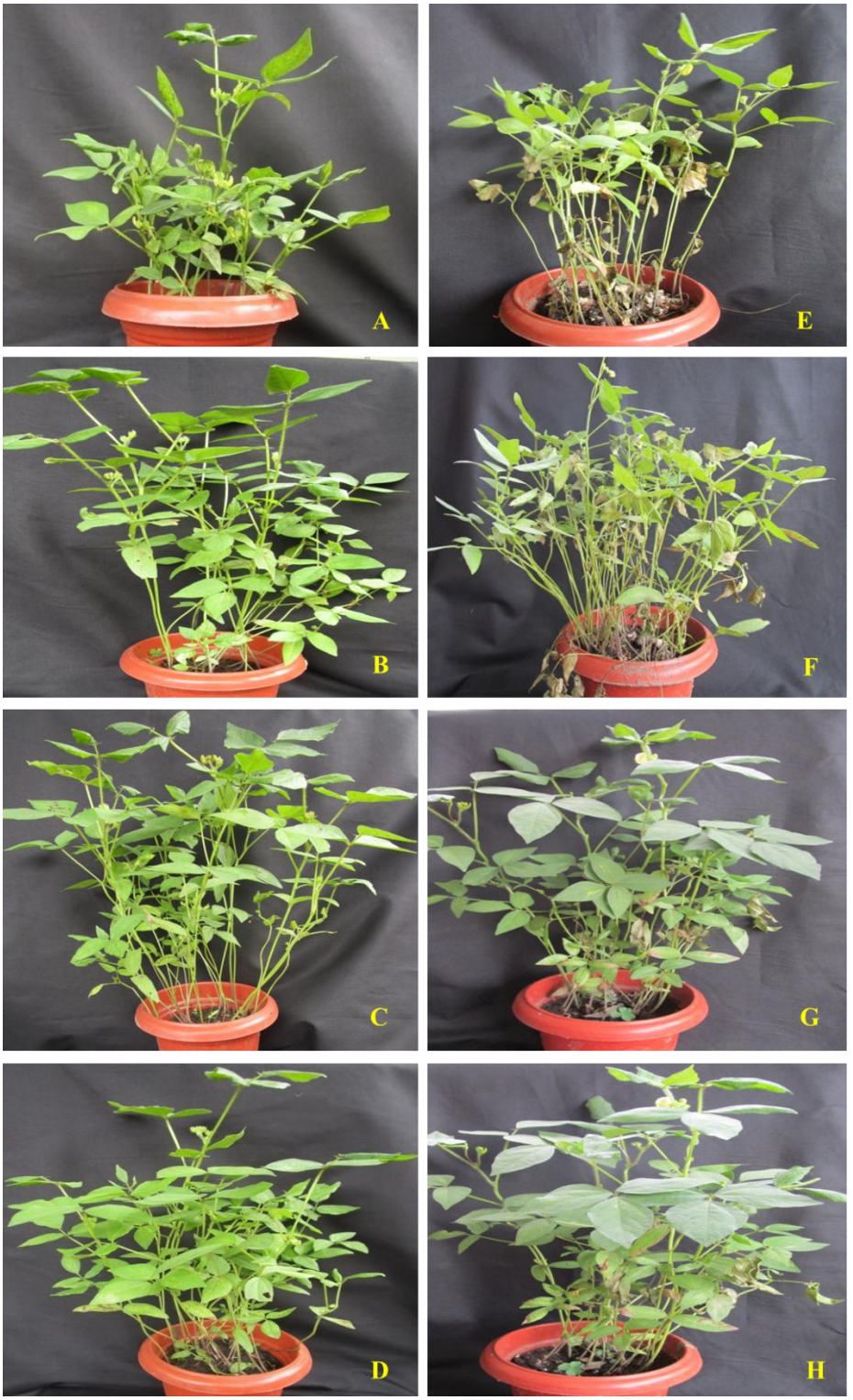


Figure 50: (A-D) *Vigna radiata*, in pot condition, A- Control Untreated Healthy (UH), B- T1 Treated Healthy (T1H), C- T2 Treated Healthy (T2H), D- T3 Treated Healthy (T3H). T1= treated with *Streptomyces griseus*, *T2=Streptomyces tricolor*, T3= *Streptomyces flavogriseus*. (E-H) *Vigna radiata*, in pot condition, E- Control Untreated Inoculated (UI), F- T1 Treated Inoculated (T1I), G- T2 Treated Inoculated (T2I), H- T3 Treated Inoculated (T3I), challenge pathogen *Sclerotium rolfsii* after 7 days under glass house condition.

Table 35. Protein content in root of varieties of *P. vulgaris* treated with actinomycetes formulation before and after inoculation with pathogen.

Cultiver	Treatments	Activity (mg/gm tissue)
Jwala (CV2)	UH	6.06±0.02
	UI	9.15±0.25
	T1H	11.33±0.11
	T2H	16.25±0.03
	T3H	18.20±0.11
	T1I	11.83±0.25
	T2I	18.25±0.18
	T3I	22.33±0.13
Kholar(CV3)	UH	11.48±0.05
	UI	12.10±0.13
	T1H	14.36±0.16
	T2H	15.19±0.14
	T3H	14.48±0.16
	T1I	19.34±0.18
	T2I	24.72±0.04
	T3I	20.43±0.11
CD(P=0.05)	Treatments	4.76
	Varieties	2.38

UH=Untreated healthy, UI= Untreated Inoculated, T1=treated with *Streptomyces griseus*, T2= treated with *S. tricolor*, T3= treated with *S. flavogriseus*

Table 35 a. ANOVA of the data in table 35 (Total Protein content)

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	321.5544	7	45.93634	11.30142	0.002424	3.78704354
Columns	29.43063	1	29.43063	7.240623	0.031048	5.591447848
Error	28.45258	7	4.064654			
Total	379.4376	15				

4.13.2. Total protein in *Vigna radiata*

Total protein was extracted from root and leaf tissue of *Vigna radiata* plants. Result showed that protein content was higher in treated plants in comparison to the untreated healthy plant. The increase was highest in case of plants challenge inoculated with the pathogen *Sclerotium rolfsii*. Protein content in the leaves was higher than the root. Detailed information can be obtained from the Figure 51.

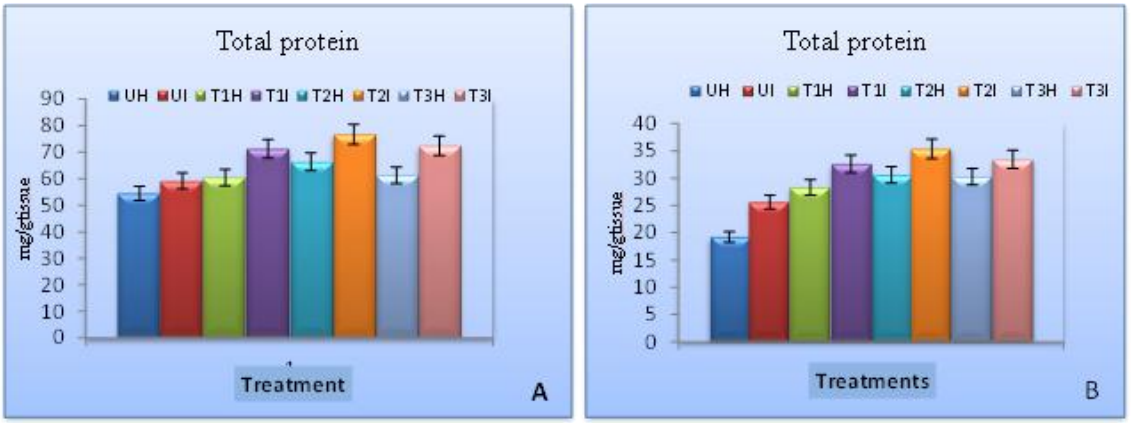


Figure 51: Total protein content in (A) leaf & (B) roots of varieties of *V radiata* treated with actinomycetes formulation before and after challenge inoculation with *Sclerotium rolfsii* **.** UH= Untreated healthy, UI=Untreated Inoculated, T1= Treated with *Streptomyces griseus,* T2= Treated with *Streptomyces tricolor,* T3=Treated with *Streptomyces flavogriseus.*

4.14. Tissue and cellular location of Chitinase and Glucanase enzyme by FITC labelling in *Phaseolus vulgaris*

Apart from the quantitative assessment of plant defence enzyme ,localisation of chitinase enzyme in the plant tissue was also observed by FITC labelling. Root and leaf samples were collected from treated and inoculated plants. Samples from healthy plants without any treatment were also collected for immunofluorescence study. Main objective of the study was to localize chitinaseat the cellular level in the root and leaf tissue of *Phaseolus vulgaris*. Cross section of the root and leaf tissue were treated with normal antisera and PAb of Chitinase and labeled with FITC. The treated root and leaves showed bright apple green fluorescence in the epidermal and cortical tissue which is indication of localization of Chitinase in this region. So, strong reaction with FITC in plant tissue indicated the induction of chitinase (PR 3) in *Phaseolus* (Figs. 52-54).

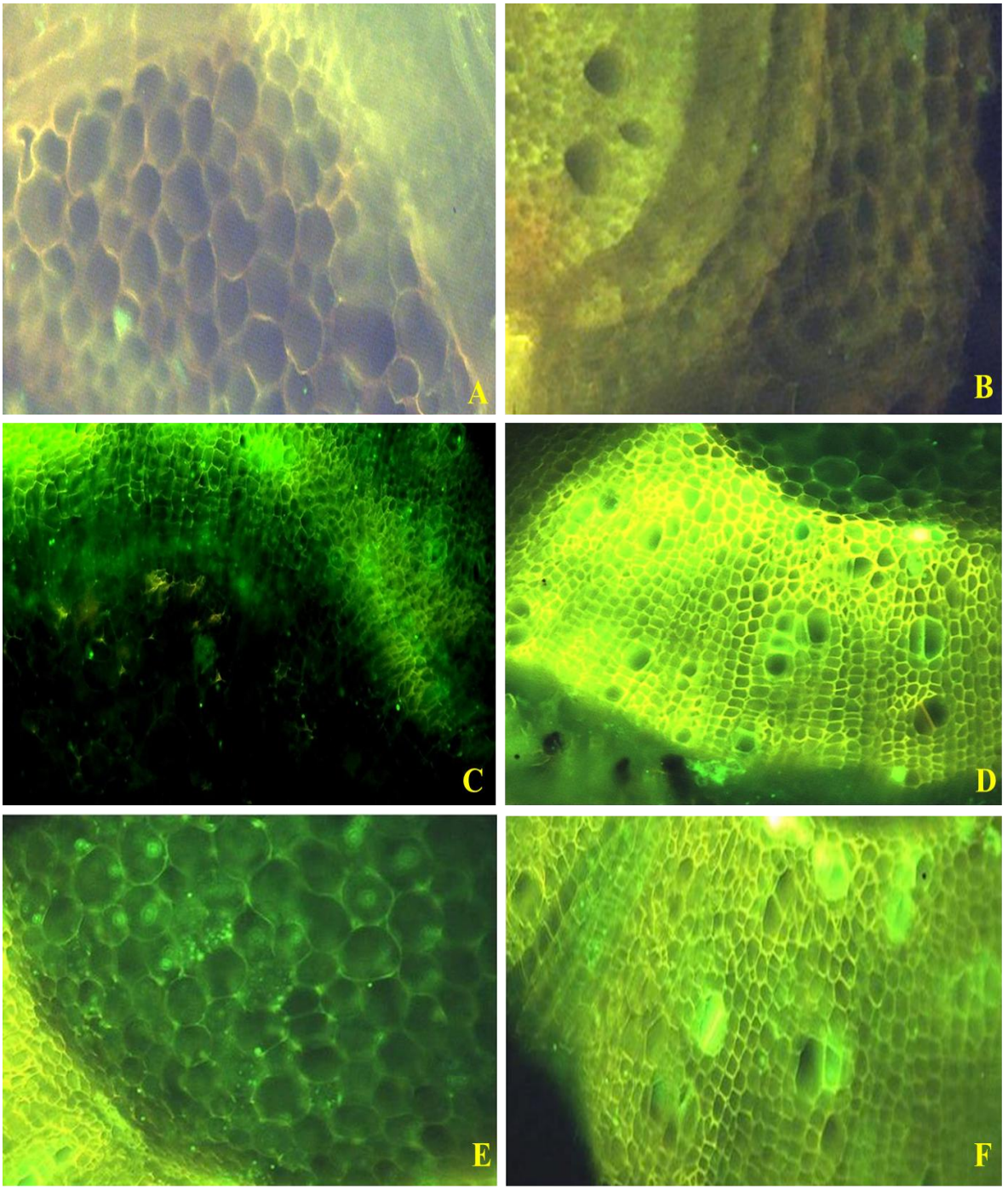


Figure 52: (A-F) FITC labeling of root tissue of *Phaseolus vulgaris* with Pab of Chitinsae enzyme after treatment with *S.flavogriseus* (KX894281) and pathogen challenge. A. transverse section (TS) of cortical tissue of control; B-TS of vascular tissue of control; (C & D) TS of cortical & vascular tissue of root of CV2 plants;(E&F) TS of cortical & vascular tissue of root of CV3 plants

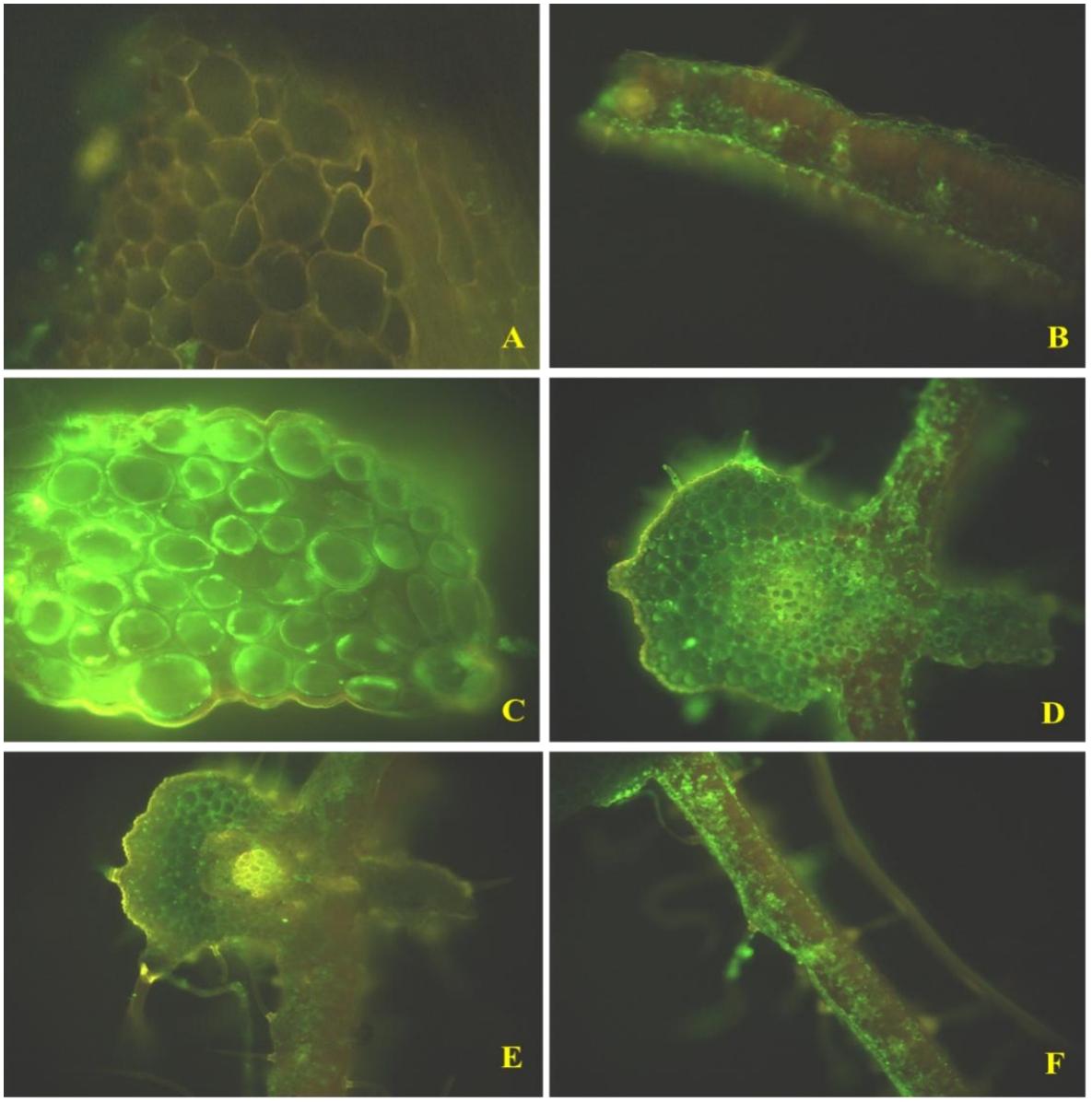


Figure 53: . (A-F) FITC labeling of leaf tissue of *Phaseolus vulgaris* with Pab of Chitinsae enzyme after treatment with *S.flavogriseus* (KX894281) and pathogen challenge . (A&B) TS of leaf tissue in untreated control; (C&D) TS of leaf tissue in CV2; (E&F) TS of leaf tissue in CV3, showing localization of chitinase enzyme.

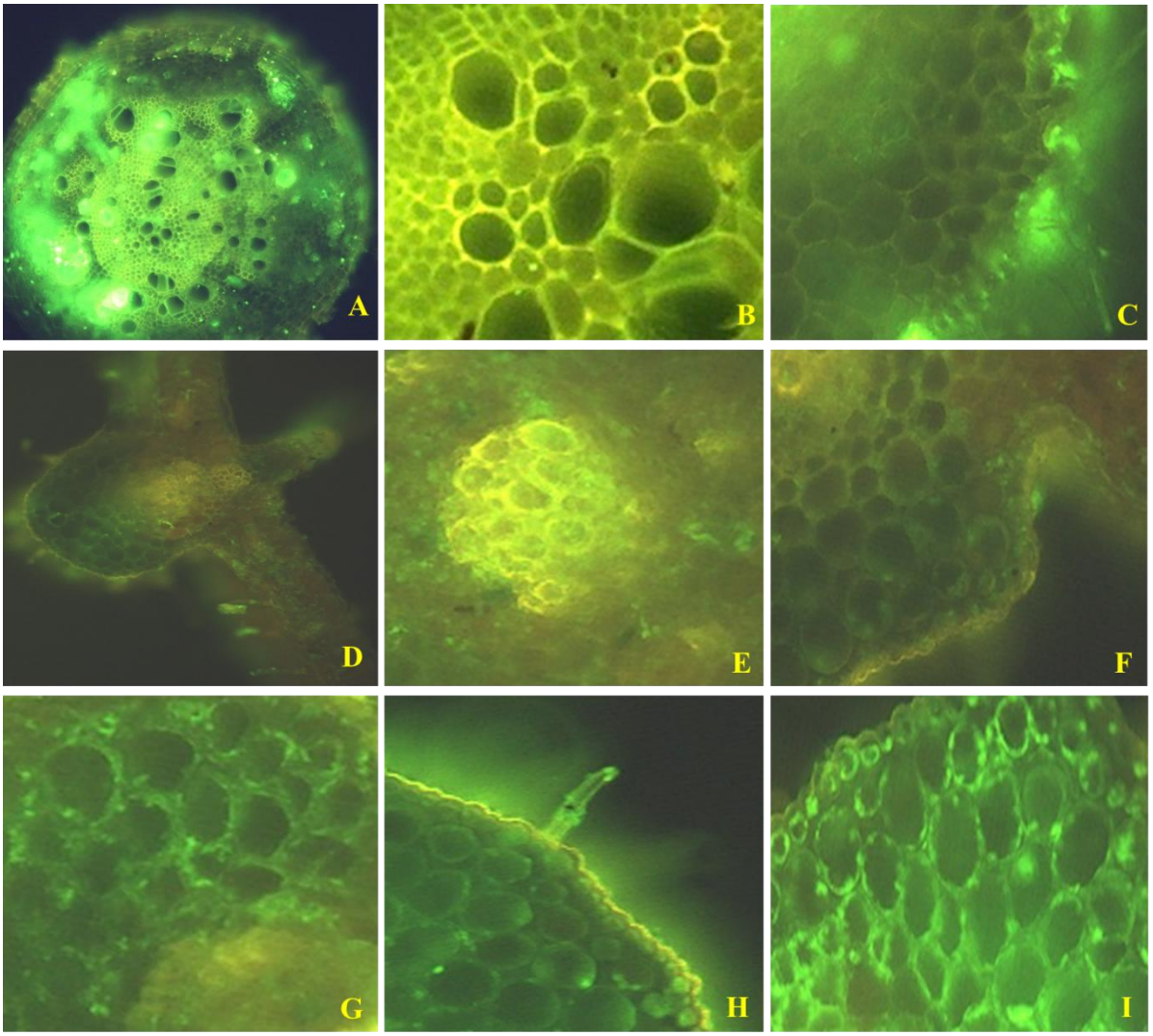


Figure 54: FITC labeling of leaf and root tissue of *Phaseolus vulgaris* with Pab of glucanase enzyme after treatment with *S.flavogriseus* (KX894281) and pathogen challenge . (A-C) TS of root tissue ; (D-F) TS of leaf tissue ; (G&F) Localization of glucanase enzyme, in cortical tissue of root (G), root hair and epidermal layer in root(H) and vascular tissue in leaf midrib area (I).

4.15. Tissue and cellular location of Chitinase and Glucanase enzyme by FITC labelling in *Vigna radiata.*

Apart from the quantitative assessment of plant defence enzyme, localisation of chitinase enzyme in the plant tissue was also observed by FITC labelling Root and leaf samples were collected from treated and inoculated plants. Samples from healthy plants without any treatment were also collected for immunofluorescence study. Main objective of the study was to localize chitinase at the cellular level in the root and leaf tissue of *Vigna radiata*. Cross section of the root and leaf tissue were treated with normal antisera and PAb of Chitinase and labelled with FITC. The treated root and

leaves showed bright apple green fluorescence in the epidermal and cortical tissue which is indication of localization of Chitinase in this region. So, strong reaction with FITC in plant tissue indicated the induction of chitinase (PR 3) in *Vigna radiata* (Fig 55).

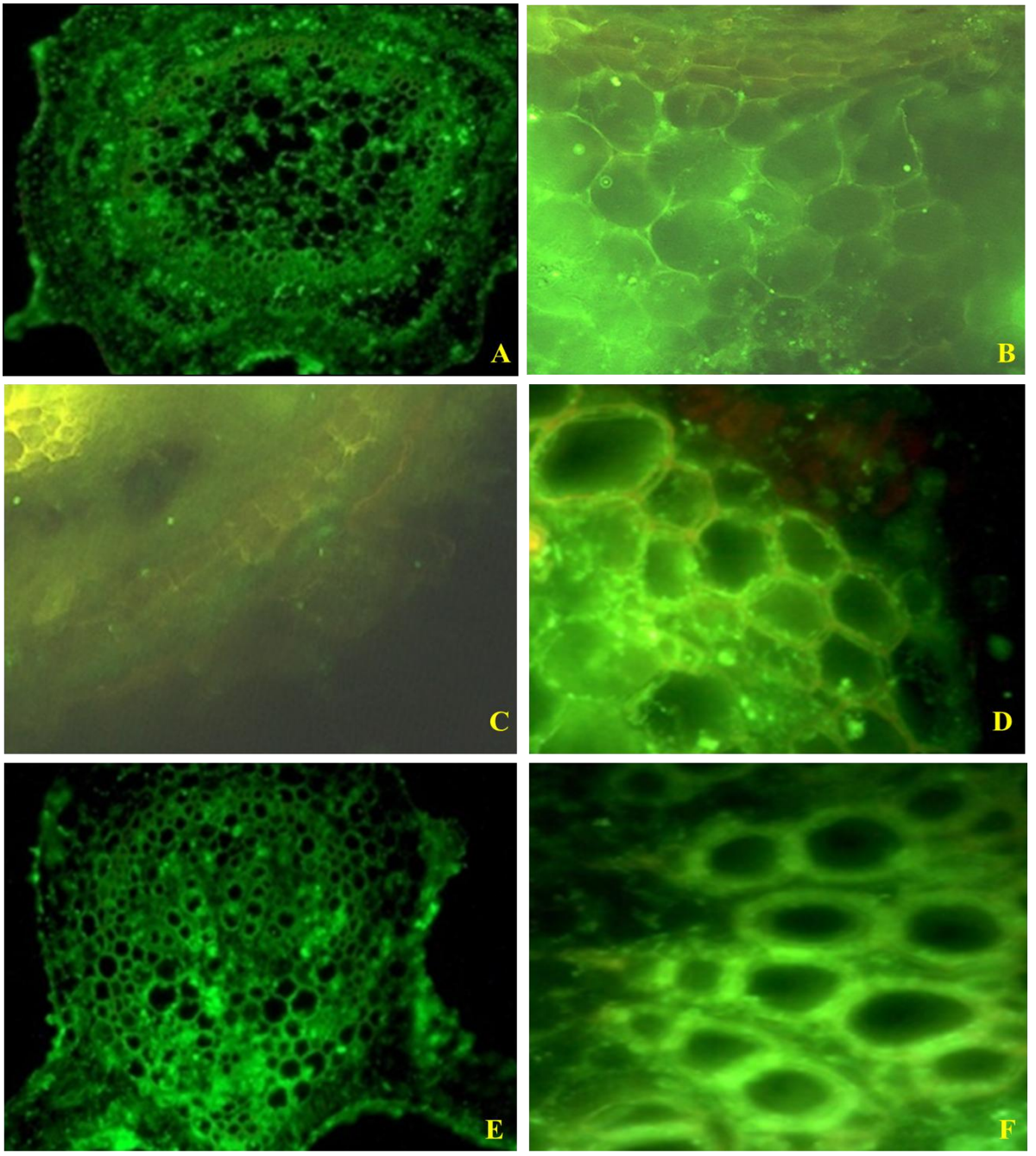


Figure 55: (A-F) FITC labeling of root & leaf tissue of *Vigna radiata* with Pab of glucanase enzyme & Pab of chitinase enzyme after treatment with *S. tricolor* (KX894280) and pathogen challenge . A-TS of root tissue of control ; (B-C & D)-TS of cortical & vascular tissue in roots ; (E & F) TS of leaf & localization of glucanase enzyme after pathogen challenge.

Chapter 5
DISCUSSION

The alternative to a safe world is safe food which can only be obtained by organic and natural way of cultivation. However some proponents may argue that the process is a slow one and may not be enough to cater to the needs of the millions of people. The fact cannot be wholly denied that it is slow but it can be long lasting as stated by Ramanathan *et al.* (2002). Use of microorganisms as biocontrol agent as insecticides, pesticides or as growth enhancer and promoter has proven to provide positive results in disease controlling and maintaining plant vigour thus ensuring sustainable and balanced environment (Kumari *et al*., 2012, Ara *et al.*, 2012a , Mishra et al., 2011, Leo *et al* 2010, Dieudonne *et al.*, 2007). As in results by Doolotkeldieva *et.al.*, (2015) wherein S*treptomyces fumanas* when used as a biofertilizer was instrumental in providing increased overall biomass and grain yield in all phases of growth of soybean aided by stimulated growth and reproduction of soil microbes. Couillerot *et al*., (2013) reported the biocontrol and biofertilizer activity of *Streptomyces anulatus* s37. A broad spectrum of microbes from dissimilar sources have been time and again,used by researchers to either totally destroy, inhibit or minimize the deleterious effect of pathogens on plants. The same holds true and tested when the arrangement of factors causing disease, is prevalent in crops which are important from the point of view of food security and of commercial exploitation. The near recent research and its application, say about a decade or two , countries were mainly concentrating on the application of mediums to contain disease and proportionally increase the production with an eye for better productivity. If the example of India itself is taken, it can be stated that green revolution wherein the medium of disease control and crop production was of inorganic/chemical origin was highly successful and to an extant has been able to the cater to the need of a large population. However, advances in conventional, clinical and medical science has proven beyond doubt, the negative effect of long term usage of chemical pesticides, insecticides or fertilizers is irreversible not only to the eco system but also other biological entities prevalent in the biota that makes life possible on the planet. Hence a sustainable approach by far is finding agents of pathogen control and plant growth promoters of biological origin .

Since the agent of potential biocontrol and growth enhancer taken for this study is actinomycetes, due consideration has been taken to relate findings of facts and figures

of works carried out by others , which do indicate the relevance as well as the importance of the role play of actinomycetes , wherein it can be used as on organic tool for not only disease conrol , or plant growth promotion but at the same time a long awaited solution to bring in the balance in agriculture fields or holistically put the environment.

There has been extensive research carried out throughout the world with different microorganisms isolated from as many locations, plants, animals and areas of interest like root rhizosphere, rhizoplane etc. some of which have shown promising results like *Trichoderma* sp. in plant disease controlling. Matloob *et al*., (2013) reported biological control of bean root rot pathogen *Rhizoctonia solani* by *Trichoderma harzianium*., Mishra *et al*., (2011) reported biocontrol activity of *Trichoderma viride* against fungal pathogens *like Rhizoctonia solani, Sclerotium rolfsii, Macrophomina phaseolina,* which cause disease in *Vigna radiata*. Others like *Rhizobium* or *Glomus* are giving constant support to the plant ecosystem through various means. (Lambais *et al*., 2003). Singh *et al*., (2011) used combination of *Bradyrhizobium* and *Glomus* to improve the cultivation of *Vigna radiata* in saline areas of West Bengal. El-Batanony *et al*., (2007) found inhibitory effect of *Rhizobium* on bean root rot pathogens, However the search for even better agents of biological origin has led researchers to look into actinomycetes as a potent vector as biocontrol and plant growth promoting agent. According to Sharma (2014) actinomycetes are durable organisms. They can survive in any kind of soil for a long time and form endospore like structure . As a result they are suitable for soil application. On the other hand they have other properties like production of a huge number of antibiotics, volatile organic compounds, secondary metabolites plant growth promoting hormones which render them very much indispensable. They also have properties for bioremediation, biocorrosion. They can also be used as biopesticide. Janaki *et al*., (2016) have reported that actinomycetes isolated from mangroves have secondary metabolites that act as antibiotics. The ability of actinomycetes group and particularly streptomycetes has been proved in medical as well as clinical sector and the same is true against many plant pathogens also. According to Chaudhary *et al.* (2013) they are the potential producer of antibiotics and therapeutic compounds. Biocontrol activity and plant growth promoting activity of actinomycetes was reported by Soares *et al*.(2006), Meguro *et al.* (2006), Heng *et al*.(2015) and Srividya *et al.(*2012). Hasegawa *et al*. (2006) reported the capability of

actinomycetes in suppressing the disease caused by the plant pathogenic bacteria and fungi . The attribute that actinomycetes have shown in the general verdict of their application is that almost all show dual mode of action, in not only containing the harmful invasive approach of its target pathogen but at the same time maintaining the host system of soil and tissue in a way directly enhancing the plant vigor, thus strengthening the primary level of defence in host plants.

The present study conducted for isolation of actinomycetes from the plains of North Bengal has been instrumental in collecting 17 isolates of actinomycetes and three isolates having biocontrol and plant growth promoting activity have been identified up to the species level using the conventional and molecular detection keys and tools. The isolates were collected mainly from agricultural fields as the first phase of the work under investigation. The site of sample collection was farming field wherein vast array of crops were cultivated. The main aim of the study was to choose organisms from the natural microflora of the crops rhizosphere. Actinomycetes are one of the most widely distributed groups of microorganisms which are omnipresent. In the soil ecosystem they are present in the root rhizospheric region of cultivated and non cultivated lands in different regions of the world (Goodfellow *et al*., 1987), Oskey *et al.,* (2004) isolate actinomycetes from farming lands in Turkey, Mohseni *et al*., (2013) isolated actinomycetes from sediments of caspian sea, Ningthoujam *et al*., (2009) isolated actinomycetes from various locations like agricultural soil, forest soil, caves, lake, river sediments in India. The present study was aimed at isolating novel actinomycetes from soil system for the disease suppression and health improvement of two crop plants *Phaselus vulgaris* and *Vigna radiata* by biological method. Various workers have worked on the biocontrol of root rot diseases by plant growth promoting bacteria or fungi. There are reports of controlling root rot of *Phaseolus vulgaris* caused by *Fusarium solani* by biocontrol agent of *Trichoderma harzianum(*El-Mohamedy *et al*., 2013) and boicontrol of root rot of *Vigna* caused by *Sclerotium rolfsii* by *Trichoderma viride* (Mishra *et al*., 2011). Inhibition of sclerotial root rot of *Vigna* by *Streptomyces* sp. was also reported by Ray *et al.,* (2016b). Pattanapipitpaisal and Kamlandharn (2012) reported the ability of *Streptomyces hygroscopicus* PACCH24 to reduce the growth of *Sclerotium rolfsii* and control the stem rot disease of Chilli caused by *S. rolfsii*.

Actinomycetes were isolated from the rhizosphere soil of the cultivated lands in Jalpaiguri district in West Bengal, which is a prominent agriculture based region. For

the isolation process soil dilution technique was followed and a total number of 17 actinomycetes isolates were obtained, which were then characterized according to their morphological, biochemical and physiological properties. The morphological characters revealed the typical properties of Streptomycetes in formation of aerial spore mass, substrate hyphae, production of melanine pigment, diffusible pigment. On the basis of the preliminary results the isolates were identified as streptomycetes. All the isolates were Gram positive. Further experiments of the isolates for different biochemical properties like starch hydrolysis, catalase production, gelatine liquefaction ,H_2S production were performed and the results confirmed their identity as streptomycetes. Dochhil *et al* (2013), Oskey *et al* (2004) performed the same biochemical tests for identification of the *Streptomyces* sp.Physiological tests for growth in different environmental conditions like different salt concentration to test the halo tolerance, in different pH to identify the acidophilic and the basophile isolates, growth in different temperature, growth in presence of different antibiotics were also performed. The result revealed that almost all the isolates were able to grow in1% to 5% NaCl concentration but none of the isolate were able to grow in the media supplemented with 10% NaCl. The optimum temperature for the growth of the isolates is 28°C -35°C . But below 15°C the growth of the isolates are hindered. The isolates are highly resistant to amphicilin, moderately resistant to streptomycin but susceptible to kanamycin as was also observed by Gopalakrishnan *et al* (2013a). Growth of the isolates were optimum in Starch casein nitrate media, sporulation was highest in Oatmeal agar whereas in Nutrient agar media(NA) the isolates growth was hampered to some extent and sporulation was not to the optimum level. Gebreyohannes *et al.*, (2013) also reported the efficacy of Starch Casein media and Oatmeal agar media for excellent growth of actinomycetes. Sowndhararajan and Kang (2012) reported the culture characteristics *of Streptomyces* sp.AM-S1on different media.

Screening of the isolates for production of chitinase production of Indole acetic acid (IAA) was done. Chitinase production by endophytic Streptomyces and its antagonism against phytopathogenic fungi is reported by Taechowisan *et al*. (2003). Though *Streptomyces tricolor* did not produce extracellular chitinase it was able to check the growth of phytopathogenic fungi. Jog *et al* (2014) earlier reported of antifungal activity of Strptomyces mchr0817 despite lacking chitinolytic activity. Manulis *et al.*,(1994), El Tarabily (2008) reported the ability of *Streptomyces* sp. to

produce IAA thereby improving plant growth. For Chitinase production qualitative estimation was performed which revealed that 7 out of 17 isolates were able to produce Chitinase..Though all the isolates were able to produce IAA in the qualitative estimation the quantitative result revealed that only some of the isolates produced higher level of IAA. Shrivastava, *et al*.(2017) have shown that a halotolerant *Streptomyces* strain K20 possess ability of plant growth promotion through production of IAA, siderophore and ammonia, with added character of phosphate solubilization.

Screening of the Isolates for phosphate solubilising activity in PKV media was also undertaken. Among the total isolates, 13 isolates were able to solubilise phosphate in the PKV Media. Jog *et al.* (2014) reported the mechanism of phosphate solubilisation by endophytic and rhizospheric *Streptomyces* spp. and their use as plant growth enhancer. According to Hamdali *et al*.(2008) the rock phosphate solubilising actinomycetes strains have the ability to stimulate the plant biomass production. Further screening of the isolates in liquid media supplemented with tri calcium phosphate and rock phosphate resulted in quantitative assessment of the ability of the isolates for phosphate solubilization. Siderophore producing activity of the isolates were also verified.

Microscopic observation of the isolates were also helpful in identifying the isolates . Under bright field microscopy the isolates showed typical arrangement of spore chain which is characteristic of *Streptomyces* sp. The isolates were grouped into three main sections namely Spirales(S), Rectiflexible (RF) and Retiacanalipetri (RA) based on their spore chain Morphology according to the method described by Shirling and Gottleib (1966), Sharma (2014).

Screening of the isolates for *in vitro* antagonistic test against root rot pathogen of *Phaseolus vulgaris* and *Vigna radiata* were done. Bilgi *et al*.(2008) reported root rot of *Phaseolu*s by the pathogen *Fusarium solani*. Mishra and coworkers (2011) reported sclerotial root rot of *Vigna radiata*. Isolates showing antagonistic activity against *Fusarium solani,* root rot pathogen of *Phaseolus vulgaris* and *Sclerotium rolfsii,* the root rot pathogen of *Vigna radiata* were obtained and further study with these isolates were carried out. *Streptomyces tricolor* (ARHS/PO/26) and *Streptomycs flavogriseus* (ARHS/PO/27) this two isolates proved to be most potent isolates for *in vitro* antagonism against plant pathogenic fungi. Antagonism of the selected isolates in solid media as well as with secondary metabolites produced by the isolates were performed

and the result showed the effectiveness of the secondary metabolites against the pathogens. Similar result was found by Soarse *et al.,* (2006) in controlling of yam pathogens *Curvularia* and *Colletotrichum.* Ara *et al,* (2012b) found that crude extract of antagonistic *Streptomyces* isolates caused swelling and distortion of fungal hyphae. Role of secondary metabolites of *Streptomyces aureofaciens* in inhibition of dumping off pathogen was also reported by Taechowisan *et al* .(2005).

Actinomycetes are mainly soil inhabiting microorganisms. There are other microbes also present in the same environment which are beneficial to the plants. PGPR, PGPF are present in the root rhizosphere of the crop and other plants and affecting the plants health. When the actinomycetes are present in the same environment with the beneficial bacteria and fungi, the interaction between the different groups can have positive or negative effect in the mechanism of the other microbes. So in the present study the isolates were screened for anti bacterial activity also to assess the possibility of negative or positive control of the isolates upon the activity of plant growth promoting Rhizobacteria mainly *Bacillus megaterium* and *Bacillus pumilus* and human pathogenic bacteria *Escherichia coli*. Oskay *et. al,* in 2004 reported antibacterial activity of actinomycetes. when grown *in vitro* dual culture method the isolates were not inhibiting the growth of the plant growth promoting Rhizobacteria although the growth of *E.coli* was checked to some extent. The result was supported by Lu *et al*,(2008) who reported that *Streptomyces lydicus* which has antifungal properties do not have any antibacterial activities.

With the help of 16S rDNA gene sequence identification of the isolates up to species level is possible. Intra *et al*. (2011) used 16S rDNA gene sequence for identification of actinomycetes up to genus level. In the present study the molecular identification of the selected isolates with help of 16S rDNA gene sequencing was carried out. PCR products of selected isolates were sequenced in commercially available automated DNA sequencing facility (CROMAS). BLAST programme was carried out to identify the homologous sequences present in the GenBank. Multiple sequence alignment was carried out using the ClastalW algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1. Phylogeneic analysis was carried out using extype strain sequences obtained from NCBI GenBank database which showed maximum homology with the selected isolates. The evolutionary history was inferred using the UPGMA method. The final result indicated

that the isolates were *Streptomyces* spp of the streptomycetes group of actinomycetes. Three of the isolates were identified upto the species level and these are *Streptomyces griseus*(KX894282), *S, tricolor*(KX894280) and *S. flavogriseus*(KX894281). 16S rDNA gene sequence of these three isolates have been submitted to NCBI database and compared and confirmed with other *Streptomyces* sequences from NCBI database (Ray *et al*., 2016a). As these three isolates are identified as *Streptomyces* spp. and their relatedness is confirmed, this genomic study can be used in future for Co-culturing the isolates to enhance bioactivity of potential actinomycetes, as suggested by Ravi *et al*., (2017).

The present work also aimed at obtaining potent isolates for the overall growth improvement and disease control of the crop plants, *Phaseolus vulgaris* and *Vigna radiata*. Merriman *et al*., (1974) had reported the effectiveness of *Streptomyces griseus* as growth enhancer in form of seed treatment of various crop plants like barley, oat, wheat and carrot. Gopalakrishnan *et al*., (2013b) evaluated *Streptomyces* spp for growth enhancement of rice plants. Effect of *Streptomyces* formulation on growth of *Vigna radiata* was evaluated by Ray *et al*.,(2016b). In the present study the effect of the actinomycetes application on the plants were carried out in pot and in field condition. The actinomycetes formulations were used in form of seed coating, foliar spray and soil drench. The result showed the positive effect of the isolates in increasing the overall shoot length, root length, total leaf area of the plants in field as well as in pot condition. It was found that *Streptomyces tricolor* was the most potent growth enhancer when seed coating or soil drench is the mode of application. When foliar spray was done *Streptomyces griseus* showed maximum activity. *In vitro* antagonistic activity of the isolates were also carried out and the result showed that *Streptomyces tricolor* has maximum inhibitory activity against *Sclerotium rolfsii* whreas *Streptomyces flavogriseus* has maximum inhibitory activity against *Fusarium solani*. Errakhi *et al*., (2007), El-Mohamedy and Abd Alla (2013) successfully inhibited root rot of sugar beet by seed treatment with *Streptomyces* sp. Karimi *et al*.,*(*2012) used soil treatment with *Streptomyces* isolate S2 to inhibit root rot of sugar beet. Danaei *et al*., (2014), Anitha and Rebeeth (2010), reported the biocontrol activity of *Streptomyces griseus* against *Penicillium, Botrytis, Fusarium, Rhizoctonia* and *Alternaria*.

Leguminous plants have the capacity of forming root nodules which help in increasing soil fertility and thereby increasing the plant health. Tokala *et al. (*2002)

reported the efficacy of *Streptomyces lydicus* in nodule formation in pea plants. Soe *et al*., (2010) evaluated the effect of endophytic actinomycetes on nodule formation in soybean plants. Likewise the effect of application of actinomycetes isolates in increasing the number of root nodule was evaluated in the present investigation. Though nodulation frequency was higher in the plants treated with actinomycetes formulation the nodulation index was not improved in the treated plants in comparison to the plants which were untreated control.

Disease symptom was established in the plants by artificial inoculation of the plants with inoculum of *Fusarium solani* and *Sclerotium rolfsii* prepared in sand maize meal media. The inoculum was mixed thoroughly with the root rhizosphere of the target plant in replicate. Disease symptom was evident within 7 days after inoculation. It was found that the level of disease incidence was higher in the untreated plants than the treated plants. The untreated plants were more prone to the disease.

In *Phaseolus vulgaris*, which is susceptible to root rot caused by *Fusarium solani*, the untreated plants showed disease symptoms within 7 days after artificial inoculation. Plants treated with *Streptomyces griseus* (KX894282), *Streptomyces tricolor* (KX894280) and *Streptomyces flavogriseus* (KX894281) showed lower level of disease symptom. Resistance against fusarial root rot in *Phaseolus vulgaris* was highest in plants treated with *Streptomyces flavogriseus* (KX894281) followed by *S. tricolor* and *S. griseus*. Among the two cultivars, Jwala (Cultiver 2) and Kholar (Cultiver 3), Kholar was more resistance to the disease than Jwala.

In *Vigna radiata* root rot disease is caused by *Sclerotium rolfsii*. When the plants were artificially inoculated with the pathogen disease development was higher in untreated inoculated plants compared to the treated inoculated plants. When the plants were treated with the actinomycetes formulations they showed higher degree of resistance. Resistance against sclerotial root rot was highest in *Streptomyces tricolor* (KX894280) treated plants followed by *Streptomyces flavogriseus* (KX894281) and *Streptomyces griseus* (KX894282) treated plants. Similar resuls was found by Srividya *et al*., (2012) during the study of the effect of *Streptomyces* sp.9p on chilli wherein the study revealed that seed bacterization of chilli with *Streptomyces* sp.9p grown in presence of *Collectotrichum* showed a considerable decline in disease symptoms, increase in biocontrol efficiency and germination properties.

Activation of defence response in form of increased level of defence enzymes was observed in the root and leaves of plants treated with actinomycetes and artificially inoculated with *Fusarium solani* and *Sclerotium rolfsii*

In *Phaseolus vulgaris* increase in level of key defence enzymes phenylalanine ammonia lyase , peroxidase, chitinase and β-1,3 glucanase were observed in treated and treated inoculated plants in comparison to the untreated and untreated inoculated plants. Broetto *et al*., (2005) reported changes in phenylalanine ammonia lyase , peroxidase activity in *Phaseolus* after infection by *Fusarium oxysporum f. sp. phaseoli.* Allay and Chakraborty, (2010) also reported enhanced activities of defence enzymes chitinase, glucanase and peroxidase in mandarin plants during disease suppression against fusarial root rot. Similar result was obtained in the present study where enzyme activities were higher in treated inoculated plants than the untreated inoculated or the untreated ones. Application of the plants with *Streptomyces flavogriseus* (KX894281) showed maximum level of enzyme activity followed by *S. tricolor* and *S. griseus.* Enzyme activity was higher in the leaf than the root. Total phenol content of the plants was also measured and the result showed presence of increased level of phenolic compound in the root tissue than the leaf of the treated inoculated plants. Enzyme activity was higher in Kholar (Cultiver 3) plants than Jwala (Cultiver 2) plants.

In *Vigna radiata* increase in level of key defence enzymes phenylalanine ammonia lyase , peroxidase, chitinase and β-1,3 glucanase were observed in treated and treated inoculated plants in comparison to the untreated and untreated inoculated plants. Enzyme activity were higher in treated inoculated plants than the untreated inoculated or the untreated healthy ones. Nandi *et al* (2013) reported induction of defence related enzymes like phenyl alanineammonia lyase, chitinase, β-1,3 glucanase, oxidative enzymes like peroxidases , poly phenol oxidases and phenolics after inoculation of *Sclerotium rolfsii* in collar region of 30 days old cowpea(*Vigna*) plant. Parihar *et al* (2012) also reported increase in PAL, PPO and peroxidase activity in *Brassica juncea* plants infected with *Alternaria* blight. Ramanathan *et al* (2001) reported activation of pathogenesis related peroxidase in *Vigna radiata* after infection by *Macrophomina phaseolina.* Changes in levels of different defense related enzymes, viz. Phenylalanine ammonia lyase (PAL), Peroxidase (POX), Chitinase (CHT) and β-1,3Glucanse (GLU) was also studied in som plants following treatment with bioinoculants and infection with *Colletotrichum gloeosporioides* by Chakraborty *et al* (2016). These findings are in

accordance with the result obtained in the present study. In the present study plants treated with *Streptomyces tricolor* (KX894280) showed maximum level of enzyme activity followed by *S. flavogriseus* and *S. griseus.* Enzyme activity was higher in the leaf than the root. Total phenol activity of the plants were also measured and the result showed presence of increased level of phenolic compound in the root tissue than the leaf of the treated inoculated plants.

Total protein content of the treated and untreated plants before and after pathogen inoculation was also noted. In both *Phaseolus vulgaris* and *Vigna radiata* total protein activity increased in treated inoculated plants in comparison to only treated or untreated plants.

Induction of the defence enzymes, chitinase and glucanase in the leaf and root tissue of both of the plants, were studied using fluorescent antibody staining technique. In 2009 Chakraborty and co-workers studied the expression of chitinase in leaves of treated tea plants following induction with salicylic acid using immunofluorescent techniques. Following the technique, expression of chitinase and glucanase in *Phaseolus vulgaris* and *Vigna radiata* were observed. As the leaves and roots of treated plants in both *P. vulgaris* and *V. radiata* showed higher level of chitinase activity than the untreated control plants so leaves and roots of the plants treated with actinomycetes formulation were reacted with Pab of chitinase and glucanase followed by labelling with FITC. Strong bright apple green fluorescence was observed in the epidermal and in mesophyll tissues of the treated leaves and roots which indicate the presence of chitinase and glucanase in the tissue.

From the study it was observed that that actinomycetes isolates obtained from the agricultural fields have the potential of inducing overall plant growth by means of phosphate solubilization , IAA production and increase various defence enzyme activity like peroxidase, chitinase and Glucanase as well as increased level of phenolics in broad bean and mung plants thereby giving the plant resistance against the root rot diseases caused by plant pathogenic fungi *Fusarium solani* and *Sclerotium rolfsii*..

Chapter 7
CONCLUSION

Agricultural land in the district of Jalpaiguri in West Bengal was the main area of the following study. A total number of 17 actinomycetes isolates were obtained from rhizospheric soil of potato fields. All the isolates were characterized by their morphological, biochemical and physiological attributes. The actinomycetes isolates were identified initially as belonging to the group Streptomycetes .

Further study of the isolates with both normal bright field microscopy and Scanning Electron microscopy revealed about their spore chain morphology and identified the isolates as *Streptomyces* spp.

In vitro screening of the isolates for phosphate solubilisation activity was done. Out of 17 isolates 13 isolates were found to be phosphate solubilizer. *Streptomyces tricolor* (ARHS/PO/26) was found to be the most efficient phosphate solubilizer among the isolates. This isolate was able to solubilise both tricalcium phosphate and rock phosphate efficiently.

The isolates were also tested for their growth promoting attribute i.e. production of Indole Acetic Acid (IAA). All the isolates were found to produce IAA but the level of the hormone was highest in the isolate ARHS/PO/26, ARHS/PO27 and ARHS/PO/15 respectively. In the other isolates the amount of IAA was not very high.

In vitro screening of the isolates for antifungal activity was evaluated with two fungal root pathogens *Fusarium solani* and *Sclerotium rolfsii*. The fungal pathogens *Fusarium solani* and *Sclerotium rolfsii* cause root rot disease of *Phaseolus vulgaris* and *Vigna radiata* respectively. From the *in vitro* study it was found that ARHS/PO/26 showed antagonist activity against *Sclerotium rolfsii* whereas ARHS/PO/27 was highly antagonist against *Fusarium solani*.

Selected isolates were identified successfully by their 16S rDNA sequences. Three isolates were identified as being *Streptomyces griseus* (ARHS/PO/15), *Streptomyces tricolor* (ARHS/PO/26) and *Streptomyces flavogriseus* (ARHS/PO/27). Genetic relatedness of the three isolates was also measured. The sequences have been

deposited to NCBI GenBank and Accession numbers of the same were obtained. The accession numbers for the isolates are *Streptomyces griseus* ARHS/PO/15 (KX894282), *Streptomyces tricolor* ARHS/PO/26 (KX894280) and *Streptomyces flavogriseus* ARHS/PO/27 (KX894281).

Growth promoting attributes of the three isolates *Streptomyces griseus* (KX894282), *Streptomyces tricolor* (KX894180) and *Streptomyces flavogriseus* (KX894281) were tested in both pot and open field conditions. Though two of the isolates *Streptomyces griseus* (KX894282) and *Streptomyces flavogriseus* (KX894281) could not solubilise phosphate *in vitro* they successfully contributed in plant growth *in vivo* condition. The effect of the isolates on nodulation of the leguminous plants were also studied. Though the nodulation frequency increased in the treated plants in comparison to the untreated plants the nodulation index variation was not that much.

Disease symptoms were observed in plants artificially inoculated with the test pathogens. The histo-pathological study of the infected root tissue was done. Plants were treated with *Streptomyces griseus* (KX894282), *Streptomyces tricolor* (KX894280) and *Streptomyces flavogriseus* (KX894281) in open field and pots, and activities of various defence enzyme activities, PAL, POX, Glucanase, Chitinase were assayed in both untreated and treated plant samples. Increase in the level of defence enzymes were observed in the treated plants in comparison to the untreated control plants. Application of the fungal pathogen by artificial inoculation method to both untreated and treated plants increased the level of defence enzymes furthermore.

In *Phaseolus vulgaris* after the pathogen inoculation it was found that levels of defence enzymes was highest in the treated plants after pathogen inoculation followed by the treated plants, inoculated plants and untreated control plants. The defence enzyme level was highest in the plants treated with the isolate *Streptomyces flavogriseus* (KX894281). Levels of all the defence enzymes were higher in the leaves than the roots. Total phenol content of the root and leaves tissue were also assayed. Root tissues showed higher phenol activity. Total protein content of the plants were also assayed which was much higher in the leaf tissues than the root tissue.

In *Vigna radiata* also the level of defence enzyme increased in treated inoculated plant samples. Here also the lowest levels of enzymes were found in untreated control plant samples. The enzyme level was highest in plants treated with

Streptomyces tricolor (KX894280) and inoculated with the pathogen *Sclerotium rolfsii*. Total phenol content of the root and leaves tissue were also assayed. Root tissues showed higher phenol content. Total protein content of the plants were also assayed which was much higher in the leaf tissues than the root tissue.

Cellular localization of the two defence enzymes Chitinase and Glucanase were also studied by indirect immunofluorescence. Expressions of these enzymes were observed in treated leaf sections, in accordance to the earlier results obtained.

The study undertaken is indicative of the positive role of actinomycetes in crop cultivation and very much supports the aim of the study, that they play a significant role towards plant growth promotion and selected strains of locally isolated groups can also act as antagonist against fungal plant pathogens. The findings of the study can be exploited to explore the potential application of actinomycetes formulation, for dual application, as plant protector and growth enhancer of plant, not just for the pulse crop like *Phaseous* or *Vigna*, the crop plants identified for the present study but also for plantation crops like tea and Large cardamom in the near future.

Chapter 7
BIBLIOGRAPHY

Ababutain IM, Aziz ZKA, Al-Meshhen NA. Lincomycin antibiotic biosysthesis produced by *Streptomyces* sp. isolated from Saudi Arabia soil: taxonomical, antimicrobial and insecticidal studies on the producing organism. *Can J Pure Appl Sci.* **6(1):** 1739- , 2012.

Abawi GS, Pastor-Corrales MA. Root rots of beans in Latin America and Africa: Diagnosis, research methodologies, and management strategies. CIAT Publication No. **35**, Cali, Colombia.1990

Abbas IH. Biological and Biochemical Studies of Actinomycetes isolated from Kuwait Saline Soil. *J Appl Sci Res*. **2(10):** 809-815, 2006.

Abeysinghe S. Biological control of *Fusarium solani* f. sp. *phaseoli* the causal agent of root rot of bean using *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01. *Ru J Sci.* **2**: 82-88. 2007.

Agrios G. *Plant Pathology*, 5th edition. Elsevier Academic Press. 2005.

Akram A, Iqbal MS, Qureshi RA, Rauf CA.Variability among isolates of *Sclerotium rolfsii* associated with coller rot disease of chickpea in Pakistan. *Pak. J. Bot,* **40(1):** 453-460.2008.

Allay S and Chakraborty BN. Activation of defense response of mandarin plants against Fusarium root rot disease using *Glomus mosseae* and *Trichoderma hamatum. J Mycol Pl Pathol,* **40 (4):** 499-511. 2010.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.***25:**3389-3402, 1997.

Anitha A and Rabeeth M. *In vitro* Antifungal Activity of *Streptomyces griseus* Against Phytopathogenic Fungi of Tomato Field. *Aca J Pl Sci* **2** (**2**): 119-123.2009

Anitha A and Rabeeth M. Degradation of fungal cell walls of phytopathogenic fungi by lytic enzyme of *Streptomyces griseus. African J Pl Sci*. **4 (3):**061-066, 2010

Ara I, Bukhari NA, Perveen K and Bakir MA. Antifungal activities of some actinomycetes isolated from Riyadh soil, Saudi Arabia: An evaluation for their ability to control *Alternaria* caused tomato blight in green house pot trial. *Afr J Agric Res.* **7(13):** 2042-2050, 2012a

Ara I, Rizwana H, Al-Othman MR and Bakir MA. Studies of actinomycetes for biological control of *Colletotrichum musae* pathogen during post harvest anthracnose of banana. *Afr. J. Microbiol.Res*. **6(17**)3879-3886, 2012b.

Ayari A, Morakchi H. and Djamila KG Identification and antifungal activity of *Streptomyces* sp. S72 isolated from Lake Oubeira sediments in North- East of Algeria. *Afr J Biotechnol*.**11(2):** 305-311. 2012.

Aycock R. Stem rot and other diseases caused by *Sclerotium rolfsii*. *NC Agric Exp Stn Tech Bull*, 174- 202. 1966.

Balakrishna G, Shiva SA and Kumar PP. Isolation of Phosphate Solibulizing Actinomycetes from Forest Soils of Mahabubnagar District. *IOSR J Pharma*. **2(2)** : 271-275, 2012

Baniasadi F, Bonjar GH, Shahidi BA, Nik KA, Jorjandi M, Aghighi S. and Rashid PF .Biological Control of *Sclerotinia sclerotiorum*, Causal Agent of Sunflower Head and Stem Rot Disease, by Use of Soil borne Actinomycetes Isolates. *American J Agri Biol Sci* **4 (2):** 146-15,2009.

Barakate, M, Ouhdouch Y , Oufdou Y and Beaulieu C. Characterization of rhizospheric soil streptomycetes from Moroccan habitats and their antimicrobial activities. *World J. Microbiol. Biotechnol*. 18: 49-54.2002

Barka A, Belarbi EA, Hachet C, Nowak J, and Audran JC. *Enhancement of in vitro growth and resistance to gray mould of Vitis vinifera cocultured with plant growth-promoting rhizobacteria. FEMS Microbiol. Lett.* **186:**91-*95,* 2000.

Basha CSK and Rao SKRS. An intrinsic assessment of bioactive potentiality of mangroves actinomycetes. *J. Entomol and Zool. Studies* . **5(1):** 20-26, 2017

Basilio A, Gonzalez I, Vicente MF, Gorrochategui, J, Cabello, A, Gonzalez, A, Genilloud O. Patterns of antimicrobial activities from soil actinomycetes isolated under different condition of pH and salinity. *J. Appl. Microbiol.* **95,** 814–823, 2003.

Behal V, Bioactive products from *Streptomyces. Adv Appl Microbiol*.**47:**113-157,2000

Ben Rebah F, Tyagi RD, Prevost D. Wastewater sludge as a substrate for growth and carrier for rhizobia: the effect of storage conditions on survival of *Sinorhizobium meliloti*. *Bioresour Technol*.**83**:145–151, 2002.

Berdy J. Bioactive Microbial metabolites, a personal view. *J Antibiotics*. **58:** 1-26, 2005

Bignell DR, Seipke RF, Huguet-tapia JC, Chambers AH, Parry RJ and Loria R. *Streptomyces scabies* 87-22 contains a coronafacic acid like biosysthetic clusterthat contributes to plant microbe interactions. *Mol Plant Microb Intract.***23:**161-175,2010

Bilgi VN, Bradley CA, and Khot SD. Response of Dry Bean Genotypes to Fusarium Root Rot, Caused by *Fusarium solani* f. sp. *phaseoli*, Under Field and Controlled Conditions . *Plant Disease.* **92 (81):**197 -1200, 2008.

Biswas KK and Sen C. Management of stem rot of groundnut caused by *Sclerotium rolfsii* through *Trichoderma harzianum*. *Indian Phytopath*., **53 (3)** : 290-295,2000

Bloemberg, G. V. and **Lugtenberg, B. J. J.** Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr. Opin. Plant Biol*, 2001**, 4,** 343 352.

Boller,T. and Mauch, F. Colorimetric assay for chitinase. *Meth. Enzymol*. **161**:430-435.1998

Bressan W. Biological control of maize seed pathogenic fungi by use of actinomycetes. *Biocontrol*. **48**: 233-240, 2003

Broetto F, Marchese JA, Leonardo M, Regina M. Fungal elicitor-mediated changes in polyaminecontent, phenylalanine-ammonia lyase and peroxidase activities in bean cell culture. *Gen. Appl. Plant Physiology.* **31(3-4):**235-246, 2005.

Buchanan RE and Gibbons NE . *Bergey's Manual of Determinative Bacteriology*. (Eighth Edition), The Williams & Wilkins Co., Baltimore 1974

Castillo UF, Strobel GA, Ford EJ, Hess WM Porter H, Jensen JB, Albert H, Robison R, Condron MAM, Teplow DB, Steevens D and Yaver D *Munumbicins, wide-spectrum antiobiotics produced by Streptomyces NRRL 30562, endophytic on Kennedia nigriscans. Microbiol.* ***1*48***:*2675-*2685.* 2002.

Chakraborty BN. Scoping the potential uses of beneficial microorganisms for biopesticide industry and entrepreneurship development in crop protection.In: Prespectives of plant pathology in Genomic Era (Eds) P Chowdappa, P Sharma, D Singh and AK Misra, Today & tomorrow's printers and publishers, New Delhi. 335-355, 2016.

Chakaraborty BN, Basu P, Das R, Saha A and Chakraborty U. Detection of cross reactive antigen between *Pestalopsis theae* and tea leaves and their cellular location. *Ann. Appl. Biol.***207**:11-21, 1995.

Chakraborty BN and Saha A. Detection and cellular location of cross-reactive antigens shared by *Camellia sinensis* and *Bipolaris carbonum.Physiol Mol Plant Pathol*.**44:** 403–416, 1994.

Chakraborty BN, Acharya A and Chakraborty U. Biochemical changes in *Persea bombycina* following infection with *Colletotrichum gloeosporioides*. *J. Mycopathol. Res,* **54(1):**127-133, 2016.

Chakraborty BN, Sengupta D and Sharma M. Serological relationship between *Exobasidium vexans* and *Camellia sinensis*. *Ind phytopathol* **62(1)**: 19-26, 2009.

Chakraborty, U. Chakraborty, B.N. and Kapoor, M.. Changes in levels of peroxidase and phenylalanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptoshaeria maculans. Folia Microbiol*. **38**:491-496, 1993

Charoensopharat K, Thummabenjapone P, Sirithorn P, Thammasirirak S. Antibacterial substance produced by Streptomyces sp. No87. *Afr J Biotechnol* **7(9):** 1362-1368. 2008.

Chaudhary HS, Soni B, Shrivastava AR and Shrivastava S. Diversity and versatility of actinomycetes and its role in antibiotic production. *J. Appl Pharma sci.* **3(8):** 83-94, 2013

Compant S, Duffy B, Nowak J, Clement C, Barka EA. Use of plant growth promoting bacteria for Biocontrol of plant diseases: principles, mechanisms of action and future prospects. *Appl. Environ.Microbiol*. **71 (89)**: 4951- 4959. 2005.

Coombs JT, Franco CMM. Isolation and identification of actinobacteria from surface sterilized wheat roots. *Appl Environ. Microbiol*. **69(9):** 5603-5608, 2003

Costa GF, Zucchi DT and **Melo de SI.** Biological Control of Phytopathogenic Fungi by Endophytic Actinomycetes Isolated from Maize (*Zea mays* L.) *Brazilian arc Biol Technol* **56(6):**948-955, 2013.

Couillerot O, Vatsa P, Loqman S, Ouhdouch Y, Jane H, Renault JH, Clement C and Barka EA. Biocontrol and biofertilizar activities of the *Streptomyces anulatus* S37: an endophytic actinomycetes with biocontrol and plant growth promoting activities. *Biological Control of Fungal and bacterial plant Pathogens IOBC- WPRS Bulletin*. **86**:271-276, 2013

Crawford DL, Lynch JM, Whipps JM and Ousley MA.. Isolation and characterization of Actinomycetes antagonists of a fungal pathogen. *Appl.Environ.Microbiol*. **59(11)** : 3899-3905.1993

Cummins, C.S. and **Harris, H. A.** Comparison of cell wall composition in Nocardia, Actinomyces, Mycobacterium and Propionbacterium. *J. Gen. Microbiol*, **15(9).**19-56, 1956.

Dahiya N, Tewari R, Hoondal GS. Biotechnological aspects of chitinolytic enzymes. *Appl. Microbiol. Biotechnol*. **71**: 773-782. 2006.

Danaei M, Baghizadeh A, Pourseyedi S, Amini J and Yaghoobi MM. Biocontrol of plant fungal diseases using volatile substances of *Streptomyces griseus*. *Euro J Experimental Biol*. **4(1):** 334-339, 2014.

Dastager SG, Wen-Jun Li , Dayanand A, Shu-Kun Tang, Xin-Peng Tian, Xiao-Yang Zhi, Li-Hua Xu and Cheng-Lin Jiang .Separation, identification and analysis of pigment (melanin) production in *Streptomyces. Afr J Biotechnol*. **5 (8):**1131- 1134. 2006.

Davis NK, and **Chater KF.** Spore colour in *Streptomyces coelicolor* A 3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. *Mol Microbiol***. 1(10):**1679-91,1990.

De Boer W, Gunnewick, P.J.A.K, Lafeber, P, Janse, J.D, Sujit, D.E. and **Woldendrop, J.W**. Antifungal properties of chitinolytic dune soil bacteria. *Soil. Biol. Biochem.* **30**: 193-203. 1998.

Dhanasekharan D, Sivamani P, Panneerelvam A, Thajuddin N, Rajakumar G, Selvamani S .Biological control of tomato seedling damping off with *Streptomyces* sp. *Pl Pathol Journal* **4 (2):** 91-95. 2005.

Dieudonné N, Henri F, Germain EO, Laurette NN and Jean SR. Pseudomonads and symbiotic micro-organisms as biocontrol agents against fungal disease caused by *Pythium aphanidermatum Afr J Biotechnol*. **6 (3**): 190-197, 2007.

Dobbelaere S, Croonenberghs A, Thys A, Van De Broek A. Photostimulatory effects of *Azospirillum brasiense* wildtype and mutant strainsaltered in IAA production in wheat. *Plant soil,* **212**:155-164, 1999

Dochhil H, Dkhar MS and Barman D. Seed germination enhancing activity of endophytic *Streptomyces* isolated from indigenous ethno-medicinal plant *Centella asiatica. Int J Pharma Bio Sci.* **4(1):** 256-262, 2013.

Doolotkeldieva T, Bobusheva S and Konurbaeva M. Effects of *Streptomyces* biofertilizer to soil fertility and rhizosphere's functional biodiversity of agricultural plants. *Advances in Microbiol*. **5**: 555-571, 2015

Doumbou, CL, V. Akimov, M. Côté, P.M. Charest, and C. Beaulieu. Taxonomic study on nonpathogenic streptomycetes isolated from common scab lesions on potato tubers. *Syst. Appl. Microbiol*. **24**: 451-456. 2001.

Duffy, B. Schouten, A and Raaijmakers.J.M. Pathogen self defense: mechanisms to counteract microbial antagonism. *Annu. Rev. Phytopathol*. **41:**501- *538.* 2003.

El-Batanony NH, Massoud ON, Mazen MM and El-Monium A. The inhibitory effects of cultural filtrates of some wild *Rhizobium* spp. on some faba bean root rot pathogens and their antimicrobial synergetic effect when combined with Arbuscular mycorrhiza(AM). *World J Agri.Sci*. **3(6):** 721-730, 2007.

El-Mohamedy RSR and Abd Alla MA. Bio-priming seed treatment for biological control of soil borne fungi causing root rot of green bean (*Phaseolus vulgaris* L.) *J Agri.Technol.* **9(3):** 589-599, 2013

El-Nakeeb MA and **Lechevalier HA.** Selective isolation of aerobic actinomycetes. *Appl. Microbiol.* **11**:75-77. 1963.

El-Tarabily KA, Solimana MH, Nassara AH, Al-Hassania HA, Sivasithamparam K, McKennad F, Hardy GE Biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. *Plant Pathol*. **49**: 573-583. 2000.

El-Tarabily KA. Promotion of tomato plant growth by rhizosphere competent 1-aminocyclopropane-1-carboxylic acid deaminase producing-streptomycetes Actinomycetes. *Plant soil*. **308:** 161-174, 2008.

Errakhi R, Lebrihi A, and **Barakate M.** *In vitro* and *in vivo* antagonism of actinomycetes isolated from Moroccan rhizospherical soils against *Sclerotium rolfsii*: a causal agent of root rot on sugar beet (*Beta Vulgaris* L.) *J Appl Microbiol.* **107:** 672–681. (2009).

Errakhi, R, Bouteau, F, Lebrihi, A, Barakate, M. Evidences of biological control capacities of *Streptomyces* spp. against *Sclerotium rolfsii* responsible for damping-off disease in sugar beet (*Beta vulgaris* L.). *World J. Microbiol. Biotechnol*, **23**: 1503–1509. 2007.

Frankowski, J. Lorito, M Scala, F. Schmidt, R. Berg, G. and Bahl. H *(*2001). *Purification and properties of two chitinolytic enzymes of Serratia plymuthica HRO- C48. Arch. Microbiol*. **176:**421-*426, 2001*

Fridlender M, Inbar J and Chet I. Biological control of soil borne plant pathogens by a β-1,3 glucanase producing *Pseudomonas cepacia. Soil Biol Biochem.***25:**1211-1221, 1993.

Fuentesa, M.S, Benimelia, C.S, Cuozzoa, S.A. and **Amorosoa, M.J.** Isolation of pesticide-degrading actinomycetes from a contaminated site: Bacterial growth, removal and dechlorination of organochlorine pesticides. International Biodeterioration & Biodegradation **64:** 434–441. 2010.

Gamliel A, Katan J and Cohen E. Toxicity of chloronitobenzenes to *Fusarium oxisporum* and *Rhizoctonia solani* as related to their structure. *Phtoparasitica*. 17:101- 105, 1989.

Gebreyohannes G, Moges F, Sahile S, Raja N . Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia. *Asian Pac J Trop Biomed.* **3(6):** 426-435. 2013.

Goodfellow M, Sympson KE. Ecology of *Streptomyces. Frontiers Appl. Microbiol.* **2**:97- 125, 1987

Gopalakrishnan S, Ranga Rao GV, Ratna Kumari B, Vijayabharathi R, Srinivas V and Gowda CLL. Development of broad-spectrum actinomycetes for biocontrol and plant growth promotion of food crops. Recent Advances in Biofertilizers and Biofungicides (PGPR) For Sustainable Agriculture. *Proceedings of 3rd Asian Conference on Plant Growth-Promoting Rhizobacteria (PGPR) and other Microbials*. 75-100, 2013a

Gopalakrishnan S, Vadlamudi S, Apparla S, Bandikinda P, Vijayabharathi R, Bhimineni RK, Rupela O. Evaluation of *Streptomyces* spp. for their plant-growth- promotion traits in rice. *Canadian J. Microbiol*. **59(8):** 534-539, 2013b

Gopalakrishnan S, Pande S, Sharma M, Humayun P, Kiran B K, Dsyam S, Vidya, Sree Kanala D M, Om Rupela Evaluation of actinomycete isolates obtained from herbal vermicompost for the biological control of *Fusarium*

wilt of chickpea. Crop Protection ,open access repository ,*International crops research institute for Semi-Arid Tropics.* 2011.

Gupte M, Kulkarni P, Ganguli BN Antifungal antibiotics. *Appl.Microbiol. Biotechnol.***58**: 46-57. 2002.

Hamdali H, Hafidi M, Virolle MJ and Ouhdouch Y. Rock phosphate solubilising actinomycetes: screening for plant growth promoting activity. *World J Microbiol Biotechnol*. **24:** 2565-2575, 2008.

Hasegawa S, Meguro A, Shimizu M, Nishimura T and Kunoh H. Endophytic Actinomycetes and Their Interactions with Host Plants *Actinomycetologica* **20(2)** :72–81.2006.

Hastuti RD, Lestari Y, Suwanto A and Saraswati R. Endophytic *Streptomyces* spp. As biocontrol agents of Rice Bacterial Leaf blight Pahogen (*Xanthomonas oryzae* pv. *oryzae*). *Hayati J Biosci*. **19(4):**155-162,2012

Hathout TA, Felaifel MS, El-Khallal SM, Abo-Ghalia HH and Gad RA. Biocontrol of *Phaseolus vulgaris* root rot using arbuscular mycorrhizae. *Egypt J Agric Res*. **88(1):** 15-29**,** 2010

Hayakawa, M, Otoguro, M, Takeuchi, T, Yamazaki, T. and **Iimura, Y**. Application of a method incorporating differential centrifugation for selective isolation of motile actinomycetes in soil and plant litter. Antonie Van Leeuwenhoek. **78:**171–85. 2000.

Helmy S. , Zeinat K. , Mahmoud S. , Moataza S. , Nagwa M. , Amany H. *streptomyces nigellus* as a biocontrol agent of tomato damping-off disease caused by *pythium ultimum*, ejpau **13(4).** 2010.

Heng JLS, Md Shah UK, Rahman NAA, Shaari K, Hamzah H *Streptomyces ambofaciens* S2 -A Potential Biological Control Agent for *Colletotrichum gleosporioides* the Causal Agent for Anthracnose in Red Chilli Fruits*. J Plant Pathol Microbiol* S1: **006.** doi:10.4172/2157-7471.S1-006.2015.

Hong-Sik Oh and **Yong-Hwan Lee** .A Target-Site–Specific Screening System for Antifungal Compounds on Appressorium Formation in *Magnaporthe grisea* Phytopathology Vol. 90, No. 10, 1162-1168. 2000.

Hsu SC and Lockwood JL .Powdered Chitin Agar as a Selective Medium for Enumeration of Actinomycetes in Water and Soil, *Appl. Microbiol* **29(3**): 422- 426. 1975.

Igarashi Y, Iida T , Yoshida R and Furumai T. Tpteridic acids A and B, novel plant growth promoters with auxin-like activity from *Streptomyces hygroscopicus* TP-A0451. *J. Antibiot.* **55**: 764–767. 2002a.

Igarashi Y, Iida T, Sasaki Y , Saito N, Yoshida R and Furumai T. Isolation of actinomycetes from live plants and evaluation of antiphytopathogenic activity of their metabolites. *Actinomycetologica* **16**: 9–13. 2002b.

Ilic S.B, Konstantinovic S.S, Todorovic Z.B. UV/ Vis analysis and antimicrobial activity of Streptomyces isolates. *Facta universitatis; series:Medicine and Biology* **12(1**):44-46. 2005.

Inrta B, Mungsuntisuk I, Nihira T, Igarashi Y and Panbangred W. Identification of actinomycetes from plant rhizospheric soils with inhibitory activity against *Colletotricum* spp., the causative agent of anthracnose disease. *BMC Rearch Notes*. **4**:98, 2011

Jami M, Ghanbari N, Kneifel W and Domig KJ. Phylogenetic diversity and biological activity of culturable Actinobacteria isolated from freshwater fish gut microbiota. *Microbiological Res*. **175:**6-15, 2015.

Janaki T, Nayak BK, Ganesan T . Antifungal activity of soil actinomycetes from the mangrove *Avicennia marina. J. Medicinal Pl. Studies*. 2016; **4(2)**: 05-08, 2016

Jog R, Pandya M, Nareshkumar G, Rajkumar S. Mechanism of phosphate solubilization and antifungal activity of *Streptomyces* spp. isolated from wheat roots and rhizosphere and their application in improving plant growth. *Microbiol* . **160**: 778–788, 2014

Joo JG. Purification and characterization of an extracellular chitinase from the antifungal biocontrol agent *Streptomyces halstedii*. *Biotechnol. Lett.* **27:** 1483-1486. 2005

Kalakoutswl V and Agre NS. Endospores of actinomycetes: dormancy and germination. In The Actinomycetales: Characteristics and Practical Importance, pp. I79-1 **95.** Edited by G. Sykes and F. A. Skinner. London and New York: Academic Press. 1973.

Kamal R, Gusain YS and Kumar V. Interaction and symbiosis of AM fungi, Actinomycetes and Plant Growth promoting Rhizobacteria with plants: Strategies for the improvement of plants health and defense system. *Int J Curr Microbiol App Sci***. 3(7):** 564-585, 2014

Kamil Z, Rizk M, Saleh M and Moustafa S. Isolation and identification of rhizosphere soil chitinolytic bacteria and their potential in antifungal biocontrol. *Global J Mol Sci.* **2**:57-66, 2007

Kampfer P, Kroppenstedt RM, Dott W.A Numerical classification of the genera *Streptomyces* and *Streptosverticillium* using miniaturized physiological tests. *J. Gen. Microbiol.* 137: 1831-1891.1991

Karandikar, A, Sharples, G.P. and Hobbs.G. Differentiation of *Streptomyces coelicolor* A3(2) under nitratelimited conditions. *Microbiol.* **143**:3581–3590. 1997.

Karimi E, Sadeghi A, Dehaji PA, Dalvand Y, Omidvari M and Nezhad KM. Biocontrol activity of salt tolerant *Streptomyces* isolates against phytopathogens causing root rot of sugar beet. *Biocontrol Sci Technol.* **22(3):**333-349, 2012

Kaushik B.D. Role of rhizoshpere microorganisms in sustainable crop production. Chapter 2, -Rhizospere biotechnology plant growth- retrospect and prospect. 7-21. 2007.

Kavitha A, Vijayalakshmi M, Sudhakar P, Narasimha G.Screening of Actinomycete strains for the production of antifungal metabolites, *AfrJ Microbiol Res.* **4 (1):** 27-032. 2010

Khanna M, Solanki R and Lal R. Selective Isolation of rare Actinomycetes producing novel Antimicrobial Compounds. *Inter. J. Adv. Biotechnol. Res.***2(3):**357-375. 2011.

Kilicoglu MC and Ozkoc I. Phylogenetic analysis of *Rhizoctonia solani* AG-4 isolates from common beans in Black sea coastal region, Turkey, based on ITS-5.8rDNA. *Turk J Biol.* **37**: 18-24, 2013.

Kumari R, Shekhawat KS, Gupta Renu and Khokhar.MK Integrated Management against Root-rot of Mungbean [*Vigna radiata* (L.) Wilczek] incited by *Macrophomina phaseolina*. *J Plant Pathol Microb.* **3:**5. 2012.

Kundsen, D and Beegle, D. Recommended phosphorous tests. P.122-15, In: W.C.Dahnke(ed.) Recommended chemical soil tests procedures for the north central region. Bulletin no. 499 (revised) North Dakota agric.exp.sta., Fargo, North Dakota, 1988.

Labeda DP, Doroghazi JR, Ju KS and Metcalf WW. Taxonomic evaluation of *Streptomyces albus* and related species using multilocus sequence analysis and proposals to emend the description of *Streptomyces albus* and describe *Streptomyces pathocidini* sp. nov. *Int. J Syst. Evolutionary Microbiol***.64:**894-900, 2014.

Laidi RF, Amany L, Kansoh A.K. Elshafei, A.M. and Cheikh.B Taxonomy, identification and biological activities of a novel isolate of *Streptomyces tendae.Arab J Biotec*.9(3); 427-436, 2006.

Lam KS. Discovery of novel metabolites from marine actinomycetes. *Curr Opin Microbiol.* **9(3):** 245-251, 2006.

Lambais MR, Ríos-Ruiz WF and Andrade RM. Antioxidant responses in bean (*Phaseolus vulgaris*) roots colonized by arbuscular mycorrhizal fungi. *New Phytologist.* **160**: 421–428,2003

Leo D, Amalraj E , Kumar GP, Desai S and Ahmed SK. *In vitro* Characterization of *Trichoderma viride* for Abiotic Stress Tolerance and Field Evaluation against Root Rot Disease in *Vigna mungo L.Biofertil Biopestici*, **2**:3.2010.

Lertcanawanichakul M and Sawangnop S. A comparison of two methods used for measuring the antagonistic activity of *Bacillus* species. *Walailak J Sci. Tech*. **5**:161- 171, 2008

Li Q, Xiu C, Jiang Y and Jiang C. Morphological Identification of Actinobacteria. *Actinobacteria-Basics and Biotechnological Applications*. InTecOpen(NY) : 59-86, 2016

Lim HS, Kim YS and Kim SD. *Pseudomonas stutzeri YPL-1 genetic transformation and antifungal mechanism against Fusarium solani, an agent of plant root rot. Appl. Environ. Microbiol*. **57:**510-*516.* 1991.

Lin QS, Chen SH, Hu MY, Haq UI, Yang MR L, and Li H. Biodegradation of cypermethrin by a newly isolated actinomycetes HU-S-01 from wastewater sludge. *Int. J. Environ. Sci. Technol*. **8:** 45–56. 2011.

Lo CW, Lai NS, Cheah HY, Wong NKI and Ho CC. Actinomycetes isolated from soil samples from the crocker range sabah. *ASEAN Review of Biodiversity and Environmental Conservation* (ARBEC). 2002.

Lowry OH, Rosenbrough NJ, Farr AL and Randell RJ. Protein measurement with folin phenol reagent. *J. Biol. Chem*. **193**:265-275.1951

Lu CG, Liu WC, Qiu JY, Wang HM, Liu T and Liu DW. Identification of an antifungal metabolite produced by a potential biocontrol actinomyces strain a01. *Brazilian J. Microbiol*. **39**:701-707, 2008

Mahadevan N and Shridhar R. Methods in physiological plant pathology.2nd Ed. Sivakani Publ., India -242, 1982.

Manulis S, Shafir H, Epstein E, Lichter A. and Barash I. Biosynthesis of indole-3-acetic acid via the indole-3-acetamide pathway in *Streptomyces* sp. *Microbiology* **140**:1045–1050. 1994

Marinelli F. Antibiotics and *Streptomyces*: The future of antibiotic discovery. *Microbiol. Today* **36**:21–22. 2009.

Masand M and Menghani E. Antimicrobial activity of isolates of actinomycetes from soils of semi arid regions of Rajasthan. *W J pharma pharmaceutical Sci*, **4(4):** 1549- 1556, 2015

Masayuki H, Ishizawa K, Nonomura H, Distribution of rare actinomycetes in Japanese soils. *J. Fermentation Technol.* **66(4):** 367-373, 1988.

Mathew K.A and Gupta SK. Studies on web blight of French bean and its management. *Indian J Mycol Pl Pathol* **26**: 171-177, 1996

Mathivanan N, Kabilan V and Murugesan K. Production of chitinase by *Fusarium chlamydosporum*, a mychoparasite to groundnut rust, *Puccinia arachidis*. *Indian J Exp Biol*. **35**:890-893, 1997

Matloob AH and Kamil JS. Biological control of bean root rot diseases caused by *Rhizoctonia solani* under green house and field conditions. *Agric. Biol. J.N.Am.* **4(5):**512-519, 2013

Matsumoto A, Takahashi Y, Mochizuki M, Seno A, Iwai Y and Omura S. Characterization of actinomycetes from fallen leaves. *Actinomycetologica*. **12:**46-48, 1998.

Meguro, A. Ohmura Y, Hasegawa S, Shimizu M, Nishimura T and Kunoh H An endophytic actinomycete, *Streptomyces*sp. MBR-52, that accelerates emergence and elongation of plant adventitious roots. *Actinomycetologica* **20**: 1–9. 2006.

Merriman P R, Price R D, Price KF (1974) *Aus J Agri Res.* **25**:213-218

Mishra BK, Mishra RK , Mishra RC, Tiwari AK, Yadav RC and Dikshit A. Biocontrol efficacy of *Trichoderma viride* isolates against fungal plant pathogens causing disease in *Vigna radiata.* L. *Arch. Appl. Sci. Res*: **3 (2):**361-369, 2011.

Mohseni M, Norouzi H, Hamedi J and Roohi A. Screening of Antibacterial Producing Actinomycetes from Sediments of the Caspian Sea. *Int. J.Mol. Cell. Med*. **2(2):** 64-71, 2013

Moncheva PT , Dimitrova S , Chipeva N , Antonova-Nicolova VS. and **Bogatzevska, N.** Characteristics of soil actinomycetes from Antarctica. *J culture collections* **3**: 3-14. 2002.

Mukankusi CM, Melis RJ, Derera J, Buruchara RA and Mark DA. Screening technique for resistance to *Fusarium* root rot of common bean. *Afr J Pl Sci.* **5 (3):** 152-161. 2011.

Muthu MR, Subbaiya R, Balasubramanium M, Ponmurugan P and Selvam M. Isolation and Identification of Actinomycetes *Isoptericola variabilis* From Cauvery River Soil Sample. *Int.J.Curr. Microbiol. App. Sci.* **2(6):** 236-245, 2013.

Nandi S, Dutta S, Mondal A, Adhikari A, Nath R, Chattopadhaya A and Chaudhuri S. Biochemical responses during the pathogenesis of *Sclerotium rolfsii* on cowpea. *Afr. J. Biotechnol.* **12(25):** 3968-3977, 2013

Newman, D.J. and **Cragg, G.M.** Natural products as sources of new drugs over the last 25 years. *J Nat Prod* **70:** 461–477. 2007.

Ningthoujam DS, Sanasam S, Tamreihao K and Nimaichand S. Antagonistic activities of local actinomycetes against rice fungal pathogens. *Afr J Microbiol Res.* **3(11**): 737-742, 2009

Okazaki T, Takahashi K, Kizuka M, Enokita R Studies on actinomycetes isolated from plant leaves. *Annu Rep Sankyo Res Lab* **47:**97–106, 1995.

Okazaki, T. Studies on actinomycetes isolated from plant leaves. In Selective Isolation of Rare Actinomycetes. National Library of Australia, (Ed. *I.* Kurtboke)102–121. 2003.

Ordentlich, A, Elad, Y. and Chet. I . *The role of chitinase of Serratia marcescens in biocontrol of Sclerotium rolfsii. Phytopathol*. **78:**84-*88*, 1988.

Oskay M, Usame Tamer A and Azeri C. Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. *African J Biotechnol*. **3(9):** 441-446,2004

Pan SQ, Ye XS and Kuc J. A technique for detection of chitinase,β-1,3 glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis for isoelectric focussing. *Phytopathology*, **81:** 970-974, 1991.

Pandey A, Ali I, Butola K.S. and Chatterji T, Singh V. Isolation and characterization of actinomycetes from soil and evaluation of Antibacterial activities of Actinomycetes against pathogens. *Int J Appl Biol PharmaTechnol*.**2(4):** 384-392. 2011.

Parihar PS, Prakash O and Punetha H. Investigation on defensive enzymes activity of *Brassica juncea* genotypes during pathogenesis of *Alternaria* blight. *Nat Sci.* **10(2):** 64-68, 2012.

Park JO, El-Tarabily KL, Ghisalberti EL. and Sivasithamparam K. Pathogenesis of *Streptoverticillium albireticuli* on *Caenorhabditi elegans* and its antagonism to soil-borne fungal pathogens. *Letters in Appl Microbiol.* **35**: 361–365. 2002.

Patel JD, Parmar M, Patel P, Rohit P, Taviyad R, Ansari P, Bhattacharya B, Vyas D, Kumar V, Sahay NS and Singh PK. Dynamism of Antimicrobial activity of Actinomycetes- A case study from Unisturbed Microbial Niche. *Advances in Microbiol.* **4:** 324-334, 2014.

Pattanapipitpaisal P, Kamlandharn R. Screening of chitinolytic actinomycetes for biological control of *Sclerotium rolfsii* stem rot disease of chilli. *Songklanakarin J. Sci. Technol*. **34 (4**), 387-393, 2012

Persello-Cartieaux F, Nussaume L. and Robaglia C. Tales from the underground: Molecular plant rhizobacteria interactions. Review. *Plant, Cell Environ.* **26**: 189 - 199, 2003.

Pikovskaya RI. Mobilization of phosphorus in soil connection with the vital activity of some microbial species. *Microbiol*. **17**: 362-370.1948

Polti MA, Amoroso MJ, and Abate CM. Chromium(VI) resistance and removal by actinomycete strains isolated from sediments. Chemosphere **67:** 660–667. 2007.

Prapagdee B, Kuekulvong C, and Mongkolsuk S . Antifungal Potential of Extracellular Metabolites Produced by *Streptomyces hygroscopicus* against Phytopathogenic Fungi *Int. J. Biol. Sci.* **4(5):**330-337. 2008.

Pridham TG. Color and Streptomycetes. *Appl. Microbiol.* **13(1):**43-61. 1965.

Pridham, TG, Lindenfelser LA, Shotwell OL, Stodola FH, Benedict RG, Foley C, Jackson RW, Zaumeyer WJ, Preston WH and Mitchell JW. Antibiotics against plant disease. I. *Laboratory and greenhouse survey. Phytopathol.* **46**:568–575. 1956.

Prinsen E, Costacutra A, Michielis K, Vanderleyden J and Van Onckelen H. *Azospirillum brasilense* indole-3-acetic acid biosynthesis: evidence for a nontryptophan dependent pahway. *Mol.Plant-microbe.Interact*.**6:**609-615, 1993

Punja ZK. *Sclerotium (Athelia) rolfsii*, a pathogen of many plant species. In: *Genetics of plant pathogenic fungi*. (Ed.): G.S. Sidhu. London: Academic Press. **6**:523-534. 1988.

Punja ZK and Rahe JE. *Sclerotium*. In: *Methods for research on soilborn phytopathogenic fungi*. (Eds.): L.L. Singleton, J.D. Mihail and C.M. Rush. St. Paul: APS Press. 166-170.1992.

Punja ZK. Biology, ecology and control of *Sclerotium rolfsii*. *Ann Rev Phytopathol*, **23**: 97-127. 1985.

Ramanathan A, Shanmugam V, Raghuchander T and Samiyappan R. Induction of systemic resistance in ragi againt blast disease by *Pseudomonas Fluorescens*. *Ann PC Prot Soc*, **10**: 313-318, 2002

Ramanathan A, Vidhyasekaran P, Samiyappan R. Two pathogenesis-related peroxidases in greengram (*Vigna radiata* (L.) wilczek) leaves and cultured cells induced by *Macrophomina phaseolina* (Tassi) Goid. and its elicitor. *Microbiol. Res.***156**: 139–144, 2001

Ravi L, Baskar R, Sarveswari S, Krishnan K. Co-culturing of Marine and Terrestrial Actinomycetes to Obtain Novel Secondary Metabolites. *Ann Pharmacol Pharm.* **2(7)**: 1041, 2017

Ray P, Chakraborty AP and Chakraborty BN. Biochemical and Molecular Characterization of *Streptomyces* species isolated from Agricultural field of North Bengal and evaluation for growth improvement and suppression of sclerotial blight diseases of *Vigna radiata. J Mycol Pl Pathol*. **46(4):** 219-229, 2016a.

Ray P, Chakraborty AP and Chakraborty BN. Evaluation of *Streptomyces* and non-streptomyces actinomycetes isolates for growth promotion in *Vigna radiata* and their use as biocontrol agent against *Sclerotium rolfsii*. *NBU J Pl Sci*. **10(1):**73-79, 2016b.

Reddi GS and Rao AS. Antagonism of soil actinomycetes to some soil-borne plant pathogenic fungi. *Indian Phytopathol*. **24**:649–657. 1971.

Redshaw PA, McCann PA, Sankaran L and Pogell BM. Control of differentiation in streptomycetes: involvement of extra chromosomal deoxyribonucleic acid and glucose repression in aerial mycelia development. *J. Bacteriol.***125**:698–705. 1976.

Reissig JL, Strominger JL and Leloir LF. A modified colorimetric method for the estimation of N-acetylamino sugars. *J Biol.Chem*. **217:** 959-966, 1959.

Roiger DJ, Jeffers SN. Evaluation of *Trichoderma* spp. for biological control of *Phytophthora* crown and root rot of apple seedlings. *Phytopathol* **81**, 910- 917. 1991.

Rothrock CS and Gottlieb D. Importance of antibiotic production in antagonism of selected streptomycetes species to two soil bourne plant pathogens. *The J antibiotics* .**44 (7):** 830-835,1981.

Sahaya MR and Dhanaseeli M. Biocontrol otential of selected Actinomycetes and its Metabolites against *Rhizoctonia solani*. *Ind J Natural Sci***. 3(12):** 2012

Sahin N, Ozturk E, Isik K, Kariptas E, Ozkanca R. Selective Isolation and Numerical Classification of Novel Thermophilic *Streptomycetes*. *Turk.J.Biol* **26**:13-24.2002

Sahin N, Ugur A. Investigation of the Antimicrobial Activity of Some *Streptomyces* Isolates. *Turk J Biol* **27**:79-84. 2003.

Sangmanee P, Bhromsiri A and Akarapisan A. The potential of endophytic actinomycetes, (*Streptomyce*s sp.) fpr the biocontrol of powdery mildew disease in sweet pea (*Pisum sativum*). *As J Food Ag-Ind*. Special issue, 93-98, 2009

Sarma BK, Singh UP and Singh KP. Variability in Indian isolates of *Sclerotium rolfsii* *Mycologia,* **94(6)**:1051–1058, 2002

Sastrahidayat IR, Djauhari S, Prasetya B and Saleh N. Biocontrol of damping of disease(*Sclerotium rolfsii* sacc.) using actinomycetes and VAM fungi on soybean and impact to crop production and microorganism diversity in rhizosphere zone. *Int J Academic Res*. **3(6):** 114-119, 2011.

Satya VK, Vijayasamundeeswari A, Paranidharan V and Velazhahan R. *Burkholderia* sp. strain TNAU-1 for biological control of root rot in Mung bean(*Vigna radiata* L.) caused by *Macrophomina phaseolina*. *J Pl Prot Res.* **51(3):** 273-278,2011

Schlegel HG. General microbiology,7thedition, Cambridge University Press.104-108.1992.

Schon R and Groth I. Practical thin layer chromatography techniques for diaminopimelic acid and whole cell sugar analysis in the classification of environmental Actinomycetes. *J. Basic Microbiol*. **46 (3):** 243-249, 2006

Selman A, Wakson and Hubert A. Lechevalier. Guide to the Classification An Identification of the Actinomycetes and Their Antibiotic. The Williams & Wilkins Co., Baltimore, 1953.

Sharma M. Actinomycetes: source, identification and their application, *Int J Curr Microbiol App Sci,* **3(2):**801-832, 2014

Shirling EB, Gottleib D. Methods for characterization of *Streptomyces* sp.*Int J Syst Bacteriol* **16:**313-340, 1966

Shrivastava P, Kumar R, Yangdigeri MS. *In vitro* biocontrol activity of halotolerant *Streptomyces aureofaciens* K20: A potent antagonist against *Macrophomina phaseolina* (Tassi) Goid. *Saudi J Biol Sci***. 24(1):** 192-199, 2017

Shrivastava S, D'Souza SF, Desai PD. Production of indole-3-acetic acid by immobilized actinomycete (*Kitasatospora* sp.) for soil applications *Curr Sci.* **94(12**): 1595-1604. 2008.

Simizu M. Endophytic Actinomycetes: Biocontrol Agents and Growth Promoters. *Bacteria in Agrobiology: Plant Growth responses*. D K Maheshwari (Ed.). Springer. 2011.

Singh N, Samajpati N and Paul AK. Dual inoculation of salt tolerant *Brady rhizobium* and *Glomus mosseae* for improvement of *Vigna radiata* L. cultivation in saline areas of west Bengal, india. *Agri. Sci*. **2(4):**413-423, 2011.

Singh PP, Shin YC, Park CS and ChungYR. *Biological control of Fusarium wilt of cucumber by chitinolytic bacteria. Phytopathol* **89:**92-*99.* 1999.

Singh UP and Pavgi. **MS.** Spotted leaf rot of plants, a new sclerotial disease. *Pl. Dis.Reptr*, **49** :58-59. 1965.

Sivakumar K, Sahu MK and Kathiresan K. Isolation and Characterization of Streptomycetes producing antibiotic from a mangrove environment. *Asian J Microbiol, Biotechnol and Environ Sci*, **7**: 457-464, 2005

Sneath PHA and Sokal RR. Numerical taxonomy: Principles and practice of numerical taxonomy, (W H Freeman & Co, San Francisco, USA). 1973

Soares ACF, Sousa CS, Garrido M, Perez JO, Almeida NS. Soil S*treptomycetes* with *in vitro* activity against the yam pathogens *Curvularia eragrostides* and *Colletotrichum gloeosporioides*. *Brazilian J. Microbiol.* **37:** 456-461, 2006.

Soe KM, Bhromsiri A and Karladee D. Effect of selected endophytic actinomycetes (*Streptomyces* sp.) and bradyrhizobia from Myanmar on growth, nodulation, nitrogen fixation and yield of different soybean varieties. *CMU. J. Nat. Sci.* **9(1):** 95-109, 2010.

Sowndhararajan K and Kang SC. *In vitro* antgonstic potential of *Strptomyces* sp. AM-S1 against plant and human pathogens. *J Agric Chem and Environ*. **1(1**): 41-47, 2012

Sprusansky O, Stirrett K, Skinner D, Denoya C, Westpheling J. The bkdR gene of *Streptomyces coelicolor* is required for morphogenesis and antibiotic production and encodes a transcriptional regulator of a branched-chain amino acid dehydrogenase complex. *J Bacteriol.*187:664-71. 2005.

Sreeja SJ And Surendra GK. Bio-efficacy of endophytic actinomycetes for plant growth promotion and management of bacterial wilt in tomato Pest Management in Horticultural Ecosystems. Ph.D. Thesis Kerala Agri Univ. 2013.

Srividya S, Thapa A, Bhat D, Golmei K and Dey N, *Streptomyces* sp. 9p as effective biocontrol against chilli soilbourne fungal phytopathogens. *Euro j Exp Bio* **2(1)**: 163- 173, 2012.

Sunar K. Studies on soil microbial diversity of Darjeeling hills and their evaluation for utilization in the improvement of crop health. Ph.D. Thesis, University of North Bengal, Siliguri , India.2014.

Suwan N, Boonying W and Nalumpang S. Antifungal activity of soil actinomycetes to control chilli anthracnose caused by *Colletotrichum gloeosporioides*. *J Agri Technol.* **8(2):** 725-737, 2012.

Taechowisan T, Lu C, Shen Y, Lumyong S. Secondary metebolites from endophytic *Streptomyces aureofaciens* CMUAc130 and their antifungal activity. *Microbiol.* **151**: 1691-1695, 2005.

Taechowisan T, Peberdy JF, Lumyong S. Chitinase production by endophytic *Streptomyces aureofaciens* CMUAc130 and its antagonism against phytopathogenic fungi. *Annals of Microbiol.* **53(4):** 447-461, 2003.

Takahashi Y and Ômura S. Isolation of new actinomycetes strains for the screening of new bioactive compounds. *J. Gen. Appl. Microbiol,* **49:** 141–154. 2003.

Tamura K, Dudley J, Nei M and Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0, *Mol Biol Evol* **24**:1596-1599.2007

Tamura K, Nei M and Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method, *Proc Natl Acad Sci USA*, **101**:11030-11035, 2004

Thompson J, Higgins D and Gibson T. CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res* 22: 4673-4680, 1994.

Tokala RK, Strap JL, Jung CM, Crawford DL, Salove MH, Deobald LA, Bailey JF, Morra MJ. Novel plant microbe rhizosphere interaction involving *Streptomyces l ydicus* WYEC108 and the pea plant (*Pisum sativum*) *Appl. Environ.Microbiol*. **68(5):** 2161-2171, 2002.

Van Peer R, Niemann GJ and Schippers B. Induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by *Pseudomonas* sp. Strain WCS417r. *Phytopathol*. **81**: 728-734, 1991.

Verma VC, Gond SK, Kumar A, Mishra A, Kharwar RN, Gange AC. Endophytic actinomycetes from *Azadirachta indica A. Juss*.: isolation, diversity, and anti-microbial activity. *Microb Ecol.* **57**(4):749-56. 2009.

Viswanathan R and Samiyappan R . Induction of systemic resistance by plant growth-promoting rhizobacteria against red rot disease caused by *Colletotrichum falcatum* went in sugarcane. In Proceedings of the Sugar Technology Association of India, **61***:24-39*,. 1999.

Wang, Y, Zhang, Z. S, Ruan Wang, JS, Y.M. and Ali SM. Investigation of actinomycete diversity in the tropical rainforests of Singapore. *J Industrial Microbiol Biotechnol.* **23**: 178–187. 1999.

Warcup JH. The origin of colonies of fungi on soil dilution plates. *Trans. Br.Mycol.Soc.*,**38**,298, 1955

Wei G, Kloepper JW, TuZun S. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. *Phytopathol*. **81:**1508–1512, 1991

Xiao K, Kinkel LL, Deborah A, Samac DA Biological Control of *Phytophthora* Root Rots on Alfalfa and Soybean with *Streptomyces Biological Control* **23**:285–295. 2002.

Xue L, Xue Q, Chen Q, Lin C, Shen G and Zaho J. Isolation and evaluation of rhizospheric actinomycetes with potential application for biocontrol of Verticillium wilt of cotton. *Crop Protection*. **43:** 231-240, 2013.

Yabe S, Aiba Y, Sakai Y, Hazaka M. and Yokota A. *Thermasporomyces composti* gen. nov, sp. nov, a thermophilic actinomycete isolated from compost. *Int J Syst Evol Microbiol*. **61:**86–90. 2011.

Yaqub F and Shahazad S. Pathogenecity of *Sclerotium rolfsii* on different crops and effect of inoculum density on colonization of mungbean and sunflower roots. *Pak. J. Bot*, **37(1):** 175-180. 2005

Yu J, Zhang L, Liu Q, Qi X, Ji Y and Kim BS. Isolation and characterization of actinobacteria from Yalujhang costal wetland, North China. *Asian Pac J Trop Biomed*. **5(7):** 555-560, 2015.

Yuan WM, Crawford DL. Characterisation of *Streptomyces lydicus* WYEC108 as a potential Biocontrol agent against fungal root rot and seed rot. *Appl. Environ.Microbiol*. **61 (8):** 3119- 3128. 1995.

Zarandi ME, Bonjar Shahidi GH, Dehkaei PF, Ayatollahi MSA, Farokhi RP and Aghighi S. Biological Control of Rice Blast (*Magnaporthe oryzae*) by use of *Streptomyces sindeneusis* isolate 263 in Greenhouse. *Am J Appl Sci* **6 (1):** 194-199. 2009.

APPENDIX A: List of Thesis related publications

In Journals

1. **Ray P**, Chakraborty AP and Chakraborty BN. Biochemical and Molecular Characterization of *Streptomyces* species isolated from Agricultural field of North Bengal and evaluation for growth improvement and suppression of sclerotial blight diseases of *Vigna radiate. J. Mycol. Pl. Pathol.,* **46(4):** 219-228, 2016.

2. **Ray P**, Chakraborty AP and Chakraborty BN. Evaluation of streptomyces and non-streptomyces actinomycetes isolates for growth promotion in *Vigna radiata* and their use as biocontrol agent against *Sclerotium rolfsii. NBU J. Pl. Sci.,* **10(1):**73-79,2016

APPENDIX B: List of Abbreviation

1. BLAST- Basic local alignment search tool
2. BSA- Bovine serum albumin
3. Ca3(PO4)2 - Tri-calcium phosphate
4. CaHPO4 - Calcium phosphate
5. CAS- Chrome azurol S
6. CAT- Catalase
7. CDA- Chitinase detection agar
8. cfu- Colony forming unit
9. CHT- Chitinase
10. CuSO4- Copper sulphate
11. DAPG- 2,4-Diacetylphloroglucinol
12. dATP- Deoxy adenosine tri-phosphate
13. dCTP- Deoxy cytosine tri-phosphate
14. DEAE cellulose- diethyl aminoethyl cellulose
15. dGTP- Deoxy guanosine tri-phosphate
16. DMAB- Di methyl amino benzaldehyde
17. DNA- Deoxyribonucleic acid
18. dNTPs- Deoxy nucleotide tri-phosphates
19. DNSA- Dinitro salicylic acid
20. dTTP- Deoxy thymidine tri- phosphate
21. EDTA- Ethylene diamine tetra acetic acid
22. FeCl3- Ferric chloride
23. FITC- Fluorescein isothiocyanate
24. g- gram
25. GlcNAc- N-acetyl glucosamine
26. H_2O_2 - Hydrogen peroxide
27. H2SO4- Sulphuric acid
28. HCl- Hydrochloric acid
29. HCN- Hydrocyanic acid
30. IAA- Indole acetic acid
31. ISR- Induced systemic resistance

32. ISP3 –International *Streptomyces* project media 3
33. KCl- Potassium chloride
34. KH2PO4 – Potassium dihydrogen phosphate
35. MEGA 4- Molecular Evolutionary Genetics Analysis 4
36. mg- Mili gram
37. MgCl2- Magnesium chloride
38. ml- Mili litre
39. Na2CO3- Sodium carbonate
40. Na2HPO4- Di sodium hydrogen phosphate
41. Na2MoO4- Sodium molybdate
42. NaCl- Sodium chloride
43. NaN3- Sodium azide
44. NaNO2- Sodium nitrite
45. NaOH- Sodium Hydroxide
46. NA- Nurient Agar
47. NB- Nutrient Broth
48. NBT/BCIP substrate- Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl)
49. dihydrogen phosphate substrate
50. NCBI- National Center for Biotechnology Information
51. NCM- Nitrocellulose membrane
52. NH4Cl- Ammonium chloride
53. PAL- Phenylalanine ammonia lyase
54. PBS-Tween- Phosphate buffer saline- Tween
55. PCA- Phenazine-1-carboxylic acid
56. PCI- Water saturated phenol: Chloroform: Isoamyl alcohol
57. PCR- Polymerase chain reaction
58. PDA- Potato Dextrose agar
59. PGPR- Plant Growth Promoting Rhizobacteria
60. POX- Peroxidase
61. PPO- Polyphenol oxidase
62. PR proteins- Pathogenesis related proteins
63. PVK- Pikovskaya’s agar
64. PVP- Poly vinyl- pyrollidone
65. RNA- Ribonucleic acid
66. RP- Rock phosphate

67. RS- Rhizosphere soil
68. SA- Salicylic acid
69. SAR- Systemic acquired resistance
70. SDS- Sodium dodecyl sulphate
71. SCN- Starch casein nitrate agar
72. TAE buffer- Tris Acetic Acid and EDTA buffer
73. TE buffer- Tris-EDTA buffer
74. TEMED- N,N,N',N'-Tetramethylethylenediamine
75. Tris Hcl- Tris hydrochloric acid
76. UPGMA- Unweighted Pair Group Method with Arithmetic Mean
77. yr- Year.
78. β-1,3-GLU- β-1,3 glucanase
79. µl- Micro litre.

APPENDIX C: List of Chemicals

1. Ammonium chloride
2. Bovine serum albumin
3. Calcium phosphate
4. Carboxy methyl cellulose
5. Chrome azurol S
6. Colloidal chitin
7. Copper sulphate
8. Deoxy nucleotide tri-phosphates
9. Di methyl amino benzaldehyde
10. Di sodium hydrogen phosphate
11. Diethyl aminoethyl cellulose
12. Dinitro salicylic acid
13. Ethylene diamine tetra acetic acid
14. Ferric chloride
15. Fluorescein isothiocyanate
16. Helicase (3%)
17. Hexa-decytrimethyl ammonium bromide
18. Hydrochloric acid
19. Hydrocyanic acid
20. Hydrogen peroxide
21. Indole acetic acid
22. Magnesium chloride
23. N-acetyl glucosamine
24. Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate
25. O-dianisidine (5 mg/ml methanol)
26. p- nitrophenyl phosphate
27. Phosphate buffer saline- Tween
28. Potassium chloride
29. Potassium dihydrogen phosphate
30. Sodium azide
31. Sodium carbonate
32. Sodium chloride
33. Sodium dodecyl sulphate

34. Sodium Hydroxide
35. Sodium molybdate
36. Sodium nitrite
37. Sulphuric acid
38. Tri-calcium phosphate
39. Tris Acetic Acid and EDTA buffer
40. Tris hydrochloric acid
41. Tris-EDTA buffer
42. Water saturated phenol: Chloroform: Isoamyl alcohol
43. 0.05(M) sodium phosphate buffer (pH 6.8)
44. 0.1M sodium phosphate buffer pH (7.4)
45. 0.1(M) sodium acetate buffer (pH 5.0)
46. 0.2M Na-phosphate buffer (pH 5.4)
47. 0.3mM borate buffer (pH 8.0)
48. 1 M K-PO4 buffer (pH 7.1)
49. 1-amino-cyclopropane-1-carboxylic acid hydrochloride 1M Na-acetate buffer (pH 4) Sodium borate buffer (pH 8.8)
50. 2 mM β- mercaptoethanol
51. 1 M Na-borate buffer (pH 9.8)
52. 2% L-phenylalanine
53. 2,4-Diacetylphloroglucinol
54. 4 mM H_2O_2.
55. 4% laminarin
56. 30%-90% absolute alcohol
57. 1% aqueous toluidine blue solution
58. 0.2% lead acetate

Research Article

Biochemical and Molecular Characterization of *Streptomyces* species isolated from Agricultural field of North Bengal and evaluation for growth improvement and suppression of sclerotial blight diseases of *Vigna radiata*

Pushpanjali Ray, Arka Pratim Chakraborty and Bishwanath Chakraborty*

*Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal, Siliguri-734013, West Bengal, *email:bncnbu@gmail.com*

Abstract

Seventeen streptomycetesisolates were obtained from the rhizosphere of *Solanum tuberosum*. Their morphological attributes such as the colony colour, pigment production in culture media, colour of the aerial spore mass and a number of biochemical tests were executed to determine whether the isolates have the ability to hydrolyze starch, produce catalase and indole ring, which confirmed them as being streptomycetes.Among these, 15 isolates showed the ability of solubilizing phosphate by producing a transparent halo zone around the colony in Pikovskaya agar medium. *In vitro* antagonistic activity of some of these isolates against fungal pathogens (*Fusarium graminarium, F. solani* and *Sclerotium rolfsii)* were also confirmed by dual culture method. Three potent isolates were identified as *Streptomyces tricolor* (NCBI KX894280)*, S. flavogriseus* (NCBI KX894281) *and S. griseus* (NCBI KX894282) by 16S rDNA technology. Phylogenetic analysis of the three isolates were conducted in MEGA4 and *Streptomyces tricolor* (NCBI KX894280), *S. flavogriseus* (NCBI KX894281) *and S. griseus* (NCBI KX894282) showed similarity with D44434.1 (*S. tricolor*), KC113491.1 (*S. flavogriseus*) and KJ623766.1 (*S.griseus*). *In vivo* evaluation of the growth promoting activity of the isolates on *Vigna radiata* revealed that *S.tricolor* (NCBI KX894280) and *S. flavogriseus* (NCBI KX894281) showed comparatively better growth promotion in comparison with the untreated control. These three *Streptomyces*isolates also enhances key defense enzymes like chitinase, β 1-3 glucanase, and peroxidase in field condition which help in suppression of Sclerotial blight disease.

Key words: Gram positive actinomycetes, *Fusarium graminarium, F. solani* and *Sclerotium rolfsii, Streptomyces griseus, S. flavogriseus, S. tricolor,* 16S rDNA sequences

Citation: Ray P, Chakraborty AP and Chakraborty BN. 2016. Biochemical and Molecular Characterization of *Streptomyces* species isolated from Agricultural field of North Bengal and evaluation for growth improvement and suppression of sclerotial blight diseases of *Vigna radiata*. *Mycol Pl Pathol*

Streptomycetesaremainly found in the rhizosphere of plants in association with other microorganisms like rhizobacteria and fungi. Streptomyce are a group of Actinobacteria. These are Gram positive filamentous soil inhabiting bacteria. Streptomyces sp. and other filamentous actinobacteria have been explored extensively over several decades for antibiotic production (Jog et al 2014). Actinomycetes are important producers of bioactive compounds and constitute a potential group of biocontrol agents. They have the capability to synthesize wide varieties of biologically active secondary metabolites such as antibiotics, pesticides, anti- parasitic compounds and enzymes like cellulase, xyla-nase, proteinase and chitinase (Sowndhararajan et al 2012).Some endophytic streptomycetes can also be used as biological control agents against plant pathogens.They utilize humic acid and other organic matter in soil. Patil et al (2010) isolated and characterized antagonistic actinomycetes- *Streptomyces tricolor* vh85 against *Rhizoctonia solani*. Dezfully and Ramanayaka

(2015) reported antimicrobial activity of *Streptomyces flavogriseus* ACTK2 (KC990785). Phosphate is an important part of plant system. In its absence plant growth is retarded. Streptomycetes having the phosphate solubilizing ability can help the plant to get more phosphate from the soil thereby increasing plant growth. Couillerot et al (2013) also reported biofertilizer and plant growth promoting activities of *Streptomyces anulatus*S37 in agriculture. Actinobacteria, especially Streptomyces, also exhibit immense biocontrol action against a range of phytopathogens (Wang et al 2013).

The present study aims at identifying streptomycetes isolates from the agricultural fields of North Bengal having plant growth promoting characteristics like Phosphate solubilizingactivityas well as antagonistic effect against plant pathogens as well as *in vivo* efficacy against *Vigna radiata* after application of potential isolates of Streptomycetes obtained from rhizosphere soil.

Material and Methods

Collection of soil samples. Soil samples were collected from various locations of Jalpaiguri and Darjeeling district. Soil samples were obtained from a depth of 6-10 cm in the rhizosphere of agricultural crops. The soil samples were allowed to air dry at room temperature for a week. Soil samples were collected from the rhizosphere soil of potato.

Isolation. The standard serial dilution plate technique by Warcup (1955) was used for isolation of streptomycetes. 5 grams of soil sample was suspended in 25 ml sterile distilled water. The suspension was stirred for 3 hour in magnetic stirrer and left for 30 minute so that soil particles settle down. The supernatant was used as stock solution which was diluted upto 10^{-6} level by serial dilution method. 1 ml of the dilutions were spread on Starch Casein Nitrate agar (SCN) medium, the plates were incubated at 35^0C for 7 days.

Biochemical tests. Biochemical tests were executed to determine whether the isolates have the ability to hydrolyze starch, and production of Catalase and indole ring. .

Starch hydrolysis. Isolates were streaked on sterilized starch agar plate and incubated for five days at 37º C. Then plates were flooded with Lugol's iodine solution. A clear zone underneath and around the growth indicates positive result

Indole test. 10ml of Davis Mingoli's broth supplemented with 0.1% tryptophan was inoculated with the isolates. Incubation was done anaerobically at 37º C for 7 days. The culture were flooded carefully with 2 ml of Ehrlich- Bobme (P-dimethylaminobenzaldehyde 10g, concentrated HCL 100ml) reagent on the surface,left for a few minutes and observed for the formation of a ring at the medium reagent interface indicating the production of indole.

Catalase. 24 hour old cultures were flooded with 0.5 ml 10% H_2O_2 solution. Production of gas bubbles indicated positive reaction.

Screening for phosphate solubilizing activity. Streptomycetes isolates were screened in Pikovskaya medium for phosphate solubilization activity (Pikovskaya 1948). Isolates were inoculated in Pikovskaya media and incubated for 7 days. A halo zone around the growth indicates phosphate solubilization activity.

***In vitro* antagonistic effect on fungal pathogen.** Streptomycetes isolates were tested for antagonistic effect against fungal pathogens of *Fusarium graminarium, F.solani* and *Sclerotium rolfsii* by dual culture method (Skidmore and Dickinson 1976). Streptomycetes isolates were placed in both PDA and SCN plates in a circle and the set up was incubated for 8 days. Blocks cut off from 8 day old fungal pathogen culture were transferred to the center of Petri dishes inoculatedwith streptomycetes isolates.Inhibitions of the radial growth of fungal pathogen by the isolates confirm antagonism. In another setup streptomycetes isolates were streaked in one side of the petridish and block of fungal pathogen put on other side. Inhibition of radial growth of fungal pathogen by the isolates confirmed antagonism.

Genomic DNA extraction. The broth cultures of isolates were centrifuged at 10,000 rpm at 28°C for 5 mins and the pellets were collected by discarding

the supernatant. The pellets were washed thrice with distilled water and resuspended in 0.5ml of lysis solution (100mM Tris Hcl, pH 7.5, 20mM EDTA, 250mM NaCl, 2% SDS, 1mg/ml lysozyme). To it 5 µl of R Nase (50mg/ml) was added and incubated at 37C for 3 hrs. Then 10 µl proteinase K solution (20mg/ml) was added and it was allowed to incubate at 65C for 3min. The lysate was extracted with equal volume of tris water saturated phenol: chloroform: isoamylalcohol (25:24:1) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was collected in clean tube and 2 volume of chilled absolute ethanol was added to this aqueous phase. The mixture was centrifuged at 10,000 rpm for 5 mins at 4C, the pellet was air dried and finally dissolved in 40µl TE buffer and stored at 4°C (Bollet et al 1991).

Amplification of 16S rDNA by PCR. For ITS-PCR amplification, DNA was amplified by mixing the template DNA (50 ng) with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction (PCR) was performed in a total volume of 100 µL, containing 78 µL deionized water, 10 µL 10× Taq polymerase buffer, 1 µL of 1U Taq polymerase enzyme, 6 µL 2 m*M* dNTPs, 1.5 µL of 100 m*M* reverse and forward primers and 3.5 µL of 50 ng template DNA. For amplification of the ITS region of *B. altitudinis* isolate, the primer pair, Forward primer: 5'-AGAGTRTGATCMTYGCTWAC-3' and Reverse primer: 5'-CGYTAMCTTWTTACGRCT-3', was used. The PCR was programmed with an initial denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. The PCR product (20 µL) was mixed with loading buffer (8 µL) containing 0.25% bromophenol blue, 40% w/v sucrose in water, and then loaded in 2% agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis. The PCR product was sent for sequencing to Credora Life Science, Bangalore, India.

16 S rDNA Sequence Analysis. The sequenced PCR product was aligned with extypeisolates' sequences from NCBI GenBank for identification as well as for studying phylogenetic relationship. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal 1973). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al 2004) and these are represented in the units of number of base substitutions per site. Codon positions included were 1st +2nd+3rd+ noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA-4 (Tamura et al 2007). 16S rDNA of all the three streptomyces isolates were aligned to study the range of homology present in the conserved regions following the Clustal W algorithm (Thompson et al 1994) using the Bioinformatic tool BioEdit.

Inoculation technique and disease assessment. Pot-grown 2-3 week old plants (*Vigna radiata*) was used for artificial inoculation with fungal pathogen. Sand maize meal media containing fungal inoculum were added carefully in the rhizosphere and ensured that inocula were attached to healthy roots. Disease assessment was done 15 d after inoculation. In order to determine the effects of Streptomycetes on disease reduction, three treatments were taken in each case: untreated control, inoculated with pathogen and inoculation with both test isolate and fungal pathogen. Percentage disease incidence was recorded while disease intensity was calculated using a 0-6 scale (Mathew and Gupta 1996).

Assay of enzyme activities.β-1, 3-glucanase. Estimation of β-1, 3-glucanase activity was done using laminarin dinitrosalicylate method described by Pan *et al* (1991).

Chitinase. Chitinase activity was measured following the method Boller and Mauch (1988).

Peroxidase. Estimation of peroxidase activity was done following the method described by Chakraborty et al (1993).

Results and Discussion

Morphological identification of streptomyces isolates. A total number of 17 isolates were isolated from starch casein nitrate agar medium. Manyof the

isolates showed typical morphology of streptomycetesgroup. The colony colour varies from white, pink, grey and red. Some of the isolates produced soluble pigments which diffuse in the media (Fig. 1; Table 1).

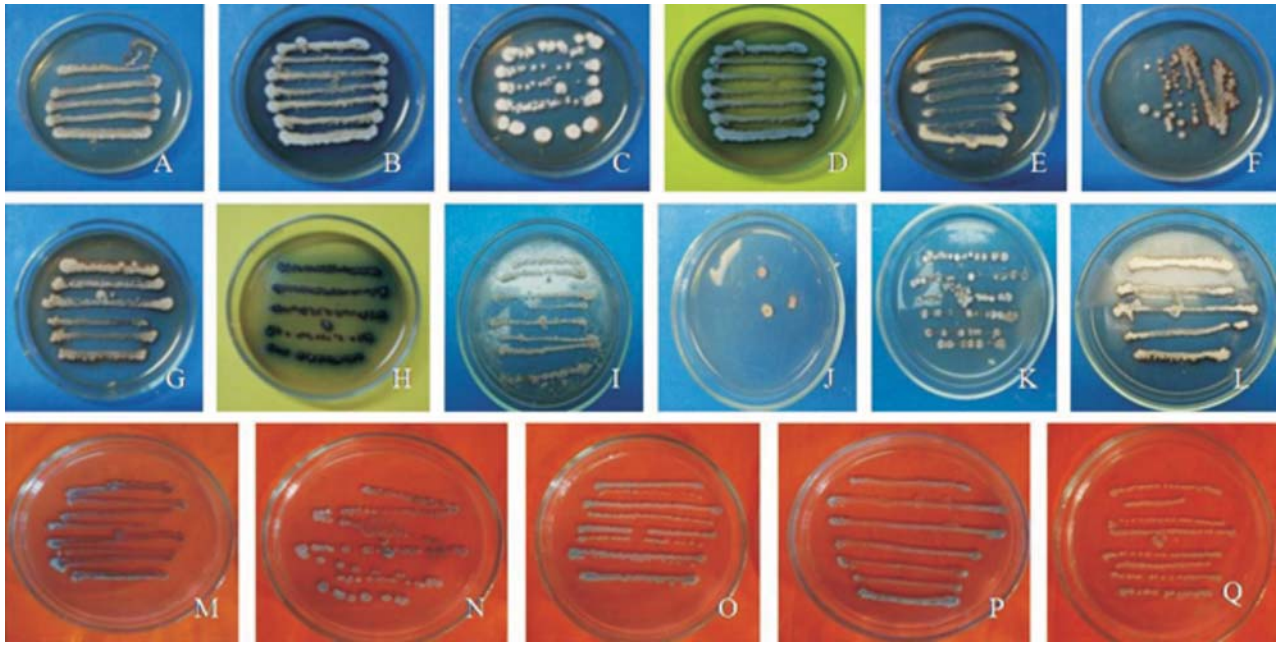


Figure 1. Morphological characteristics of seventeen different streptomycetes isolates– ARHS/PO11(A); ARHS/PO12(B); ARHS/PO13(C); ARHS/PO14(D); *Streptomyces griseus* (ARHS/PO15) (E); ARHS/PO16(F); ARHS/PO17(G); ARHS/PO18(H); ARHS/PO20(I); ARHS/PO22(J); ARHS/PO23(K); ARHS/PO24(L); ARHS/PO25(M); *Streptomyces tricolor* (ARHS/PO26)(N); *Streptomyces flavogriseus* (ARHS/PO27) (O); ARHS/PO28(P) and ARHS/PO24(Q)

Biochemical tests and phosphate solubilization activity. All of the isolates showed positive result for biochemical tests which confirmed that the isolates are streptomycetes. 15 isolates out of 17 showed phosphate solubilization activity by the production of clear zone around the growth in Pikovskaya medium (Table 1).

***In vitro* antagonistic effect on fungal pathogen.** Seventeen isolates were tested for *in vitro* antagonistic effect against selected plant pathogenic fungi. Three isolates, *viz*,. *Streptomyces griseus* (ARHS PO15), *Streptomyces tricolor* (ARHS/PO26) and *Streptomyces flavogriseus* (ARHS/PO27) showed antagonistic activity (Fig.2; Table2).

Molecular identification of streptomyces isolates

rDNA Sequence Analysis for Identification. The BLAST query of 16S rRNA sequence of the selected, potential isolates against Gen Bank database confirmed their identity, where isolate ARHS/PO26 was identified as *Streptomyces tricolor,* ARHS/PO27 as *Streptomyces flavogriseus* and ARHS PO15 as *Streptomyces griseus.* The sequences have been deposited in NCBI, Gen Bank database under the accession Nos. KX894280, KX894281 and KX894282 for *Streptomyces tricolor, Streptomycesflavogriseus* and *Streptomyces griseus* respectively.

Phylogenetic Analysis. The sequenced PCR product was aligned with extype isolate sequences from NCBI GenBank for identification as well as for studying phylogenetic relationship with other ex-type sequences. Multiple alignment parameters were used. The use of Clustal Wdetermines that once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. The optimal tree with the sum of branch length (=107.31370584) is shown in Fig. 3.

Table 1. Morphology and biochemical tests of streptomyces actinomycetes

Code	Spore	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Urase production	Cellulase Production	Indolae Production	Identification
Potato													
ARHS/PO/11	+	+	–	+	+	+	+	+	+	–	+	–	*Streptomyces sp.*
ARHS/PO/12	+	+	–	+	+	+	+	+	+	–	+	–	*Streptomyces sp.*
ARHS/PO/13	+	+	–	–	+	+	–	–	+	–	–	–	*Streptomyces sp.*
ARHS/PO/14	+	+	–	+	+	+	–	–	+	–	–	–	*Streptomyces sp.*
ARHS/PO/15	+	+	–	–	+	+	–	–	+	–	–	–	*Streptomyces griseus* (KX894282)
ARHS/PO/16	+	+	–	+	+	+	+	+	+	–	+	–	*Streptomyces sp.*
ARHS/PO/17	+	+	–	+	+	+	–	–	+	–	–	–	*Streptomyces sp.*
ARHS/PO/18	+	+	–	+	+	+	+	+	+	–	+	–	*Streptomyces sp.*
ARHS/PO/20	+	+	–	+	+	+	–	–	+	–	–	–	
ARHS/PO/22	+	+	–	+	+	+	–	–	+	–	–	–	*Streptomyces sp.*
ARHS/PO/23	+	+	–	+	+	+	–	–	+	–	–	–	*Streptomyces sp.*
ARHS/PO/24	+	+	–	+	+	+	+	+	+	_	+	–	*Streptomyces sp.*
ARHS/PO/25	+	+	–	+	+	+	+	+	+	_	+	–	*Streptomyces sp.*
ARHS/PO/26	+	+	–	+	+	+	–	–	+	–	–	–	*Streptomyces tricolor* (KX894280)
ARHS/PO/27	+	+	–	–	+	+	+	+	+	–	+	+	*Streptomyces flavogriseus* (KX894281)
ARHS/PO/28	+	+	–	+	+	+	–	–	+	–	+	+	*Streptomyces sp.*
ARHS/PO/29	+	+	–	+	+	+	–	–	+	–	+	+	*Streptomyces sp.*

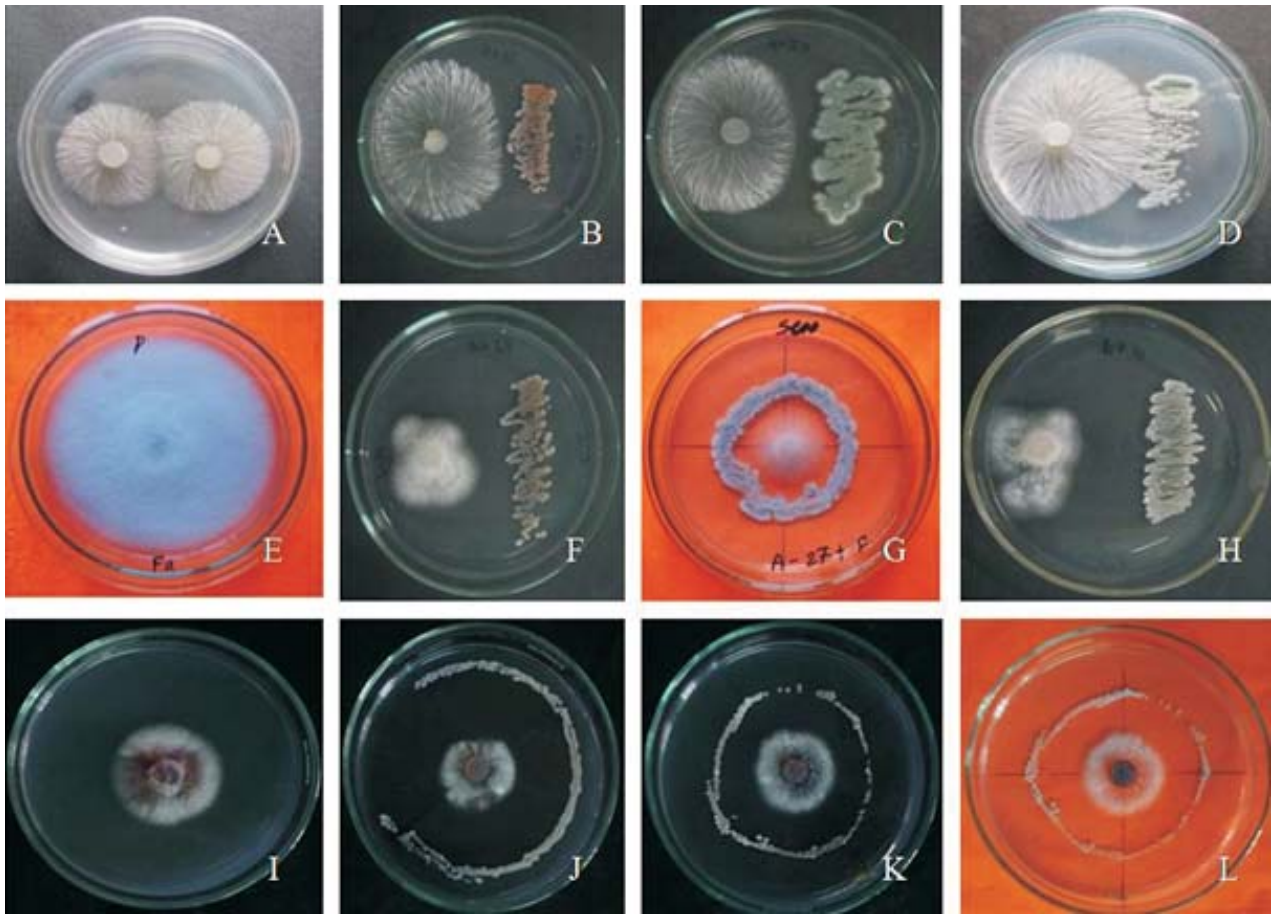


Figure 2. Antagonistic activities of three streptomyces actinomycetes isolates against three fungal pathogens: *Sclerotiumrolfsii* (A); *Streptomyces tricolor* (ARHS/PO26) + *S. rolfsii* (B); *S. flavogriseus* (ARHS/PO27)+ *S.rolfsii* (C) ; *S.griseus* (ARHS/PO15) + *S.rolfsii* (D) *Fusariumsolani* (E); *Streptomyces tricolor* (ARHS/PO26)+*F.solani* (F); *S. flavogriseus* (ARHS/PO27) + *F.solani* (G) ; *S.griseus* (ARHS/PO15) + *F.solani* (H) *Fusariumgraminearum* (I); *Streptomyces tricolor* (ARHS/PO26) + *F. graminearum* (J); *S.flavogriseus* (ARHS/PO27) + *F.graminearum* (K); *S. griseus* (ARHS/PO15) + *F.graminearum*(L)

Phylogenetic analyses were conducted in MEGA4. Multiple sequence alignment revealed that there were regions in the sequences which were not similar and, hence, gaps were introduced in these regions. Presence of regions with similar sequences indicated relationships among the three isolates.

Evaluation of three streptomyces actinomycetes on plant growth promotion. In preliminary screening experiments three isolates: *Streptomyces griseus* (ARHS PO15), *Streptomyces tricolor* (ARHS/PO26) and *Streptomyces flavogriseus* (ARHS/PO27) showed phosphate solubilization, starch hydrolysis activities *in vitro* conditions. These isolates were also found to inhibit *Sclerotium rolfsii.* Marked increase in attributes of parameters in growth of *V. radiata*was noticed when three isolates were applied in the rhizosphere of plant. Increase in the growth was observed in terms of increase in height of saplings, number of shoots and number of leaves and roots (Table 3). Percentage increase in terms of growth parameterssuch as , height of the plants, number of leaves and leaf length, and root length were recorded at 7day interval upto 20 days. Since plant growth promotion could also be due to induction of biochemical responses within the host, experiments were conducted to assess the effect of three streptomyces isolateson the accumulation of defense enzymes in 7 day old seedlings of *V.*

Table 2. Result of *in vitro* antagonistic effect of streptomycesactinomycetes on fungal pathogens

Isolates	Code	Inhibition (%)		
		Fusarium graminearum	*F. solani*	*Sclerotium rolfsii*
Streptomyces sp	ARHS/PO/11	66.7	67.8	41.66
Streptomyces sp	ARHS/PO/12	56.7	57.8	56.18
Streptomyces sp	ARHS/PO/13	56.7	67.8	46.66
Streptomyces sp	ARHS/PO/14	66.7	60.0	53.72
Streptomyces griseus	ARHS/PO/15	60.0	62.2	51.81
Streptomyces sp	ARHS/PO/16	60.0	60.0	40.95
Streptomyces sp	ARHS/PO/17	62.2	60.0	44.90
Streptomyces sp	ARHS/PO/18	66.7	56.7	40.90
Streptomyces sp	ARHS/PO/20	56.7	66.7	46.66
Streptomyces sp	ARHS/PO/22	57.8	66.7	58.22
Streptomyces sp	ARHS/PO/23	67.8	60.0	45.45
Streptomyces sp	ARHS/PO/24	62.2	62.2	45.77
Streptomyces sp	ARHS/PO/25	60.0	67.8	48.25
Streptomyces tricolor	ARHS/PO/26	67.8	66.7	70.26
Streptomycesflavogriseus	ARHS/PO/27	85.6	88.5	64.86
Streptomyces sp	ARHS/PO/28	67.8	56.7	54.05
Streptomyces sp	ARHS/PO/29	57.5	63.4	56.45

Table 3. Effect of foliar application of *S.tricolor, S.flavogriseus and S. griseus*on the growth of *Vigna radiata*

Treatment	Days	*Vigna radiata*			
		Total height (cm)	Number of leaves	Leaf area (cm^2)	Root length (cm)
Control	7	09±1.73	4	6.25±1.24	2.0±0.67
	14	14±0.21	06	6.85±0.25	2.85±0.55
	20	16±1.62	16	8.64±1.05	3.25±0.76
S. triolor	7	13±1.05	6	6.45±1.22	2.5±3.46
(ARHS/PO26)	14	18±1.75	10	8.55±1.24	4.5±1.22
	20	23±1.00	18	09.12±1.25	8.2±3.36
S. flavogriseus	7	14±1.73	7	6.5±0.73	2.3±1.22
(ARHS/PO27)	14	17±1.54	12	8.6±0.54	4.6±1.33
	20	22±1.14	19	9.3±1.12	8.5±1.54
S.griseus	7	15±1.14	6	6.3±1.14	2.6±0.93
(ARHS/PO15)	14	19±1.63	15	8.8±1.15	4.7±1.12
	20	24±1.73	20	9.5±1.63	8.5±1.54

±= SE; Values are mean of 10 replicate plants

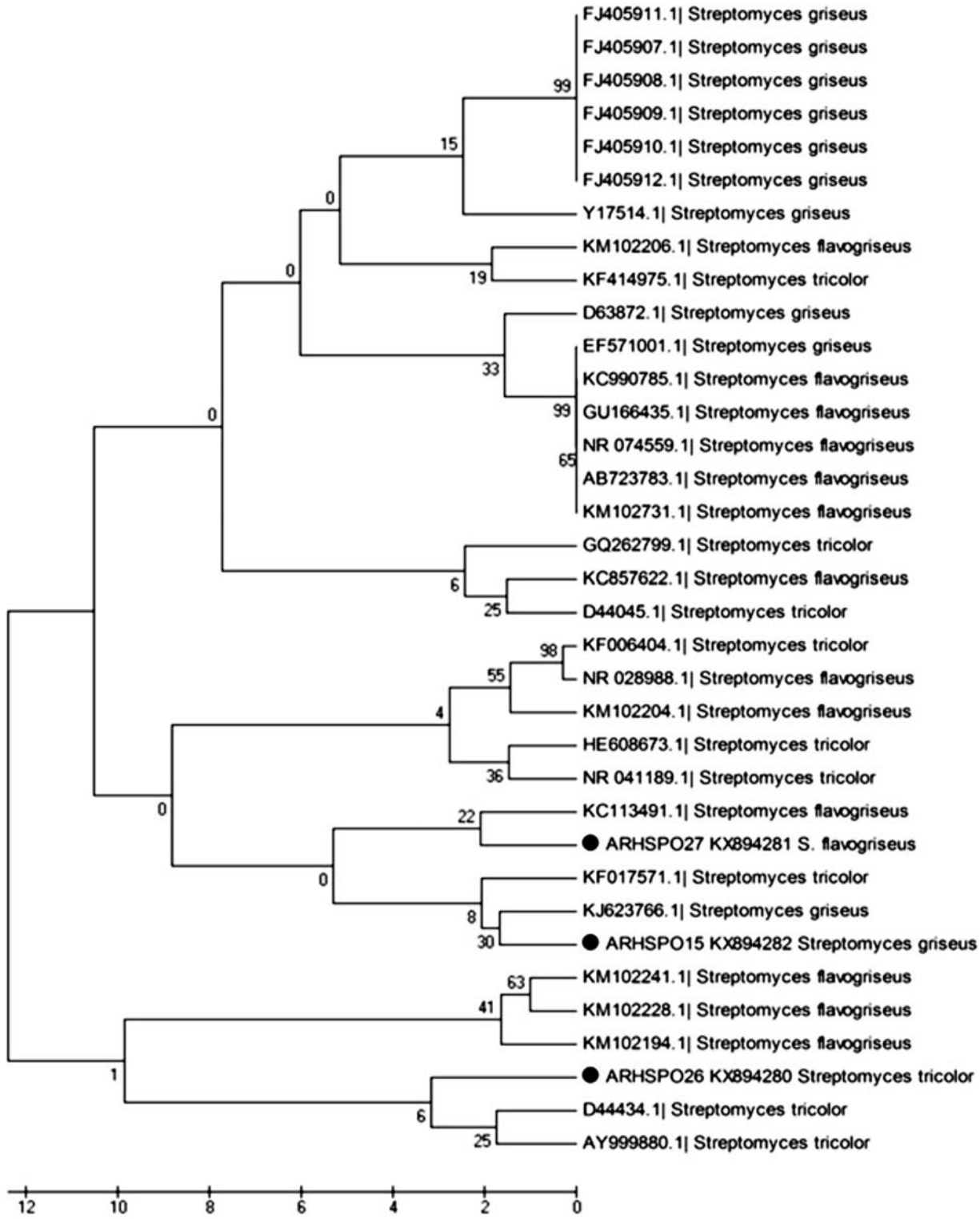


Figure 3. Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationship between *Steptomyces griseus* (ARHS/PO15), *Streptomyces tricolor* (ARHS/PO26) and *Streptomyces flavogriseus* (ARHS/PO27) with closely related species were used as out–groups in the tree. The optimal tree with the sum of branch length = 107.31370584 is shown

radiata. Three specific defense enzymes like peroxidase, ß-1, 3- glucanaseand chitinase activities were determined and compared with healthy control. Results revealed enhanced production of peroxidase, chitinase and ß-1, 3- glucanasein the leaf of the plant following application of *Streptomyces griseus* (ARHS PO15), *Streptomyces tricolor* (ARHS/PO26) and *Streptomyces flavogriseus* (ARHS/PO27) (Table 4).Shimizu (2011) reported role of endophytic actinomycetes as plant growth promoters as well as biocontrol agents. Induced Systemic Resistance (ISR) is effective against different types of pathogens but differs from Systemic Acquired Resistance (SAR) in that the inducing PGPR does not cause visible symptoms on the host plant (Van Loon *et al* 1998). Pieterse *et al* (2002) confirmed that to protect themselves from the disease, plants have evolved sophisticated defense mechanisms in which the signal molecules salicylic acid, jasmonic acid and ethylene often play crucial roles. This resistance was correlated with the accumulation of pathogenesis related (PR) proteins, generally assumed to be the markers of defense response (Ward *et al* 1991). Chitinase are a functionally and structurally diverse group of enzymes that can hydrolyse chitin and are believed to contribute to the defence of plants against certain fungal pathogens (Sahai and Manacha 1993; Jackson and Taylor 1996).

Biocontrol potential of three streptomyces isolates. In order to assess the biocontrol potential of *Streptomyces* against *Sclerotium rolfsii*, three potent isolate**s-** *Streptomyces griseus* (ARHS PO15), *Streptomyces tricolor* (ARHS/PO26) and *Streptomyces flavogriseus* (ARHS/PO27) were selected on thebasis of their antagonistic activity in *Vigna radiata*. Results revealed that sclerotial blight disease development was markedly reduced with prior applications of *Streptomyces griseus* (ARHS PO15), *Streptomyces tricolor* (ARHS/PO26)and *Streptomyces flavogriseus* (ARHS/PO27) (Table 5).There are reports about antagonistic activities of actinomycetes isolates against rice fungal pathogens (Ningthoujam et al 2009). Pattanapipitpaisal and Kamlandharn (2012) screened chitinolytic actinomycetes and isolated one potent isolate- *Streptomyces hygroscopicus which reduced stem rot disease of chilli caused by Sclerotium rolfsii. Gopalakrishnan* et al (2011) isolated actinomycetes isolates from herbal vermicompost and those actinomycetes were used as biocontrol agents in controlling *Fusarium* wilt of chickpea.

No disease was observed in uninoculated control, or those inoculated with either *T. harzianum* alone.

[a]0 = No symptoms;

1 = Small roots turn rotten lesion appeared at the

Table 4. Changes in defense enzymes of *Vigna radiata*seedlings following foliar application of *S.tricolor, S.flavogriseus and S. griseus* in field condition

Plants treated with	Defense enzymes	*Vigna radiata*
Untreated control	Peroxidase (ΔOD/gm/min)	68.0±1.06
	Chitinase (mg Glc NAC/g/hr)	07.3±0.78
	Glucanase (µg gliucose/g/min)	0.285±0.002
Treated with *Streptomyces tricolor* (ARHS/PO26)	Peroxidase (ΔOD/gm/min)	85.0±2.50
	Chitinase (mg Glc NAC/g/hr)	08.6±0.845
Treated with *Streptomyces flavogriseus* (ARHS/PO27)	Glucanase (µg glucose/g/min)	0.300±0.006
	Peroxidase (ΔOD/gm/min)	87.0±2.35
	Chitinase (mg Glc NAC/g/hr)	08.9±0.54
	Glucanase (µg glucose/g/min)	0.354±0.005
Treated with *Streptomyces griseus* (ARHS/PO15)	Peroxidase (ΔOD/gm/min)	86.0±2.73
	Chitinase (mg Glc NAC/g/hr)	08.8±0.73
	Glucanase (µg glucose/g/min)	0.301±0.007

±= SE; Values are replicate of three samples

Table 5 Evaluation of *Streptomyces* isolates on the development of sclerotial blight incidence of *Vigna radiata*

Disease Index[a]	
Treatments	*Vigna radiata*
S. rofsii	6.35
S. rofsii + *S. griseus* (ARHS/PO15)	0.84
S. rofsii + *S. flavogriseus* (ARHS/PO27)	0.83
S. rofsii + *S. tricolor*(ARHS/PO26)	0.80

collar region; 2 = Middle leaves start wilting and 10–20% of root turn brown; 3 = Leaves wilted and 20–40% roots become dry with browning of shoot; 4 = Extensive rotting at the collar region of roots, 60–70% of roots and leaves wilted, browning of shoot over 60%; 5 = 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80% 6 = Whole plants die, since 100% roots were average of 3 separate inoculated plants

The result of the present study is indicative thatpotent*Streptomyces* species could induce plant growth promotion and disease reduction in *Vigna radiata.* Streptomyces actinomycetes can be used as potent biocontrol agent for agriculture.

Acknowledgement

Financial support from ICAR, as a part of network project entitled Application of Microorganisms in Agriculture and Allied Sectors (AMAAS) of National Bureau of Agriculturally Important Microorganisms (NBAIM), is gratefully acknowledged.

References

Bollet C, Gevaudan MJ, Lamballerie X de, Zandotti C and Micco P de. 1991. A simple method for the isolation of chromosomal DNA from Gram positive or acid-fast bacteria. *Nucleic Acids Res* 19:55.

Chakraborty U, Chakraborty BN and Kapoor M. 1993.Changes in levels of peroxidase and phenylalanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptoshaeria maculans.Folia Microbiol* 38:491-496.

Couillerot O, Vatsa P, Loqman S, Ouhdouch Y, Jane H, Renault JH, Clement C and Barka EA. 2013. Biocontrol and biofertilizer activities of the *Streptomyces anulatus* S37: an endophytic actinomycete with biocontrol and plant-growth promoting activities.*Biological Control of Fungal and Bacterial Plant Pathogens IOBC-WPRS Bulletin.* 86: 271-276.

Dezfully NK and Ramanayaka JG. 2015. Isolation, Identification and Evaluation of Antimicrobial Activity of *Streptomyces flavogriseus*, strain ACTK2 from Soil Sample of Kodagu, Karnataka State (India).*Jundishapur J Microbiol* 8: e15107.

Gopalakrishnan S, Pande S, Sharma M, Humayun P, Kiran BK, Sandeep D, Vidya MS, Deepthi K and Rupela O. 2011. Evaluation of actinomycete isolates obtained from herbal vermicompost for the biological control of *Fusarium* wilt of chickpea. *Crop Protect* http://dx.doi.org/10.1016/j.cropro.2011.03.006.

Jackson AO and Taylor CB. 1996. Plant-microbe interactions: life and death at the interface. *The Plant Cell* 8:1651-1668.

Jog Rahul, Pandya Maharshi, Nareshkumar G and Rajkumar Salini. 2014. Mechanism of phosphate solubilization and antifungal activity of *Streptomyces* spp. isolated from wheat roots and rhizosphere and their application in improving plant growth *Microbiology* (2014), 160 : 778–788.

Mathew K. A and Gupta SK 1996. Studies on web blight of French bean and its management. *Indian J Mycol Pl Pathol* 26: 171-177.

Ningthoujam DS, Sanasam S, Tamreihao K and Nimaichand S. 2009. Antagonistic activities of local actinomycete isolates against rice fungal pathogens. *Afr J Microbiol Res* 3:737-742.

Pan QS, Ye SX and Kuc J. 1991. A technique for detection of chitinase, β-1,3-glucanase, and protein pattern safter a single separation using

polyacrylamid e gel electrophoresis or iso electro focusing. *Phytopathol*81: 970-974.

Patil HJ, Srivastava AK, Kumar S, Chaudhari BL and Arora DK. 2010.Selective isolation, evaluation and characterization of antagonistic actinomycetes against *Rhizoctonia solani*.*World J Microbiol Biotechnol* 26:2163–2170.

Pattanapipitpaisal P and Kamlandharn R. 2012. Screening of chitinolytic actinomycetes for biological control of *Sclerotium rolfsii* stem rot disease of chilli. *Songklanakarin J Sci Technol* 34: 387-393.

Pieterse CMJ, Van Wees SCM, Ton J, Van Pelt JA and Van Loon LC. 2002. Signalling in rhizobacteria induced systemic resistance in *Arabidopsis thaliana.Pl. Biol.* 4:535– 544.

Pikovskaya RI. 1948. Mobilization of phosphorus in soil connection with the vital activity of some microbial species. *Microbiologiya* 17: 362-370.

Sahai AS and Manocha MS. 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction *FEMS Microbiol Rev*11: 317-338.

Shimizu M. 2011. Endophytic Actinomycetes: Biocontrol Agents and Growth Promoters. In: DK Maheshwari (Ed.),*Bacteria in Agrobiology: Plant Growth Responses*, Springer-Verlag Berlin Heidelberg,pp 201-220.

Skidmore AM and Dikinson CH.1976.*Trans Br Mycol Soc* 66: 57-64.

Sneath PHA and Sokal RR. 1973. *Numerical taxonomy:Principles and practice of numerical taxonomy,*(W H Freeman & Co, San Francisco, USA).

Sowndhararajan K andKang SC. 2012. *In vitro* antagonistic potential of *Streptomyces* sp. AM-S1 against plant and human pathogens. *J Agric. Chem. Environ.*1:1 41-47

Tamura K, Dudley J, Nei M and Kumar S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0, *Mol Biol Evol* 24:1596-1599.

Tamura K, Nei M and Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method, *Proc Natl Acad Sci USA*, 101:11030-11035.

Thompson J, Higgins D and Gibson T. 1994. CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res* 22: 4673-4680.

Van Loon LC, Bakker PAHM and Pieterse CMJ. 1998 Systemic resistance induced by rhizosphere bacteria.*Annu Rev Phytopathol* 36:453-83.

Wang C, Wang Z, Qiao X, Li Z, Li F, Chen M , Wang Y, Huang Y and Cui H. 2013 Antifungal activity of volatile organic compounds from Streptomyces alboflavus TD-1. *FEMS MicrobiolLett* 341:45–51.

Warcup JH. 1955. The origin of colonies of fungi on soil dilution plates. *Trans Br Mycol Soc* 38:298.

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Metraux JP and Ryals JA. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3: 1085-1094.

NBU Journal of Plant Sciences
Vol. 10, No. 1, March 2016 p. 73-79

ISSN No. 0974-6927

Research Article

Evaluation of streptomyces and non-streptomyces actinomycetes isolates for growth promotion in *Vigna radiata* and their use as biocontrol agent against *Sclerotium rolfsii*

Pushpanjali Ray, Arka Pratim Chakraborty and Bishwanath Chakraborty*
Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal, Siliguri, West Bengal

Abstract

Two streptomyces (ARHS/PO26 and ARHS/PO27)and two non streptomyces (ARHS/Mn3 and ARHS/Mn7) actinomycetes isolates obtained from the rhizosphere soil of *Solanum tuberosum* and *Mangifera indica* were found to be phosphate solubilizers and showed antagonistic activity against *Sclerotium rolfsii*. Isolates ARHS/PO26 and ARHS/PO27 were identified morphologically and confirmed by the National Centre for Fungal Taxonomy, as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08). ARHS/Mn 3 and *Streptomyces griseolus* (ARHS/PO27) could inhibit 68% and 59.7% growth of *Sclerotium rolfsii in vitro*. *In vivo* evaluation of the isolates ARHS/Mn 3, *Streptomyces griseolus* (ARHS/PO27) and *Streptomyces griseus* (ARHS/PO26) showed maximum growth promotion on *Vigna radiata* by enhancing key defense enzymes like chitinase, β-1,3-glucanase, phenylalanine ammonia lyase and peroxidase. The results emphasize the fact that soil actinomycetes could be used as potential biocontrol agents.

Keywords: Non-streptomyces Actinomycetes, *Streptomyces griseus, Streptomyces griseolus*, *Vigna radiata*, Growth promotion, Defense enzymes, *Sclerotium rolfsii*

Introduction

Mung bean or Green gram *Vigna radiata* (L.) Wilczek (syn: *Phaseolus aureus* Roxb.) constitutes the important group of grain legumes which form a major source of dietary proteins of high biological value, energy, minerals and vitamins (Taylor *et al*., 2005). Those who can not eat animal protein this plant belonging to the family Fabaceae or leguminosae is a good source of protein. However, the yield of mung bean is greatly reduced due to various factors of which diseases caused by fungi and viruses are of major concern (Satya *et al.,* 2011). Now it has become necessary to find out ways of increasing yield and decreasing disease incidence in *Vigna*.

Streptomycetes are a group of actinobacteria which are part of the microbial flora of most natural substrates (Moustafa *et al.,* 1963) and mainly found in the rhizosphere of plants in association with other microorganisms like rhizobacteria and fungi. They utilize humic acid and other organic matter in soil. In their natural habitat, such as forests, the actinomycetes interact in various ways with the higher plants (Lo *et al*., 2002). These organisms are part of PGPM or plant growth promoting microorganisms. Streptomycetes affect plant health in various ways like by producing plant growth promoting hormones like IAA (Manulis *et al.,* 1994), production of siderophores (Tokala *et al.,* 2002) which influence plant growth or by protecting the plant against plant pathogenic microorganisms. It has been reported that secondary metabolites produced by some *Streptomyces* spp. inhibit growth of phytopathogenic fungi like *Colletotrichum musae* and *Fusarium oxysporum* (Taechowisan *et al.,* 2005).Many of non-streptomycete actinomycetes (NSA) taxa are therefore rarely reported in literature dealing with routine isolations of biocontrol agents and plant growth promoters from plant and soil. Seed-coating with powder formulation of the biocontrol agent was as effective as drench application of the fungicide, oxine benzoate (No-Damp), in controlling *Rhizoctonia* damping-off and superior to the commercial biocontrol agent, *Streptomyces*

**Corresponding author*
E-mail: bncnbu@gmail.com

griseoviridis (Mycostop), applied to tomato seeds as seed-coating. Ensign *et al.* (1993) reviewed the physiology of some NSA as a component of soil microflora. Although Lechevalier (1988) and Doumbou *et al.* (2001) reviewed the literature on the biological control of soil-borne fungal plant pathogens and plant growth promotion by actinomycetes, they covered activities mainly of *Streptomyces* spp.

The present study reflects the role of non-streptomyces strains- ARHS/Mn 3 and 7 and streptomyces strains (*Streptomyces griseus* and *S. griseolus*) as plant growth promoters and biocontrol agents in reducing sclerotial disease in *Vigna radiata.*

Material and Methods

Isolation of actinomycetes

Actinomycetes were isolated by the standard serial dilution plate technique by Warcup (1955) using starch casein nitrate agar (SCN) medium.

Biochemical Characterization

Biochemical characterization of actinomycetes isolates were performed including starch hydrolysis, catalase and indole tests.

Screening for phosphate solubilizing activity

Isolates were screened in Pikovskaya medium for phosphate solubilization activity (Pikovskaya, 1948). Isolates were inoculated in Pikovskaya media and incubated for 7 days. A halo zone around the growth indicates positive result for phosphate solubilization activity.

Selection of two non-Streptomyces and two Streptomyces actinomycetes strains

On the basis of *in vitro* plant growth promoting activities out of the isolates of actinomycetes, two non-streptomyces strains (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected. ARHS/PO26 and ARHS/PO27 were morphologically identified and confirmed by the National Centre for Fungal Taxonomy, Delhi as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08).

In vitro antagonistic effect on Sclerotium rolfsii

Streptomycetes and non-streptomycetes isolates were tested for antagonistic effect against *Sclerotium rolfsii* by dual culture method (Skidmore and Dickinson, 1976). Inhibitions of the radial growth of fungal pathogen by the isolates confirm antagonism.

Inoculation technique and disease assessment

15 days old plant (*Vigna radiata*) was used for artificial inoculation with fungal pathogen. Sand maize meal media containing fungal inoculum were added carefully in the rhizosphere and ensured that inocula were attached to healthy roots. Disease assessment was done 15 days after inoculation. In order to determine the effects of two non-streptomyces and two streptomyces strains on disease reduction, four treatments were taken in each case: untreated control; inoculated with pathogen; inoculation with test isolates and inoculation with both test isolate and fungal pathogen. Percentage disease incidence was recorded while disease intensity was calculated using a 0-6 scale (Mathew and Gupta, 1996).

Field Trial

Two non-streptomyces (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected for *in vivo* evaluation of the growth promoting activity on *Vigna radiata*. For seed coating the seeds were soaked in cell suspension overnight. For preparation of cell suspension 7 days old broth culture were centrifuged at 10000rpm for 10 min and the cell pellet was dissolved in 250ml sterile distilled water and tween-20. Growth measurement were observed 15 days after inoculation and dry biomass were measured after three months of inoculation.For growth promotion average root length, shoot length, total height, fresh weight and dry weight were measured against control.

Biochemical analyses

Leaves of *Vigna* plants treated with actinomycetes were used for all biochemical analyses. Leaves were collected for assay 15days after inoculation.

Enzyme assays

Peroxidase (POX, EC1.11.1.7.)

Extraction and assay of peroxidase was done following the method described by Chakraborty *et al* (1993). The plant tissues were macerated to powder in liquid nitrogen and extracted in 5 ml of chilled 0.05(M) sodium phosphate buffer (pH 6.8) containing 2 mM β-mercaptoethanol. One ml of 0.2(M) Na-phosphate buffer (pH 5.4), 1.7 ml dH_2O, 100µl crude enzyme, 100 µl O-dianisidine (5mg/ml methanol) and 0.1 ml of 4mM H_2O_2 were used in the reaction mixture. Activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2.

Chitinase (CHT, EC 3.2.1.14)

Chitinase was extracted and assayed from leaves following the method of Boller and Mauch (1988). 10µl of 1M Na-acetate buffer (pH 4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin were used in the reaction mixture. Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 rpm for 3 min. 0.3 ml supernatant, 30µl of 1M K-PO_4 buffer (pH 7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl of 1M Na-borate buffer (pH 9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C.The amount of GlcNAc released was measured spectrophotometrically at 585 nm and activity was expressed as µg GlcNAc released /min/ g fresh wt. tissue.

Phenylalanine Ammonia Lyase (PAL, EC 4.3.1.5)

Enzyme was extracted and assay was done following the method described by Bhattacharya and Ward (1987). The assay mixture contained 500µl crude enzyme, 300µl of 0.3mM borate buffer (pH 8.0), 300µl of 2% L-phenylalanine and 1.9 ml distilled water. The mixture was allowed to incubate for 1 hr at 40°C and then absorbance value was measured at 290 nm. The enzyme activity was described as the amount of cinnamic acid produced from L-phenyl alanine by the enzyme from 1 g tissue/min.

β- 1,3-glucanase (β-GLU, EC 3.2.1.39)

β-1,3-glucanase was extracted and assayed from leaf samples following the method of Pan *et al.* (1991).The reaction mixture consisted of 62.5µl crude enzyme and 62.5µl 4% laminarin which was incubated at 40°C for 10 min and 375µl DNSA (dinitro salicylic acid) added to the mixture following incubation for 5 min on a boiling water bath. Finally the colored solution was diluted with 4.5 ml water and the amount of glucose liberated was determined spectrophotometrically. Activity was expressed as µg glucose released /min/g tissue.

Results and Discussion

Actinomycetes isolates were characterized by morphologically as well as biochemically. Out of the isolated isolates, two non-streptomyces and two streptomyces actinomycetes isolates showed positive result for biochemical tests.On the basis of *in vitro* plant growth promoting activities and phosphate solubilising activities *in vitro* two non-streptomyces strains (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected for further studies (Table 1, Fig. 1). ARHS/PO26 and ARHS/PO27 were identified and morphological identification were confirmed by the National Centre for Fungal Taxonomy, Delhi as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08).

Isolates were tested for *in vitro* antagonistic effect against *Sclerotium rolfsii*. ARHS/Mn 3 and *Streptomyces griseolus (*ARHS/PO27) were comparatively more effective to control *S. rolfsii*. ARHS/Mn3 and *S. griseolus (*ARHS/PO27) could inhibit 68 % and 59.7% growth of *Sclerotium rolfsii* (Table 2, Fig. 2). Results revealed that among the isolates tested, sclerotial blight disease development was markedly reduced with prior applications of isolates of *S. griseolus* (ARHS/PO 27) and ARHS/Mn 3 in comparison to *S. griseus* (ARHS/PO26) and ARHS/Mn7 (Table 3). Increase in the growth was observed in terms of increase in height of

Table 1: Biochemical tests of two non-streptomyces actinomycetes (NSA) and two streptomyces isolates

Isolates Code	Catalase test	Indole test	Starch hydrolysis	Phosphate solubilising activity
ARHS-Mn3	+	+	+	+
ARHS-Mn7	+	+	+	+
Streptomyces griseolus (ARHS/PO27)	+	+	+	+
Streptomyces griseus (ARHS/PO26)	+	+	+	+

Mn- Rhizosphere soil of *Mangifera indica* (25°32'12"N .88°24'45" E); PO- Rhizosphere soil of *Solanum tuberosum* (26°33.676'N 89°03.149'E).

Table 2: *In vitro* antagonistic activity of Actinomycetes isolates against *Sclerotium rolfsii*

Isolates	% of inhibition of *Sclerotium rolfsii*
ARHS-Mn 3	68.00
ARHS-Mn 7	56.00
Streptomyces griseolus (ARHS/PO27)	59.70
Streptomyces griseus (ARHS/PO26)	57.00

Table 3: Evaluation of isolates on the development of sclerotial blight incidence of *Vigna radiata*

Treatments	Disease Index* of *Vigna radiata*
S. rolfsii	6.35
S. rolfsii + *S. griseus* (A/RHS/Po26)	0.84
S. rolfsii + *S. griseus* (A/RHS/Po27)	0.81
S.rolfsii+ ARHS/MN 3	0.80
S.rolfsii+ ARHS/Mn 7	0.85

*0 = No symptoms; 1= Small roots turn rotten lesion appeared at the collar region; 2= Middle leaves start wilting and 10-20% of root turn brown; 3= Leaves wilted and 20-40% roots become dry with browning of shoot; 4= Extensive rotting at the collar region of roots, 60-70% of roots and leaves wilted, browning of shoot over 60%; 5= 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80%; 6= Whole plants die. Average of 3 separate inoculated plants (15 days after inoculation)

Evaluation of selected isolates on the growth and development of *Vigna radiata* was conducted in *in vivo* conditions. Marked increase in attributes of parameters in growth of *V. radiate* was noticed when actinomycetes were applied in the rhizosphere of plants. saplings, number of shoots and number of leaves and roots. Growth parameters were recorded from 15 days onwards (Fig. 3 and 4). Better growth enhancement was observed by *S. griseolus* (ARHS/PO27) in comparison to other streptomyces isolate. ARHS/Mn 3 also showed promoted better growth in mung bean in respect to control as well as ARHS/Mn 7 strain.

Activities of POX, PAL, chitinase and glucanase were also observed after application of bacterial strains. POX acitivities were increased more in ARHS/Mn 3 and ARHS/Mn 7 treated plants. In PAL acitivity, *Streptomyces griseolus* (ARHS/PO27)strain showed better results in comparison to other strains and control. Similarly, chitinase and glucanase activities were increased after application of ARHS/Mn 3 strain (Fig. 5).Induced systemic resistance (ISR) is effective against different types of pathogens but differs from systemic acquired resistance (SAR) in that the inducing PGPR does not cause visible symptoms on the host plant (Van Loon *et al.,* 1998). Pieterse *et al.* (2002) confirmed that to protect themselves from the disease, plants have evolved sophisticated defense mechanisms in which the signal molecules salicylic acid, jasmonic acid and ethylene often play crucial roles. The phenomenon of SAR suggests that there is a signal that originates at the site of elicitor (biotic or abiotic) application and moves throughout the plant. The activation of SAR turns the compatible plant-pathogen interaction into an incompatible (Uknes *et al*., 1992) one. This resistance was correlated with the accumulation of pathogenesis related (PR) proteins, generally assumed to be markers of defense response (Ward *et al*., 1991).

It can be concluded from the results of the present study that ARHS/Mn 3 and *S.*

*griseolus (*ARHS/Po27) can be used as good growth promoters as well as biocontrol agents against *S. rolfsii* in *Vigna radiata* in comparison to other selected non-streptomyces and streptomyces actinomycetes strains. Non-streptomyces actinomyces (NSA)

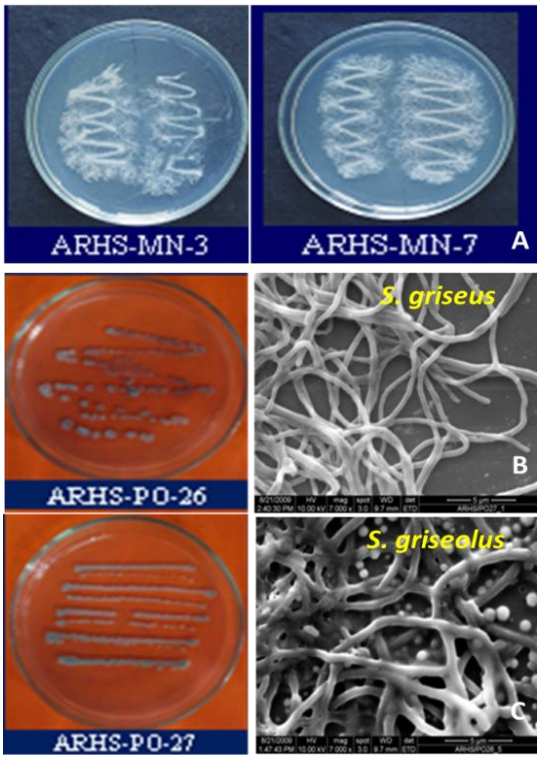


Fig. 1: Actinomycetes strains on starch casein nitrate agar (SCN) medium (A); Scanning electron microscopic view of *Streptomyces griseus* (ARHS/PO26) (B) and *Streptomyces griseolus* (ARHS/PO27) (C).

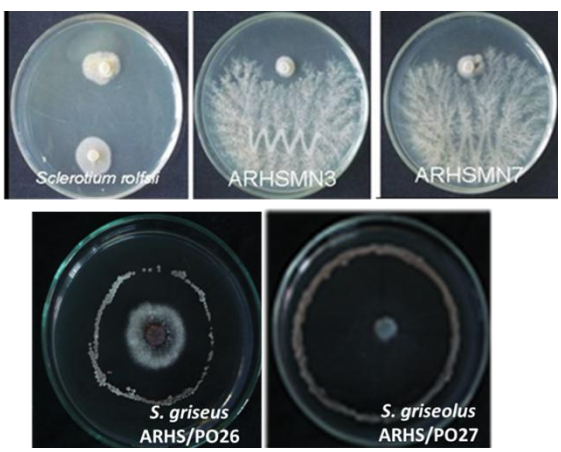


Fig. 2: Antagonistic activity of streptomyces and non-streptomyces actinomycetes isolates against *Sclerotium rolfsii*

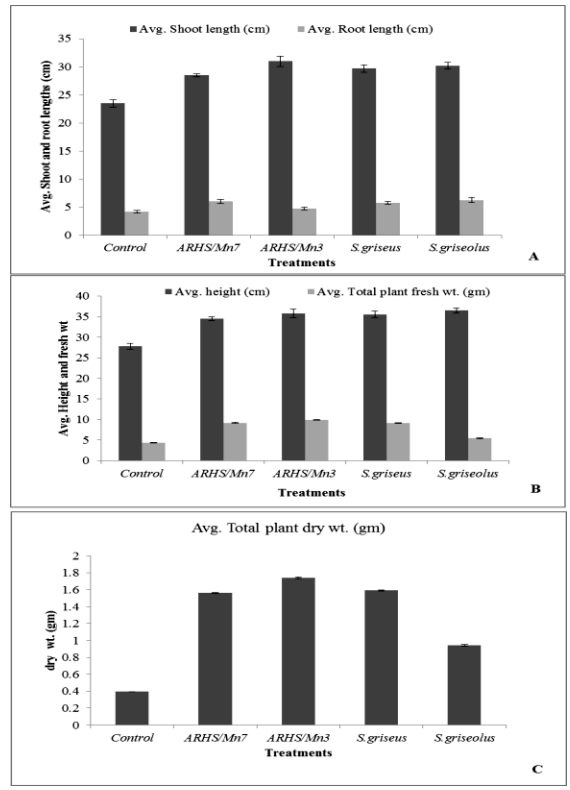


Fig. 3: Growth promotion in *Vigna radiata* after 15 days of treatment with streptomyces and non-streptomyces actinomycetes

Fig. 4: Growth enhancement of *Vigna radiata* after application of ARHS/Mn 3 strain (B) and *Streptomyces griseolus* (ARHS/PO27) (C) in comparison to control (A).

have great potential as candidates for the biocontrol of soil-borne fungal plant pathogens and also as plant growth promoters. With better understanding and screening of NSA, successful candidates from among NSA for biocontrol and plant growth promotion could be sourced.

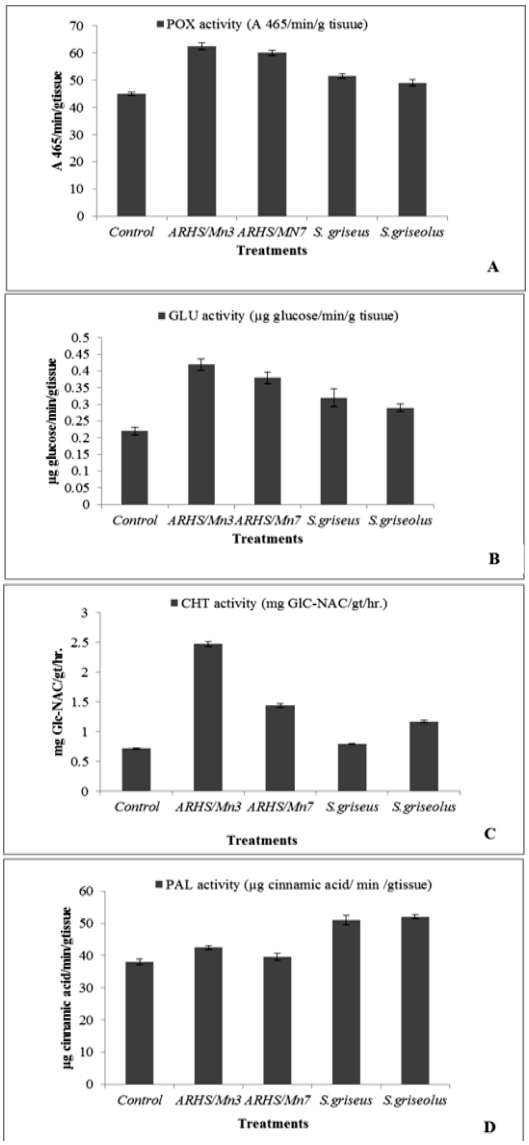


Fig. 5: Changes in defense enzyme activities in *Vigna radiata* after application of streptomyces and non-streptomyces actinomycetes isolates.

Acknowledgement

Financial support from National Bureau of Agriculturally Important Microorganisms (NBAIM), ICAR, as a part of network project entitled "Application of Microorganisms in Agriculture and Allied Sectors (AMAAS)" is gratefully acknowledged.

References

Bhattacharya, M.K., and Ward, E.W.B. (1987). Biosynthesis and metabolism of glyceolin I in soybean hypocotyls following wounding or inoculation with *Phytophthora megasperma* f. sp. *Glycinea*. *Physiol. Mol. Plant. Pathol.,* 31: 401-409.

Boller, T., and Mauch, F. (1988). Colorimetric assay for chitinase. *Meth. Enzymol*., 161: 430-435.

Chakraborty, U., Chakraborty, B.N., and Kapoor, M. (1993). Changes in the levels of peroxidase and phenyl alanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. *Folia. Microbiol*., 38: 491-496.

Doumbou, C.L., Akimov, V.,Côté, M., Charest, P.M., and Beaulieu, C. (2001). Taxonomic study on nonpathogenic streptomycetesisolated from common scab lesions on potato tubers. *Syst. Appl. Microbiol*., 24: 451-456.

Ensign, S.A., Hyman, M.R., and Arp, D.J. (1993). *In vitro* activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper. *J. Bacteriol*., 175: 1971-1980.

Lechevalier, M.P. (1988). Actinomycetes in agriculture and forestry. *In*: Good fellow MS, Williams, T., and Mordarski, M. (eds.), *Actinomycetes in Biotechnology*, Academic Press, New York. pp. 327-358.

Lo, C.W., Lai, N.S., Cheah, H.Y., Wong, N.K.I., and Ho, C.C. (2002). Actinomycetes isolated from soil samples from the crocker range sabah. *Asean Rev Biodiv Environmen Conserv,* pp. 1-7.

Manulis, S., Shafir, H., Epstein, E., Lichter, A., and Barash, I.(1994). Biosynthesis of indole-3-acetic acid via the indole-3-acetamide pathway in *Streptomyces* sp. *Microbiology,* 140:1045-1050.

Mathew, K.A., and Gupta, S.K. (1996). Studies on web blight of French bean and its management. *Indian J. Mycol. Pl. Pathol.,* 26(2): 171-177.

Moustafa, A., El-nakeeb, and Hubert, A.L. (1963). Selective isolation of aerobic actinomycetes. *Appl. Microbiol*., 11: 75-77.

Pan, S.Q., Ye, X.S., and Kue, J. (1991). Atechnique for detection of chitinase, β-1,3- glucanase and protein patterns after a single separation using polyacrylamide

gel electrophoresis and isoelectric focusing. *Phytopathology*, 81: 970-974.

Pieterse, C.M.J., Van Wees, S.C.M., Ton, J., Van Pelt, J.A., and Van Loon, L.C. (2002). Signalling in rhizobacteria induced systemic resistance in *Arabidopsis thaliana*. *Plant.Biol*., 4: 535-544.

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil connection with the vital activity of some microbial species. *Microbiologiya*, 17: 362-370.

Satya, V.K., Vijayasamundeeswari, A., Paranidharan, V., and Velazhahan, R. (2011). *Burkholderia* sp. strain TNAU-1 for biological control of root rot in mung bean (*Vigna radiate* L.) caused by *Macrophomina phaseolina*. *J. Plant. Protect. Res.,* 51(3): 273-278.

Skidmore, A.M., and Dikinson, C.H. (1976). Coony interactions and hyphal interference between *Septorianodorum* and phylloplane fungi. *Trans.Br. Mycol. Soc*., 66: 57-64.

Taechowisan, T., Lu, C., Shen, Y., and Lumyong, S. (2005). Secondary metabolites from endophytic *Streptomyces aureofaciens* CMUAc130 and their antifungal activity. *Microbiology,* 151: 1691-1695.

Taylor, S.R., Weaver, B.D., Wood, W.C., and van Edzard, S. (2005). Nitrogen application increases yield and early dry matter accumulation in late-planted soybean crop*. Sci. J.* 45: 854-858.

Tokala, R.K., Strap, J.L., Jung, C.M., Crawford, D.L., Salove, M.H., Deobald, L.A., Bailey, J.F., and Morra, M.J. (2002). Novel plant microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). *Appl. Environ. Microbiol*., 68 (5): 2161-2171.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in *Arabidopsis*. *Plant.Cell*., 4: 645-656.

Van Loon, L.C., Bakker, P.A.H.M., and Pieterse. C.M.J. (1998). Systemic resistance induced by rhizosphere bacteria. *Ann. Rev. Phytopathol*., 36: 453-483.

Warcup, J.H. (1955). The origin of colonies of fungi on soil dilution plates. *Trans. Br.Mycol.Soc.,* 38: 298.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P., and Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant. Cell*., 3: 1085-1094.