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*..