BIOLOGICAL CONTROL OF IMPORTANT FUNGAL PATHOGENS OF TEA BY ANTAGONISTIC MICROORGANISMS ISOLATED FROM TEA RHIZOSPHERE

A Thesis submitted to the University of North Bengal for the award of Doctor of Philosophy in Biotechnology

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DECLARATION

I hereby declare that the work embodied in my thesis entitled, **"BIOLOGICAL CONTROL OF IMPORTANT FUNGAL PATHOGENS OF TEA BY ANTAGONISTIC MICROORGANISMS ISOLATED FROM TEA RHIZOSPHERE"** has been carried out by me under the supervision of Dr. Dipanwita Saha, Assistant Professor of Department of Biotechnology, University of North Bengal and co-supervision of Dr. Aniruddha Saha, Associate Professor, Department of Botany, University of North Bengal for the award of the Degree of Doctor of Philosophy in Biotechnology. I also declare that, this thesis or any part thereof has not been submitted for any other degree/diploma either to this or any other university.

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ABSTRACT

The present study deals with "Biological control of important fungal pathogens of tea by antagonistic microorganisms isolated from tea rhizosphere". Tea is one of the important plantation crops of north-east India. Fungal pathogens of tea plants pose severe threat to its cultivation. Tea growers largely depend on the synthetic chemicals for disease management. Use of chemicals not only allows the development of resistance in the pathogens but also disturbs the normal soil microflora as well as create health hazard to human beings. Therefore, an eco-friendly approach is essential to control the fungal diseases of tea in a more cost-effective and efficient manner. The rhizosphere inhabiting microorganisms are being used increasingly for combating the plant pathogens for improving plant health worldwide. Several beneficial soil microbes protect plants by inducing resistance and thus indirectly antagonizing the pathogens through the secretion of several antifungal metabolites. Additionally, several plant growth promoting factors are also produced by the soil microbes.

Different features of soil inhabiting bacteria and fungi capable of suppressing several fungal pathogens of tea have been studied in the present work. The major aspect of the study was designing of a stable bioformulation for plant protection and improvement of tea plant health and characterization of the antifungal metabolites associated with it. The main objectives of the study were: (i) To isolate antagonistic bacteria and fungi inhabiting tea rhizosphere; (ii) To study the antifungal activity of the isolated strains against several foliar and root pathogens of tea *in vitro*; (iii) To identify the antagonistic bacterial and fungal isolates; (iv) To characterize the antifungal metabolites produced by the antagonistic microbial strains; (v) To purify and chemically characterize the major antifungal metabolite produced by the most potent biocontrol bacterium; (vi) To study the biocontrol efficiency of selected bacteria and fungus in suppressing tea root pathogen *in vivo*.

Rhizosphere soil samples were collected from several tea gardens of West Bengal and Assam for the isolation of microbial strains. A total of 212 bacterial isolates and 72 fungal isolates were found. They were tested for *in vitro* antagonistic activity against eight tea pathogens viz. *Lasiodiplodia theobromae*, *Colletotrichum camelliae*, *Fomes lamaoensis*, *Pestalotiopsis theae*, *Poria hypobrunnea*, *Rhizoctonia solani*, *Sphaerostilbe repens* and *Ustulina zonata.* From the above study, 35 bacterial and 10 fungal isolates were found to be strong antagonists.

Morphological and biochemical characterization of the antagonistic bacterial isolates revealed that the strains belonged to diverse genera comprising mostly of the genus *Bacillus* followed by *Micrococcus*, *Pseudomonas*, *Serratia*, *Enterobacter*, *Acinetobacter*, *Citrobacter*, *Cedecea*, *Enterococcus*, *Aerococcus*, *Paenibacillus, Gemella* and *Actinobacillus.* Four most potential strains were identified by further phylogenetic studies involving 16S rRNA gene (NCBI GenBank accession numbers: HM150755, JX566992, HM150756 and HM150757). These were found to be *Serratia marcescens* ETR17*, Bacillus subtilis* KTR6, *Citrobacter freundii* ETR20 *and Enterobacter* sp. D7. Ten fungal antagonistic isolates were identified based on their morphological characters and phylogenetic data derived from 18S rRNA gene sequences. Most of the antagonistic fungal strains belonged to the genus *Penicillium* followed by *Aspergillus*, *Paecilomyces* and *Trichoderma* (accession numbers: KF836742, KF453975, KF836741, KF453974, KF453972, KF453976, KF453973, KF866294, KF866295 and KF866296).

The isolated antagonistic strains were tested for the production of antifungal metabolites such as extracellular lytic enzymes, HCN and siderophores and occurrence of PGPR traits such as phosphatase and IAA production. A large number of bacterial isolates were amylase and protease producers whereas a few produced chitinase, cellulase, lipase and hydrogen cyanide and none produced pectinase. Most of the fungal antagonists produced chitinase and cellulase while none produced pectinase and DNase. Siderophore production was exhibited by twenty five bacterial and three fungal isolates in CAS agar medium. Siderophore quantification by CAS shuttle assay showed that the bacterial and fungal isolates could produce siderophore units up to 71% and 70% respectively. IAA production was detected in most of the bacterial and fungal strains while phosphate solubilization by production of phosphatase was limited to only five bacterial isolates. Nineteen bacterial strains produced catecholate type of siderophore, nine bacterial and three fungal strains produced hydroxamate type of siderophore and eight bacterial strains produced carboxylate type of siderophore.

Biofilm formation by antagonistic bacterial strains which is also an important biocontrol mechanism was exhibited by all 35 bacterial isolates. Maximum production of biofilm was recorded in Luria Bertani broth. The interaction of two potent antagonistic bacterial and one fungal isolate with

the fungal pathogen *R. solani* was also studied by SEM where severe deformities and lysis of pathogen mycelia were observed.

Antibiotic production in the culture supernatant of twenty bacterial antagonists was detected spectrophotometrically on three different media, CPM-Ca^{2+}, PPM and YEM. Pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol (DAPG) and phenazine were found in the gram negative isolates while lipopeptide or peptide antibiotics in gram positive isolates. The extracellular cell free culture extracts obtained from two potential isolates, *S. marcescens* ETR17 and *B. subtilis* KTR6 produced clear inhibition zones against phytopathogenic fungi in *in vitro* plate assays. The results were very encouraging to exploit the microbial strains as biocontrol agents against phytopathogens.

The antifungal antibiotic was isolated from the most potential bacterial isolate *Serratia marcescens* ETR17 grown in PPM medium by fractionating the crude concentrated culture extract by silica gel column chromatography. Four purified fractions showed antagonistic activity *in vitro* against *L. theobromae*, *R. solani* and *S. repens.* On TLC-monitoring of the fractions, two compounds: at R_f 0.6 (detected as prodigiosin) and R_f 0.8 (detected as pyrrolnitrin) were found. Bioactivity was tested by bioautography which produced two inhibition zones at similar R_f thereby confirming their antifungal property. The identity of the antibiotic compounds pyrrolnitrin and prodigiosin was confirmed by UV-spectrophotometry and HPLC by comparing with standards. Finally LC-ESI-MS confirmed the presence of pyrrolnitrin in the culture extract.

Talc-based formulation of *S. marcescens* ETR17 showed promising results in controlling root rot of tea as well as in plant growth promotion. Formulated product of another isolate *B. subtilis* KTR6 also showed comparable results *in vivo.* However, the combination of two strains reduced the root rot disease to a higher extent compared to individual formulations and similar observations were also evident in growth promotion. When formulation of the bacterial isolate ETR17 was used, the bacterium survived in the soil at a high concentration (8×10^8 cfu/g of soil) which was detected by ELISA after 45 days of treatment. ETR17 could survive up to six months in talc-formulation, when packed in polypropylene bags kept at room temperature. SEM studies of the roots inoculated with suspension of ETR17 revealed the localization of bacterial cells on the root surface. *In vivo* biocontrol of *Rhizoctonia* root rot disease in tea was also demonstrated when the most promising antagonistic

fungus *Trichoderma viride* AD10 was used. The root rot disease was reduced significantly compared to the commonly used chemical fungicide thiophanate methyl.

The present study reports the isolation of some potential soil inhabiting antagonistic bacteria and fungi harboring multiple bioactive principles. The major findings of the study suggest an eco-friendly, cost-effective means of fungal disease control. The use of these microbial strains as bioinoculants provides an alternative for culminating the use of harmful pesticides and providing an efficacious control of fungal diseases.

PREFACE

Survey on the world population in the present scenario indicates around 7 billion people residing in the world which is expected to increase to approximately 8 billion by 2020. Considering the increase in human population and the environmental damage resulting from higher level of urbanization, it can be expected that it would be a real challenge to feed the world population with the present agricultural and horticultural productivity including important beverages like tea. Therefore, very shortly the scientists should handle the situation in a sustainable and eco-friendly manner. The existing agricultural practices include the overuse of chemical fertilizers, fungicides, pesticides, herbicides and insecticides which needs to be controlled. For sustainable and environment friendly agriculture, the use of disease resistant transgenic plants and plant growth promoting microorganisms alongwith judicious use of chemicals can sustain high economic returns without disturbing the ecological balance.

Tea serves as a vital, medicinally important beverage consumed by two-third of the global population. India is one of the largest tea growing countries in the world with an annual turnover of 955.5 million kilograms of tea which contributes to 27.1% of the global tea production and its North-eastern part contributes substantially both in terms of liquor and flavour. Tea leaves contain several essential vitamins, amino acids, caffeine, flavanoides and polysaccharides which are of extreme health importance. The backbone of economy of the people of Darjeeling hills and Dooars of West Bengal and upland and lowland areas of Assam and Tripura in the North-east India lies in tea cultivation. But, the tea growers face a crop loss between 15-20% due to pests, diseases and weeds. The varying climatic conditions, soil and several environmental stresses under which the tea plants are cultivated makes them prone to a wide variety of pathogen attack. Presently, around 300 species of pests/insects and 100 pathogenic fungi known to attack different plant parts are recorded from tea plants in India. As an approach to combat the pathogens tea growers relied predominantly on the chemical pesticides/fungicides over the decades. But the chemicals were found to have severe drawbacks like development of pesticide/fungicide resistance, resurrection of pests, outbreak of secondary pathogens or pests and most importantly, the harmful effects on human health and environment due to the presence of undesirable residues

in the tea leaves and surrounding areas of the plant. However, the growing concern about the harmful chemical residues in processed tea leaves, toxic effects on the control since they can aggressively colonize the plant roots and eliminate the pathogens by producing several allelochemicals. Production of antifungal compounds like antibiotics, siderophores and hydrolytic enzymes by plant beneficial microbes restrict the pathogen growth in the same environment and thereby provides an ecofriendly and cheaper disease management strategy.

The research work compiled in this thesis was commenced in late 2006 with the main objective of protecting the tea plantation from the attack of fungal pathogens using biocontrol agents harbouring multifaceted mechanisms of antagonism. Additionally, the PGPR strains have also been used to improve the health of tea plants. The current thesis initiates with an introduction to the subject of study and an immense literature survey of the past in two different chapters. The experimental procedure, results and the inferences obtained from the present study is represented in seven major chapters and the additional supplementary materials are provided in the appendices at the end of the thesis.

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ABBREVIATIONS

A_r	Absorbance of reference
A_s	Absorbance of sample
BCA	Biocontrol agent
bp	base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
^0C	Degree Celcius
CAS	Chrome Azurol S
cfu	Colony forming unit
cm	centimeter
CMC	Carboxy methyl cellulose
Cont.	Continued
CTAB	Cetyl trimethyl ammonium bromide
CPM-Ca^{2+}	Cassamino acid peptone mannitol dibasic-calcium ion
C.V.	Crystal Violet
d	days
DAPG	Diacetlylphloroglucinol
dapi	days after pathogen inoculation
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dATP	deoxy Adenosine triphosphate
dCTP	deoxy Cytidine triphosphate
dGTP	deoxy Guanidine triphosphate
dNTP	Deoxy nucleotide triphosphate
dTTP	deoxy Thymidine triphosphate
dsDNA	Double stranded DNA
EDTA	Ethylene diamino tetraacetic acid
Ed.	Editor
Eds.	Editors
ELISA	Enzyme linked immunosorbent assay
et al.	*Et alia*
F	Fraction
Fig.	Figure
g	gram
GIS	Geographical Information System
h	hour
HCN	Hydrogen cyanide
HDTMA	Hexadecyltrimethyl-ammonium bromide
HPLC	High performance liquid chromatography/High pressure liquid chromatography
HRP	Horseradish Peroxidase
IAA	Indole-3-acetic acid
IARI	Indian Agricultural Research Institute
IgG	Immunoglobulin G
ISR	Induced Systemic Resistance

ITCC	Indian Type Culture Collection
ITS	Internal transcribed spacers
L	litre
LB	Luria Bertani
LC-ESI-MS	Liquid chromatography-electrospray ionization-mass spectrometry
LSD	Least significant difference
M	Molar
MDI	Mean Disease Index
MEGA	Molecular Evolutionary Genetics Analysis
MIC	Minimum Inhibitory Concentration
µg	microgram
µl	microlitre
mcg	microgram
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
m/z	Mass/charge ratio
min	minutes
MR	Methyl Red
M9YE	Minimal 9 Yeast extract medium
VP	Voges–Proskauer
N	Normal
NA	Nutrient Agar
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
No.	Number
nm	nanometer
ng	nanogram
OD	Optical Density
OD_{260}	Absorbance at 260 nm
OD_{280}	Absorbance at 280 nm
O-F	Oxidation-Fermentation
ONPG	*Ortho*-nitrophenyl-β-D-galactopyranoside
' (prime)	denotes a truncated gene at the indicated side
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PCA	Phenazine-1-carboxylic acid
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PEDC	Percent Efficacy of Disease Control
PGPR	Plant Growth Promoting Rhizobacteria
PGPM	Plant Growth Promoting Microorganism
PGPF	Plant Growth Promoting Rhizofungi
PIPES	Piperazine-1.4-bis (2- ethanesulphonic acid)
Plt	Pyoluteorin
Prn	Pyrrolnitrin
psi	per square inch
psu	percent siderophore units
PPM	Pigment producing medium

R_f	Relative front	TMB	Tetramethylbenzidine
rRNA	Ribosomal ribonucleic acid	TRIS	Tris hydroxymethyl amino methane
rpm	rotation per minute	TS	Tea seed variety
RT	Retention time	TSA	Tryptic Soy Agar/ Tryptone Soy Agar
SDS	Sodium dodecyl sulphate	TSI	Triple Sugar Iron
SEA	Soil Extract Agar	TSIA	Triple Sugar Iron Agar
SEM	Scanning Electron Microscopy	U	Unit
Soln.	Solution	UV	Ultra violet
spp.	Species	UV-Vis	Ultra violet visible
SPSS	Statistical Package for the Social Sciences	V	volt
TAE	Tris acetic acid EDTA	Viz.	Videlicet/namely
TE	Tris HCl EDTA	v/v	volume by volume
T.E.	Tea Estate	w/v	weight by volume
TLC	Thin Layer Chromatography	W.B.	West Bengal
		YEMB	Yeast Extract Mannitol Broth

TABLE OF CONTENTS

1.1. Tea: An important plantation crop of North-East India

Tea is considered as the most important health beneficial non-alcoholic beverage in the world. It is consumed as morning drink by nearly two-third of the world population daily (Islam et al., 2005). Tea is prepared from the leaves and young leaf buds of the *Camellia sinensis* (L.) O. Kuntze plant belonging to the Theaceae family. A total of 325 species of *Camellia* has been described by Mondal (2002) but the present day 'tea' are the progenies and hybrids of *C. sinensis* (L.) O. Kuntze, *C. assamica* sub spp. *lasiocalyx* and to some extent of *C. irrawadiensis* (Islam et al., 2005). India is the second largest producer and exporter of tea after China recording an annual turnover of 3000 crores for 164 M kg tea export in 2012-2013. A report also suggests that India is the largest consumer of tea using nearly 30 percent of the global output (http:// www.assocham.org/prels/shownewsarchive.php? id=3238&month=12 &year=2011). Tea is under the consideration of the Department of Commerce in India to be declared as the National drink of India shortly (http:// www.worldteanews.com/news/tea-under-consideration-as-national-drink-of-india#sthash.KBA0Bphm.dpuf). In India, tea plants are cultivated in distinct regions, 'Western India', 'South India' and 'North' and 'North-East India' (Fig. 1.1 and 1.2). In the North-Eastern region of India especially the sub-Himalayan region of West Bengal and Assam, tea cultivation serves as the backbone of economy. This region acts as a substantial producer of this widely consumed beverage both in terms of aroma (Darjeeling tea) and liquor (Assam tea).

1.2. Origin and distribution

The tea plant is presumed to have originated in the mountain range between Yunnan in China and Assam in India and has been cultivated for more than two thousand years. However, considerable difference in character between tea plants indigenous to Assam and tea plants existing in China was noticed (Cohen Stuart, 1919), and it was accepted that small leaf variety in China originated in eastern and south-eastern China while large-leaf varieties originated independently in India and Yunnan (Harler, 1933). Tea plants were therefore classified into two major varieties var. *sinensis* from temperate regions and var. *assamica* from the tropical regions. Eden (1958), in his book "Tea", described the spread of tea as a fan-like movement with the centre near the source of Irrawaddy river in south-east China to Indo-China and Assam in India. The *C. sinensis* plants are distributed worldwide from China to India, to

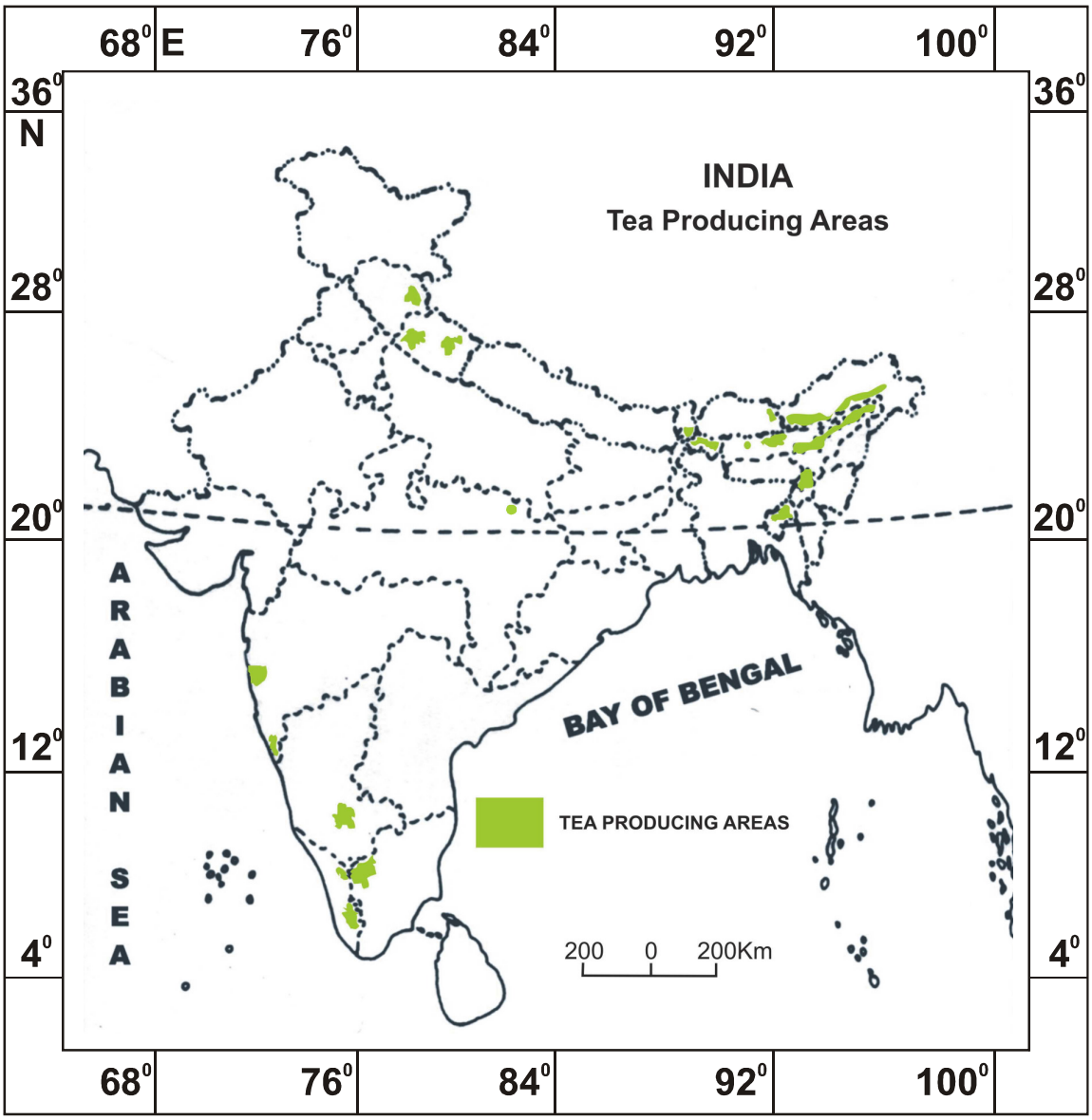


Fig. 1.1: Tea growing regions of India.

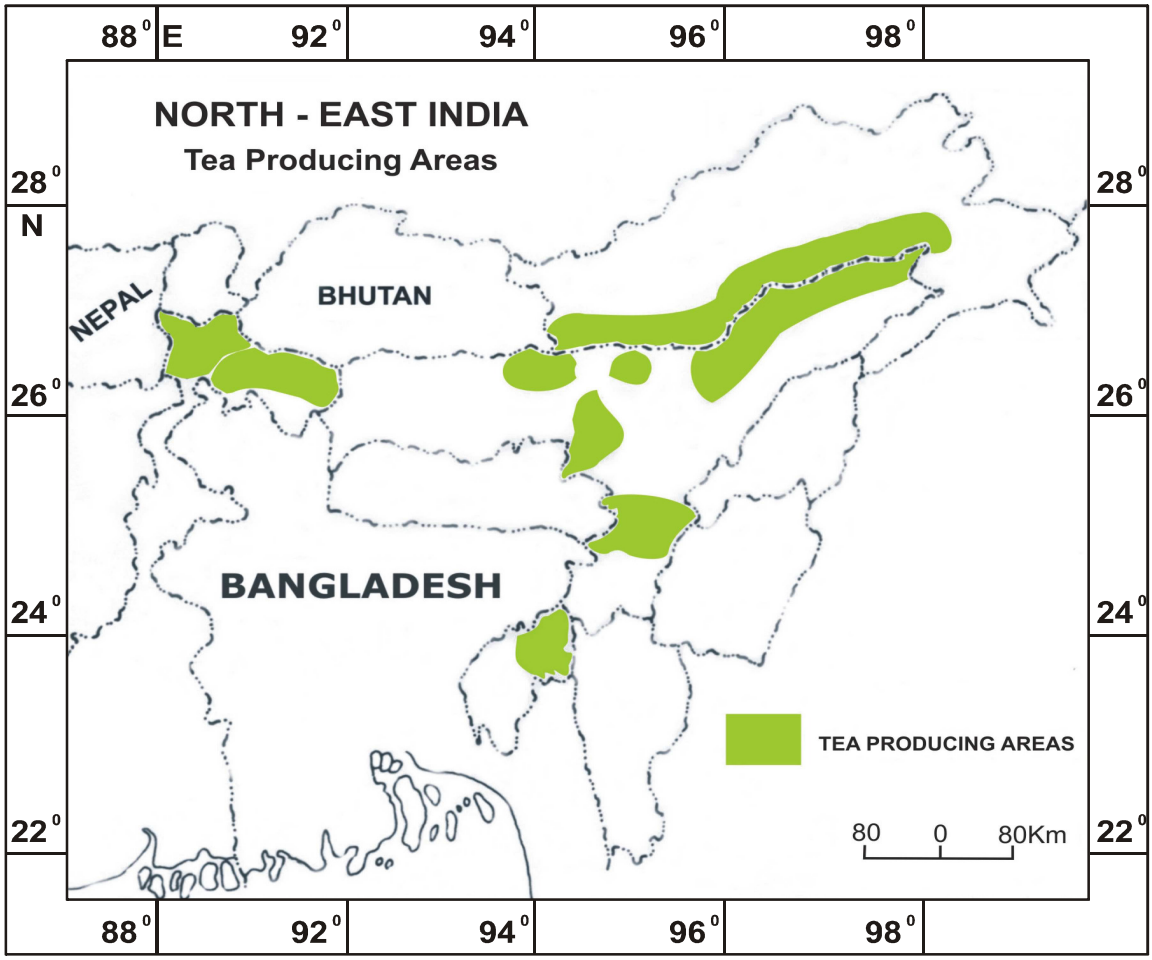


Fig. 1.2: Tea growing regions of North-East India.

Japan, then to Europe and Russia and later in the late 17th century to rest of the countries. Tea drinking in India dates back to 750 B.C. and was accidentally discovered when the natives used wild tea plants for brewing the beverage. The British East India Company started the tea cultivation in Assam and in 1837 the first English tea garden was established there. By the end of 19th century Assam became the leader in the entire world, for tea production. Although tea plantation is native to east, south and south-east Asia but presently it is cultivated across the globe including the tropical and subtropical regions (Wight, 1959; Islam et al., 2005). Around fifty countries are growing tea worldwide amongst which India, China, Sri Lanka, Kenya, Russia, Japan, Argentina and Uganda contribute substantially to the global production.

1.3. Beneficial effects of tea

Tea has earned its popularity worldwide due to the immense medicinal benefits associated with it. Tea leaves contain more than 700 different chemicals including flavanoides, amino acids, vitamins (C, E, K), caffeine and polysaccharides which are extremely health beneficial. Interestingly, the vitamin C content in tea leaves is comparable to the citrus fruits. The beverage is associated with cell-mediated immune responses of the human body, protection against intestinal disorders by enhancing the growth of beneficial intestinal microflora, reduction of blood pressure and blood-glucose activity, prevention of coronary heart diseases and also acts as antioxidant by protecting the cell from oxidative damages (Islam et al., 2005). The green and black tea also contains catechins having anti-tumorous, anti-carcinogenic and antimutagenic properties which were supported by the data of several epidemiological studies on prevention of human cancer (Islam et al., 2005). Apart from the anti-inflammatory qualities of black tea, it also helps in proper functioning of our digestive tracts and reduces stroke risks as it balances the blood cholesterol level. Fluoride is also an important constituent present mostly in black tea which is beneficial for oral and bone health. Recent research studies also prove that the caffeinated beverage like tea keep our body hydrated and moisturizes our skin by contributing to our fluid needs. People nowadays, are mostly concerned about the obesity related problems due to the slow metabolic rate. It has been estimated that by drinking five cups of green tea one can increase the metabolic rate to an extent of 70 to 80 calorie burning

per day which estimates to the loss of eight pounds body weight in a year (http://www.ivillage.com/top-10-health-benefits-drinking-tea/4-a-108301).

1.4. Diseases of tea and their control

Tea plants are cultivated either from seeds or from vegetative clones. The clonal plants have less adaptability for different environmental and soil conditions due to their genetic homogeneity and therefore, become more susceptible to different types of diseases and pests. On the contrary, the progenies of seed varieties are more resistant to the pathogen attack compared to their parents (Barbora et al., 1996). Additionally, the varying conditions of climate, soil and several environmental stresses under which the *C. sinensis* plants are cultivated the plantation is prone to a wide variety of pathogen attack. Unlike the bacterial, algal and viral pathogens, the fungal pathogens pose significant threat to tea cultivation (Saha et al., 2001; Chakraborty et al., 2009; Saikia et al., 2011). The most important leaf and root diseases affecting tea plants in North East India are blister blight, black rot, red spot, grey blight, brown blight, charcoal stump rot, brown rot, black root rot, violet root rot and diplodia (Saha et al., 2008; Barthakur, 2011). These diseases are caused by fungal species except the red spot, which is caused by an alga.

Root diseases in general are more damaging to plants since they are very hard to detect at an early stage because the above ground portion of the plant shows no symptoms and the disease spreads to the neighbouring plants by direct contact or through air-borne spores. Since, the death of tea plants caused by root pathogens takes six months to four years depending on the age and size of the plant, by the time the cause is noticed the neighbouring plants become affected.

The root pathogens like *Ustulina zonata* (causing charcoal stump rot), *Fomes lamaoensis* (causing brown rot), *Poria hypolateritia* (causing red root rot), *Rosellina arcuata* and *Armillaria mella* (causing root damage) are more prevalent in North-East India (first reported from Assam) causing severe damage to tea cultivation (Barthakur, 1999). Barthakur (2011) also described some other diseases prevalent in North-East India which are detrimental to the tea cultivation. These include leaf diseases like blister blight (*Exobasidium vexans*), black rot (*Corticium thea*), grey blight, brown blight, red rust (*Cephaleorus mycoidae*), black spot and red spot; stem diseases like *Fusarium* die back (*Fusarium solani*), red rust (*Cephaleorus parasiticus*), *Poria* branch canker (*Poria hypobrunnea*) and thorny

stem blight; and root diseases like violet root rot. Brown root rot of tea caused by *F. lamaoensis* has also been described as the commonly occurring primary root disease of tea which is more prevalent in low elevation tea growing areas mainly in the hilly tea garden areas (Morang et al., 2012). The brown root rot disease is usually slow progressing and passes from one tea bush to the other through the roots which are in direct contact with each other. An important plant root pathogen *Fusarium solani* was also found to cause detrimental effects on some tea varieties like TS-491, TS-520 and S_3A_1 (Barthakur et al., 1998). The occurrence of a secondary root disease of tea named diplodia disease caused by *Lasiodiplodia theobromae* has been found to affect tea nurseries in North-East India (Chandramouli, 1988; Saha et al., 2008). A brief account on the biotic and abiotic stresses associated with the nurseries in India alongwith the remedial measures against fungal diseases were provided by Chandramouli (1999). He observed several diseases like stalk rot caused by *Pestalotia theae,* brown blight by *Colletotrichum camelliae*, root rot caused by *Pythium* spp./ *Cylindrocladium* spp./*Fusarium* spp., blister blight caused by *Exobasidium vexans* and leaf spot caused by *Cercosporella thea* affecting tea plants in nurseries. The factors associated with spore germination and appressoria formation of *Glomerella cingulata*, causal agent of brown blight disease of tea were studied by Chakraborty et al. (1995). The temperature, pH and light conditions were reported as 25°C, 5.0 and 7 hours light /day respectively for optimum growth of the pathogen. Chakraborty et al. (2006) reported for the first time a severe foliar infection of the nursery-grown tea plants in the Dooars region of North Bengal, India caused by *Alternaria alternata*. Brown and grey blight diseases are also prevalent in the southern tea-growing regions of India (Joshi et al., 2009; Kuberan et al., 2012). Joshi et al. (2009) described *P. theae* as the second most important pathogen causing grey blight disease in tea which leads to 17% of crop loss in South India. This group isolated around 42 *Pestalotiopsis* strains from different tea growing areas of South India. Pallavi et al. (2012) also reported the isolation of the grey blight pathogen *P. theae* from the infected tea leaves in different tea gardens of south India viz. Anamallais, Coonoor, Munnar and Wayanad. Another important fungal pathogen *Phomopsis theae* casing stem canker was isolated from infected tea stems in different tea gardens of South India (Poovendran et al., 2011).

Apart from India, fungal pathogens infecting tea plantations have been reported from other tea growing regions of the world. Muraleedharan and

Baby (2007) have reported severe root diseases like red root rot, brown root rot and charcoal stump rot caused by *Poria hypolateritia*, *Phellinus noxius* and *Ustulina zonata* respectively in Sri Lanka. Field survey of tea gardens was carried by Khodaparast and Hedjaroude (1996) in north of Iran for identifying the major pathogens affecting tea plantations during 1991-1993. They demonstrated that the major pathogens in the region were several fungi like *Botrytis* sp., *Glomerella cingulata, Fusarium solani, Botryodiplodia theobromae, Pestalotiopsis longiseta, P. nattrassii, P. theae, Phyllosticta theacearum* and *Corticium rolfsii*. Tea cultivation in China, one of the largest producers of tea, is massively affected by the fungal pathogens like *Poria hypolaterite* Berk, *Phellinus noxius* Corner, *Ustulina zonata* (Lev) Sacc (Premkumar et al., 2006). Tea nurseries in South-Eastern China were found to be affected by the damping off disease caused by *Hypochnus centrifugus* (Lev.) Tul (Chen and Chen, 1989). They also reported the presence of crown gall disease caused by the bacterial pathogen *Agrobacterium tumefaciens* Smith on tea cuttings. The white root rot and brown root rot diseases affecting tea shrubs caused by soil borne pathogens *Rosellinia necatrix* (Hartig) Berl and *Phellinus noxius* respectively was reported from Taiwan (Ann et al., 2002; Sun et al., 2007). Red root disease of tea caused by three different strains of *Poria hypolateritia* isolated from different climatic conditions was reported from Sri Lanka (Wijesundera and Kulatunga, 1993). Red root disease is considered as a serious disease affecting tea plants in Sri Lanka and occurs at elevated (between 750m and 2000m) regions with diverse climatic conditions. Wijesundera and Kulatunga (1993) observed the differences in growth and secretion of enzymes involved in pathogenesis by the *Poria* isolates. The isolates showed significant differences in growth rate in different media and pH conditions. But the optimum temperature of growth was 25°C for all the three isolates tested. All the three isolates secreted similar type of polygalacturonase enzyme and different isoforms of α-glucosidase. They suggested that the isolates of *P. hypolateritia* were significantly different from each other. Fungal pathogens *Armillaria heimii* and *A. mellea* were reported to cause root rot diseases in African countries like Tanzania and Kenya (Onsando et al., 1997; http:// www.mycobank.org). Charcoal stump rot caused by *U. deusta* (Fr.) Petrak, red rot by *P. hypolateritia* and *Armillaria* root rot by *A. mellea* have been reported from Indonesia (Muraleedharan and Baby, 2007). Charcoal stump rot caused by *U. deusta* have also been reported from Africa (Muraleedharan and Baby, 2007).

Considerable amount of work has been done for controlling the tea plant pathogens but very little work has been conducted on the seed borne diseases in tea and their control measures. Earlier studies suggested that the pathogens, *Rhizoctonia solani*, *Fusarium solani, Nigrospora sp., Aspergillus niger, Pestalozzia theae, Penicillium sp.* and *Verticillium* sp. caused diseases in tea seeds and tea seedlings (Barthakur et al., 1998; Mandal et al., 2006). Mandal et al. (2006) observed that the seed borne pathogen *R. solani* affected the tea seedlings of different varieties causing severe root rot. Since *R. solani* has a very wide host range and survives in soil it affects all the plants (belonging to the same or different genera) growing in a particular cropping system (Biswas and Samajpati, 2007), it is very essential to adopt a strong control strategy against the pathogen.

1.5. The potential of biological control

Chemical compounds have been used increasingly for the control of plant diseases but their overuse has favored the development of resistant strains of pathogens. It has been observed that more specific the effect of a chemical on a pathogen, greater is the risk of decrease in its effect which is caused by genetic shift in the pathogen population. Additionally, the broad spectrum fungicides produce undesirable effects on the non-target organisms like the normal soil microflora (Mendgen et al., 1992; Dias, 2012). To address the crisis of an appropriate disease management strategy laid by the chemical agents, scientists have developed the concept of 'Biological control'. The use of microorganisms antagonizing the plant pathogen is risk-free and ecofriendly. Moreover, they can be used in combination with low doses of chemicals thus achieving a very high level of disease suppression.

The soil is an extremely rich source of diverse microbes which may be exploited to develop realistic alternative to chemical fungicides. Literature reports show that a wide range of soil inhabiting microorganisms has been utilized as biocontrol agents for management of plant diseases. They provide an efficient and ecofriendly means of disease management which is often found to be longlasting (Cawoy et al., 2011; Raghavendra and Newcombe, 2013) because apart from inhibiting phytopathogens, they sometimes induce systemic resistance in the plant and also stimulate its growth. These microbes are known as biocontrol agents (BCA) or plant growth promoting bacteria (PGPR). The success of biocontrol agents involves multiple mechanisms like rhizosphere

competence, interactions with the natural microbiota, competition for rare but essential nutrients, adaptation to the environmental conditions and above all protection of the host plant against pathogens (Weller, 1988; Pal and Gardener, 2006).

Direct mechanism involves the production of antimicrobial substances such as antibiotics, cell wall degrading enzymes and hydrogen cyanide (Pal and Gardener, 2006) while the antagonism is achieved indirectly by the secretion of iron chelating siderophores and induction of resistance in host plants (Anke et al., 1991; Wei et al., 1991; Hermosa et al., 2012). Recent research studies have focused on the isolation and structure elucidation of the secondary metabolites, especially the antibiotics produced by the biocontrol agents and studying their role in pathogen suppression (Sayyed et al., 2005; Velusamy et al., 2011).

A number of commercial biocontrol products involving individual or mixed biocontrol microorganisms have been developed and used successfully under *in vivo* conditions (Nandakumar et al., 2001; Rasu et al., 2013). Some well known commercially available biocontrol rhizobacteria include *Bacillus subtilis* strains (Kodiak, Gustafson, Integral®); *B. pumilus* strain GB34 (YieldShield, Gustafson); *B. licheniformis* strain SB3086 (EcoGuard, Novozymes); a mixture of *B. subtilis* and *B. amyloliquefaciens* strains (BioYield, Gustafson); *Streptomyces griseoviridis* K61 (Mycostop, AgBio development) and *Pseudomonas fluorescens, P. putida* and *P. chlororaphis* (Cedomon, BioAgri) (Paulitz and Belanger, 2001; Schisler et al., 2004; Kumar et al., 2011). The biofungicides developed from the fungal biocontrol agent, *Trichoderma* spp. (Plant Shield, SoilGard and T-22) are widely used for suppression of fungal diseases in plants worldwide. At present, more than 50 different bioformulations have been developed from *Trichoderma* and are successfully applied in agricultural fields for disease control (Woo et al., 2006). Mostly the earlier interests on biological control was focused on *in vitro* antagonistic mechanisms but the inconsistency in field performance of biocontrol agents led to the understanding of the importance of *in vivo* studies. Till date, only a few effective formulated biocontrol products are available, but unfortunately, they do not always produce predictable results (Pal and Gardener, 2006; Heydari and Pessarakli, 2010). Recent research involving advanced techniques are based on the field performances of the

BCAs rather than relying on the experiments done under controlled conditions in the greenhouse. The research in biocontrol nowadays is more focused on obtaining information related to the factors of disease suppression, ecological fitness of antagonistic microorganisms and genes regulating the disease suppression mechanism (Nandakumar et al., 2001; Karthikeyan et al., 2006). Extensive work should be undertaken in our country in order to understand the biosynthesis of novel antifungal metabolites and upregulation of their expression in rhizosphere competent biocontrol bacterial strains under field conditions. Designing of stable and effective bioformulations involving single or multiple microbial strains harboring multifaceted mechanisms of biocontrol is essential for achieving enhanced level of plant protection and growth. BCAs characterized in the present study can serve as potential alternative to the harmful chemical fungicides for controlling fungal diseases of tea.

1.6. Objectives

Tea diseases caused by fungal pathogens are affecting the production and quality of the beverage at an alarming rate and the chemical control measures adopted by the tea growers are posing serious threat to human health and environment. Application of eco-friendly and non-toxic biological control measures aims at greater suppression of plant diseases than its chemical counterparts but its success depends on having a proper knowledge of the relationship between the BCAs, pathogens, host plant and the environment. The present study aims at the utilization of indigenous soil microflora for restricting the fungal diseases of tea. For achieving higher success rate of disease suppression after application of the BCAs to the rhizosphere of tea plants, it is necessary to detect and characterize the factors involved in biocontrol like antibiotics, lytic enzymes and siderophores. Development of an effective formulation is also essential for the ease of application and further commercialization of the potential microbial antagonists but prior to that, their shelf-life and soil sustainability needs appropriate evaluation. Therefore, after immense literature review on the present scenario of biocontrol of tea and other economically important crops at the national and international level, the present study was designed focusing on the following objectives:

1. Isolation of microorganisms antagonistic to pathogens of tea.
2. Biochemical and phylogenetic characterization of the antagonistic bacterial isolates.

3. Identification of antagonistic fungal isolates.
4. Identification of antifungal metabolites from potential microbial antagonists.
5. Bioformulation and *in vivo* application of selected microbial isolates for biological control of tea disease.

2.1. Biological control: a concept

'Biological control' may be broadly defined as the suppression of damaging activities of one organism by one or more other organisms, often referred to as natural enemies. However, in terms of plant pathology, the definition of biological control refers to the purposeful utilization of introduced or resident living organisms, other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens (Pal and Gardener, 2006). The biological control of soil borne plant pathogens by introduced microorganisms has been investigated over 80 years and rhizospheric microorganisms are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for root against attack by pathogens (Suprapta, 2012). Biocontrol may be considered as a multitrait phenomenon whose success depends on the rhizosphere competence of the microbial inoculants, interactions with the natural microbiota, ability to compete for nutrients, adaptation to the changes in environmental conditions and above all protection of the host plant against pathogens (Weller, 1988; Pal and Gardener, 2006). In order to interact, plant beneficial organisms need to have some form of direct or indirect contact with the plant pathogens. The different types of interactions were named as mutualism, commensalism, neutralism, competition, amensalism, parasitism and predation. These terminologies originated in macroecology but all of these types of interactions exist in the natural world at both the macroscopic and microscopic level. Since, the development of plant diseases involves both plants and microbes, the interactions that lead to biological control take place at different levels and rate (Pal and Gardener, 2006). It has now been well established that the naturally occurring bacteria and fungi that have antagonistic potential towards plant pathogens, can provide the best alternative to chemical fungicides.

2.1.1. Bacteria as biocontrol agents

Although bacteria belonging to diverse genera have been reported to be antagonistic to phytopathogens, maximum attention has been focussed on the gram-negative genera *Pseudomonas* and *Serratia* and gram-positive genus *Bacillus* (Harman et al., 2004). A number of biocontrol products based on these genera have been commercially developed and used as biocontrol agents. Several other bacteria like *Enterobacter* (Chernin et al., 1996), *Citrobacter* (Purkayastha

et al., 2010), *Acinetobacter* (Xue et al., 2009), *Pantoea* (Trotel-Aziz et al., 2008), *Streptomyces* (Raatikainen et al., 1993) and *Myxococcus* (Bull et al., 2002) have also been proved to suppress a wide variety of fungal pathogens of various crops.

In this section the status of research conducted in the past 15 years on utilization of various bacterial strains in controlling plant diseases have been reviewed. Dedicated sub-sections on *Pseudomonas*, *Bacillus* and *Serratia* have been included.

2.1.1.1. *Pseudomonas*

Pseudomonas spp. is ubiquitously present in the soil and has been used as effective BCA over the years (Nandakumar et al., 2001; Nihorimbere et al., 2009; Akhtar et al., 2010). These bacteria are fast growing, good colonizers and possess appropriate ecological and rhizosphere competence. Their biocontrol activity is reproducible in the rhizosphere of multiple crops and applicable against a wide spectrum of diseased plants. Besides, they colonize with high density, compete successfully with other microbes in the rhizosphere and produce secondary metabolites with strong antifungal activity (Mark et al., 2009; Bhattacharyya and Jha, 2012). Fluorescent pseudomonads in particular have contributed substantially to the understanding of the various mechanisms involved in the inhibition of diseases of crops.

Pseudomonas strains with antifungal activity have been detected in tea rhizosphere (Saikia et al., 2011) and found to control blister blight disease in tea under field conditions (Saravanakumar et al., 2007). Among the bioformulations tested by the authors, foliar application of *P. fluorescens* Pf1 at 7-d intervals consistently reduced blister blight incidence and also increased tea yield significantly compared to the untreated control (Saravanakumar et al., 2007). Besides tea, fluorescent pseudomonads have been used successfully against several other crops including rice (Nandakumar et al., 2001; Chaiharn et al., 2009), wheat (de Werra, 2009) tomato (Jayaraj et al., 2007; Srinivasan et al., 2009; Puopolo, 2011), chickpea (Saikia et al., 2005), green gram (Sahu and Sindhu, 2011), pepper (Rajkumar et al., 2008), soyabean (Mishra et al., 2011), groundnut (Rakh et al., 2011).

Nandakumar et al. (2001) demonstrated that the application of two *P. fluorescens* strains (Pf1 and Pf7) as bacterial suspension or talc-based

formulation through seed, root, soil and foliar application either individually or in combination effectively reduced sheath blight disease incidence caused by *R. solani*, promoted plant growth and ultimately increased rice yields under glasshouse and field conditions. The biocontrol efficacy was comparable to that of the fungicide carbendazim. Jayaraj et al. (2007) used *Pseudomonas fluorescens* strain (PfT-8) in paste formulations based on peat, lignite, fly-ash and bentonite which significantly suppressed damping-off incidence in tomato caused by *Pythium aphanidermatum*. A study with antagonistic fluorescent pseudomonad strains SE21 and RD41 showed an increase in plant growth and control of the damping off of pepper caused *by R. solani* under *in vivo* conditions (Rajkumar et al., 2008). Saraf et al. (2008) demonstrated that co-inoculation of *Pseudomonas* M1P3 and fungal pathogens (*Macrophomina* and *Aspergillus*) successfully reduced the diseases of chickpea plant. In another case, Srinivasan et al. (2009) studied the greenhouse biocontrol activity of several *Pseudomonas* strains including a commercial product of *Pseudomonas chlororaphis* MA 342 (Cedomon) along with other bacterial genera against *Fusarium* wilt of tomato. Amongst different trials, the lowest disease incidence (3.3) was recorded with a single application of *P. putida* FC-6B at 10^8 cfu ml^{-1}. de Werra (2009) evaluated the role of *P. fluorescens* CHA0 against root diseases caused by pathogenic fungi. The bacterial strain showed improved biocontrol activity against take-all disease of wheat, caused by *Gaeumannomyces graminis* var. *tritici*. *Pseudomonas chlororaphis* strain M71 drastically reduced *Fusarium oxysporum* f. sp. *radicis-lycopersici* pathogenicity on tomato plantlets in seed assays and greenhouse trials (Puopolo, 2011). In another study, the same strain (*P. chlororaphis* subsp. *aureofaciens* strain M71) was able to completely inhibit the mycelial growth and conidium germination of *L. Seiridium cardinal,* the causal agent of bark canker of common cypress (*Cupressus sempervirens*) *in vitro* and prevented canker induction in field trials (Raio et al., 2011). The antagonist showed epiphytic fitness since it was able to establish itself on the crown of cypress plants and survive on it for more than three months. In another instance, Sahu and Sindhu (2011) observed that siderophore producing *Pseudomonas* strains reduced root rot disease in green gram caused by *R. solani* from 33.4 to 100% in pot experiments. Rakh et al. (2011) reported that *Pseudomonas* cf. *monteilii* showed decrease in incidence of stem rot disease (*Sclerotium rolfsii*) in groundnut by 45.45 to 66.67% in pot assay.

2.1.1.2. *Serratia*

Serratia is also a well studied gram negative genus which has been explored well for its potential since 1980's (reviewed by Saha et al., 2012c) and are considered promising antagonists and markedly inhibited soil borne pathogens as well as foliar fungal diseases in a wide variety of crops including rice (Jaiganesh et al., 2007), potato (Berg et al., 2005; Gould et al., 2008), citrus (de Queiroz and de Melo, 2006), cucumber (Kamensky et al., 2003; Roberts et al., 2005), tomato (Yazici et al. (2011), strawberry (Kurze, 2001), pepper (Shen et al., 2002, Kim et al., 2008), oilseed rape (Muller and Berg, 2008). *Serratia* sp. is isolated most often from the rhizosphere and has been found to control plant diseases associated with the roots. Chakraborty et al. (2010b) studied the *in vivo* biocontrol and plant growth promoting potential of *S. marcescens* (TRS-1) in tea plants as aqueous suspensions or as bioformulations in different carriers. The bacterium reduced brown root rot of tea caused by *Fomes lamaoensis*.

In a study conducted by Kurze et al. (2001), plant growth promoting *Serratia plymuthica* strain HRO-C48 was found to control *Verticillium* wilt and *Phytophthora* root rot in strawberry. In another instance, Shen et al. (2002) observed 100% control of *Phytophthora* blight incidence in pepper by *S. plymuthica* strain A21-4 in pot trials and substantial disease suppression in green house studies (12.6% in *S. plymuthica* treated plants compared to 74.5% in non-treated control plants). Kamensky et al. (2003) observed that *S. plymuthica* IC14 isolated from rhizosphere of melon protected cucumber against *Botrytis cinerea* gray mold and *Sclerotinia sclerotiorum* white mold diseases of leaves upon foliar application under greenhouse conditions, reducing the disease incidence by 76 and 84%, respectively. Roberts et al. (2005) reported that *S. marcescens* N1-14 provided signiûcant suppression of *R. solani* and *P. ultimum* causing damping-off disease of cucumber. Berg et al. (2005) found *S. plymuthica* 3Re4-18 to be the most effective isolate among 2648 bacterial strains isolated from potato rhizosphere in controlling soilborne pathogens *Verticillium dahliae* and *Rhizoctonia solani.* de Queiroz and de Melo (2006) used *S. marcescens* strain R-35 isolated from washed root surface of healthy citrus plants for controlling root rot disease in citrus. In greenhouse trials, the strain suppressed more than 50% of the root rot caused by *Phytophthora parasitica*. Jaiganesh et al. (2007) observed that foliar spraying (2.5kg/ha) of talc based formulations of *S. marcescens* in the field

produced maximum disease reduction of rice blast caused by *Pyricularia oryzae*. In pot experiments, maximum efficacy for controlling blast was achieved when talc-based inoculum was applied on seeds at 10g/kg. Muller and Berg (2008) used bio-priming and pelleting treatments of *S. plymuthica* HRO-C48 against *V. dahliae* in oilseed rape which showed statistically significant biocontrol. Another study reports the suppression of rice sheath blight caused by the pathogen *R. solani* using *S. marcescens* B2. The bacterium survived in soil under glasshouse conditions at concentration of 10^8 colony forming units g^{-1} of soil for 4 weeks after application. *Serratia grimessi* and *S. plymuthica* were studied for suppressing dry rot of potato caused by *Fusarium sambucinum* by Gould et al. (2008). *In vivo* studies indicated significant reduction (upto 77%) in dry rot formation by both the strains. Yazici et al. (2011) in another study, observed the biocontrol potential of *S. plymuthica* IK-139, *S. marcescens* and some other bacteria in whole plant test for protecting tomato plants against early blight disease caused by *Alternaria solani*. The bacterial isolates reduced the disease severity of early blight significantly when compared with control.

2.1.1.3. *Bacillus*

Bacillus spp. in particular are gaining recognition as safe biocontrol agents in a variety of crops, specifically as seed protectants and antifungal agents (Asaka and Shoda, 1996; Stein, 2005). Moreover, they are spore-formers, which impart a natural formulation advantage over other microorganisms (Emmert and Handelsman, 1999; Romero et al., 2007; Haas and Defago, 2005). It has been found that members of this genus has successfully controlled plant diseases in a wide variety of crops including rice (Peng et al., 2014), wheat (Liu et al., 2009), potato (Balabel et al., 2013), brinjal (Saha et al., 2012b), tomato (Domenech et al., 2006), chickpea (Karimi et al., 2012), groundnut (Manjula and Podile, 2001), cucumber (Huang et al., 2012) However, there is no report on use of *Bacillus* strains against diseases of tea.

Manjula and Podile (2001) observed that chitin supplemented peat formulations of *Bacillus subtilis* AF 1 showed better suppression of crown rot of groundnut caused by *A. niger* and *Fusarium* wilt of pigeonpea caused by *F. udum* than AF 1 culture alone. Additionally, the formulation promoted seed germination and biomass of both plants even under pathogen pressure. Cavaglieri et al. (2005) reported that *B. subtilis* CE1 was able to reduce

rhizoplane and endorhizosphere colonization of *F. verticillioides* in maize roots in greenhouse trials and promoted plant growth. Leclere et al. (2005) observed that a mutated *B. subtilis* derivative (BBG100) having 15-fold more mycosubtilin production capability than wild-type strain (ATCC 6633) also produced a marked increase in the germination rate of tomato seeds and reduction of *Pythium* infection of tomato seedlings when compared to the wild type. Greenhouse and field studies conducted with the biocontrol agent *B. amyloliquefaciens* BS6 and E16 against *Sclerotinia sclerotiorum* stem rot in canola plants revealed that disease control with BS6 using single and double spray application method was comparable to that achieved with the fungicide Rovral Flos (iprodione) (Fernando et al., 2007). In a similar study, *B. amyloliquefaciens* isolates inhibited the growth and production of mycelia and sclerotia of *S. sclerotiorum in vitro* and protected tomato, squash and eggplant seedlings inoculated with *S. sclerotiorum* to the extent (over 80%) that was comparable to the commercial products, PlantShield and SoilGard (Abdullah et al., 2008). Hernández-Suárez et al. (2011) applied microcapsules containing biocontrol *B. subtilis* strains to tomato seeds which resulted in reduction in incidence and disease severity caused by *R. solani* and *F. oxysporum* and also stimulated tomato growth and fruit yield. Saha et al. (2012b) reported significant reduction of wilt (*F. solani*) incidence under greenhouse conditions (up to 72% reduction) in eggplant seedlings pre-treated with two potential *B. subtilis* isolates AI01 and AI03 showing *in-vitro* antifungal activity against ten major phytopathogens. Kim et al. (2013) showed that an isolated *Bacillus* sp. BS061 inhibited the mycelial growth of *B. cinerea* and significantly reduced disease incidence of gray mold and powdery mildew in cucumber and strawberry. In another study, Park et al. (2013) found that soft rot disease (*Pectobacterium carotovorum* SCC1) was completely suppressed in tobacco seedlings with combined application of *B. subtilis* strain B4 and a fungicide (BTH) when compared to individual application upon pathogen challenge.

2.1.1.4. Other bacteria

Studies on biocontrol of fungal diseases affecting plantation crops showed that a diverse group of rhizobacteria apart from the most widely studied genera *Pseudomonas*, *Serratia* and *Bacillus* has also proved their efficacy as powerful biocontrol agents. A limited number of bacterial genera with immense biocontrol potential have been reviewed under this section (Table 2.1). The role of

Ochrobactrum anthropi in plant growth promotion and biocontrol of brown root-rot disease of tea was evaluated by Chakraborty et al. (2009). Application of aqueous suspension of *O. anthropi* to the rhizosphere of tea seedlings of different varieties substantially reduced the brown root-rot disease in tea caused by *Phellinus noxius* and also increased the plant growth. Similarly, the talc formulation of *O. anthropi* when applied in field also reduced the root rot disease considerably.

Arora et al. (2001) observed that two antagonistic *Rhizobium meliloti* isolates (RMP_3 and RMP_5) inhibited charcoal rot (*M. phaseolina*) in groundnut and showed increase in seed germination, seedling biomass and nodule formation in *Macrophomina* infected groundnut seedlings. Li et al. (2002) found that cucumber and soybean seed coated with peat-based formulation of *Burkholderia ambifaria* isolate BC-F significantly increased in biomass and caused a reduction in damping-off disease caused by *Pythium ultimum* compared to the non-treated pathogen controls. In a similar study, Yang et al. (2004) noted that *P. polymyxa* PKB1-coated cucumber seeds had significantly higher germination and survival rates than uncoated seeds and the coating reduced severity of seedling blight caused by *Pythium* spp. in cucumber plants in both the circulated and non-circulated hydroponic systems. Xin et al. (2005) found that the biocontrol bacterium *Brevibacillus brevis* ZJY-116 had significant colonization and disease suppression effect on barley spikes against *Fusarium* head blight (FHB) disease caused by *F. graminearum* when observed by tracking the bacterial transformants with GFP expression. Ryu et al. (2006) applied formulations of *Paenibacillus polymyxa* strain E681 by seed pelleting using clay and vermiculite in sesame which reduced damping-off and wilt incidence both in disease-conducive soil in the greenhouse and in the field and also promoted the plant growth and grain yield. Kim et al. (2008) reported that the chitinolytic bacterial strains *Chromobacterium* sp. (strain C-61) and *Lysobacter enzymogenes* (strain C-3) used in combination effectively suppressed *Phytophthora* blight incidence in pepper caused by *P. capsici* than the application with individual biocontrol strains and the efficacy of the bioformulated product depended on both dose and timing of application. Satya et al. (2011) observed that seed treatment with the talc-based powder formulation of *Burkholderia* sp. TNAU-1 significantly reduced the incidence of root rot caused by *M. phaseolina* and increased the germination percentage and plant height in mung bean plants, but the efficiency of disease control increased when seed treatment was combined with soil application under greenhouse conditions.

Table 2.1: Bacterial strains* reported as biocontrol agents against fungal diseases of plants

Bacterial Strains	Tested Plant/Disease	Target Pathogen	References
Micrococcus sp.	Tomato/Tomato-rot disease	*Fusarium solani*	Hamed, 1996
Micrococcus luteus	Tea/Brown blight disease	*Glomerulla cingulata*	Chakraborty et al., 1998
Micrococcus varians	Potato peels	*Phytophthora infestans, Fusarium sambucinum, F. avenaceum, F. oxysporum*	Sturz et al.,1999
Micrococcus varians	Potato peels	*Phytophthora infestans, Fusarium sambucinum, F. avenaceum, F. oxysporum*	Sturz et al.,1999
Burkholderia cepacia AMMDR1	Pea/*Pythium* damping-off and *Aphanomyces* root rot	*Pythium aphanidermatum, Aphanomyces euteiches*	Heungens and Parke, 2001
Pantoea agglomerans (CPA-2)	Apples/Blue mold and grey mold	*Penicillium expansum, B. cinerea, R. stolonifer*	Nunes et al., 2002
Burkholderia ambifaria isolate BC-F	Cucumber and soybean/Damping- off	*Pythium ultimum*	Li et al., 2002
Enterobacter cloacae	Damping-off	*Pythium ultimum*	Kageyama and Nelson, 2003; van Dijk and Nelson, 2000
Burkholderia cepacia strain 5.5B	Poinsettia/Stem rot	*Rhizoctonia solani*	Hwang et al., 2002
Burkholderia cepacia strain BY	Tomato/Damping- off	*Rhizoctonia solani*	Szczech and Shoda, 2004

* *Pseudomonas*, *Bacillus* and *Serratia* strains not included

Cont...

Table 2.1 (cont.): Bacterial strains* reported as biocontrol agents against fungal diseases of plants

Bacterial Strains	Tested Plant/Disease	Target Pathogen	References
Brevibacillus brevis ZJY-1	Barley/*Fusarium* head blight	*Fusarium graminearum*	Xin et al., 2005
Pantoea agglomerans	Wheat root pathogen	*Rhizoctonia solani* AG-8	Barnett et al., 2006
Exiguobacterium acetylicum			
Paenibacillus polymyxa E681	Sesame/Damping-off	*Pythium debaryanum; P. ultimum*	Ryu et al., 2006
Collimonas fungivorans	Tomato/foot and root rot	*Fusarium oxysporum*	Kamilova et al., 2007
Brevibacillus brevis	Strawberry/Gray mold	*Botrytis cinerea*	Haggag, 2008
Paenibacillus polymyxa			
Chromobacterium sp. C-61	Pepper/blight	*Primary pathogen: Phytophthora capsici, Secondary pathogens: Rhizoctonia solani, Fusarium oxysporum, F. solani*	Kim et al.,2008
Lysobacter enzymogenes C-3			
Paenibacillus polymyxa BRF-1	Soybean/Root rot	*Phialophora gregata*	Zhou et al., 2008
Azospirillum brasilense SBR	Cucumbers/damping -off	*Fusarium oxysporum* f. sp. *lycopersici, Rhizoctonia solani* and *Pythium* sp.,	Hassouna et al. 1998
Azotobacter chroococcum ZCR,			
Klebsiella pneumoneae KPR			
Ochrobactrum anthropi TRS-2	Tea/brown root rot	*Phellinus noxius*	Chakraborty et al., 2009

* *Pseudomonas*, *Bacillus* and *Serratia* strains not included

Cont…

Table 2.1 (cont.): Bacterial strains* reported as biocontrol agents against fungal diseases of plants

Bacterial Strains	Tested Plant/Disease	Target Pathogen	References
Pantoea agglomerans	Banana/crown rot	*Colletotrichum musae* and *Lasiodiplodia theobromae*	Gunasinghe ar Karunaratne, 2009
Flavobacterium sp.			
Brevibacillus brevis	Tomato	*Fusarium oxysporum* f.sp. *lycopersici*	Chandel et al., 2010
Citrobacter freundii ETR20	Tea/root rot	*Lasiodiplodia theobromae*	Purkayastha et al., 2010
Rhizobium leguminosorum	Chickpea/ *Fusarium* wilt	*Fusarium oxysporum f. Sp. ciceris*	Singh et al., 2010
Brevibacillus laterosporus strain BPM3	Rice/blast disease	*Rhizoctonia oryzae*	Saikia et al., 2011
Burkholderia sp. strain TNAU-1	Mung bean (*Vigna radiata* L.)/root rot	*Macrophomina phaseolina*	Satya et al., 2011
Paenibacillus	Strawberry/gray mold	*Botrytis cinerea* Pers. ex Fr.	Donmez et al., 2011
Paenibacillus alvei K-165	Cotton/black root rot	*Thielaviopsis basicola*	Schoina et al., 2011
Azospirillum brasilense	Strawberry/anthracnose	*Colletotrichum acutatum*	Tortora et al., 2011
Azotobacter chroococcum	Cotton and rice	*Rhizoctonia solani*	Chauhan et al. 2012
Azospirillum spp.	Tomato	*Fusarium oxysporum*	
Gluconacetobacter diazotrophicus			

* *Pseudomonas*, *Bacillus* and *Serratia* strains not included

Cont...

Table 2.1 (cont.): Bacterial strains* reported as biocontrol agents against fungal diseases of plants

Bacterial Strains	Tested Plant/Disease	Target Pathogen	References
Azotobacter spp. *Streptomyces* spp. *Chaetomium* sp.	Tomato	*Fusarium oxysporum f. splycopersici; Alternaria solani*	Kamel et al., 2012
Pantoea ananatis BLBT1-08	Grapevine/bunch rot	*Botrytis cinerea*	Gasser et al., 2012
Pantoea agglomerans PTA-AF1 and PTA-AF2 *Acinetobacter lwoffii* PTA-113 and PTA-152	Grapevine/ gray mold	*Botrytis cinerea*	Trotel-Aziz, 2012
Rhizobium japonicum	Soybean/Root rot	*Fusarium solani; Macrophomina phaseolina*	Al-Ani et al., 2012
Ochrobactrum anthropi BMO-111	Tea/blister blight	*Exobasidium vexans*	Sowndhararajan et al., 2013

* *Pseudomonas*, *Bacillus* and *Serratia* strains not included

Donmez et al. (2011) reported the antagonistic potential of *Paenibacillus* in suppressing gray mold (caused by *B. cinerea*) in strawberry plants. Saikia et al. (2011) found that the strain *Brevibacillus laterosporus* strain BPM3 suppressed blast disease of rice caused by *Rhizoctonia oryzae* by 30-67% and protected the weight loss by 35-56.5% in the green house but maximum disease protection (67%) and weight loss protection (56.5%) were recorded when the bacterium was applied before 2 days of the pathogen inoculation. Al-Ani et al. (2012) observed that inoculation of the soil infested by *F. solani* and *M. phaseolina* causing soybean root rot disease with the culture suspension of the biocontrol bacterium, *Rhizobium japonicum*, or sowing of the rhizobial culture coated seeds in pathogen infected field resulted in significant reduction in disease index and increase in the percentage of seed germination.

2.1.2. Fungi as biocontrol agents

Fungi are taxonomically and biologically diverse and many of its members appear to inhibit growth of other fungi including serious phytopathogens. A lot of attention has been focused on developing fungal strains as biocontrol agents of plant diseases. The most studied members include those belonging to the genus *Trichoderma.* Other fungi such as *Penicillium* (Alam et al., 2010), *Gliocladium* (Agarwal et al., 2011b), *Aspergillus* (Adebola and Amadi, 2010), *Saccharomyces* (Shalaby et al., 2008; Nally et al., 2012) have been reported to possess antagonistic activities against several plant fungal pathogens including *Alternaria*, *Pythium*, *Aspergillus*, *Fusarium*, *Rhizoctonia*, *Phytophthora*, *Botrytis*, *Pyricularia* and *Gaeumannomyces* (Pal and Garderner, 2006) (Table 2.2).

Trichoderma spp. are active mycoparasites against a range of economically important aerial and soil-borne plant pathogens, and has been successfully used as a biocide in greenhouse and field applications (Chet, 1987; Papavizas 1985; Howell, 2003; Kredics et al., 2003; Harman et al., 2004). Members of the genus such as *T. harzianum* (Khalili et al., 2012), *T. viride* (John et al., 2010), *T. koningii* (Tsahouridou and Thanassoulopoulos, 2002) and *T. glaucum* (Prince et al., 2011) has been reported widely as efficient biocontrol agents in plant disease management. For example, Podile and Kishore (2002) studied the role of *Trichoderma* spp. in controlling soil/seed borne disease in groundnut.

Jegathambigai et al. (2009) reported that the isolates *T. harzianum* and *T. viride* inhibited the leaf spot disease of cane palm caused by *Bipolaris* in Sri Lanka. Anand and Reddy (2009) isolated forty two strains of *Trichoderma sp.* from cultivated lands around Bangalore and analyzed their antagonistic potential against *Sclerotium rolfsii* and *Fusarium ciceri*. Martínez-Álvarez et al. (2012) demonstrated a method of biological control for the pitch canker disease caused by *Fusarium circinatum* with *T. viride*. Patale and Mukadam (2011) observed that three *Trichoderma* strains, *T. viride*, *T. harzianum*, and *Trichoderma* sp. could effectively suppress the growth of seven pathogenic fungi. In another study, Khalili et al. (2012) tested 45 *Trichoderma* isolates of rice fields of Iran against *Bipolaris carbonum* by antagonism tests including dual culture, volatile and nonvolatile metabolites and hyperparasitism. They observed that two strains of *T. harzianum* significantly controlled the disease and one strain of *T. atroviride* increased the seedling growth. Patil et al. (2012) reported the involvement of

diffusible and volatile metabolites in mycoparasitism of *Trichoderma* species (*T. harzianum T. flavofuscum*, and *T. viride*) against *Pythium* causing tomato root rot.

Table 2.2: Fungal strains reported as biocontrol agents against fungal diseases of plants

Fungal Strains	Tested Plant/Disease	Target Pathogen	References
T. koningii T8	Pea/Seed rot	*Pythium* spp.	Hadar et al., 1984
T. harzianum T12			
Trichoderma spp.	Apple/Root and crown rot	*Phytophthora cactorum*	Smith et al., 1990
Gliocladium spp.			
Penicillium janthinellum	Azalea/Root Rot	*Phytophthora cinnamomi*	Ownley and Benson 1992
Trichoderma koningii	Wheat/root rot	*Rhizoctonia solani*	Worasatit et al., 1994
Penicillium funiculosum	*Azalea* and Citrus/Root rot	*Phytophthora* spp.	Fang and Tsao, 1995
T. harzianum T39	Bean/necrosis	*Botrytis cinerea*	Zimand et al., 1996
Penicillium purpurogenum	Tomato/wilt	*Monilinia laxa*; *Fusarium oxysporum f. lycopersici*	Larena and Malgarejo 1996
Verticillium lecanii	Cucumber/ powdery mildew	*Sphaerotheca fuliginea*	Verhaar et al., 1996
Sporothrix rugulosa			
Trichoderma virens	Cotton	*Rhizoctonia solani*	Howell et al., 2000
Trichoderma spp. isolates P1 and T3	Cucumber	*P. ultimum*	Thrane et al., 2000
T. harzianum	Cowpea/damping-off	*Macrophomina phaseolina*	Adekunle et al., 2001
T. koningii			
T. koningii	*Onion/white rot*	*Sclerotium cepivorum*	Metcalf and Wilson, 2001

Cont...

Table 2.2 (cont.): Fungal strains reported as biocontrol agents against fungal diseases of plants

Fungal Strains	Tested Plant/Disease	Target Pathogen	References
T. koningii	Tomato/damping-off	*Sclerotium rolfsii*	Tsahouridou and Thanassoulopoulos, 2002
T. harzianum	Pepper/root rot	*Phytophthora capsici*; *Rhizoctonia solani*	Ahmed et al., 2003
Trichoderma harzianum	Soybean/damping-off and root-rot	*Macrophomina phaseolina; Rhizoctonia solani*	Hashem, 2004
Epicoccum nigrum			
Paecilomyces lilacinus			
Trichoderma atroviride	Beans	*Botrytis cinerea*	Brunner et al., 2005
T. atroviride C52	Onion/white rot	*Sclerotium cepivorum*	McLean et al., 2005
T. virens	Cucumber/damping -off	*R. solani*	Roberts et al., 2005
Penicillium oxalicum	Tomato/wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Sabuquillo et al., 2006
T. harzianum	Pepper/root rot	*Phytophthora capsici*	Ezziyyani et al., 2007
T. harzianum T-39	Strawberry/ Anthracnose	*Colletotrichum acutatum*	
	Gray mould	*Botrytis cinerea*	
T. viride	*Tomato*	*Fusarium solani*	Morsy et al., 2009
Penicillium sp. EU0013	Tomato and cabbage/wilt	Fusarium oxysporum	Alam et al., 2010
A. fumigatus	Cocoa/black pod	*Phytophthora palmivora*	Adebola and Amadi, 2010
A. repens			

Cont...

Table 2.2 (cont.): Fungal strains reported as biocontrol agents against fungal diseases of plants

Fungal Strains	Tested Plant/Disease	Target Pathogen	References
T. viride	Soybean	*Fusarium oxysporum* f. sp.*adzuki* and *Pythium arrhenomanes*	John et al., 2010
Trichoderma viride	Moong bean *(Vigna radiata L.)*	*Rhizoctonia solani; Sclerotium rolfsii; Macrophomina phaseolina; Alternaria alternata; Fusarium solani* and *Colletotrichum capsici*	Mishra et al.,2011
Trichoderma spp.	Tobacco/root rot	*R. solani*	Gveroska and Ziveroski,2011
T. harzianum	Lentil/foot and root rot	*F. oxysporum*	Kashem et al.,2011
T. harzianum	Rice/brown spot	*Bipolaris oryzae*	Khalili et al.,2012
T. atroviride			
T. asperellum T8a	Mango/anthracnose	*C. gloeosporiodes*	Santos-Villalobos et al., 2013

Several other fungi such as *Acremonium alternatum* (Heydari and Passarkali, 2010), *Acrodonitum crateriforme* (Heydari and Passarkali, 2010), *Ampelomyces* sp. (Heydari and Passarkali, 2010; Kiss, 2003) and *Cladosporium oxysporum* (Heydari and Passarkali, 2010) were found to be involved in physical interaction with the fungal pathogens and degrade the pathogen's mycelia. Kavkova and Curn (2005) showed biocontrol of *Sphaerotheca fuliginea*, a pathogen of cucumber powdery mildew by *Paecilomyces fumosoroseus*, which controlled the pathogen by mycoparasitism. Similarly, a strain of *Aspergillus terreus* showing *in vitro* antagonism against *Sclerotinia sclerotiorum* sporulated abundantly on the sclerotial surface and degraded the cell wall of pathogen hyphae (Melo et al., 2006). Prince et al. (2011) studied the antagonistic potentiality of several soil fungi (*Botrytis cinerea, Penicillium chrysogenum, P. citrinum, Gliocladium virens, Trichoderma glaucum, T. harzianum, T. koeningii* and *T. viride*) against *Colletotrichum falcatum*.

2.2. Mechanisms of biocontrol

The study of the underlying mechanisms of the biocontrol process is necessary to understand and formulate optimum conditions required for implementing biocontrol in a given pathosystem (Pal and Gardener, 2006; Glick, 2012). Intense research in the past two decades has focussed on various aspects of antifungal activity, rhizosphere colonization and beneficial impacts on plant health (Avis et al., 2008; Compant et al., 2010). Studies on properties that allow the biocontrol agents to dominate in the rhizosphere and simultaneously exhibit antagonism towards fungal pathogens while promoting plant growth have been of specific interest to the scientists worldwide. The overall mechanisms of biocontrol is based mainly on antibiosis, competition for micronutrient like iron, mycoparasitism, production of hydrolytic enzymes, induction of systemic resistance in host plants and rhizosphere competence. However, the best studied mechanism is the one mediated by different compounds that possess antifungal properties (Haas and Keel, 2003). As there is no scarcity of literature in this field, under this section, an attempt has been made to selectively summarize the present status of research on various mechanisms employed by BCA during manifestation of biocontrol of plant diseases.

2.2.1. Antibiosis

Antifungal antibiotics are the most widely studied metabolites produced by the biocontrol agents to combat the plant pathogens. The contribution of multiple antibiotics involved in biocontrol of plant diseases has been established by several groups worldwide (Delaney et al., 2001; Duffy et al., 2004; Krishnan et al., 2007; Kadir et al., 2008; Calderon et al., 2013). The bacterial cell growth is preferably higher during the trophophase of cell growth but the secondary metabolites like antibiotics are produced during the idiophase when the nutrients become depleted and the cell density has increased considerably (de Kievit et al., 2011).

2.2.1.1. Production of antibiotics by biocontrol bacteria

Antibiotics kill pathogens directly but for successful disease control, an antibiotic producing bacterium should be able to produce the antibiotic in the right microniche on the root surface (Lugtenberg and Kamilova, 2009). Literature studies show that several antibiotics that are produced *in vitro* are

also produced *in vivo*. Mutagenesis has been successfully used to demonstrate the role of antibiotics produced by the biocontrol bacterial isolates in the biological control of plant diseases (Rodriguez and Pfender, 1997; Huengens and Parke, 2001; Duffy et al., 2004; Liu et al., 2007). For instance, Leclere et al. (2005) observed that overproduction of mycosubtilin by *B. subtilis* mutant strain BBG100 enhanced the biocontrol potential of the bacterium against the fungal pathogen *Pythium aphanidermatum* and antagonistic potential against *Sachharomyces cerevisiae.*

The role of bacterial antibiotics like pyrrolnitrin (Prn) (Ovadis et al., 2004; Zhang et al., 2006), 2,4-diacetylphloroglucinol (DAPG/Phl) (Keel et al., 2000; Duffy et al., 2004), pyoluteorin (Plt) (Fuente et al., 2004), phenazine-1-carboxylic acid (PCA) (Liu et al., 2007; Zhang et al., 2006), kanosamine (Milner et al., 1996), oligomycin A (Kim et al., 1999), oomycin A (Compant et al., 2005), xanthobaccin (Hashidoko et al., 1999), and zwittermycin A (Zhang et al., 2006) and several other uncharacterized moieties as an active antifungal metabolite in inhibiting pathogen growth has been well documented and many have been found to be active *in vivo*. Pyrrolnitrin is a secondary metabolite which is synthesized from tryptophan and exhibit strong antifungal activity (Kirner et al., 1996). A narrow range of gram negative bacteria such as *Pseudomonas*, *Burkholdaria*, *Serratia* and *Enterobacter* has been found to produce Prn (Chernin et al., 1996, Costa et al., 2009). It has been correlated with the potential of some bacteria to control fungal diseases in plants (Hammer et al., 1997, Costa et al., 2009). Kadir et al. (2008) reported the production of thermostable pyrrolnitrin by *Burkholderia cepacia* B23 and the crude supernatant containing the antifungal metabolites showed upto 100% inhibition of the pathogen *Colletotrichum gloeosporoides* infecting papaya (*Carica papaya* L.) plants. The polyketide antibiotic DAPG was regarded as the primary biocontrol mechanism of *P. fluorescens* strain Q2-87 against *Fusarium oxysporum* f. sp. *radicis-lycopersici* affecting tomato as determined by mutational analysis (Duffy et al., 2004). Partial sequencing of the phlD gene involved in biosynthesis of DAPG from *P. fluorescens* UP61 that reduced the disease incidence in beans and tomato caused by *Sclerotium rolfsii* and *Rhizoctonia solani* revealed the similarity of UP61 with other biocontrol strains isolated worldwide that are able to produce these antibiotics (Fuente et al., 2004). Disruption of biosynthetic genes of *P. fluorescens* Pf-5 producing a wide range of antibiotics (Plt, Prn, and DAPG) by Tn5 mutagenesis showed that while both pathogens *Sclerotinia homoeocarpa* and

Drechslera poae were inhibited by the wild-type strain, pyrrolnitrin and DAPG mutants were less inhibitory, which demonstrated the positive role of these antibiotics in biocontrol.

A major class of antifungal antibiotics are the peptides or the lipopeptides which are synthesized either ribosomally or non-ribosomally in bacteria mainly belonging to the genus *Bacillus*. These include mycosubtilin (Leclere et al., 2005), subtilosin (Stein et al., 2004), TasA (Stein, 2005), sublancin (Paik et al., 1998; Leclere et al., 2005) which are synthesized ribosomally and bacilysin (Rajavel et al., 2009), chlorotetain (Arguelles-Arias et al., 2009), mycobacillin (Majumdar and Bose, 1958), rhizocticins (Borisova et al., 2010), bacillaene, difficidin (Chen et al., 2009), lipopeptides surfactin (Kim et al., 2002), iturin (Tsuge et al., 2001; Bernal et al., 2002) and fengycin families (Kim et al., 2010) are of nonribosomal origin. Besides, cyclic lipopeptides (CLPs) with antifungal and biosurfactants properties such as viscosinamide, tensin and amphisin produced by *Pseudomonas* antagonistic against *Pythium ultimum* and *R. solani* are reported by several authors. Production of CLPs by both gram-positive and gram-negative bacteria has been widely reported to induce antifungal, swarming and biosurfactant properties (Nielsen et al., 2000; Nielsen et al., 2002; Fernando et al., 2005). Different strains of *P. fluorescens* were found to produce CLPs like viscosinamide, tensin and amphisin which exhibited antagonistic activity against *P. ultimum* and *R. solani* (Henriksen et al., 2000; Nielsen et al., 2000; Nielsen et al., 2002; Sorensen et al., 2001).

Prodigiosin, the tripyrrole red pigment antibiotic is produced by three species of *Serratia* (*S. marcescens, S. rubidaea* and *S. plymuthica*) and some other gram positive (*Nocardia* sp., *Streptomyces longisporus, Streptoverticillium* sp.) and gram negative (*Pseudomonas*, *Vibrio*, *Alteromonas*, *Rugamonas*) bacteria. It is reported to possess antifungal, antibacterial, anti-proliferative and immunosuppressive activity (Saha et al., 2012c). The role of prodigiosin as an effective biocontrol metabolite of *S. marcescens* has been assessed (Okamoto et al., 1998; Someya et al., 2001, 2003; Someya and Akutsu, 2009). Someya et al. (2001) observed that prodigiosin purified from *S. marcescens* strain B2 inhibited spore germination of the grey mould pathogen *B. cinerea.* The authors suggested a synergistic antifungal action of chitinolytic enzymes and prodigiosin when both factors were applied in concert.

Abiotic factors such as constituents of the growth media of antibiotic producing isolates have been shown to influence the biosynthesis of antibiotics. For instance, alteration of media constituents like minerals, inorganic phosphate and carbon source affected the production of DAPG, Plt, Phl and Prn antibiotics by *Pseudomonas fluorescens* strain CHA0 (Duffy and Defago, 1999). The authors found that the level of DAPG and its precursor mono-acetylphloroglucinol increased in the presence of Zn^{2+}, ammonium molybdate (NH_4Mo^{2+}) and glucose in the media and repressed by inorganic phosphate. However, the production of Plt was repressed by glucose and inorganic phosphate and elevated by Zn^{2+}, Co^{2+} alongwith glycerol as the carbon source. Prn production was triggered by fructose, mannitol, Zn^{2+} and NH_4Mo^{2+} and remained unaffected by inorganic phosphate (Duffy and Defago, 1999). The level of iturin production by *Bacillus* mutant strain M40 increased manifold when cultured on CPM-Ca^{+2} (Casaminoacid, peptone, mannitol and $CaCl_2$) media indicating that mannitol was a better carbon source for iturin biosynthesis compared to glucose and the Ca^{2+} was thought to increase the membrane permeability resulting in enhanced antibiotic secretion (Bernal et al., 2002).

Use of molecular techniques and recombinant DNA technology has provided an insight into the involvement of multiple genes in the biosynthesis of a particular antibiotic in the producer cell (Whistler et al., 2003; Brodhagen et al., 2004; de Kievit et al., 2011; Bakthavatchalu et al., 2013). For example, the biosynthetic gene cluster analysis of prodigiosin showed 14 candidate genes arranged in pigA to pigN fashion and was successfully expressed in *Erwinia carotovora* subsp. *carotovora* but not in other members of *Enterobacteriaceae* including *E. coli* (Thomson et al., 2000; Cerdeno et al., 2001). Studies on the biosynthetic and regulatory genes of DAPG in the biocontrol agent *Pseudomonas fluorescens* CHA0 that also produces pyoluteorin, and HCN showed that the biosynthetic gene PhlA was autoinduced by 2,4-DAPG and strongly repressed by the bacterial extracellular metabolites salicylate and pyoluteorin as well as by the fungal metabolite fusaric acid and this regulatory mechanism was mediated, at least in part, by the transcriptional repressor PhlF (Keel et al., 2000). Ge et al. (2007) found that mutation of the pltZ gene in a Plt and PCA producing wild type strain of *Pseudomonas* sp. M18 generated M18T strain which resulted in normal PCA production but lacked the Plt production. Similarly, insertional inactivation of the PCA biosynthetic gene generated the M18Z1 capable of producing more Plt than the wild type strain but less PCA.

They indicated that the production of PCA was not affected by Plt; however Plt biosynthesis was influenced by altered PCA production. Studies on production of lipopeptides of iturin and surfactin classes by strains of *Bacillus* sp. that inhibited *Xanthomonas oryzae* showed that 33 isolates harbour the operon for iturin biosynthesis and six of them carry the *sfp* gene, responsible for the biosynthesis of surfactin (Beric et al., 2012).

The use of reporter genes to demonstrate the effects of antibiotic production in rhizosphere has been described worldwide (Raaijmakers et al., 1999; Powell et al., 2000). The role of antibiotics in biocontrol under *in vivo* conditions was confirmed by the isolation of Phl and PCA from the rhizosphere of wheat following inoculation with antagonistic strains of *Pseudomonas*. Additionally, the production of Phl in the wheat rhizosphere was strongly related to the density of the bacterial population present and the ability to colonize roots (Raaijmakers et al., 1999). Phenazine-1-carboxylic acid from *Pseudomonas aureofaciens* Tx-1 has been used as a direct field treatment for the control of dollar spot (*Sclerotinia homeocarpa*) on creeping bentgrass (*Agrostis palustris*) (Powell et al., 2000). Although the effects of antibiotic producing bacterial strains in soil is studied by using the reporter gene systems but unfortunately they fail to provide an accurate measure of the amount of the antibiotic produced *in situ* (Raaijmakers et al., 2002). Bioanalytical techniques like thin layer and high-pressure liquid chromatography are being increasingly used to detect and quantify antibiotics produced by microorganisms *in situ* (Delaney et al., 2001; Fuente et al., 2004; Krishnan et al., 2007; Raaijmakers et al., 2012).

The expression of extracellular factors in many bacteria is only at a high bacterial cell density that is, when adequate level of quorum-sensing molecules such as N-acylhomoserine lactone (AHLs) accumulate in the medium (Bassler, 1999). Several antibiotics secreted by biocontrol strains also require minimal critical bacterial cell density. Biosynthetic genes for the phenazine antibiotics in *P. aureofaciens* 30-84, in *P. chlororaphis* PCL1391 and in *P. aeruginosa* PAO1 are under quorum sensing control (Chin-A-Woeng et al., 2001; Chugani et al., 2001). *Serratia plymuthica* strain HRO-C48 was found to produce several AHLs including N-butanoyl-HSL, N-hexanoyl-HSL and N-3-oxo-hexanoyl-HSL (OHHL) (Liu et al., 2007). They found that a mutant AHL-4 *S. plymuthica* strain was incapable of producing pyrrolnitrin (Prn) and thus lacked the ability to suppress several fungal pathogens. However, Prn production was restored

in the same strain by incorporating splIR genes (analogues of luxI and luxR genes) cloned in pUCP26 plasmid. Absence of the global regulator genes grrA, grrS and rpoS in a biocontrol *S. plymuthica* strain IC1270 also restricted the production of Prn and AHL quorum sensing autoinducer molecules. As a result, the ability to antagonize two fungal pathogens *R. solani* and *P. aphanidermatum* reduced under greenhouse conditions (Ovadis et al., 2004). Haas and Keel (2003) observed that rhizobacteria may interfere with biocontrol, either by degrading AHLs or by producing AHL antagonists.

Random mutagenesis using mini-Tn5 in the effective biological control agent *Pseudomonas fluorescens* 2P24 disrupted the hfq gene required for the expression of pcoI, a synthase gene for the LuxI-type quorum-sensing signaling molecule N-acyl-homoserine lactone. Additionally, the hfq mutation drastically reduced biofilm formation and impaired the colonization ability of 2P24 on wheat rhizospheres (Xiao-Gang et al., 2010). Berry et al. (2010) used transposon mutagenesis to created two mutants of *Pseudomonas* sp. DF41, originally capable of suppressing *Sclerotinia sclerotiorum*-mediated stem rot in canola, which lost its protective function in green-house but were able to sustain in the canola phyllosphere. The first mutant disrupted the GacS/GacA regulatory system which controlled secretion of HCN, protease, alginate, and LP molecules; while the second mutation was involved in lipopeptide (LP) synthesis, thereby indicating that the loss of biocontrol activity was due to reduced production of antifungal compounds and not a declining population size. Maeyer et al. (2011) found two quorum-sensing (QS) systems in a phenazine producing fluorescent *Pseudomonas* strain CMR12a with antifungal activity against *Pythium myriotylum*; the conserved PhzI/PhzR system for phenazine production and the newly characterized CmrI/CmrR system; both of which are controlled by the GacS/ GacA two-component regulatory system. Morohoshi et al. (2013) found that *Pseudomonas chlororaphis* subsp. *aurantiaca* StFRB508 produced three major AHLs and harboured two sets of AHL-synthase and AHL-receptor gene, *phzIR* and aurIR. While the mutation of phzI drastically decreased PCA production, *aurI* mutantions did not affect it. However, the PCA production which was absent in the *phzI* and aurI double mutant was restored by exogenous AHLs along with restoration of antifungal activity against *Fusarium oxysporum* which demonstrated that the multiple quorum-sensing system play an important role in PCA production and antifungal activity

2.2.1.2. *Antibiotics by fungal antagonists*

Biocontrol fungi like *Trichoderma* species are widely found in soil. They produce volatile (e.g., ethylene, hydrogen cyanide, alcohols, aldehydes and ketones) and nonvolatile (e.g., peptides) compounds that are able to inhibit the mycelial growth of fungi. This ability gives the *Trichoderma* species an ecological advantage in soil and the rhizosphere of cultivated plants and trees. Antibiosis is one of the main antagonistic interactions between fungal plant pathogens and *Trichoderma* species, and this species with adequate antibiotic production can be used as biological control agents (BCAs) for several economically important plant-pathogenic fungi (Howell, 1998).

Marfori et al. (2002) showed that the dual culture of *Trichoderma harzianum* and *Catharanthus roseus* callus produced an antimicrobial compound with a remarkable activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis.* Structural elucidation revealed that this compound, named trichosetin, is a novel tetramic acid (2,4-pyrrolidinedione) antibiotic and a homolog of the fungal metabolite equisetin. Vinale et al. (2009) reported the production of the antibiotics, T22azaphilone, 1-hydroxy-3-methyl-anthraquinone, 1,8- dihydroxy-3-methyl-anthraquinone, T39butenolide, harzianolide, harzianopyridone by *T. harzianum* strains T22 and T39. Assays for antifungal activity showed that T22azaphilone and harzianopyridone inhibited the growth of the pathogens *Leptosphaeria maculans, Phytophthora cinnamomi*, *Botrytis cinerea, Rhizoctonia solani*, and *Pythium ultimum* at 1-10µg/ plug. The accumulation of these metabolites was quantified by LC/MS.

Trichoderma species produces trichothecenes (trichodermin and harzianum A) by a biosynthetic pathway involving several proteins (Tijerono et al., 2011; Malmierca et al., 2012). Tijerono et al. (2011) observed that overexpression of the tri5 gene in *Trichoderma brevicompactum* IBT 40841 that codes for trichodiene synthase, which catalyses the first step in the trichothecene biosynthesis, resulted in an increase of the trichodermin production and increase in the antibiotic activity against a large number of yeast, and it negatively affected tomato plant growth and the lesions caused by the pathogen *Botrytis cinerea*. In a similar study, *T. arundinaceum* IBT 40837 transformants which have a disrupted or silenced tri4, a gene encoding a cytochrome P450 monooxygenase that oxygenates trichodiene, resulted in a reduced antifungal activity against *Botrytis cinerea* and *R. solani* and also in a

reduced ability to induce expression of tomato plant defense-related genes belonging to the salicylic acid and jasmonic acid pathways against *B. cinerea*, in comparison to the wild type strain (Malmierca et al., 2012).

Many antifungal antibiotics like gliotoxin, gliovirin, viridin, viridiol, heptelidic acid and valinotrocin, have been reported to be produced by the biocontrol fungus, *Gliocladium virens.* Amongst them, gliotoxin and glioviridin were known as major antifungal substances against *Rhizoctonia solani* and *Pythium* species. In addition, these antifungal substances showed synergistic effect for disease control with intercellular enzymes produced by *G. virens* (Lumsden et al., 1992; Pietro et al., 1993; Wilhite et al., 1994; Wilhite and Straney, 1996). Singh et al. (2005) showed that antibiotic gliotoxin was effective in controlling numerous fungal pathogens such as *Rhizoctonia bataticola*, *Macrophomina phaseolina*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Sclerotium rolfsii*, and *Rhizoctonia solani*. Osamu et al. (2006) reported that the antifungal antibiotic gliotoxin were produced by different types of fungi such as *Aspergillus, Fusarium* and *Trichoderma*. El-Shami (2008) found that presowing seed soaking treatment of bean seeds (*Phaseolus vulgaris*) by gliotoxin (1-15 μgml^{-1}) reduced damping off and root rot caused by *Fusarium solani*. Shanthiyaa et al. (2013) tested several *Chaetomium globosum* isolates for antibiotic production and found that three isolates, Cg-6, Cg-7 and Cg-5 produced the antibiotic Chaetoglobosin A in the culture filtrate which appeared blue colour under UV spectrum with a wavelength of 250nm.

2.2.2. Hydrolytic enzymes produced by bacterial antagonists

Considerable effort has been put into the identification of cell wall degrading enzymes produced by bacterial BCAs although relatively little evidence about their presence and activity in the rhizosphere has been obtained. Several genera of antagonistic bacteria including *Serratia* (Kurze et al., 2001; Someya et al., 2001), *Bacillus* (Manjula et al., 2004), *Streptomyces* (Prapagdee et al., 2008), *Enterobacter* (Chernin et al., 1995) and *Pseudomonas* (Velusamy et al., 2011) produce high levels of chitinolytic enzymes. *In vitro* assays on the role of crude chitinase isolated from *Pseudomonas* sp. A3 revealed the suppression of *F. oxysporum* (Velusamy et al., 2011). Liu et al. (2011) observed that the main antifungal protein in a chitinolytic *Bacillus subtilis* SL-13 isolate which controlled tomato *Rhizoctonia* rot was the chitinase which

was then purified with DEAE-Sepharose fast flow ion exchange column chromatography and Sephadex G-75 gel filtration for further characterization. Kumar et al. (2012) also evaluated the production of extracellular lytic enzymes like chitinase, protease, cellulase and β-1,3 glucanase by *Bacillus* isolates.

Members of the genus *Serratia* are well known for their chitinolytic activity which has often been implicated in biocontrol of plant pathogens. For example, culture filtrates of *S. marcescens* B2 showed the presence of chitinolytic enzymes which suppressed the germination of *R. solani* sclerotia *in vitro* (Someya et al., 2000). Chitinolytic enzymes endochitinase (58-kDa) and chitobiase (98-kDa) purified from B2 showed inhibitory effects on the spore germination of pathogenic fungus *Botrytis cinerea* (Someya et al., 2001). An endochitinase CHIT100 purified from *S. plymuthica* HRO-C48 at a concentration of 100μgml^{-1} inhibited spore germination and germ tube elongation of the phytopathogenic fungus *Botrytis cinerea* by 28% and 31.6 % respectively (Frankowskii et al., 2001). Gel filtration chromatography was used for purifying chitinase in a single stem process from soil bacterium *S. marcescens* NK1 with a high yield of upto 92% (Nawani and Kapadnis, 2001). The enzyme was stable over a wide range of pH (3-10) and temperature (28-50ÚC) for longer time period. Exo and endochitinases have also been detected in *S. plymuthica* strain IC1270 and the production of ChiA endochitinase was regulated by the expression of global regulator genes (Ovadis et al., 2004). Mehmood et al. (2009) characterized a 60 kDa chitinase from *Serratia proteamaculans* 18A1 and demonstrated that the purified enzyme has antifungal activity against the pathogenic fungi *Fusarium oxysporum* and *Aspergillus niger*.

Prapagdee et al. (2008) observed that *Streptomyces hygroscopicus* strain SRA14 produced extracellular chitinase and β-1,3-glucanase during the exponential and late exponential phases, and their culture filtrates inhibited the growth of *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* when collected at this stage. Studies conducted by Anitha and Rabeeth (2010) found that *Streptomyces griseus* strains produced chitinase enzyme in liquid medium supplemented with cell walls of several phytopathogenic fungi as the sole source of carbon.

Poritsanos et al. (2006) evaluated the role of lipases and proteases produced by *Pseudomonas chlororaphis* PA23 in biocontrol apart from the antibiotics. Pliego et al. (2007) found variable production of exoenzymes like

protease, lipase in three potent biocontrol bacteria, two *Pseudomonas* (*P. putida* AVO102 and *P. pseudoalcaligenes* AVO110) and a *Stenotrophomonas*, which were antagonistic towards *Rosellinia necatrix*, the causal agent of avocado white root rot and demonstrated significant protection of avocado plants against the disease. Pastor et al. (2012) reported that the biocontrol fluorescent *Pseudomonas* strains which controlled tomato damping-off caused by *Sclerotium rolfsii* were capable of producing exoenzymes like proteases (30% isolates scored positive) and siderophores. Yang et al. (2012) isolated several antagonistic bacteria including *Bacillus cereus* and *Chryseobacterium* sp. from the rhizosphere, phyllosphere, endorhiza and endosphere of pepper with proteolytic, chitinolytic, cellulolytic and siderophorogenic activities and with an ability to reduce *Phytophthora* blight of pepper in greenhouse.

2.2.2.1. Mycoparasitism and production of hydrolytic enzymes by biocontrol fungus

Production of cell-wall degrading enzymes like chitinase, β-1,3-glucanase, cellulase is the major biocontrol mechanism associated with the antagonistic activities exhibited by fungal biocontrol agents. The production of hydrolytic enzymes by *Trichoderma* sp. led to the parasitization of the pathogen due to partial degradation of the pathogen cell wall (Lorito et al., 1998; Kubicek et al., 2001).

The production of chitinases to break down the mycelial cell walls of fungal plant pathogens by antagonistic fungal isolates has been implicated as a major cause of biocontrol activity (Inbar and Chet, 1995). Lorito et al. (1998) reported that the purified hydrolytic enzymes obtained from *Trichoderma* spp. were capable of lysing soft hyphal tip as well as hard chitin structure of mature hyphae, conidia, chlamydospores and sclerotia of many fungal pathogens. Transgenic tobacco and potato plants containing the endochitinase encoding gene were found to be highly tolerant or resistant towards the foliar pathogens *Alternaria alternata*, *A. solani*, *B. cinerea* and a soilborne pathogen *R. solani* (Lorito et al., 1998). Manjula et al. (2004) reported the role of extracellular chitinase produced by *T. viride* pq1 in the parasitism of the pathogen *Sclerotium rolfsii*. Anand and Reddy (2009) found that *Trichoderma* strains T35 and T6 displayed maximum endochitinase and exochitinase activity that positively correlated with their bioefficacy.

The antifungal activity of the cell-wall degrading enzymes of *Trichoderma* has been reported to increase synergistically when used with PR proteins, commercial fungicides, biocontrol bacteria or cell-membrane damaging toxins (Lorito et al., 1993; 1996; 1998; Woo et al., 2002). Woo et al. (2002) demonstrated that the cell wall degrading enzymes (CWDEs) produced by *Trichoderma* spp. increased the biocontrol efficacy of *Pseudomonas* spp. against *Botrytis cinerea*. The *Trichoderma* CWDEs and the membrane-disrupting lipodepsipeptides: syringotoxins and syringomycins produced by *Pseudomonas* spp. synergistically inhibited the growth of fungal pathogens. Djonovic et al. (2006) demonstrated the positive role of a β-1,6-glucanase, Tvbgn3 produced by *T. virens* in biocontrol of *P. ultimum* by mutant studies and showed the role of β-1,6-glucanase in mycoparasitism. Zembek et al. (2011) enhanced the biocontrol abilities of *T. atroviride* P1 against *P. ultimum* by overexpressing the *Saccharomyces cerevisiae DPM1* gene coding for dolichyl phosphate mannose (DPM) synthase, a key enzyme in the O-glycosylation pathway. The transformants obtained showed enhanced (doubled) DPM synthase activity and, at the same time, significantly elevated cellulolytic activity.

Mycoparasitism is the most salient feature of biocontrol exhibited by the members of the fungal family Hypocreaceae, to which the most common biocontrol fungi *Trichoderma* spp. belong (Atanasova et al., 2013). Mycoparasitism involves directional growth of *Trichoderma* spp. towards a pathogen followed by attachment, coiling around the pathogen hyphae, formation of hooked branches and appressorium like structures, penetration and subsequently dissolution of pathogen cytoplasm (Benhamou and Chet, 1993; Howell, 2003; Williams et al., 2003). *Trichoderma* spp. initially recognizes a pathogen using lectin and penetrates into the host cell wall by using enzymatic and mechanical components (Williams et al., 2003). John et al. (2010) observed that a *T. viride* isolate which showed mycoparasitism and destructive control against two fungal pathogens, *F. oxysporum* f. sp. *adzuki* and *Pythium arrhenomanes*, infecting soybean also showed growth promoting action along with biocontrol activity on the soybean plant during pot assay. Santos-Villalobos et al. (2013) found that cellulases from *Trichoderma* isolates showing complete overgrowth of the pathogen *C. gloeosporioides* ATCC MYA 456 *in vitro* played a major role in biological control *in vivo* in mango more than chitinase or glucanase.

2.2.3. Iron chelation by production of siderophore

Soil inhabiting microorganisms obtains their intracellular iron from mineral deposits in the soil. Iron (Fe) exists in its divalent (Fe^{2+}) and trivalent (Fe^{3+}) insoluble state as iron hydroxides in the soil (pH 7.0). As a result, Fe is unavailable to the microorganisms. The microorganisms have evolved a mechanism to uptake and store the insoluble Fe sources present in the soil by producing low molecular weight iron chelating secondary metabolites called siderophores (Renshaw et al., 2002; Winkelmann, 2007). The mechanism of biocontrol involving siderophores is based on the fact that the ferric iron is being sequestered in rhizosphere by the siderophores produced by biocontrol agents thereby making it unavailable to pathogenic fungi, leading to their growth inhibition or impaired metabolic activities (Khokhar et al., 2012; Loper and Henkels, 1999). The dynamics of iron competition in the rhizosphere have been found to be complex because it is influenced by environmental factors (Duffy and Défago, 1999). Besides, some siderophores can be used by many different bacteria or even the plant (Leeman et al., 1996; Ongena et al., 1999; Loper and Henkels, 1999).

2.2.3.1. Siderophore production by bacteria

The role of iron-chelating siderophore as an efficient antifungal metabolite conferring the competitive mode of biocontrol to the rhizobacteria is well documented (Haas and Defago, 2005). Siderophores have been demonstrated to play a major role in plant disease suppression by several bacterial biocontrol agents including *Pseudomonas* (Chaiharn et al., 2009), *Enterobacter* (Costa and Loper, 1994), *Agrobacterium* (Penyalver et al., 2001), *Serratia* (Purkayastha et al., 2010), *Streptomyces* (Macagnan et al., 2008) and *Bacillus* (Yu et al., 2011; Almoneafy et al., 2012; Saha et al., 2012a).

Iron competition in pseudomonads has been studied in detail and pyoverdine siderophore produced by many *Pseudomonas* species has been clearly shown be involved in the control of *Pythium* and *Fusarium* species (Loper and Buyer, 1991; Duijff et al., 1993). Pyochelin and its precursor salicylic acid are two other siderophores which are thought to contribute to the protection of tomato plants from *Pythium* infections by *Pseudomonas aeruginosa* 7NSK2 (Buysens et al., 1996). The role of siderophore in the biocontrol of bacterial wilt in tomato was studied by Jagadeesh et al. (2001) using Tn^5 mutants of

fluorescent pseudomonad. The hyperactive mutants resulted in suppression of wilt disease to a greater extent than the wild-type. Meziane et al. (2005) found that Tn5 transposon mutant of the biocontrol strain strain *Pseudomonas putida* WCS358 defective in biosynthesis of the ûuorescent siderophore pseudobactin lost its effectivity in inducing disease resistance in tomato against *Botrytis cinerea* infection. Sharifi et al. (2010) demonstrated the role of pyoverdine production in *Pseudomonas fluorescens* UTPF5 in suppression of common bean damping off caused by *Rhizoctonia solani* (Kuhn). The siderophore mutant strain, MPFM1, did not exhibit satisfactory disease inhibition and growth promotion activity.

Costa and Loper (1994) observed that the bacterium *Enterobacter cloacae* EcCT-501 having biocontrol efficacy against *Pythium* dumping-off of cucumber produced hydroxamate siderophore aerobactin and catecholate siderophore enterobactin. They created enterobactin and aerobactin deletion double mutants and demonstrated that both aerobactin and enterobactin produced by the bacteria function in iron uptake. Sayyed and Chincholkar (2009) reported the in vitro phytopathogen suppression activity of siderophoregenic preparations of *Alcaligenes feacalis*. Siderophore-rich culture broth exhibited most potent antifungal activity followed by siderophore-rich supernatant, and purified siderophore preparation produced antifungal activity against *Aspergillus niger* NCIM 1025, *A. flavus* NCIM 650, *Fusarium oxysporum* NCIM 1008, and *Alternaria alternata* IARI 715. Yu et al. (2011) observed that a siderophore producing *Bacillus subtilis* CAS15 reduced the disease incidence of *Fusarium* wilt in pepper significantly in pot culture experiments and also promoted plant growth. Solanki et al. (2013) reported the isolation of nine potent antagonistic siderophore producing rhizobacteria strains including *Alcaligenes* sp., *Enterobacter* sp., *Pseudomonas* sp., *P. aeruginosa* and *P. fluorescens*. *P. fluorescens* (MPF47) and *P. aeruginosa* (MB123) showed significant disease reduction in glasshouse conditions against *R. solani* in tomato.

2.2.3.2. Siderophore production by fungi

Fungi exploit their surrounding soil environment by hyphal extension and secrete siderophores into the soil for iron chelation or binds the Fe tightly which is subsequently taken inside the cell by specific mechanisms. The fungal species mostly produces hydroxamate type of siderophore with rare exceptions like Zygomycetes producing polycarboxylate siderophore named rhizoferrin

(Renshaw et al., 2002). The major classes of fungal hydroxamate-type of siderophores include ferrichromes, coprogens and fusarinines (Renshaw et al., 2002; Winkelmann, 2007).

Although secondary metabolites such as antibiotics and cell wall degrading enzymes have been studied thoroughly, siderophores have yet to be analyzed beyond their general presence in most *Trichoderma* strains (Djonovic et al., 2006; Wei et al., 2005; Djonovic et al., 2007). Nevertheless, presence of siderophore is considered as a beneficial property and has been thought to contribute to the rhizosphere competence and growth promoting abilities of *Trichoderma* strains (Hoyos-Carvajal, 2009). Jalal et al. (1986) demonstrated that the culture filtrate of the fungus *T. virens* contained high amount of siderophores such as cis- and trans-fusarinine (cF, tF) and dimerum acid (DA) while low amounts of some trihydroxamates like coprogen, coprogen B, and ferricrocin. Anke et al. (1991) found that the culture filtrate of nine siderophore producing strains of *Trichoderma* contained coprogen, coprogen B and ferricin siderophores or their derivatives under iron-deficient conditions. Additionally, *T. longibrachiatum* and *T. pseudokoningii* produced fusigen type of siderophore. The authors reported palmitoylcoprogen as the first fungal siderophore which is found in the fungal cells and is not excreted extracellularly. Segarra et al. (2010) observed that the suppression of *Fusarium oxysporum* f.sp. *lycopersici* infections in tomato plants by the siderophore producing *Trichoderma asperellum* strain T34 was significant only at low levels of iron thereby concluding that Fe competition is one of the key factors in the biocontrol activity exerted by the strain. Qi and Zhao (2013) observed that the growth of cucumber seedlings was increased and the percentage of wilted cucumber seedlings was decreased in the treatment of siderophore-containing culture filtrate of isolated *Trichoderma* strain Q1 with insoluble Fe^{3+} under salt stress indicating that *Trichoderma* spp. can stimulate growth and plant resistance to biotic and abiotic stresses.

2.2.4. Induction of Systemic resistance

Induced resistance is a state where defense mechanism is developed in plants when it is properly stimulated (van Loon et al., 1998). Induced systemic resistance (ISR) was first described as a mode of action of disease control by plant beneficial rhizobacteria (van Peer et al., 1991; Wei et al., 1991). Several studies on ISR have been carried out since then with biocontrol microorganisms.

The ability to develop ISR in response to certain rhizosphere microbes has been demonstrated in several species of plants and appears to depend on the specificity of the interaction between microorganisms and plants. Failure to elicit ISR in certain hosts was attributed to the absence of inducing components in the rhizosphere or an inability of the particular plant species to perceive such compounds (van Loon, 2007).

2.2.4.1. ISR by bacteria

ISR is dependent on jasmonic acid and ethylene signaling in the plant. Several bacterial components individually induce ISR, such as lipopolysaccharides, flagella, salicylic acid, and siderophores. More recently, the signal molecule AHLs, the antifungal factor Phl, cyclic lipopeptides, volatile blends produced by *B. subtilis* GB03 and individual volatiles acetoin and 2,3-butanediol have been added to the list (Ongena et al., 2007; Lugtenberg and Kamilova, 2009). The mechanisms of biocontrol like antibiosis and induced resistance has been thought to act synergistically to elevate the level of biocontrol by bacteria. It has been proposed that, at the beginning the bacterial antibiotics weaken and subsequently, when the weakened population attacks a plant whose defense mechanism is already stimulated, greater efficacy of disease suppression is achieved (Bakker et al., 2003).

Duijff et al. (1998) observed that the suppression of *Fusarium* wilt by a non-pathogenic, root-colonizing *P. fluorescens* WCS417r was due to systemic induced resistance without any involvement of the PR-proteins (PR-1 and chitinases). Pieterse et al. (2001) observed that the selected strain of rhizobacterium *Pseudomonas fluorescens* WCS417r activated rhizobacteria-mediated induced systemic resistance (ISR) in the plant. In another study, this strain was found to trigger ISR in *Arabidopsis thaliana* which was phenotypically similar to pathogen-induced SAR, but in contrast to SAR, WCS417r-mediated ISR was controlled by salicylic acid (SA)-independent signalling pathway which involves the stimulation by the plant hormones jasmonic acid and ethylene (Ton et al., 2002).

Role of siderophores in induced systemic resistance (ISR) in plants has also been documented (de Meyer et al., 1999; Bakker et al., 2003; Fallahzadeh-Mamaghani et al., 2009). Ran et al. (2005) demonstrated the role of salicylic acid and siderophore produced by fluorescent pseudomonads in inducing

resistance (ISR) in *Eucalyptus urophylla* against bacterial wilt caused by *Ralstonia solanacearum.* They also observed that a pseudobactin siderophore deficient mutant strain could not induce ISR while purified siderophore did. Fallahzadeh-Mamaghani et al. (2009) reported that siderophores are important determinants of fluorescent pseudomonads in induction of systemic resistance (ISR) of cotton plants against bacterial blight disease.

Kyungseok et al. (2008) demonstrated the role of exopolysaccharides (EPSs) from a plant growth-promoting rhizobacterium, *Burkholderia gladioli* IN26, on elicitation of induced systemic resistance. A purified EPS induced expression of PR- 1a::GUS on tobacco and elicited induced resistance against *Colletotrichum orbiculare* on cucumber. Verhagen et al. (2010) demonstrated that *Pseudomonas fluorescens* CHA0 and *Pseudomonas aeruginosa* 7NSK2 induces resistance in grapevine against *Botrytis cinerea*. Both strains also triggered an oxidative burst and phytoalexin accumulation in grape cells and primed leaves for accelerated phytoalexin production upon challenge inoculation with B. cinerea. They also observed through mutation studies that although salicylic acid, pyochelin, and DAPG are potentially effective in inducing or priming defence responses but the role of these chemicals in ISR inducing capability observed in *P. fluorescens* WCS417, *P. putida* WCS358 and *P. fluorescens* Q2-87 was strain dependent.

Schiliro et al. (2012) used quantitative real-time PCR (qRT-PCR) analysis to observe that the elicitation of defense enzymes by *Pseudomonas fluorescens* PICF7 against *Verticillium* wilt in an economically important woody crop, olive involved the induction of lipoxygenase, phenylpropanoid pathway, terpenoids and plant hormone biosynthesis transcripts. Weller et al. (2012) observed that phlD orphlBC mutants of *P. fluorescens* strains Q2-87 (2,4-DAPG negative strain) possessed significantly reduced level of ISR activity in *A. thaliana* against bacterial speck caused by *P. syringae* pv. tomato and genetic complementation of the mutants restored ISR activity back to wild-type levels. Alizadeh et al. (2013) observed that a combination of *Trichoderma harzianum* Tr6, and *Pseudomonas* sp. Ps14 induced resistance in cucumber against *Fusarium oxysporum* f. sp. *radicis cucumerinum* and in the model plant *A. thaliana* against *Botrytis cinerea*. Magnin-Robert et al. (2013) showed that several bacteria

including *Acinetobacter lwoffii*, *Bacillus subtilis*, *Pantoea agglomerans* and *Pseudomonas fluorescens* and their mixtures induced systemic resistance in grapevine against *Botrytis cinerea* and a particular mixture containing selected strains of *P. agglomerans* was most effective in triggering systemically the plant defense reactions and reducing the symptoms of grey mould disease

2.2.4.2. ISR by fungi

In addition to plant growth-promoting rhizobacteria, the anamorphic stages of several fungi, including *Trichoderma* spp., *Fusarium* spp., binucleate *Rhizoctonia*, and *Pythium oligandrum*, is another group of root-colonizing beneficial microorganisms that have been found to induce plant resistance to pathogens and are commonly found in most soils throughout the world (Hwang and Benson, 2003; Harman et al., 2004; Le Floch et al., 2005). However, the induction mechanisms as well as the fungal elicitors and the plant signals involved are largely undefined. More recently, a JA/ET signaling pathway and a mitogen-activated protein kinase signaling pathway of both the plant and the fungus were identified to be important for the *Trichoderma* spp.-mediated ISR in cucumber (*Cucumis sativus*) plants (Shoresh et al., 2005, 2006; Viterbo et al., 2005).

Early studies have shown that members of the genus *Trichoderma* were capable of inducing plant defence. For example, de Meyer et al. (1998) reported that a biocontrol treatment with *T. harzianum* T39 in tomato, lettuce, pepper, bean and tobacco, reduced grey mould (*Botrytis cinerea*) symptoms by inducing systemic resistance that was similar to that of the rhizobacterium *Pseudomonas aeruginosa* KMPCH. PR-proteins involved in plant defense mechanism against pathogens were found to be stimulated by the biocontrol fungi, *T. harzianum* (Jangid et al., 2004). Djonovic et al. (2007) found that a *T. virens* isolate secreting hydrophobin-like elicitor Sm1 and inducing systemic disease resistance in the dicot cotton (*Gossypium hirsutum*) also induced resistance in monocot maize (*Zea mays*) against the pathogen *Colletotrichum graminicola*.

Korolev et al. (2008) tested thirty-six phytohormone-affected mutants of A. thaliana and their wild-types for resistance/susceptibility to grey mold disease caused by *Botrytis cinerea* and the induction of resistance by *T. harzianum*

T39. Most of the *Arabidopsis* genotypes which were unable to express *Trichoderma*-mediated ISR exhibited enhanced susceptibility to *B. cinerea*. However, *T. harzianum* treatments enhanced the growth of *Arabidopsis* plants regardless of genotype or ISR inducibility. Palmieri et al. (2012) studied the mechanism of induced resistance mediated by *T. harzianum* T39 which reduced the severity of downy mildew disease caused by *Plasmopara viticola* in susceptible grapevines. Most of the proteins directly affected by T39 were found to be involved in signal transduction, indicating activation of complete microbial recognition machinery. Moreover, T39-induced resistance was associated with rapid accumulation of reactive oxygen species and callose at infection sites, as well as changes in abundance of proteins involved in response to stress and redox balance, indicating an active defence response to downy mildew. In another study, soil treatment by T39 was found to enhance resistance to *B. cinerea* infection in tomato leaves that was proportional to the concentration of the T39 suspension and changes related to jasmonic acid signalling were induced by the application of a 0.4% T39 suspension (Harel et al., 2013).

Apart from *Trichoderma* strains, several other fungi have also been reported to induce defence in the plants against pathogen attacks. Olson and (2007) found that a particular *Rhizoctonia* isolate suppressed *Botrytis* blight in geranium by induction of host systemic resistance. Murali et al. (2013) observed that a PGPF, *Penicillium chrysogenum* (PenC-JSB9) induced resistance against downy mildew caused by *Sclerospora graminicola*. In Northern blot analysis, transcript accumulation of resistant and PenC-JSB9 induced susceptible cultivars showed higher basal levels of defence gene expression than non-pretreated susceptible controls. Kojima et al. (2013) found that a PGPF *Fusarium equiseti* isolate GF19-1 was able to induce resistance in *A. thaliana* challenged with *P. syringae* pv. tomato DC3000. Examination of ISR in various signalling mutants and transgenic plants showed that GF19-1-induced protection was observed in the jasmonate response mutant jar1 and the ethylene response mutant etr1, whereas it was blocked in *Arabidopsis* plants expressing the NahG transgene thereby indicating that induced protective effect conferred by GF19-1 against the pathogen requires responsiveness to an SA-dependent pathway.

2.3. Rhizosphere competence

The rhizosphere which was first described by Hiltner (1904) is the zone of soil surrounding plant roots, and a hot spot of microbial activity. Plants store a substantial amount of fixed carbon in the root system and releases about 20% as root exudates (Nguyen, 2003). The density of microbial population is higher at the rhizosphere. Potential colonization of the rhizosphere niche and protection of the root system against phytopathogens is the prerequisite of rhizobacteria and it is called rhizosphere competence. The rhizosphere competence involves the ability to successfully establish a population of microorganisms on plant roots or in their vicinity. Colonization of the roots is achieved by growth, chemotaxis and motility of the bacterial strains (de Weert and Bloemberg, 2006).

Nautlyal et al. (2002) used a rifampicin-marked mutant NBRI2650R, to monitor the survival and colonization of the soil isolate *P. fluorescens* NBRI2650 antagonistic to several phytopathogens (*F. oxysporum*, *R. bataticola* and *Pythium* sp.) in the phytosphere of chickpea, cotton, cucumber and tomato and found that the strain varied in its ability to invade and survive in the phytosphere of different plants. Adesina et al. (2009) determined the rhizosphere competence of 15 *in vitro* antagonists of *Rhizoctonia solani* and found that only *Pseudomonas jessenii* RU47 showed effective and consistent suppression of bottom rot caused by *R. solani* on lettuce out of eight strains which were selected based on their colonization ability, for growth chamber experiments. Das et al. (2010) found that the IAA producing biocontrol strain *Bacillus subtilis* SRB28 colonised on sorghum root, formed micro-colonies and survived in the sorghum rhizosphere and increased the number of adventitious root and number and length of root hairs in seedlings in the presence of tryptophan.

Bjorkman et al. (1998) reported that the breeding of a common beneficial fungus, *Trichoderma harzianum* produced a new strain, T-22 with exceptionally high rhizosphere competence, which was resistant to edaphic conditions that reduce the colonization by indigenous *Trichoderma* sp. in field experiments. McLean et al. (2005) observed by UP-PCR band profiling that propagation of *T. atroviride* isolate C52 in the rhizosphere of onion was formulation-dependent: a pellet formulation of the biocontrol fungus maintained a high concentration

(10^5 cfu per g soil), whereas solid-substrate (10^4 cfu per g soil) and seed-coating (10^1 cfu per g soil) formulations resulted in lower concentrations. Pellet treatment into *Sclerotium cepivorum* -infested soil caused doubling of the percentage of healthy plants when compared to control. Jash and Pan (2007) isolated a *T. harzianum* strain from Kalyani in West Bengal which colonized the rhizosphere and maintained high population density at 30 days after sowing apart from being strongly antagonistic to *R. solani* and promoting growth in Bengal gram (*Cicer arietinum* Linn.). The authors found that the isolates showing high antagonism were not always highly rhizosphere competent.

2.4. Isolation of antifungal metabolites secreted by bacteria

Antifungal compounds are generally secondary metabolites that are secreted outside the cell by antagonistic bacteria. These accumulate in the culture media and may be recovered by multiple separation techniques. The metabolites are extracted from the media which are often manipulated for adequate production of the antifungal compounds. Spectrophotometric and several chromatographic techniques are generally used for detecting and isolating the antifungal antibiotic compounds from biocontrol agents (Chernin et al., 1996; Fuente et al., 2004; Ge et al., 2007; Liu et al., 2007).

Pyrrolnitrin [3-chloro-4-(28-nitro-38-chlorophenyl) pyrrole] is an antibiotic with broad spectrum antifungal activity which is produced by several bacteria some of which have been competently utilized as biocontrol agents against phytopathogens. Several pyrrolnitrin producing strains of genera *Pseudomonas* (Hammer et al., 1997) and *Burkholderia* (Hwang et al., 2002) are reported as biocontrol agents. Isolation of pyrrolnitrin from *Enterobacter agglomerans* strain IC1270 was reported for the first time by Chernin et al. (1996). The authors isolated an antifungal compound by silica gel column chromatography and purified it by TLC and HPLC. The purified compound was further analyzed by UV, IR, MS and NMR and identified as pyrrolnitrin. The purified antibiotic showed broad spectrum antagonistic activity against many pathogenic bacteria and fungi under *in vitro* conditions.

The production of antibiotics phenazine-1-carboxylic acid (PCA), pyrrolnitrin, DAPG and pyoluteorin were monitored at the spectral peak maxima

of 250, 255, 270 and 308 nm respectively in the designated solvent system by Fuente et al. (2004). Liu et al. (2007) extracted the secondary metabolites of *Pseudomonas chlororaphis* GP72 by solvent extraction method, purified the crude metabolite by HPLC, bioassayed the fractions for antifungal activity and analyzed each fraction by APCI-MS and NMR (1H and 13C) which resulted in isolation of two compounds identified as phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine. Zhang et al. (2006) also detected high levels of PCA and 2-hydroxyphenazine in the culture supernatant of *P. chlororaphis* PA23 by HPLC analysis. In another study, PCA was detected along with pyoluteorin in HPLC purified elutes prepared from *Pseudomonas* sp. M18 grown in pigment producing medium (PPM) or King's medium B (KMB) (Ge et al., 2007). Shanmugaiah et al. (2010) purified and obtained the crystal structure of the extracellular product phenazine-1-carboxamide (PCN) from *P. aeruginosa* MML2212 isolated from rice rhizosphere by silica-gel column chromatography followed by mass spectrometry, IR and X-ray diffraction analysis. The authors established antifungal property of PCN against two major rice pathogens viz. *R. solani* and *Xanthomonas oryzae* pv. *oryzae*.

The red pigment antibiotic prodigiosin (methyl-3-pentyl-6 methoxy-prodigiosin), mainly produced by the genus *Serratia* and some other gram positive and gram negative bacteria is an important secondary metabolite with moderate antifungal properties (Saha et al., 2012c) which has been found to be involved in suppression of phytopathogens (Someya et al., 2001). Someya et al. (2004) observed that prodigiosin production decreased in the bacterial cells during incubation of *S. marcescens* strain B2 under white or blue light while the red and far-red light had no effect. de Araujo et al. (2010) purified prodigiosin from *Serratia marcescens* UCP 1549 by TLC which on further analysis by UV-VIS spectrophotometry and mass spectrometry showed absorbance maxima at 536nm and the molecular mass was determined as 324 Da which confirmed the antibiotic as prodigiosin. Kamble and Hiwarale (2012) observed that prodigiosin production was greatly influenced by media composition, O_2 availability and incubation period and the production was higher in nutrient broth under aeration compared to the peptone glycerol broth. Park et al. (2012) extracted prodigiosin by solvent extraction method using nine different solvents from a marine bacterium *Hahella chejuensis* M3349 having biocontrol potential

against a harmful alga, *Chattonella antique* and analyzed by LC-MS which revealed that acetone was the best solvent due to its high extraction efficiency and less processing time.

2.5. Plant growth promotion by rhizosphere inhabiting microorganisms

Plant growth promoting microorganisms (PGPMs) include a varied group of microbes which represent a wide spectrum of genera. The PGPMs are characterized by (i) root colonization ability, (ii) survivability and multiplication in microhabitats associated with the root surface (iii) competition with other indigenous microorganisms (iv) induction of resistance in host plants and (v) finally, plant growth promotion (Bloemberg and Lugtenberg, 2001; Gamalero et al., 2004). Plant growth promotion by PGPMs mostly involves direct provision of a beneficial compound to the plant that is synthesized by the microbe or facilitates the uptake of specific nutrients from soil. The PGPMs include the plant-growth promoting rhizobacteria (PGPR) and the plant-growth promoting rhizofungi (PGPF) which are often found to act as biocontrol agents (BCAs) and inhibit plant pathogens. Thus many BCAs are also involved in nutrient cycling and increase plant biomass (proliferation of shoots, roots and leaves) (Bajsa et al., 2013).

Chakraborty et al. (2013) found that the rhizobacterial strains *Bacillus amyloliquefaciens, B. pumilis* and *Serratia marcescens* which showed several *in vitro* PGPR traits like siderophore production, phosphate solubilisation, IAA production and antagonism towards fungal pathogens could induce growth in tea plants in terms of increase in number of leaves, biomass and number of shoots in nursery as well as field conditions. Application of the PGPR strains also led to the increase in defense enzymes like PAL, peroxidase, chitinase and β-1,3-glucanase. Several *S. plymuthica* strains have also been demonstrated to produce plant growth-promoting effects in greenhouse and field plants (Berg et al., 2001; Kurze et al., 2001; Faltin et al., 2004). The phytohormone IAA has been linked to the plant-growth promoting ability of *Serratia*.

Nivedhitha et al. (2008) isolated three bacterial and one actinomycetes isolate from the bamboo rhizosphere capable of inhibiting the growth of the fungal pathogen *Fusarium* along with increase in plant growth. Vinale et al. (2009) reported that harzianic acid produced by a *T. harzianum* strain possessed

antifungal activity against *Pythium irregulare*, *Sclerotinia sclerotiorum*, and *R. solani* as well as growth promoting activities even at very low concentrations. The induction of shoot and root growth in *A. thaliana* and *Solanum tuberosum* plants by an auxin and gibberellin producing strain of *Aspergillus ustus* and the increase in biomass upon inoculation of *A. ustus* in hormone signalling defective mutant *Arabidopsis* lines was unrelated to hormone signalling genes but linked with the expression of salicylic acid, jasmonic acid/ethylene, and camalexin defense-related gene as it protected *Arabidopsis* plants against the necrotrophic fungus *Botrytis cinerea* and the hemibiotrophic bacterium *Pseudomonas syringae* DC3000 (Miguel Angel et al., 2011).

Chithrashree et al. (2011) observed that fresh suspensions as well as formulated *Bacillus* PGPR strains protected rice plants against bacterial leaf blight pathogen *Xanthomonas oryzae* pv. oryzae, promoted germination and growth and induced systemic resistance as was evident from an increased accumulation of defence related enzymes. Masunaka et al. (2011) analyzed the colonization of *Lotus japonicus* roots by *Trichoderma koningi*, a PGPF strain, in relation to induction of plant defense as evident from the induction of biosynthesis of the isoflavonoid phytoalexin vestitol, a major defensive response of leguminous plants by northern blot and HPLC. The authors observed that the response was equivalent to that induced by the symbiotic *Mesorhizobium loti* and thereby concluded that *T. koningii* resembled mycorrhizal fungi in the establishment of symbiotic associations rather than acting as fungal parasites. Rinu et al. (2013) found that the endophytic and psychrotolerant fungus *Trichoderma gamsii* (NFCCI 2177) isolated from the lateral roots of lentil (*Lens esculenta* Moench) was positive for the *in vitro* PGPR and biocontrol traits like phosphate solubilization, chitinase activity, and production of ammonia and salicylic acid, while negative for production of IAA, HCN and siderophores. The strain showed *in vitro* antagonism against six phytopathogenic fungi and plant growth promoting potential in wheat, maize, soyabean and lentil.

2.6. Detection of bacterial antagonist in soil by ELISA

Proper management of plant diseases requires accurate disease diagnosis and precise identification of the phytopathogens involved. One of the revolutionary steps in plant pathological research is the development of

antibody based diagnostics that overcome several demerits of traditional and conventional methods of disease diagnosis and pathogen detection (McCartaney et al., 2003). Enzyme linked immunosorbent assay (ELISA) is the most commonly used diagnostic technique that uses antibodies (Voller and Bidwell, 1985). Among the different formats of ELISA, double antibody sandwich – enzyme linked immunosorbent assay (DAS – ELISA) is widely used as a precise and rapid tool for detection of several phytopathogens due to the usefulness, specificity and sensitivity involved in the process (Langham, 2003).

Chakraborty et al. (2009) used ELISA for monitoring the population of biocontrol bacterium *Ochrobacter anthropi* in the tea rhizosphere after its application as talc-formulation for managing brown root rot disease of tea (*Phellinus noxius*) and found that the population was high in the soil up to a desirable extent even after six months. In a similar study, the authors observed that the PGPR strains, *Bacillus amyloliquefaciens, B. pumilis* and *Serratia marcescens* also multiplied and survived in the tea rhizosphere after six months of treatment with the bioformulations (Chakraborty et al., 2013).

Towsen and Korsten (1995) optimized Indirect ELISA for more effective monitoring of *Bacillus subtilis* in avocado field spray programmes using natural antagonists. They optimised ELISA protocol which gave a higher signal/ background value and also emphasized the fact that ELISA technique can be used for efficient and rapid monitoring of antagonists in field biocontrol studies. Immunological methods were used successfully by Quadt-Hallmann and Kloepper (1996) to study the colonization of internal tissues of different plant species by the endophytic bacterium *Enterobacter asburiae* JM22. Polyclonal and monoclonal antibodies applied in enzyme-linked immunosorbent assay (ELISA), dot blot assay, tissue printing, or immunogold labeling were sensitive and specific enough to detect JM22 in 10 plant tissues. Paulitz (2000) also considered indirect ELISA methods to be best for quantifying fungi and bacteria in soil, which would be crucial for proper understanding of the dynamics between a pathogen and a biocontrol agent (BCA) in soil or in an infection court such as the rhizosphere for predicting the success of biological control. They also recommended that studying the population density fluxes over time, one can observe how the BCA and pathogen influence each other's population and life cycles, and how the biological and physical environment influence this

relationship. Gyaneshwar et al. (2001) studied the colonization of rice plants by nitrogen fixing *Serratia* strain by ELISA and found that the polyclonal antibody showed strong *Literature Review* reaction with *Serratia* strain IRBG500 and its gusA-marked derivative up to a dilution of 1:15,000 (when IRBG500 polyclonal antibody was used) but did not show positive reaction with any of the other bacteria tested at any dilutions.

Ghosh and Purkayastha (2003) used polyclonal antibodies and antigens of ginger and *Pythium aphanidermatum*, a causal organism of rhizome rot disease for early diagnosis of rhizome rot disease of ginger. They detected *P. aphanidermatum* in ginger rhizome after eight weeks of inoculation by agar gel double diffusion and immunoelectrophoretic tests, but only one week after inoculation by indirect ELISA.

3.1. Collection of soil samples from tea rhizosphere

Field survey was carried out during 2007 and 2008 in several tea gardens of Darjeeling and Jalpaiguri district of West Bengal and Barak valley region of Assam (Table 3.1; Fig. 3.1, 3.2, 3.3, 3.4, 3.5 and 3.6). Soil samples were collected from rhizosphere of tea plants where biocontrol agents have never been applied. Plant roots with adherent soil were collected aseptically in sterile bags with the help of sterile forceps and scissors. Samples were transported to the laboratory within five hours and those collected from tea gardens of Assam were stored at 4°C and brought to the laboratory within 48h. Additionally, 1000g (approx) soil was collected from the same area in clean glass bottles which would be required for preparing soil extract.

3.2. Isolation of bacteria from rhizosphere soil

For isolation of bacteria, soil was collected by gently shaking the roots and vortexed for 10 min in sterile distilled water (1 g/10 ml) as depicted in "Experiments in Microbiology, Plant Pathology and Biotechnology" (Aneja, 2003). Samples were serially diluted with sterile distilled water from 10^{-1} to 10^{-6} dilutions. A 100µl aliquot of each dilution was used for spread plating on soil extract agar (SEA) plates. The SEA medium (Barrow and Feltham, 1993) was used for the selective isolation of bacteria specific for the source rhizosphere soil. For preparing the soil extract, 1000g soil of specific rhizosphere was dried, sieved through fine mesh, suspended in 1000ml tap water, autoclaved and allowed to settle for 24h. The top clean layer was decanted aseptically and the turbidity was removed by adding a pinch of calcium carbonate ($CaCO_3$). Finally, the solution was filtered (through Whatman grade II) to remove traces of fine soil particles. Soil extract of a particular rhizosphere was used to prepare the SEA medium (Appendix B) specific for that rhizosphere. This medium which was essentially enriched with indigenous minerals and ions of that soil sample was used for the culturing of bacteria present in that rhizosphere. After incubation for 72h at 30ºC in inverted position, isolated single colonies were picked from spread plates and pure cultures were obtained on NA slants.

3.3. Isolation of fungi from rhizosphere soil

Potato dextrose agar (PDA) medium was used to obtain cultures of fungal strains, which are predominant in a definite rhizosphere soil. A 100µl aliquot of soil dilution sample (10^{-1} to 10^{-7}) was inoculated on PDA plates following

Table 3.1: Geographic Information System (GIS) locations of the places of sample collection and their respective codes*

Place of sampling	Code Assigned	Location (District, State)	GIS Location (Latitude/Longitude)
Bagdogra T.E.	TBD	Darjeeling, W. B.	26°42'N/88°18′E
Bagrakote T.E.	BTRL	Jalpaiguri, W. B.	26°53'N/88°35'E
Baradighi T.E.	BTR	Jalpaiguri, W. B.	26°48′N/88°47′E
Dewan T.E.	AD	Cachar, Assam	24°52′N/93°02′E
Diana T.E.	D	Jalpaiguri, W. B.	26°51′N/89°01′E
Ellenbarie T.E.	ETR	Jalpaiguri, W. B.	26°52′N/88°54′E
Gayaganga T.E.	TGY	Darjeeling, W. B.	26°38′N/88°19′E
Good Hope T.E.	GH	Jalpaiguri, W. B.	26°58′N/88°56′E
Kharibarie T.E.	KTR	Darjeeling, W. B.	26°33′N/88°11′E
Kumbha T.E.	KV	Cachar, Assam	24°56′N/92°59′E
Labak T.E.	TLB	Cachar, Assam	24°50′N/93°01′E
Monachara T.E.	NB	Hailakandi, Assam	24°44′N/92°32′E
Bidyanagar T.E.	B2	Karimganj, Assam	24°31′N/92°29′E
Margaret Hope T.E.	M	Darjeeling, W.B.	26°55′N/88°17′E
Matigara T.E.	TMG	Darjeeling, W. B.	26°43′N/88°23′E
Matelli T.E.	MAT	Jalpaiguri, W. B.	26°56′N/88°48′E
NBU T.E.	NBT	Darjeeling, W. B.	26°43′N/88°21′E
Raya T.E.	TR	Jalpaiguri, W. B.	26°31′N/88°41′E
Red bank T.E.	TRB	Jalpaiguri, W. B.	26°52′N/89°02′E

*T.E.: Tea Estate; W.B.: West Bengal

spread plate inoculation technique and incubated at 28°C until fungal colonies developed. The isolated fungal colonies were subcultured in streptomycin supplemented PDA plates. Pure culture was obtained after placing 4mm fungal mycelial discs from these plates on fresh PDA plates and incubated for 7-10 days till the fungal mycelia covered the whole plate. Observation for pure culture was made by studying morphological characteristics under microscope.

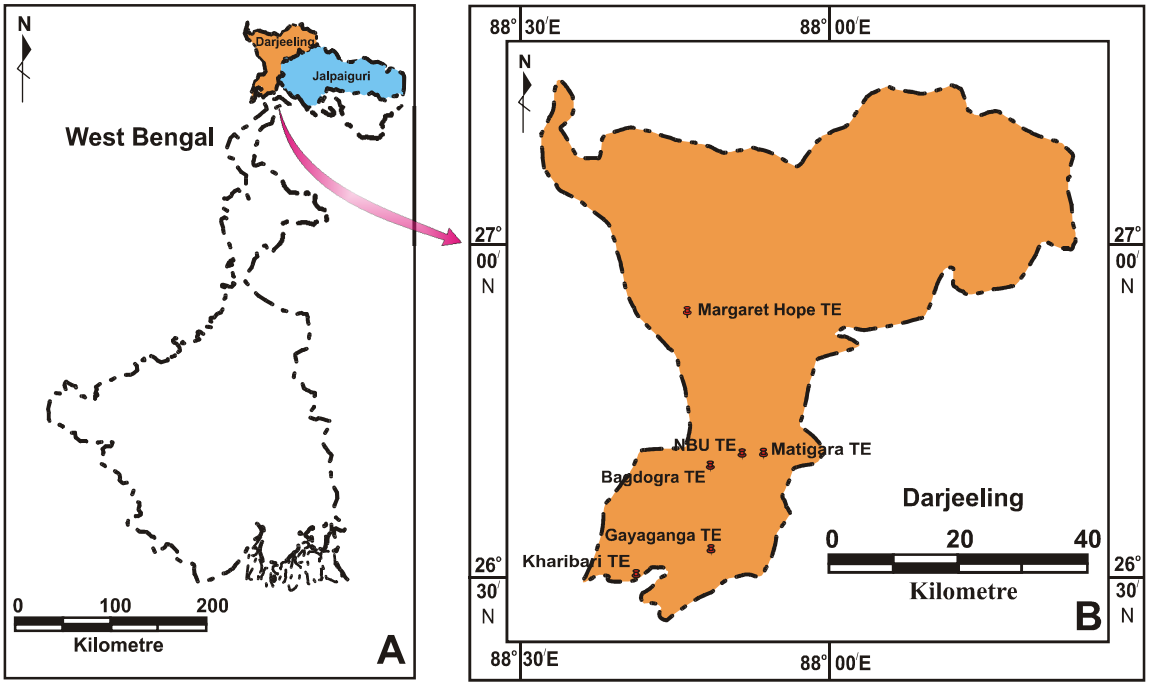


Fig. 3.1: Map of West Bengal(A) and Darjeeling district (B) showing the geographical locations of different tea estates.

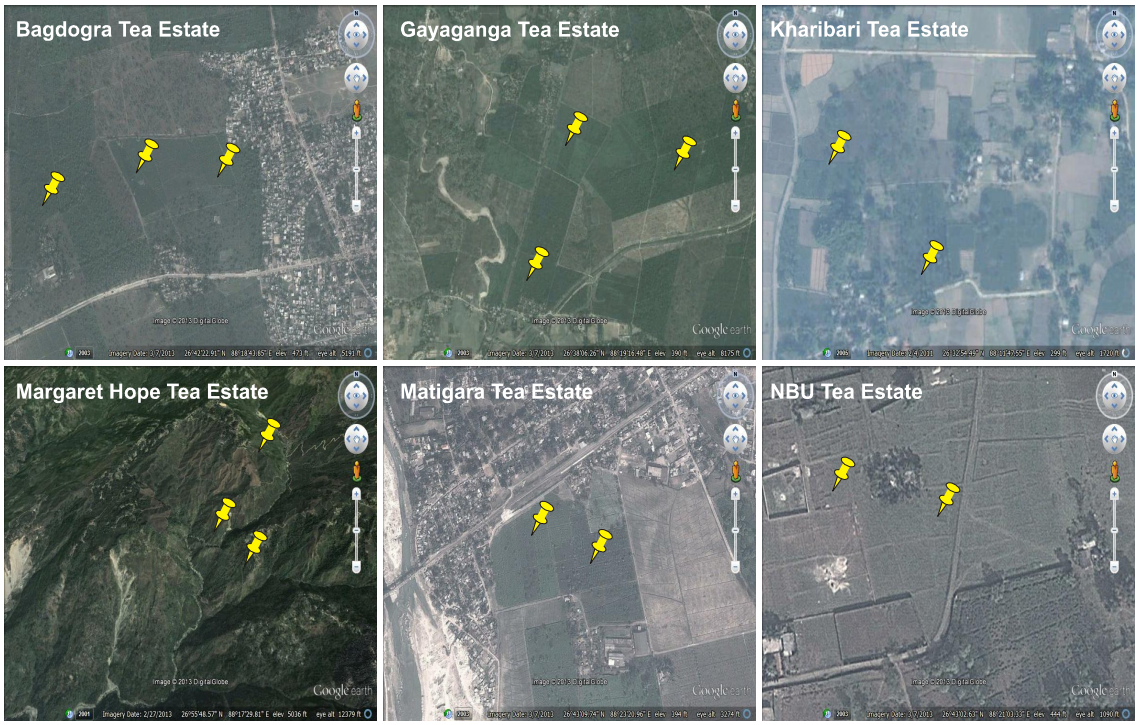


Fig. 3.2: GIS locations of sampling areas (shown in yellow marker points) of different tea gardens of Darjeeling district.

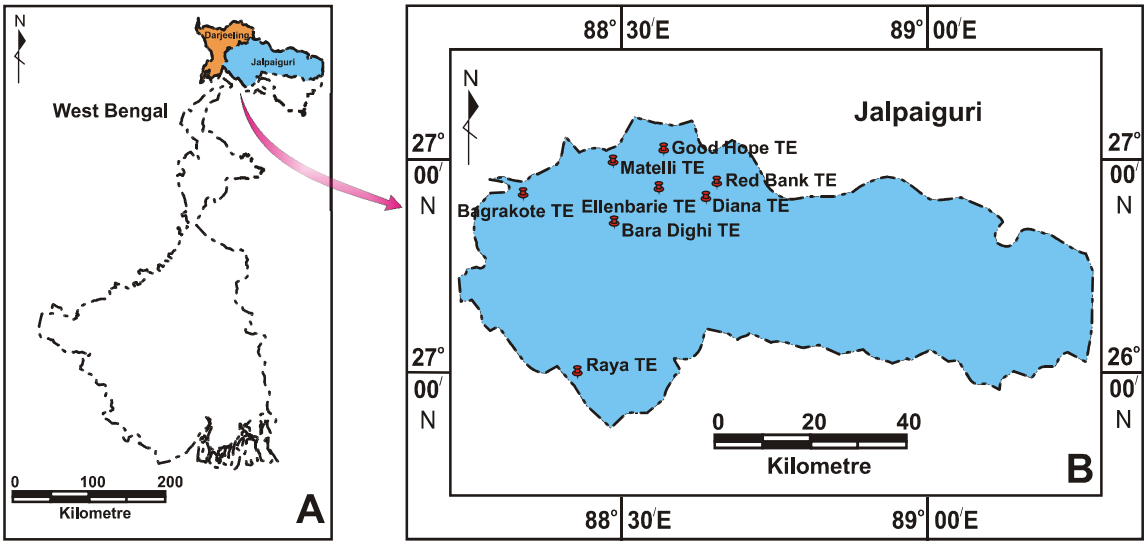


Fig. 3.3: Map of West Bengal (A) and Jalpaiguri district (B) showing the geographical locations of different tea estates.

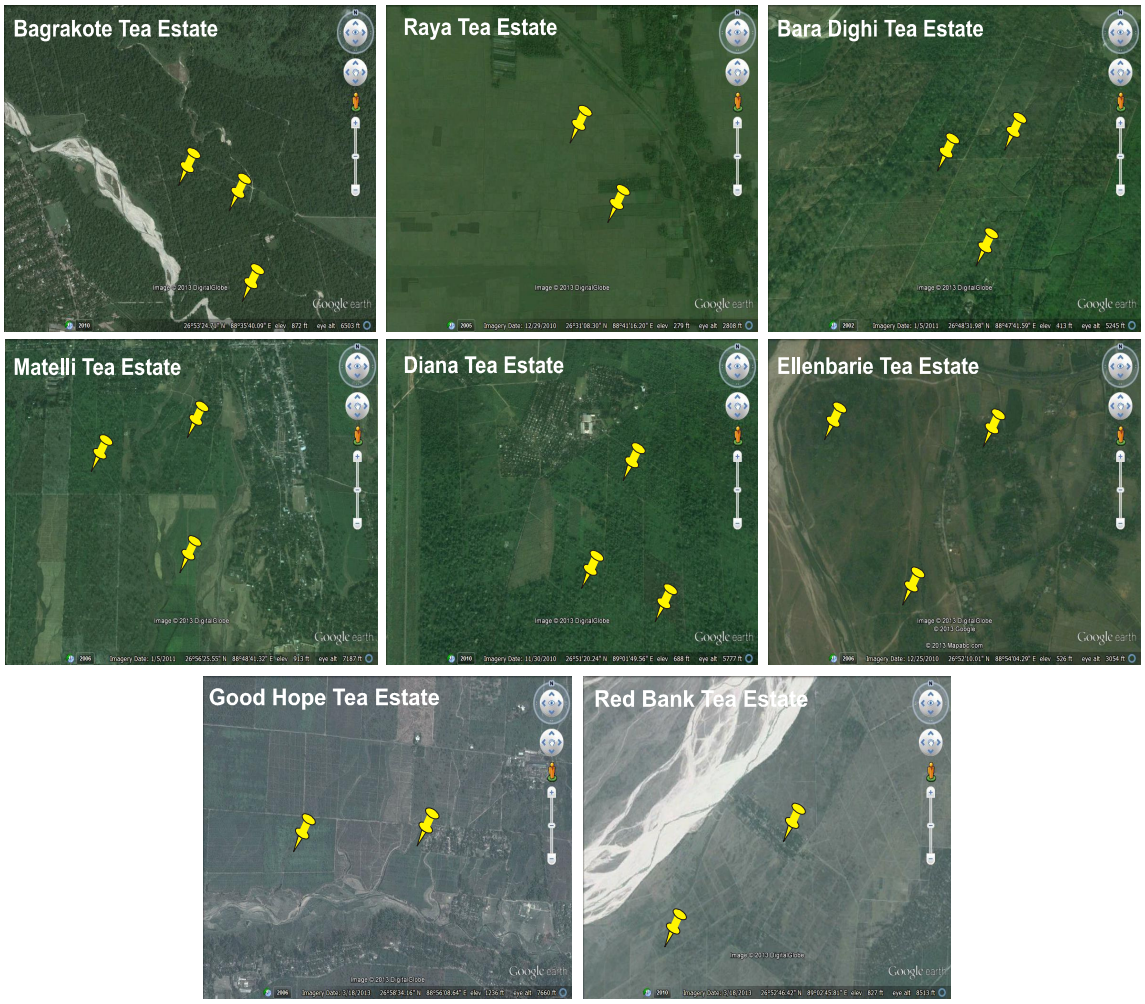


Fig. 3.4: GIS locations of sampling areas (shown in yellow marker points) of different tea gardens of Jalpaiguri district.

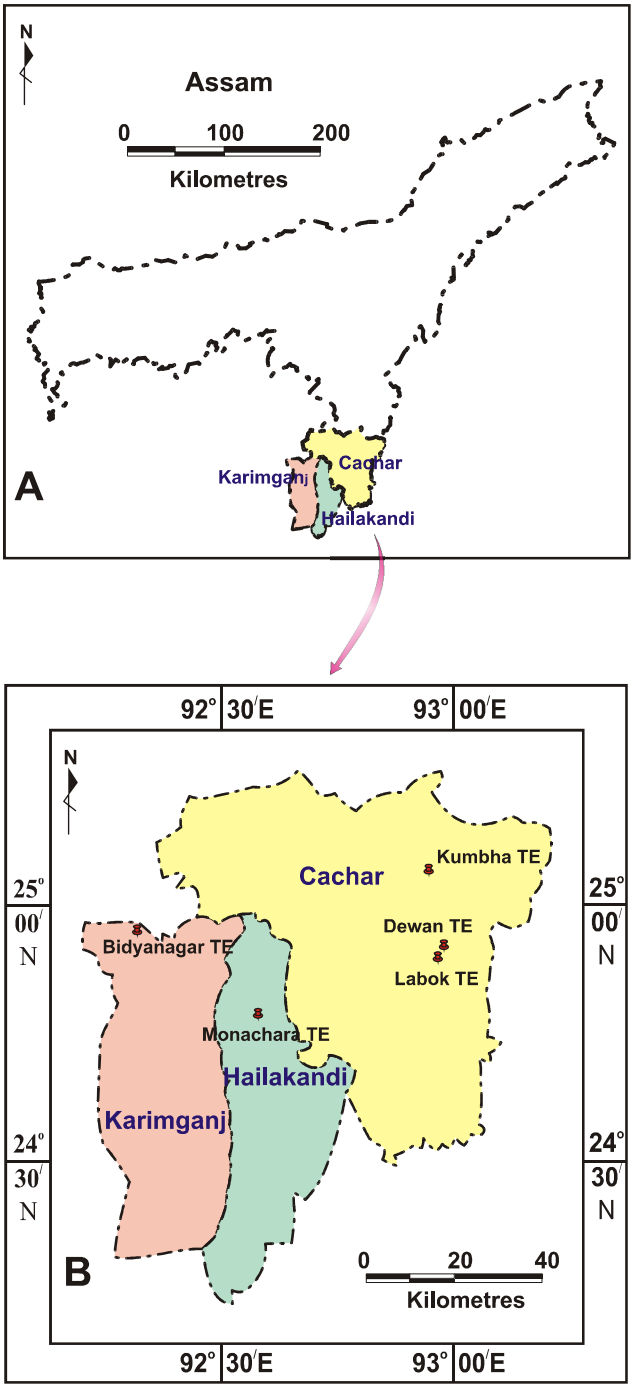


Fig. 3.5: Map of Assam (A) and Cachar, Hailakandi and Karimganj districts (B) showing the geographical locations of different tea estates.

Fig. 3.6: GIS locations of sampling areas (shown in yellow marker points) of different tea gardens of Assam.

3.4. Maintenance of pure cultures

Freshly prepared sterile NA slants were used for the maintenance of the bacterial cultures at 4^0C with sub culturing at regular intervals. The bacterial isolates were also preserved in glycerol stock which contains NB and 15-20% glycerol and stored at -20°C for future work. The fungal cultures were maintained on sterile PDA slants and stored both at room temperature and in refrigerator (4°C).

3.5. Fungal pathogens of tea

Altogether eight fungal pathogens which severely affect the tea plantation in North-Eastern region of India were used as test pathogens in the current study (Table 3.2). Of these four cultures were kindly gifted by Dr. Aniruddha Saha, Molecular Plant Pathology and Fungal Biotechnology laboratory, Dept. of Botany, University of North Bengal, Siliguri, India (Saha et al., 2005; Mandal et al., 2006; Saha et al., 2008). Five other important root pathogens of tea used in the present study were kindly provided by Tea Research Association, Tocklai Experimental Station, Jorhat, Assam. The pathogens *L. theobromae* and *R. solani* were further identified by Indian Agricultural Research Institute (IARI), New Delhi and identifications were assigned to them.

3.6. Screening of bacterial isolates for antifungal activity by dual culture test

Bacterial isolates were screened for *in vitro* antagonism against *Lasiodiplodia theobromae* following dual culture technique (Nielsen et al., 1998). A 4 mm diameter mycelial disc of the fungal pathogen was collected from advancing zone of hyphae growing in PDA plates and inoculated at the centre of a 9 cm diameter petriplate containing PDA medium. The bacterial isolate was streaked at a distance of 2-3 cm from the centre in either semi-circular pattern. The plates were incubated at 30°C and checked daily for inhibition until the fungal growth on the control plate (inoculated only with the pathogen) reached the edge of the plate. Inhibition of fungal growth along the bacterial line of streaking indicated antagonistic activity of the isolated bacterial strain.

3.7. Quantitative test for evaluation of *in vitro* antagonism

For evaluation of antagonistic potential, the fungal pathogen and the selected bacterial strain were allowed to grow simultaneously following dual

Table 3.2: Major fungal pathogens affecting tea plantations in North-East India and used during present study.

Fungal Culture	Strain identity	Disease produced	Plant part affected
Pestalotiopsis theae	PT01	Grey blight	Leaf
Colletotrichum camelliae	CC01	Brown blight	Leaf
Lasiodiplodia theobromae	5446.02	Root rot or diplodia disease	Root, stem, leaf
Rhizoctonia solani	5995.05	Root rot	Root
Sphaerostilbe repens	SR-01	Violet root rot	Root
Fomes lamaoensis	FL-01	Soft rot	Root
Poria hypobrunae	PH-01	Poria root rot and stem canker	Root and stem
Ustulina zonata	UZ-01	Stump rot	Root

culture technique as described above, but the bacteria were now streaked circularly at a distance of 2 cm from the central 4 mm fungal inoculum in 9 cm PDA plates. The control plates were inoculated only with the fungal pathogens. Radial growth of the fungal mycelia was recorded until the fungal growth in the control plates reached the edge of the plates. The antifungal activity was measured in terms of the percentage (%) inhibition over pathogen growth as [(90-d)/90] × 100 where d is the diameter (in mm) of fungal growth in the test plates. The tests were performed in three replications and the data was averaged.

3.8. Screening of fungal isolates and antifungal activity assay

Isolated fungal strains were screened for *in vitro* antagonism against following dual culture technique described by Benhamou and Chet (1993) with modifications. For examining the antagonistic activity of fungal isolates, a 4mm fungal mycelial disc of both the fungal isolate and the *L. theobromae* pathogen taken from advancing zone of growing hyphae was inoculated aseptically about 2 cm from the periphery of 9 cm diameter PDA plate in a straight line opposite to each other (at a distance of 4 cm from each other). The plates were incubated at 28°C and observed daily for inhibition until the

fungal growth on the control plate (inoculated only with the pathogen) reached the edge of the plate. Radial growth of the pathogen was measured after the desired incubation period and percent inhibition in comparison to control was determined as [(90-d)/90] × 100 where d is the diameter (in mm) of fungal pathogen growth in the test plates. The data recorded was an average of three replications.

3.9. Study of *in vitro* antagonism in liquid medium by bacterial isolates

Quantitative study of *in vitro* antagonism was carried out in PDB against all the eight test pathogens using fourteen most potent bacterial isolates (BTR19, BTR21, BTR22, D7, ETR17, ETR20, GH13, KTR06, TBD7, TGY3, TMG1, TR1, TR5 and TRB14). Fungal mycelial disc of 6mm was co-inoculated with bacterial isolates in 100 ml PDB dispensed in 250ml flasks and incubated at 28°C. Control flask was inoculated only with fungal pathogen. Mycelial dry weight (MDW) was noted at 24h interval upto a period of 7 days by straining the media through muslin cloth and then removing the excess media by blotting dry. The mycelia were then dried in hot air oven at 60^0C and dry weight was measured. Percent reduction of fungal pathogen biomass was calculated as follows: % reduction = [(MDW in control flask – MDW in dual culture flask)/ (MDW in control flask)] X100. The experiment was performed in triplicate and the mean data was recorded.

3.10. Characterization of antagonistic bacterial isolates

Several morphological and biochemical tests were performed to characterize selected number of antagonistic bacterial strains (Barrow and Feltham, 1993; Aneja, 2003). Additionally, analysis of 16S rRNA gene of four most potent isolates was done by PCR amplification followed by *in silico* studies like BLAST and generation of phylogenetic tree. To determine the identity of bacterial antagonists, “Bergey’s Manual of Systematic Bacteriology” and Cowan and Steel’s “Manual for the Identification of Medical Bacteria” were studied (Barrow and Feltham, 1993; Palleroni, 1984). The antibiotic sensitivity test towards the commercially available antibiotics was also performed with selected bacterial antagonists.

3.10.1. Morphological characterization

For studying the morphological characteristics like colony characteristics of bacteria in NA slants or plates or NB media and microscopic observation of

bacterial cell morphology, fresh cultures (16-24h old) of biocontrol bacteria were used.

3.10.1.1. Colony characteristics

Colony characteristics were studied with fresh cultures (16-24h) of selected bacterial antagonists in NA plates or slants or in NB media incubated at 30^0C. Parameters considered in the present study such as surface texture, margin/ edges, elevation, pigment production etc. were tested following Seeley and Vandemark (1972). The various parameters of colony characters were scored as follows:

Growth: Slow, moderate, fast

Surface: Smooth, rough, dry, moist, opaque, dull, glistening (shiny)

Elevation: Flat, raised, convex

Edges: Entire, undulate (swell), lobate.

Broth culture: turbid, pellicle formation

Few other characters such as odour, were also tested for distinguishing specific strains from other similar types when needed.

3.10.1.2. Shape and size

Overnight broth cultures (NB) of the bacterial strains were used for studying the cell size and shape. A drop of bacterial culture was mounted on a clean grease-free slide, heat fixed and stained with methylene blue and observed under compound microscope (Olympus, India). Cell diameter was measured after calibration of ocular micrometer with respect to the stage micrometer of the microscope.

3.10.1.3. Motility test

Detection of motility of bacterial strains was performed by stab inoculation using a straight needle in semi-solid motility medium (Barrow and Feltham, 1993). The tubes were incubated at 30°C for 48h and observed for diffused growth around the line of inoculation which indicates positive for motility test.

3.10.1.4. Endospore staining

Endospore staining of a 3-4 day old culture of selected isolates was performed using malachite green (Aneja, 2003). A loopful of bacterial culture taken from 24 h old NA slants was smeared with sterile distilled water on a

clean grease-free slide. Thereafter, the smear was heat fixed and the slide was flooded with malachite green. The slide was heated to steaming and the process was continued for 10 minutes during which stain was added occasionally. The slide was then washed carefully under tap water and was counter-stained with safranin. After 1 minute, safranin stain was washed off carefully with distilled water. The excess water was blotted dry from the slide and observed under microscope. The presence of green coloured spores inside red vegetative cell indicated endospore formation.

3.10.2. Biochemical characterization

Biochemical characterization of the antagonistic bacterial isolates was done following standard published methods as described by Barrow and Feltham (1993) and Aneja (2003). For biochemical tests, the bacteria were sub-cultured twice from stocks and 24h old fresh cultures were used. In every test, an uninoculated tube was included as negative control.

3.10.2.1. Gram characters

The smears of each of the bacterial isolates were made by placing a drop of pure bacterial suspension on a clear grease free slide, allowed to air dry and heat fixed. The dried smears were flooded with crystal violet solution for 30 seconds, whereafter slides were washed with distilled water for a few seconds, using wash bottle. Each smear was then treated with iodine solution for 30 seconds. The iodine solution was washed off with careful addition of 95% ethyl alcohol until complete discoloration. Ethyl alcohol was added dropwise on the slide which was held in a slanting position against a white background until no colour was visible in the drops falling off from the lower edge of the slide. The slides were washed with distilled water and drained off. Safranin was applied to the smears for 30 seconds, washed with distilled water and blotted dry. The stained slides were observed under microscope to study the Gram character (Aneja, 2003).

3.10.2.2. Gelatin hydrolysis

Each gelatin agar medium containing tube was inoculated with loopful of isolated bacterial strains. They were incubated at 30°C for 2 days. The cultures were then allowed to chill by incubating in ice bath for 15 minutes. The tubes that contained liquid media even after chilling were scored as positive for gelatin liquefication (Aneja, 2003).

3.10.2.3. Methyl Red (MR) and Voges-Proskauer (VP) test

MR test is performed to determine the ability of isolates to produce acid from glucose and thus reduce the pH to 4.2 and maintain the same for at least 4days. Similarly VP test determines the ability of bacteria to produce acid from glucose and subsequently convert it to acetylmethylcarbinol (a neutral substance). MR-VP tubes were inoculated with isolated bacterial strains and one uninoculated tube was kept as control. All tubes were incubated at 30ºC for 48 hours. After incubation, the culture was divided equally into two tubes for each isolate. To one of the tube marked as MR, 5 drops of Methyl red indicator was added. An appearance of red colour throughout the broth indicated positive result. To the other tube marked as VP, 12 drops of VP reagent I and 2-3 drops of V-P reagent II were added. Tubes were shakened gently for 30 seconds. The reaction was allowed to complete for 15-30 minutes. Formation of pink colored ring at the top layer was scored as positive for VP test (Aneja, 2003).

3.10.2.4. Citrate test

Simmon's Citrate agar slants were inoculated with isolated bacterial strains by streaking and one tube was kept as uninoculated control. All the slants were incubated at 30ºC for 48 hours. The indicator, bromothymol blue shows green colour in acidic pH and turns blue when alkaline. Observation of color change from green to blue indicated positive result (Aneja, 2003).

3.10.2.5. Urease test

Plates containing urea agar medium were inoculated with the isolated strains by streaking and incubated for 24-48 hours at 30ºC. Colour change of the media from yellow to pink around the bacterial inoculation line indicated positive result (Barrow and Feltham, 1993).

3.10.2.6. Oxidation and fermentation (O-F) test

Tubes containing Hugh and Leifson's O-F medium were inoculated in duplicate by each isolated strain by stabbing with a straight wire. Two uninoculated tubes were used as control. Liquid paraffin was poured over the medium to form a layer of about one cm in depth into one of the tubes of each pair. The tubes were incubated at 35°C for 24-48 hours. Oxidative reaction was shown by the change of blue color of the medium to yellow in open tube

and fermentative utilization of carbohydrate was indicated by the color change from blue to yellow in both open and sealed tubes (Barrow and Feltham, 1993).

3.10.2.7. Indole test

Test tubes containing tryptone broth were inoculated with bacterial isolates and one tube was kept as an uninoculated comparative control. Tubes were incubated at 37ºC for 48 hours. After 48 hours incubation 1 ml of Kovac's reagent was added to each tube including control. The tubes were gently shaken after intervals of 10-15 min. The test tubes were allowed to stand to permit the reagent to come to the top. A cherry red colour in the reagent layer indicated indole production (Aneja, 2003).

3.10.2.8. Catalase test

Bacterial isolates were inoculated on NA slants and incubated at 30°C for 24-48 hours. After that, 3-4 drops of 10% hydrogen peroxide was allowed to flow over the growth of each slant culture. Effervescence over the surface indicated positive result (Aneja, 2003).

3.10.2.9. Production of 3-ketolactose from lactose

The plate containing Medium 1 (specific for 3-ketolactose production from lactose) (Appendix C) was inoculated and incubated at 30ºC for 1-2 days. Then a loopful of the culture was inoculated onto a plate of Medium 2 (Appendix C) and incubated again at 30ºC for 1-2 days. The second plate was flooded with a shallow layer of Benedict's reagent and left at room temperature. Development of a yellow zone (of Cu_2O) about 1-2 cm in width around the growth within one hour indicated the pro-duction of 3-ketolactose. The yellow colour contrasted markedly with the blue reagent solution (Barrow and Feltham, 1993).

3.10.2.10. Phenylalanine deamination

Slants containing phenylalanine agar medium were inoculated with bacterial isolates and incubated at 30°C for 24-48 hrs. Then 0.2 ml of 10% aqueous solution of 10% aqueous $FeCl_3$ was added, shaken and observed for any colour change. A positive reaction was indicated by a dark green colour on the slope and in the liquid collected at the base which quickly fades.

3.10.2.11. DNase (Deoxyribonuclease) activity

Bacterial isolates were inoculated on the surface of DNase agar plates by streaking and incubated at 30°C for 36 h. The plates which appeared greenish blue were observed for the formation of a pinkish/clear halo around the bacterial growth (Barrow and Feltham, 1993).

3.10.2.12. Nitrate reduction

Nitrate Broth was inoculated with the bacterial isolates and incubated at 30°C for 48 hrs. One ml of nitrite reagent I followed by 1 ml of reagent II was added. A deep red colour indicated the presence of nitrite (positive reaction) which proves that nitrate had been reduced. To tubes, not showing a red colour within 5 minutes, powdered zinc was added and allowed to stand. Red colour formation confirmed the presence of nitrate in the medium (i.e. not reduced by the organism) (Barrow and Feltham, 1993).

3.10.2.13. Nitrite reduction

Nitrite Broth was inoculated with the bacterial isolates and incubated at 30°C for 7-14 days. Nitrite reagent Solutions I and II was added to the tubes as for the nitrate reduction test. Appearance of red colour showed the presence of nitrite. Absence of red color indicated that nitrite was reduced and therefore considered as a positive test (Barrow and Feltham, 1993).

3.10.2.14. ONPG test

Tubes of ONPG broth were inoculated with the bacterial isolates and incubated at 30°C for 48 hours. â-galactosidase activity was indicated by the appearance of a yellow colour due to the production of o-nitrophenol (Barrow and Feltham, 1993).

3.10.2.15. Oxidase activity

A fresh solution of the reagent was prepared each time of use by adding a loopful of tetramethyl-p-phenylenedi-amine dihydrochloride to about 3 ml of sterile distilled water. A filter paper disc was soaked in a sterile plastic petriplate with a few drops of the indicator solution and a 24h NA culture of each of the bacterial strains was smeared across the moist paper with a platinum loop. The appearance of a dark purple colour on the paper within 30 seconds denoted a positive reaction (Barrow and Feltham, 1993).

3.10.2.16. Growth on MacConkey agar

Bacterial strains were streak inoculated on MacConkey agar medium and the plates were incubated at 37°C for 48h. The growth on the agar plates and any change in color of the bacterial strains were recorded. The appearance of colonies were scored as positive for growth while color variation like pink colored colonies indicated lactose fermentation by bacterial strains and white or colorless colonies indicated the growth of lactose non-fermenters (Barrow and Feltham, 1993).

3.10.2.17. TSI agar Test

Tubes containing TSI agar slants were inoculated with each bacterial isolate by stabbing the butt and streaking the slope. The tubes were incubated at 30^0C for 24 h and observations were recorded. A red coloration in the medium indicated alkaline reaction and yellow indicated fermentation of sugar leading to acid formation. H_2S production was shown by blackening of the butt. The appearance of red coloration in the entire tube (both slant and butt) indicated absence of fermentation (Aneja, 2003).

3.10.2.18. Ornithine Decarboxylase Test

Decarboxylase test medium supplemented with 0.5% L-ornithine hydrochloride were inoculated with each bacterial isolate and incubated at 37°C initially for 24h. The initial color of the medium is purple due to the presence of bromocresol purple indicator which turns to yellow after 24h of inoculation with the bacteria which are positive for ornithine decarboxylation due to the fermentation of glucose (drop in pH). Under acidic condition, ornithine decarboxylase enzyme is activated. The inoculated medium is incubated for an additional 24h at 37°C to allow the bacterial isolates to utilize the ornithine. The change in color of the medium from yellow to purple indicates positive test for ornithine decarboxylase. Failure to turn yellow after 24h and again to purple from yellow after 48h indicates a negative result (Barrow and Feltham 1993).

3.10.2.19. Arginine Decarboxylase Test

For arginine decarboxylation, the decarboxylase test medium supplemented with 0.5% L-arginine hydrochloride were inoculated with each bacterial isolate as described above and incubated at 37°C initially for 24h.

The change in colour from purple to yellow after 24h of inoculation due to the fermentation of glucose and again to purple in another 24h due to utilization of the arginine indicates positive test for arginine decarboxylase (Barrow and Feltham, 1993).

3.10.2.20. Lysine Decarboxylase Test

Lysine decarboxylase test was performed as described above using decarboxylase test medium supplemented with 0.5% L-lysine hydrochloride. The medium was inoculated by the bacterial isolates, incubated and observed for colour change from purple to yellow and again to purple which would indicate positive test for lysine decarboxylase production (Barrow and Feltham, 1993).

3.10.2.21. Production of acid and gas from carbohydrates

To detect acid and gas formation from carbohydrates, fifteen different types of carbohydrates (D-Glucose, D-Mannitol, Fructose, Cellobiose, Sucrose, Adonitol, L-Arabinose, L-Rhamnose, m-Inositol, Raffinose, Maltose, Trehalose, D-Sorbitol, Lactose and D-Xylose) were used. Broth of carbohydrate fermentation medium was inoculated with the bacterial isolates and incubated at 30°C for 24-48 h. Acid production was indicated by color change from red to yellow, retaining the red color indicated negative result. The change of color was recorded by comparing with the uninoculated (control) tube. Gas production was indicated by formation of bubble in Durham's tube (Barrow and Feltham, 1993).

3.10.2.22. Screening for fluorescent Pseudomonads

For screening of fluorescent pseudomonads amongst the antagonistic bacterial isolates, bacterial strains were streaked on *Pseudomonas* Agar (for fluorescein) plates and incubated at 30°C for 48 h. The plates were then observed under UV light source at 254nm for fluorescence emission. The strains emitting fluorescence were selected as positive strains by visually comparing to the fluorescence emitted by a standard *P. fluorescens* strain (NRRL B23932), which was also inoculated as a positive control.

3.10.3. Phylogenetic characterization

Phylogenetic characterization of four most potential antagonistic bacterial strains (D7, ETR17, ETR20 and KTR6) was carried out by partial sequencing of the 16S rRNA gene. The obtained sequences were compared

with that of other sequences available in NCBI GenBank. The genomic DNA isolated from selected bacterial strains was used as template for amplification of the target gene. The amplicon sequences obtained were subjected to BLAST searches for finding similarity with other sequences of related species and the results were analysed for identification of the bacterial strains.

3.10.3.1. Isolation of genomic DNA from bacteria

Genomic DNA was isolated from selected antifungal bacterial isolates following the CTAB method (Gomes et al., 2000). A loopful of bacterial culture was inoculated to 5ml of NB media and incubated at 30ºC for 16h under constant shaking. The overnight culture was then centrifuged as 1.5ml aliquots taken in eppendorf tubes at 10,000 rpm for 10 minutes at 4ºC. The pellet was suspended in 20µl of TE buffer, 3µl Proteinase K (10 mg/ml) and 30µl of 10% SDS solution was added, mixed well and incubated at 55ºC for 16h. To the mixture, 80µl of 1%CTAB in 1M NaCl solution and 100µl of 5M NaCl solution was added. The mixture was incubated at 65ºC for 10 minutes and centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was collected carefully in fresh eppendorf tubes and 0.6 volume of 70% ethyl alcohol was added to it to precipitate the DNA. The tubes were again centrifuged at 10,000 rpm for 10 minutes at 4ºC and the supernatant was discarded. The pellet was washed with 2 volumes of 70% ethyl alcohol by centrifuging at 10,000 rpm for 10 minutes at 4ºC. Finally, the pellet was air dried by incubating at room temperature for 1h and dissolved in 50µl of TE buffer.

3.10.3.2. RNase treatment

The genomic DNA dissolved in 100 ml TE buffer was treated with 10µg ml^{-1} RNase A at 37°C for 30-60 min. Following incubation, DNA was re-extracted with PCI (Phenol: chloroform: Isoamyl alcohol 25:24:1) and purified DNA was precipitated with chilled ethanol. Ethanol wash of the DNA was carried out twice by centrifugation at 10,000 rpm for 10 minutes at 4ºC, the pellet was dried and resuspended in 30-40µl of TE buffer (Maniatis et al., 1982). The purified DNA sample was stored at -20°C for future use.

3.10.3.3. Quantification of DNA

Initially, the purity of DNA was evaluated by determining the OD_{260}/OD_{280} value. The optical density (OD) was recorded at two different wavelengths i.e. 260 nm and 280 nm. The absorbance value at 260 nm relates to the

concentration of nucleic acid in the sample whereas the value at 280 nm indicates the amount of protein in the sample. OD_{260}/OD_{280} value in the range of 1.8-2.0 indicates a pure preparation of DNA. Standard value of 1 O.D. of double-stranded (ds) DNA sample at 260nm corresponds to 50 ng/µl of dsDNA. For quantification, the purified DNA sample (1 µl) was diluted in 50 µl TE buffer (dilution factor = 50) and the absorbance was recorded in a dual beam Varian Cary 50 Bio UV-Visible spectrophotometer (Varian, Australia).

3.10.3.4. Gel electrophoresis

The quality of isolated DNA was further assessed by agarose gel electrophoresis performed in a submarine gel electrophoresis system (Bangalore Genei (India) Pvt. Ltd., India). The gel block was prepared by suspending 0.8% agarose in 1X TAE buffer and heated to boiling till a clear solution was obtained. The solution was allowed to cool to about 50-55^{0}C and then ethidium bromide (0.5µg/ml) was added, mixed properly, poured into the gel casting tray and allowed to solidify. After solidification, the gel was transferred to the electrophoresis tank in such a manner that the gel block was completely submerged in 1X TAE running buffer. DNA samples (10µl) were mixed with 2µl of gel loading buffer and loaded onto the wells. Gel electrophoresis was carried out at 55-60 volt for 1 hour and visualized for the presence of sharp and bright fluorescent orange bands under UV transilluminator (Bangalore Genei (India) Pvt. Ltd., Bangalore, India).

3.10.3.5. PCR amplification of 16S rRNA gene

PCR amplification of the 16S rRNA gene of four most potential antagonistic bacterial isolates (ETR17, KTR6, D7 and ETR20) was performed using the respective genomic DNA. PCR was performed in 25µl reaction volume containing 2.5µl of 10X Taq DNA polymerase buffer, 3µl of 2.5mM $MgCl_2$, 0.5µl each of 25mM dATP, dCTP, dGTP and dTTP, 2µM of each 16S rDNA primers fD1 and rP2 (Weisberg et al., 1991), 50ng template DNA and 3U of Taq polymerase using the following program: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min on a thermal cycler (Applied Biosystems GeneAmp PCR 2400). The sequences of the primers used were: Forward Primer, fD1 (Positions 8 to 27 of *E. coli* numbering system): 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse Primer

rP2 (Positions 1509 to 1491 of *E. coli* numbering system): 5'-ACGGCTACCTTGTTACGACTT-3' (Weisberg et al., 1991).

3.10.3.6. Detection of PCR amplicon in agarose gel

The PCR amplicon was resolved on 1% agarose gel (prepared as mentioned in section 3.10.3.4) containing ethidium bromide (0.5µg/ml concentration) at 55V for 1 h in 1X TAE running buffer along with a 500 bp DNA ladder. The resolved amplicons were observed under UV transilluminator (Bangalore Genei (India) Pvt. Ltd, Bangalore, India) and photographed.

3.10.3.7. Sequencing of PCR amplicons

The PCR amplicons were purified using PCR purification kit (Bangalore Genei (India) Pvt. Ltd., Bangalore, India) to remove the presence of any impurity like primer dimers. The purified PCR products were then sequenced at Bangalore Genei Sequencing Services, India and Xcerlis Labs, Gujarat, India. The partial 16S rRNA gene sequences obtained was submitted to NCBI GenBank. The sequences of the PCR products are listed in Appendix D.

3.10.3.8. Phylogenetic analysis of bacterial isolates

The 16S rRNA gene sequences of the bacterial isolates D7, ETR17, ETR20 and KTR6 were compared with the 16S rRNA gene sequences of related species available in GenBank databases using the BLAST search program of the National Center for Biotechnology Information (NCBI) (Zhang et al., 2000). The 16S rRNA gene sequences of the bacterial strains having similarity ranging from 98% to 100% with the target sequence were used for sequence alignment. The phylogenetic trees were constructed with neighbor-joining method by using MEGA 4.0 (Tamura et al., 2007). Bootstrap analysis using 1000 replications of the sequences was done to determine confidence in the tree topology (Felsenstein, 1985).

3.10.3.9. PCR amplification of 16S-23S ITS region of rRNA gene using *Pseudomonas* fluorescens specific primer

PCR amplification of 16S-23S rRNA gene ITS region using *P. fluorescens* specific primer was performed in order to detect *P. fluorescens* isolates, BTR8, D6 and D8. The primers used were: 16S-23S ITS forward primer: 5'AAGTCGTAACAAGGTAG-3' and 16S-23S ITS reverse primer: 5'-GACCATATATAACCCCAAG-3' (Kumar et al., 2002). The 25µl reaction mix

contained 2.5µl of 10X Taq DNA polymerase buffer with 2.5mM $MgCl_2$, 2mM dNTP mix, 2µM primer (each forward and reverse), 50 ng template DNA and 3U of Taq polymerase. PCR was performed using the following program: initial denaturation at 94°C for 5min, followed by 40 cycles at 92°C for 4min, 28°C for 1 min, 72°C for 2min and a final extension at 72°C for 10min (Kumar et al., 2002). Agarose gel electrophoresis of the amplified product was performed and the 1% agarose gel was viewed in a UV-Transilluminator (Bangalore Genei, India) along with a 100bp DNA ladder.

3.11. Characterization of antagonistic fungal isolates

3.11.1. Studies on morphological and sporulation characters

The morphology of ten most potential fungal antagonists: AD2, AD3, AD4, AD6, AD7, AD10, B2.4, KV8, NB4 and NBT1.2, was studied by inoculating the fungus on PDA plates and slants. Observations and differentiations were made on the basis of color of mycelial mat and growth pattern. For microscopic observations, mycelia were taken from pure culture on clean and grease free microscopic slides and stained using cotton-blue in lactophenol. The slides were mounted with cover glass, sealed and observed under compound microscope at 40x and 100x (oil immersion) (Olympus, India). Length and breadth of spores and breadth of mycelia were measured by calibrated ocular micrometer with respect to the stage micrometer of the microscope. The details of the morphology of the fungus were noted. Further, the pure culture slants of most potential biocontrol fungal isolates were sent to IARI, New Delhi for identification.

3.11.2. Phylogenetic characterization

Phylogenetic characterization of ten most potential antagonistic fungal isolates (AD2, AD3, AD4, AD6, AD7, AD10, B2.4, KV8, NBT1.2 and NB4) was carried out by partial sequencing of the 18S rRNA gene. The obtained sequences were compared with that of other sequences available in NCBI Genbank. The genomic DNA isolated from selected fungal strains was used as template for amplification of the target gene. The sequences obtained were subjected to Blast searches for finding similarity with other sequences of related species and the results were analysed for identification of the fungal strains.

3.11.2.1. Isolation of genomic DNA from fungi

Mycelial disc of 6mm diameter of antagonistic fungal isolates was inoculated in 100 ml PDB dispensed in 250ml flasks and incubated either under static conditions (all except AD4) or under constant shaking (AD4) at 28°C for 3-5 days. The mycelial mat was harvested by straining the media through muslin cloth, then washed with 0.5% sterilized NaCl solution to remove the excess media and subsequently re-washed thrice with sterile distilled water to remove excess salt and blotted dry. The fungal mycelium was frozen at -20^{0}C for 2 hours prior to DNA isolation. The frozen mycelium (1gm) was homogenized in a chilled mortar and pestle using 5ml of 2% CTAB DNA extraction buffer. The mycelial suspension (1.0ml) was collected in sterilized 2.0ml eppendorf tubes and incubated for 1 hour at 60°C in a water bath with occasional mixing. Following incubation, 0.6 volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tubes and mixed by inversion for 15 min. The mixture was centrifuged at 10,000 rpm for 15 min and supernatant was collected into fresh eppendorf tubes (1.5ml). Equal volume of chilled isopropanol (-20°C) was added to the tubes to precipitate the DNA, mixed gently and centrifuged at 3,000 rpm for 5 minutes at 4°C. The supernatant was discarded and the DNA pellet was washed with 70% ice cold ethanol (40µl) by incubating the sample at room temperature for 20 minutes and centrifuging at 7,000 rpm for 5 minutes at 4°C. The pelleted DNA was dried overnight at room temperature and finally dissolved in 30µl of 1X TE buffer.

3.11.2.2. RNase treatment, quantification of DNA and Gel electrophoresis

The genomic DNA was treated with RNase A to remove RNA and the pure DNA was quantified spectrophotometrically. The DNA samples were then subjected to agarose gel electrophoresis and visualized under UV light following methods as described earlier (sections 3.3.1.2.2-4).

3.11.2.3. PCR Amplification of 18S rRNA gene

PCR amplification of the 18S rRNA gene of ten most potential antagonistic fungal isolates was performed using the respective genomic DNA. The primers used were: ITS1: 5'- TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3' (White et al., 1990). PCR was performed in 25µl reaction volume containing 2.5µl of 10X Taq DNA polymerase buffer containing 2.5mM $MgCl_2$, 2.0µl of 2.5mM dNTP mix, 15 pmol of each primer

ITS1 and ITS4, 50ng template DNA and 1.5U of Taq polymerase using the following program: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 53.5°C for 45 seconds, extension at 72°C for 1 min and a final extension at 72°C for 5 min on a thermal cycler (Applied Biosystems GeneAmp PCR 2400).

3.11.2.4. Detection of PCR amplicon in agarose gel and sequencing of the amplicons

The PCR amplicon was resolved on 1% agarose gel) containing ethidium bromide along with a 100bp DNA ladder following methods described earlier (section 3.10.3.4). The resolved amplicons were observed under UV transilluminator and photographed. For sequencing, the amplicons were purified using PCR purification kit (section 3.3.1.2.7) and sequenced at Xcerlis Labs, Gujarat, India. The partial 18S rRNA gene sequence of selected fungal antagonists was submitted to NCBI GenBank. The sequences of the PCR products are listed in Appendix D.

3.11.2.5. Phylogenetic analysis

The 18S rRNA gene sequences of the fungal isolate AD10 were compared with the 18S rRNA gene sequences of related species available in GenBank databases using the BLAST search program of NCBI (Zhang et al., 2000). The 18S rRNA gene sequences having similarity of 98% with the target sequence were used for sequence alignment. The phylogenetic trees were constructed with neighbor-joining method by using MEGA 4.0 (Tamura et al., 2007). Bootstrap analysis using 1000 replications of the sequences was done to determine confidence in the tree topology (Felsenstein, 1985).

3.12. Hemolytic activity of selected antagonistic bacterial strains

Hemolytic activity of selected bacterial isolates (ETR1, ETR17, ETR20, ETR24, D7 and TR1) was assessed on Tryptone Soya Agar (TSA) medium (Himedia Laboratories, India) supplemented with 5% human blood. For this, each bacterial isolate was streaked on blood supplemented TSA plates and incubated at 30°C for 48 hours. After incubation, the plates were observed against light for halo formation around the bacterial growth. A clear halo indicated β hemolysin production, dark colored halo indicated α, α' hemolysin production and green color halo indicated γ hemolysin production (Gerhardt et al., 1994).

3.13. Fungicide sensitivity assay of antagonistic bacterial strains

Susceptibility of the antagonistic bacterial strains to the chemical fungicides, mancozeb, thiophanate methyl, bavistin and captan, which are used commonly in the sampling tea gardens was tested *in vitro*. Each bacterial strain was inoculated to 5ml of NB and incubated at 30°C for 24h under constant shaking (120 rpm). The resulting bacterial culture (1 ml) was used to inoculate molten NA medium (19 ml) by pour plate method in 9cm petriplates and allowed to solidify. To determine the minimum inhibitory concentration (MIC), each fungicide was serially double diluted in sterile distilled water (1000, 500, 250, 125, 62.5, 31.5, 15.6, 7.8, 3.9, 1.95, 1 mg/ml) to obtain 11 dilutions and 100µl of each of the different concentrations were pipetted into the wells (4mm diameter) cut in the solidified NA medium seeded with bacteria. Control wells received sterile distilled water only, without any fungicide. The plates were incubated at 30°C for 24h. Bacterial growth was monitored by observing for the formation of inhibition zone. The minimum concentration at which an inhibition zone was visible around the well was considered as the minimum inhibitory concentration (MIC) of the fungicides.

3.14. Antibiotic sensitivity of selected antagonistic bacterial strains

Antibiotic sensitivity profile of selected bacterial antagonists, D7, ETR17, ETR20 and KTR6 was determined using 20 different antibiotics. A 100µl of 16h old bacterial culture was inoculated to sterilized molten nutrient agar medium at 50°C. The inoculated media was then poured on sterile petriplates (19 cm diameter) and allowed to solidify at room temperature. After solidification, antibiotic ring (Icosa Universal-2, Himedia, India) containing twenty different antibiotic discs viz. Amikacin (30mcg), Ampicillin (10mcg), Amoxycillin (10mcg), Cefadroxil (30mcg), Cefoperazone (75mcg), Ceftazidime (30mcg), Ceftriaxone (30mcg), Chloramphenicol (30mcg), Ciprofloxacin (5mcg), Cloxacillin (1mcg), Co-Trimoxazole (25mcg), Erythromycin (15mcg), Gentamycin (10mcg), Nalidixic Acid (10mcg), Netilmycin (10mcg), Nitrofurantoin (300mcg), Norfloxacin (10mcg), Penicillin (10mcg), Tobramycin (10mcg) and Vancomycin (30mcg) was placed carefully over the inoculated media. The plates were incubated at 30°C for 24-48 hours and observed for the formation of clear zones around the antibiotic discs and the zone diameter (in cm) was recorded. Resistance and sensitivity of the isolates towards the antibiotics were assessed according to the antibiotic disc manufacturer's protocol.

3.15. Growth kinetics study of selected antagonistic bacterial strains

Nutrient broth media in sterilized test tubes were inoculated aseptically with 100µl of 24 hour old culture of the bacterial isolates. The tubes were then incubated at 30°C on an orbital shaker and bacterial growth was recorded at a regular interval of one hour by measuring the absorbance at 590nm till stationary phase was reached.

3.16. Production of antifungal metabolites by antagonistic microorganisms

The production of antimicrobial metabolites by antagonistic micoorganisms may be detected *in vitro* by specific tests. All the selected thirty five bacterial and ten fungal isolates were subjected to the tests listed below. Prior to the experiments, each bacterium and fungus was sub-cultured in NA and PDA respectively and finally 24h bacterial culture and 7d old fungal cultures were used for each assay. An uninoculated control was included in all tests for comparison.

3.16.1. Production of hydrolytic enzymes

For preparing culture filtrates, bacterial strains were grown overnight in nutrient broth and cultures were centrifuged at 10,000 rpm for 15 min. Culture supernatants were filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius) to obtain the final filtrate which was used in cellulase production and chitinase production tests.

3.16.1.1. Pectinase activity

Pectinase production by the antagonistic bacterial and fungal isolates was determined following the method described by Cattelan et al. (1999) with modifications (Kumar et al., 2005). For testing pectinase production, 9 cm petriplates containing M9 agar medium (Miller, 1974), supplemented with $10gL^{-1}$ pectin and 1.2g L^{-1} yeast extract were inoculated with bacterial cultures and incubated for 2 days at 30°C. The plates were thereafter flooded with 2M HCl. A positive result was indicated by visible clear halos around the colonies.

3.16.1.2. Cellulase activity

Production of cellulase enzyme was detected following the method of Cattelan et al. (1999) with some modifications. Antagonistic bacterial strains were grown in M9 broth medium (supplemented with 10g L^{-1} cellulose) at 30^0C

for 48h. Culture supernatants were filter sterilized by passing through cellulose acetate filter paper (pore size 0.2µm diameter) (Sartorius). The fungal strains were also grown in PDB for 6 days under constant shaking at 28^{0}C (Picard et al., 2000). The cultures were strained through a filter paper (Whatman Grade-I) for retaining the mycelia. The filtrate was sterilized by passing through a cellulose acetate filter paper as was done for bacterial strains. The sterile culture filtrates of bacterial and fungal strains were added individually to 4mm agar wells cut on M9 agar medium supplemented with 10g L^{-1} cellulose and 1.2g L^{-1} yeast extract. The plates were incubated at 30^{0}C for 5-6 days and subsequently stained with 0.1% Congo red solution overnight and destained thrice with 1M NaCl at 2hrs interval. Plates were inspected for the formation of clear halo around the wells which indicated positive result for cellulase production.

3.16.1.3. Lipase activity

Extracellular lipase activity of the bacterial antagonistic strains was tested in Tween 80 agar (Smibert and Krieg, 1994). Each bacterium was streak-inoculated on the surface of Tween 80 agar medium and incubated at 30^{0}C for 24-28h. An opaque or translucent halo formation around the bacterial colonies indicated positive lipase activity.

3.16.1.4. Protease activity

For determining protease activity, skim milk agar plates were used (Aneja, 2003). Antagonistic bacterial strains were inoculated as a single streak and incubated for 24-48 h at 30^{0}C in an inverted position. Formation of a clear halo around the bacterial line of inoculation in the background of a turbid white colored medium indicated positive result.

3.16.1.5. Chitinase activity

Chitinase activity was observed by a slight modification of the method of Bargabus et al. (2002). Antagonistic bacteria were grown overnight in nutrient broth and culture was centrifuged at 10,000rpm for 15min. The culture supernatant was filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius) and 100µl of the filtered sample was used for detection of chitinase activity. The fungal strains were grown in potato dextrose broth for 5days under constant shaking at 28^{0}C. The culture was strained through a filter paper to remove mycelia (Whatman Grade-I) and the resulting solution was

filter-sterilized in a way similar to that done for bacterial strains. The culture filtrates were used as enzyme sources for both fungus and bacteria and loaded (100ml each) on the wells cut on 1% glycol chitin supplemented M9 medium. The plates were incubated for 48h at 30^0C and thereafter stained with fluorescent brightener 28. Positive result was indicated by the presence of non- fluorescent lytic regions under UV light source.

3.16.1.6. Amylase activity

Amylase activity was determined by inoculating starch agar plates with the bacterial antagonists as single streak followed by incubation at 30^0C for 48 hours in an inverted position. The plates were then flooded with iodine solution and after 30 second the solution were drained out. Development of clear zone around the bacterial line of inoculation in a dark blue or purple background was considered as positive result (Barrow and Feltham, 1993).

3.16.1.7. DNase activity by fungal antagonists

Antagonistic fungal strains were inoculated as 4mm mycelial discs using sterile inoculation needle on the surface of DNase agar medium and incubated at 28°C for 3-4days. The plates were observed for the formation of a pinkish or clear halo around the fungal growth which indicated positive DNase activity.

3.16.2. Detection of HCN production

Test for HCN production was carried out by the method of Bakker and Schippers (1987). Bacterial isolates were inoculated heavily in nutrient agar medium and incubated at 30°C with filter paper strips soaked in picric acid solution (0.5% picric acid in 2% Na_2CO_3 aqueous solution) attached to the lids. Fungal isolates were tested by inoculating each fungus in PDA medium and incubating the plates carrying picric acid solution loaded filter paper strips for 3-4d at 28^0C. A positive result was indicated by the color shift of the filter paper strip immersed from yellow to red.

3.17. Detection of siderophore produced by microbial antagonists in CAS agar medium

Siderophore production was determined using the Universal Chrom azurol S (CAS) assay (Schwyn and Neilands, 1987). The CAS (Appendix B) medium is made from a combination of four solutions which were prepared separately and sterilized. Solution 1 contains Fe-CAS indicator solution which

was prepared by mixing 10ml of 1mM $FeCl_3.6H_2O$ (prepared in sterilized 10mM HCl), 50ml of aqueous solution of CAS (1.21mg/ml) and 40ml of aqueous solution of HDTMA (1.82mg/ml). Solution 2 is a buffer solution, prepared by dissolving 30.24 g of PIPES buffer in distilled water. The final pH of solution 2 was adjusted to 6.8 by using 50% KOH solution to obtain a final volume of 800 ml. The solution was amended with agar (15g) and autoclaved. Solution 3 consisted of 2g each of glucose and mannitol dissolved in 70 ml distilled water and autoclaved. Solution 4 was prepared by dissolving casamino acid in 30 ml of distilled water (10% w/v). The solution was filter sterilized using cellulose acetate filter paper (0.2µm). All the solutions were mixed under appropriate conditions to obtain the final medium. Solution 2 was cooled to attain a temperature of about 50-55^{0}C following which solution 3 and 4 were mixed with it. Solution 1 was further added to it and mixed carefully in order to avoid bubble formation. The final colour of the medium appeared blue to dark green due to the presence of the dye-Fe-CAS complex. Spot inoculation of bacterial culture onto CAS agar plate was done and incubated at 30^{0}C overnight for 48h. For fungal culture, 4mm mycelial discs were inoculated on CAS agar plates and incubated for 3-4 d at 28^{0}C. The formation of orange colored halo around the microbial colonies indicated siderophore production.

3.18. Characterization of microbial siderophore

Siderophores are normally produced under iron restricted conditions, therefore, siderophore production was tested in Fiss-glucose minimal medium (Vellore, 2001) which contains limited amount of iron. The medium was prepared by dissolving 5.0 g KH_2PO_4 and 5.0 g L-asparagine in 954 ml deionized water and the pH was adjusted to 6.8. After sterilization of this medium, 10 ml of each of the solutions: 50% glucose, 0.005% $ZnCl_2$, 0.001% $MnSO_4$ and 0.4% $MgSO_4.7H_2O$ (each autoclaved separately) were mixed with the 960 ml of the previously sterilized medium. Finally, filter sterilized 1mM $FeSO_4$ solution was added to the above medium. All the medium constituents were prepared in deionized water in order to minimize traces of iron and all the glassware used for preparation of the medium and growth of the microorganisms were treated with concentrated HNO_3 and further rinsed with deionized water.

The antagonistic bacterial isolates tested positive for siderophore production in Universal CAS assay were grown in Fiss-glucose minimal medium (10ml) for 24h at 30^{0}C. Following incubation, the culture was centrifuged at

10,000 rpm for 15 min to obtain the culture supernatant which was further used for siderophore characterization. In case of fungal isolates tested positive for siderophore production in CAS plates, 10ml of the Fiss-glucose minimal medium was inoculated using 4mm mycelial agar discs and incubated for 3-4d at 28^0C. The fungal mycelium was removed by straining through sterilized Whatman filter paper (Grade III) and the filtrate was centrifuged at 10,000 rpm for 15 min to obtain the culture supernatant. The culture supernatant thus obtained was used for siderophore characterization.

3.18.1. Arnow's test for catecholate siderophore

Identification of catecholate type of siderophore was carried out following the method of Arnow (1937). Arnow's test was performed by mixing 1 ml culture supernatant of bacteria and fungus separately with 1 ml 0.5M HCl, 1 ml Nitrite-Molybdate reagent and 1 ml 1N NaOH. The mixture was adjusted to 5ml volume by addition of distilled water and allowed to stand at room temperature for 5 minutes for the reaction to occur completely. Catechol production produced yellow coloration, changed to pink in presence of 1N NaOH which when reacted with nitrous acid changed to an intense orange-red when made strongly basic (pH 10) (Holzberg and Artis, 1983).

3.18.2. Detection of hydroxamate siderophore

Hydroxamate nature was examined by tetrazolium salt test (Baakza et al., 2004). To 1ml of culture supernatant (bacteria and fungus separately) a pinch of triphenyl tetrazolium chloride salt and 1–2 drops of 2N NaOH were added. Appearance of an instant deep red coloration indicated the presence of hydroxamate siderophores.

3.18.3. Ferric chloride test for hydroxamate siderophore

Production of hydroxamate siderophore was confirmed by ferric chloride test following the method described by Neilands (1981). To 1 ml of culture supernatant, 1ml of 2% $FeCl_3$ solution was added and the formation of red or purple colour indicated the presence of siderophore. Characteristic peak obtained in spectral scan between 420 and 460 nm in a Cary 50 Bio UV-Visible spectrophotometer (Varian, Australia) indicated the hydroxamate nature of siderophore.

3.18.4. Detection of Carboxylate siderophore

Spectrophotometric test was conducted following the methodology of Shenker et al. (1992). To 1 ml of 250 µM $CuSO_4$ ml and 2 ml of acetate buffer (pH 4), 1ml of culture supernatant was added. The mixture was scanned in the wavelength range of 190–280 nm and observed for the peak of absorption of siderophores. An uninoculated medium was kept as blank.

3.19. Siderophore production assay

Siderophore quantification of bacterial and fungal strains was carried out by CAS shuttle assay (Payne, 1994). Bacterial and fungal isolates were grown in Fiss glucose minimal media overnight under constant shaking. The overnight grown culture was centrifuged at 10,000 rpm for 15 minutes and the cell-free culture supernatant was mixed with an equal volume of CAS reagent (solution 1 of CAS agar medium) and incubated for one hour at 37°C. A control was kept where uninoculated broth was mixed with equal volume of CAS reagent. The absorbance was then measured at 630 nm. This assay method determined the amount of siderophore units produced by the antagonistic strains. Siderophore content was calculated by using the formula:

% siderophore units = $[(A_r - A_s)/A_r] \times 100$, where, A_r = Absorbance of reference (uninoculated medium) at 630nm (Absorbance maxima of CAS reagent is 630nm) and A_s = Absorbance of sample at 630nm.

3.20. Phosphate solubilization

Each of the selected antagonistic bacterial and fungal isolate was tested for phosphatase activity in Pikovskaya's agar (Pikovskaya, 1948) medium. The medium was streak inoculated with bacterial strains and incubated at 30^0C for at least 5 days (Kumar et al., 2005). In case of the fungal isolates, 4mm mycelial discs were inoculated on Pikovskaya's agar plates and incubated for 5-7d at 28^0C (Chakraborty et al., 2011). Development of a clear zone around the bacterial and fungal growth was considered as positive for phosphatase production.

3.21. Detection and quantification of IAA

Production of IAA (indole-3-acetic acid) was investigated by using the method described by Patten and Glick (2002). Bacteria were incubated overnight in 5 ml Luria Bertani (LB) broth supplemented with 5mM of L-tryptophan.

After incubation for 42 h, bacterial cells were removed from the medium by centrifugation at 8000 g for 10 minutes. The supernatant (1ml) was mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Weber, 1951) and allowed to stand at room temperature for 20 min following which the absorbance was measured at 535 nm. Standard curve was obtained using 10–100 µg/ml concentrations of IAA. The concentration of IAA was determined by comparison with the standard curve. In case of fungal isolates, the strains were grown in potato dextrose broth supplemented with 5mM L-tryptophan for 5 days under constant shaking at 28°C. After incubation, the suspension was filtered through Whatman filter paper (Grade III) in order to remove the mycelia and then the filtrate was centrifuged at 10, 000 rpm for 10 minutes and the supernatant was collected. The supernatant was used for IAA quantification following the method of Patten and Glick (2002) as described above.

3.22. Assessment of biofilm formation by microtitre plate assay

Biofilm formation by the antagonistic bacterial isolates was assessed in two different medium following the method of Harvey et al. (2007). Bacterial strains were inoculated to 5ml each of Luria Bertani (LB) broth and M9 Yeast extract (M9YE) broth in test tubes and incubated for 18h at 30°C. An aliquot of 125µl of the bacterial cultures from each medium was transferred separately to 5ml of growth medium (LB), vortexed for 1 min and 100µl volumes of the mixtures were transferred to the wells of sterile polystyrene microtitre plates. Uninoculated LB and M9YE were used separately as control. The microtiter plates were incubated at 30°C and incubated for 24h and 48h. The adherence of bacterial cell on the microtitre plates was observed by measuring the absorbance of crystal violet stain at 595nm at two different time periods (24h and 48h). The experiment was performed in triplicate.

3.23. Scanning electron microscopy

The effect of bacterial isolates ETR17 and KTR6 and antagonistic fungal isolate AD-10 on the mycelial growth of the pathogen *R. solani* was studied by scanning electron microscopy. The bacterial isolate and the pathogen were co-inoculated on PDA plates as a dual culture (Section 3.2.2) where a 4 mm diameter mycelial disc of *R. solani* was inoculated at the centre of sterile PDA plates (9 cm diameter) while the bacteria were streaked at a distance of 25 mm from the centre in circular pattern. Cover-slips were carefully placed in the bacterial-fungal interaction zone of the co-inoculated plates (closer to

bacterial inoculation line) and towards the edge in the control plate (inoculated only with *R. solani*). The plates were incubated at 28°C until the fungal growth on the control plate reached the edge of the plate. Similarly, a 4mm fungal mycelial disc of both the antagonistic fungal isolate AD10 and the pathogen was inoculated aseptically in sterile PDA plates at a distance of 4cm from each other to set up fungal dual culture (Section 3.2.2). Sterile coverslips were placed in the expected zone of interaction. After the interacting fungal mycelia in dual cultures grew over the cover slips, the cover slip was removed from each plate and placed on a petriplate. The mycelium on the coverslip was fixed with 2.5% glutaraldehyde solution for 1 hour followed by dehydration with ethanol in the following series: 50% ethanol for 5 minutes, 70% ethanol for 5 minutes, 90% ethanol for 30 minutes with two consecutive changes and finally absolute ethanol till microscopic observation. Prior to microscopy, the samples were air dried, adhered to stubs and coated with gold (IB2 ion coater, Japan). Finally the samples were observed under a scanning electron microscope [Model: Hitachi S-530 (Japan) 1986].

3.24. Spectrophotometric detection of antibiotics in bacterial culture supernatants

The antibiotic production capability of 35 most potential bacterial isolates was studied by spectrophotometric analysis of culture supernatant by using a newly developed protocol. Three sets of growth media were used in the study: Casamino acid peptone mannitol (CPM-Ca^{2+}), pigment producing medium (PPM) and yeast extract mannitol (YEM) broth. Gram positive strains were grown in CPMCa^{2+} broth while gram negative strains were grown in PPM and YEM broth. Test tubes containing sterile medium were inoculated with bacterial isolates and incubated at 30^0C for 72h. The bacterial cultures were centrifuged at 12,000 rpm for 10 minutes. The culture supernatant was filter-sterilized using 0.2ìm diameter cellulose acetate filter paper and the filtrate was transferred to fresh centrifuge tube and scanned between 200 to 700 nm on a dual beam Varian Cary 50 Bio UV-Visible spectrophotometer (Varian, Australia). Uninoculated broth was included as control in each case. The antibiotic standards like prodigiosin, pyrrolnitrin, pyocyanin, pyoluteorin and phenazine (purchased from Sigma Aldrich Chemicals Pvt. Ltd.) were also scanned in the same range (200-700 nm) and used as reference in this study.

3.25. Extraction of crude extracellular metabolites from bacterial cultures

For studying the effect of antifungal metabolite on biocontrol of fungal pathogens two potent bacterial isolates viz. ETR17 and KTR6 were selected. Bacterial strain ETR 17 was inoculated into semi-solid PPM (Levitch and Stadtman, 1964) while KTR6 was inoculated in $CPMCa^{2+}$ medium (Bernal et al., 2002) by pour plate method and incubated at 30^0C for 8 d. The total content (180 ml) was crushed in a blender and the extracted with 250 ml of 80% aqueous acetone for 24 hours in an orbital shaker (Shanahan et al., 1992). Agar was removed by centrifugation at 15,000 rpm for 20 min at 10^0C and the supernatant containing antibiotics was condensed at 40°C in a rotary vacuum evaporator (Eyela CCA-1110, Japan). The aqueous concentrate was filtered through cellulose acetate filter paper (Sartorius, pore size 0.2µm) and 20 ml portions of the filtrate were extracted twice with 2.5 volumes of diethyl ether. The organic phase containing antibiotics were evaporated to dryness in vacuo at 30^0C and the residue was re-extracted with 30ml of acetone, and finally evaporated to obtain a red pasty mass.

3.26. Antifungal activity assay of crude extracellular extract

Antifungal activity of the crude culture extract of bacterial isolates was assessed *in vitro* by agar cup assay on PDA plates against the fungal pathogen, *L. theobromae*. Fungal inoculum in the form of 4mm mycelial disc excised from advancing zones of hyphae growing in PDA was placed on fresh PDA plate and agar cups (4mm diameter) were cut at a distance of 2-3cm from the fungal inoculum in the same plate. Aliquots of 100 µl of the filtered crude culture extracts were loaded on each cup. The control cup received 100µl acetone. The plates were incubated at 28^0C for 3 to 7 days or till the fungal growth in the control plate (inoculated only with *L. theobromae*) reached the edge of the petriplate. Antifungal activity was indicated by clear inhibition zones around the agar cups.

3.27. Determination of minimum inhibitory concentration of the bioactive crude extract

The crude extract containing extracellular antifungal metabolites obtained from biocontrol bacterial isolate was sterilized through 0.2µm diameter cellulose acetate filter paper (Sartorius). A 750 mg of the crude extract was dissolved in 5ml of methanol to obtain a stock solution of $150mgml^{-1}$

concentration. Thereafter, various concentrations of the crude extract ranging from 5 mg ml^{-1} to 150 mg ml^{-1} were prepared by diluting the stock in methanol. Mycelial disc (4mm) of the pathogen *L. theobromae* was excised from advancing zones of hyphae growing in PDA plates and inoculated at the center of sterile potato dextrose agar (PDA) plates. Wells of 4mm diameter were cut at a distance of 2-3cm from the centre of the plate. An aliquot of 100µl of the filtered extracts were loaded on each well at different concentrations (5 mg ml^{-1} to 150 mg ml^{-1}). The plates were incubated at 28^0C for 3-5 days. The lowest concentration below which no zone of inhibition was observed was considered as the minimum inhibitory concentration (MIC).

3.28. Purification of antibiotics from crude extracellular culture extract of *S. marcescens* strain ETR17

Crude extracellular metabolite extracted from the most potential bacterial isolate *S. marcescens* strain ETR17 was purified by silica gel column chromatography followed by preparative TLC.

3.28.1. Silica gel column chromatography

The crude product (2.5g) was dissolved in 10ml methanol and the solution was passed through a petroleum ether balanced silica gel (mesh 60-120, SRL, India) column (height 60cm, diameter 3cm) to facilitate trapping of the target product within the column. In order to release the adsorbed product, the column was eluted with petroleum ether (PE): ethyl acetate (EA) (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45 and 50:50) with increasing percentage polarity. Ten fractions (F1 to F10) of 100ml volume each were collected and each fraction was concentrated to in a rotary vacuum evaporator at 35^0C and bioassayed.

3.28.2. Detection of antibiotics in column fractions by TLC

The fractions exhibiting antifungal properties were monitored by thin layer chromatography (TLC). Samples (fractions F2, F3, F4, F9 and F10 obtained from column chromatography) were loaded on TLC sheets precoated with Silica Gel 60 F254 (S.D. fine Chemicals, Mumbai, India) and co-chromatographed with standard antibiotics pyrrolnitrin, prodigiosin and phenazine (Sigma-Aldrich). The sheets were developed in benzene: acetic acid (9:1) and viewed under UV (254nm) light. The appearance of UV active spots having same R_f

value as that of the standards indicated that the corresponding antibiotic may be present in the sample.

3.28.3 Preparative TLC for purification of antibiotics in column fractions

The fractions obtained from column chromatography showing presence of antibiotics were further purified by preparative TLC. For this, larger volumes (1 ml) of the bioactive fractions (F2, F3, F4, F9 and F10) were spotted as before on glass-backed preparative TLC plates (prepared manually by coating with silica gel). The sheets were developed similarly in benzene: acetic acid (9:1) and the zone corresponding to the R_f value obtained on analytical plates were scrapped from the TLC plate, suspended in methanol and centrifuged. The supernatant was dried in vacuo and used for further spectroscopic analysis

3.29. UV-Visible Spectral analysis of partially purified antibiotics

The antifungal fractions obtained by purification through preparative TLC were scanned between 200 to 700 nm on a dual beam Varian Cary 50 Bio UV-Visible spectrophotometer (Varian, Australia) along with standard antibiotics. The maximum absorbance recorded in each case was compared to that of the antibiotic standards viz. prodigiosin, pyrrolnitrin, pyocyanin and phenazine.

3.30. Analysis of partially purified antibiotics by high performance liquid chromatography (HPLC)

High performance liquid chromatographic analysis of the antifungal metabolites purified by TLC was performed in Shimadzu SPD-20A, Japan. The antifungal fractions which appeared to contain similar antibiotic were combined based on the spectral scan results to obtain two samples. Each sample (50µg) was dissolved in 1ml methanol (HPLC grade, SRL, India) and 20µl was injected into C18 Reverse Phase column (250 x 4.6mm size and 4µm particle size) (Phenomenex, USA). The pump used was LC-20AD (Shimadzu, Japan). The eluent flow rate was adjusted to 1ml min^{-1} and analyzed isocratically in 100% methanol. Standard antibiotics were used at a concentration of 10µg ml^{-1}. Pyrrolnitrin was detected at 225nm using a D2 detector (Prominence, Shimadzu, Japan) and prodigiosin was detected at 536nm using tungsten (W) detector (Prominence, Shimadzu, Japan).

3.31. LC-ESI-MS analysis of partially purified antibiotics

The bioactive fractions F9 and F10 obtained by column chromatography were combined and directly subjected to LC-ESI-MS analysis using a gradient elution program with solvent A (methanol) and solvent D (ammonium acetate buffer, pH 6.5): 50% solvent A and 50% solvent D from 0 to 10 min; 70% solvent A, 30% solvent D at 10 min and 80% solvent A, 20% solvent D till 30 min at a flow rate of 0.8 ml min^{-1} at 254 nm. A 20µl sample was injected into the column without any dilution. The column used was Thermo ODS-2 (250 x 4.6mm size and 5µm particle size) (Thermo, India). The electrospray ionization mass spectra were recorded on a Thermo LCQ Advantage Max (Thermo, India) with the following specifications: Source voltage 5.3V, source current 80.0µA, capillary voltage 3.0 V, tube lens offset 5.0V and capillary temperature of 300^0C.

3.32. *In vitro* bioassay of column fractions

Each column eluted fraction was tested *in vitro* for antifungal activity against the pathogens *L. theobromae*, *S. repens* and *R. solani*.

3.32.1. Preparation of spore and mycelial suspension

Spore suspension was prepared by following the method of Saha et al. (2005). Potato dextrose agar plates (9 cm) were inoculated with the mycelial discs of the individual fungal pathogens (*L. theobromae*, *R. solani* and *S. repens*) and incubated for 10-12 days at 28°C for adequate sporulation. Spore suspension was prepared either in sterile distilled water or in sterile Richard's medium; which was added aseptically to the fungal cultures. The surface of the mycelial mat was brushed gently with inoculation needle to loosen the spores. The resulting suspension was filtered through four layers of muslin cloth to remove mycelial fragments. The concentration of spores in the filtrate was measured using haemocytometer and the final concentration was adjusted to 1 x 10^6 spores ml^{-1} (Saha et al., 2005). In case of *R. solani* which forms only sclerotia, the mycelium was scrapped lightly in sterile distilled water with inoculation needle and filtered through sterile double-layered muslin cloth in order to avoid any traces of agar. The mycelial suspension was used for agar cup bioassay.

3.32.2. Agar cup bioassay

Each column eluted fraction was tested in vitro for antifungal activity against the pathogens *L. theobromae*, *S. repens* and *R. solani*. Potato dextrose agar

medium was autoclaved at 121^{0}C for 15 min, cooled to 45^{0}C and 1 ml of pure spore suspensions of pathogens were mixed with 19 ml of molten medium, and poured into sterile petriplates of 9 cm diameter. After solidification of the medium in petriplates, wells were prepared with sterile cork-borer (4mm diameter) and 100µl of individual column fractions dissolved in 1 ml methanol was loaded into the wells. The plates were incubated at 28^{0}C for 2-4 days and diameter of inhibition zones formed around the wells, if any, was measured.

3.33. Bioautography

Antifungal activities of the crude culture extract and the partially purified column fractions obtained from the strain ETR17 were tested by bioautography following the method of Saha et al. (2005). Bioautography with the crude culture extract was done on glass-backed silica gel TLC plates (10 cm x 20 cm) prepared manually by coating with silica gel G (for TLC) (SRL, India). Aluminium backed precoated TLC plates (3 x 5 cm) were used for testing the partially purified column fractions (F2, F3, F4, F9 and F10). The manually prepared plates were air-dried for 2-3 days and each plate was activated by heating at 80^{0}C for 45 minutes prior to sample-loading. Concentrated test extracts were spotted on the activated TLC plates and developed either in hexane: ethyl acetate: methanol (60:40:1) (for crude extract) or in benzene: acetic acid (9:1) mixture (for column fractions). The developed chromatograms were air-dried until the solvent evaporated completely. Subsequently, spore suspension (10^6 spores/ ml) prepared from *L. theobromae* was mixed with Richard's medium (15g/L) and sprayed with an atomizer on dried TLC plates. The plates were incubated in a humid chamber at 28^{0}C for 2-5 days. Inhibition zones, which appeared as clear white spots on a background of thick mycelial growth, indicated the presence of antifungal compounds. R_f value of the inhibition zone was noted. The centre of inhibition zones where the four zone diameters meet (diameters at 45^0 angle to each other) was used for calculating R_f.

3.34. Plant material for *in vivo* biocontrol test

Tea seeds of TS-520 and TS-462 varieties were procured from Gayaganga tea estate (Darjeeling, West Bengal). The seeds were disinfected and planted in pre-sterilized sand taken in aluminium trays at one inch below the surface. The trays were watered regularly with sterile tap water. The germinated seedlings were transplanted to plastic cups (5cm x 7cm) containing presterilized

sand-soil mixture (1:1). Finally one month old seedlings with two-three leaves were transplanted to earthen pots (16cm diameter and 11cm height) containing sterile tea garden soil and maintained in the experimental garden of the Department of Botany, University of North Bengal under normal light and temperature. Tea plantlets at one-two month old stage of TS-520 variety were also directly procured from Gayaganga tea estate, maintained similarly and used for experimental purpose after acclimatization for 30 days.

3.35. Test for pathogenicity of *Rhizoctonia solani* and verification of Koch's postulates

R. solani was selected as the test pathogen for *in vivo* studies because it causes severe damage to tea seeds, seedlings and plant roots in this region. The fungus is reported to cause disease in several agricultural and horticultural crops. The present culture was isolated from infected tea seeds (Mandal et al., 2006) and used for biocontrol experiments after verification of Koch's postulates. For this, four-week old germinated tea seedlings of two widely cultivated seed varieties of tea of North-East India (TS-520 and TS-462) were used for pathogenicity test. Five seedlings potted in plastic cups (5cm x 7cm) of each variety were used for the study and five plants were kept as uninoculated control. Ten day old culture of *R. solani* (grown in 100ml PDB in 250 ml flasks) was harvested and the mycelial mat was collected by filtration through sterile muslin cloth. The mycelium (50g) was mixed with sterile distilled water (200 ml). The mixture was blended properly to obtain fragmented mycelial mat. The fragmented mycelial mat was thereafter applied to the soil where the seedlings were planted and maintained in the experimental net house under normal conditions of light and temperature. The pots were watered regularly with sterile distilled water and observed for 15 days for any symptoms of root rot. The diseased plants were uprooted and damaged regions of roots were excised into 5-10 mm long pieces. The root pieces were washed with sterile distilled water and then surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 3 min and again washed with sterile distilled water. The root pieces were transferred to the PDA slants aseptically and incubated at 28^0C for 10-15 days until the pathogen mycelium covered the entire surface of the medium. The identity of the isolates was confirmed after comparing them with the stock culture.

3.36. *In vivo* studies for management of *Rhizoctonia* root rot in tea using bio-formulated antagonistic bacterial strain *S. marcescens* strain ETR17

3.36.1. Preparation of talc-based formulation from ETR17 and KTR6

Talc-based formulation was prepared with single bacterial strains and also with a mixed culture of isolates ETR17 and KTR6 following the method of Nandakumar et al. (2001). Initially, a 100µl of 48h old nutrient broth cultures of each bacterium was used to inoculate 400ml of nutrient broth separately and grown at 30ºC on a rotary shaker at 150 rpm so as to reach a concentration of $9x10^8$ cfu ml^{-1}. One kg of talcum powder (used as carrier) was taken in an aluminium tray and its pH was adjusted to 7.0 by adding calcium carbonate ($CaCO_3$) at the rate of 15g/kg. Ten grams of carboxymethyl cellulose (CMC) was added to the above mixture, mixed well and the resultant mixture was autoclaved for 30 min at 15psi pressure at 121^0C on each of two consecutive days. The 400ml bacterial culture with a concentration of $9x10^8$cfu/ml was added to the carrier-cellulose mixture and mixed well under sterile conditions. The mixture was then dried under shade aseptically, packed in polypropylene bags, sealed and stored at room temperature for future use.

In case of mixed talc-based formulation using biocontrol strain mixture, the individual bacterial strains ETR17 and KTR6 were grown separately in nutrient broth as described above. Two hundred millilitres of each culture was mixed with 1kg of sterilized carrier-cellulose mixture and the formulation was prepared as done previously. The talc formulation was applied to the experimental pots (pot diameter 16cm and height 11cm) at the rate of 50g per pot.

3.36.2. Preparation of fungal inoculum from tea root pathogen *R. solani*

Pathogen inoculum was prepared following the method of Soares et al. (2007). For this, 300g of rice grains were soaked in 500ml of distilled water at room temperature for one hour for proper hydration. The excess water was drained off; the grains were distributed equally in three 250ml conical flasks and sterilized for 30 minutes at 15psi pressure on each of two consecutive days. Mycelia disc (6mm) of the pathogen *R. solani* excised from actively growing regions of a 7 day old fungal culture in PDA plate was used to inoculate the sterilized rice and incubated for 6-9 days at 28ºC until the pathogen mycelia covered all the rice grains. The resulting inoculum was added to the experimental pots at the rate of 10g per kg soil.

3.36.3. *In vivo* studies for management of *Rhizoctonia* root rot in tea using talc-based formulation

For *in vivo* study, soil drenching method described by Nandakumar et al. (2001) was used. In this method, 10g talc formulation was mixed with 100ml of sterile distilled water and poured into the experimental pots (16cm x 11cm) containing 2 kg of soil where the 2 month-old tea seedlings of TS-520 variety were planted as described earlier. Experiment was conducted in two separate sets containing sterile and non sterile garden soil for each treatment. Talc formulations of individual strains ETR17 and KTR6 and mixed formulation containing both KTR6 and ETR17 were applied separately to different pots. All the treatments were replicated thrice and for each treatment five healthy plants were selected. After three days of treatment with bacterial formulation, the rhizosphere surrounding soil was inoculated with the fungal pathogen *R. solani*. For this, the soil was carefully removed, mixed with the rice inoculum and replaced back to the original pots. A set of five plants each for sterilized soil and unsterilized soil was maintained as untreated control which were not treated with the talc formulation but inoculated with the pathogen only. A fungicide control included set of five plants treated only with thiophanate methyl (0.1%) prior to pathogen exposure. The plants were covered with plastic bags to maintain humid condition. All plants were kept in experimental net house under normal light and temperature conditions. Assessment of root disease was done as described earlier (Kobriger et al., 1998). The mean disease index and percent efficacy of disease control was calculated following the method described earlier.

3.37. Root disease assessment

Incidence of *Rhizoctonia* root rot was evaluated at different time intervals recorded as days after pathogen inoculation (dapi). Severity of the symptoms was graded into five disease classes (0 - 4) (Kobriger et al., 1998). The seedlings were uprooted carefully and graded as follows: 0 = no disease; 1 = slightly affected lesions on roots; 2 = moderate lesions on roots; 3 = severe lesions and 4 = completely damaged roots. Based on the classes, disease index was calculated using the following formula: Disease index = [Σ(P x DC) x 100]/(T x 4) where P = plants per class, DC = disease class and T = total number of plants. Percent efficacy of disease control was calculated using the formula: PEDC = [(Disease index in untreated control – Disease index in treated plants)/ Disease index in untreated control] x100 (Purkayastha et al., 2010).

3.38. Determination of the survivability of *S. marcescens* ETR17 in talc formulation

The population of *S. marcescens* strain ETR17 was assessed in the talc formulated product containing ETR17 alone at an interval of 30 days for one year. Samples of talc formulation (1 g) were collected aseptically and suitable dilutions (10^{-1} to 10^{-5}) were prepared in sterile distilled water. An aliquot of 100µl of the individual dilutions were inoculated in NA medium by spread plate technique. The visible number of colonies formed after incubation at 30ÚC for 24h were recorded and the bacterial population was expressed as cfu g^{-1}. The plate count was recorded as mean of three replicates and plotted against time as log cfu g^{-1}.

3.39. Sustainability of biocontrol strain ETR17 in the rhizosphere

The sustainability of talc based formulation of *S. marcescens* isolate ETR17 in the rhizosphere was determined serologically using indirect ELISA (Enzyme linked immunosorbent assay).

3.39.1. Preparation of bacterial antigen

Bacterial antigen was prepared according to the method of Sakai et al. (1991). ETR17 isolate was grown in nutrient broth for 48 hours at 30ºC on a rotary shaker. The cell culture was then centrifuged at 10,000 rpm for 10 minutes and the pellet was suspended in sterilized 0.15M phosphate buffer saline (PBS) (pH 7.2) and washed thrice by centrifugation using previous conditions. The pellet was resuspended in PBS and bacterial population was inactivated by addition of 1% formaldehyde. The concentration of bacterial suspension was adjusted to an optical density (O.D.) of 1.0 at 545 nm. The cell suspension was used as antigens for raising of polyclonal antibody (PAb).

3.39.2. Raising of polyclonal antibody

Antiserum against bacterial antigens were raised in New Zealand white male rabbits by giving intramuscular injections (1 ml) of bacterial cell suspension emulsified with equal volume of Freund's complete adjuvant (Bangalore Genei, India). Injections were administered for six consecutive weeks at 7 day intervals. Blood samples were collected in sterile centrifuge tubes by puncturing the marginal ear vein at the fourth day of the last injection, and used for preparation

of antiserum (Dasgupta et al., 2005). For preparation of normal serum, blood was collected from the rabbit prior to immunization.

3.39.3. Preparation of antiserum and normal serum

For preparation of antiserum and normal serum, the collected blood samples were kept undisturbed for an hour at 37^0C for clotting. The clot was then loosened carefully with a sterile toothpick and the tubes were centrifuged at 4^0C for 10 minutes at 3000 g to remove the cells. The serum, obtained as supernatant, was distributed in sterile cryo vials and stored at -20°C until required.

3.39.4. Preparation of soil antigens for ELISA

For preparing soil antigens, soil samples were collected from the experimental sets containing the bacterial formulation and control sets not inoculated with bacterial formulations. One gram of each soil sample was added in 1 to 10 times of PBS-Tween and mixed by vortexing for 30 seconds. The mixture was allowed to stand at room temperature until the soil particles settled down. The supernatant was filtered through sterile Whatman Grade-I filter paper and the filtrate was used as antigen for indirect ELISA (Tsuchiya et al., 1991).

3.39.5. Indirect ELISA

In order to determine the viability or presence of bacterial isolate ETR17 in the soil rhizosphere, indirect ELISA method was used (Elder et al., 1982; Dasgupta et al., 2005). One hundred microlitre of the soil antigen (10^1, 10^5 and 10^9 dilutions) was mixed with an equal volume of 0.2M carbonate buffer (pH 9.6) and coated in the wells of a microtiter plate except the air blank and antigen blank. A positive control was set with the whole cell antigen of ETR17 and ETR17 antiserum. Negative controls were set with soil antigen prepared from the pots inoculated with the KTR6 talc formulation alone and uninoculated sterilized and unsterilized soil to react with ETR17 antiserum. The plate was incubated overnight at 4°C for adsorption. The antigen was poured off the next day; the wells were dried and subsequently washed with 0.15M PBS (pH 7.2) containing 0.02% sodium azide and 0.05% Tween-20 (v/v) four to five times. The plates were again air dried and 100µl of PBS-BSA (0.15M phosphate buffered saline and 1% bovine serum albumin) solution was added to block the unbound sites and incubated for 2 hours at room temperature. The plate was rewashed

thoroughly with PBS-Tween and air dried. Two hundred microlitre of antisera (1:100 dilution; diluted with PBS-Tween) was added to the wells except air blank, antisera blank and normal sera control (1:100 dilution; diluted with PBS-Tween containing 0.5% BSA) and incubated overnight at 4ºC. The antisera were discarded and the plate was again thoroughly washed with PBS-Tween and dried. Then, 100µl of goat anti-rabbit IgG HRP (Horse Raddish Peroxidase) conjugate (1:10,000 dilution) was added to each well except the air blank and conjugate blank and incubated for 2 hours at room temperature. The plate was washed thoroughly with PBS-Tween, air dried and 100µl of TMB/H_2O_2 (1:20), a chromogenic substrate, was added to each well except the air blank. It was incubated for 30 minutes in dark at room temperature for development of blue color due to enzyme-substrate reaction. The reaction was stopped by adding 100µl of 1N H_2SO_4 and absorbance was recorded at 492 nm in an ELISA reader (Mios Junior; Merck, Darmstadt, Germany). A standard curve was plotted with varying bacterial concentrations against the corresponding ELISA values of the positive control which was used to determine the concentration of the bacterium in the soil after *in vivo* study.

3.40. Studies on antagonistic bacterial colonization on tea root by SEM

Bacterial strain ETR17 was grown in nutrient broth at 30^0C for 48h in a rotary shaker at 120 rpm. The bacterial culture was thereafter centrifuged at 10,000 rpm for 10 min and the cell pellet was resuspended in 0.1 M phosphate buffer (pH 7.0) to obtain a final concentration of 1 x 10^8 cfu/ml. Roots of two month old tea seedlings were dipped in bacterial suspension for four hours. The roots were then cut into 1cm long pieces and fixed in 2.5% glutaraldehyde solution for 1 hour. The root pieces were thereafter dehydrated with ethanol in the following series: 50% ethanol for 5 minutes, 70% ethanol for 5 minutes, 90% ethanol for 30 minutes with two consecutive changes and finally with absolute ethanol till microscopic observation. After dehydration the root sections were air dried, adhered to stubs, coated with gold and observed under a scanning electron microscope [Model: Hitachi S-530 (Japan) 1986].

3.41. Biocontrol of root rot disease in tea by potential fungal antagonist *Trichoderma viride* strain AD10

The most potential fungal antagonist AD10 was selected based on the *in vitro* tests of antagonism in PDA medium for controlling the root rot disease

caused by *R. solani* in tea seedlings. The pathogen inoculum was prepared based on the method described previously (Soares et al., 2007).

3.41.1. Preparation of the inoculum of fungal antagonist

Trichoderma viride strain AD10 was grown in potato dextrose agar plates for 10 days at 28^0C. The petriplates (9cm diameter) were thereafter rinsed for a few minutes with sterile distilled water (@ 10ml per petriplate) and the resulting suspension was filtered through double layered sterile muslin cloth. The filtrate was used as the inoculum for *in vivo* biocontrol studies (Khalili et al., 2012; Martinez-Alvarez et al., 2012). A haemocytometer count of the *T. viride* spores was obtained and 10^8 conidia/ml concentrations were used to treat the tea seedlings.

3.41.2. Biocontrol using fungal antagonist *Trichoderma viride* strain AD10

For *in vivo* biocontrol study using fungal isolate AD10, soil application or soil drenching method was used. A 100ml of spore suspension of *T. viride* strain AD10 mixed with 0.1% carboxymethyl cellulose (binder) was poured into the experimental pots (16cm x 11cm) containing 2 kg of soil where the 2 month-old tea seedlings of TS-520 variety were planted as described earlier. The pathogen *R. solani* was inoculated at the rate of 10g per pot after three days of treatment with AD10. Thiophenate methyl (0.1%), used as fungicide control, was applied prior to pathogen exposure. All experimental and control plants were kept in experimental net house under normal light and temperature conditions. Experiment was conducted in sterile and unsterile sets as done with bacterial antagonists and maintained accordingly. The plants were covered with plastic bags to maintain humid condition. The treatments were replicated thrice and for each treatment five healthy plants were selected. Assessment of root disease was done as described earlier (Kobriger et al., 1998) in section 3.37. The mean disease index and percent efficacy of disease control was calculated as described previously.

3.42. Plant growth promotion by bacterial formulations

To determine whether the isolated strains ETR17 and KTR6 were capable of plant growth promotion, 2 month old tea seedlings of TS-520 variety were treated twice at an interval of 15 days by talc formulation of the strain by soil application method in sterilized and unsterilized soil sets as described earlier. The shoot and root length of the seedlings were measured with a centimeter

scale prior to treatment with bacterial formulations and again after 45 days of treatment, by carefully uprooting the seedlings. Plants treated with sterile distilled water grown in both sterilized and unsterilized soil served as control sets. All the treatments were replicated thrice and for each treatment five healthy plants were selected.

3.43. Statistical analysis

Statistical analysis of the *in vitro* and *in vivo* biocontrol studies was done with the help of Smith's statistical package (version 2.5), developed by Dr. Gray Smith, Pomona College, Claremont-91711, USA and Statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois. Standard error was also calculated using this software. The data represented were means of three replications and for analysis, were subjected to analysis of variance and the means (± standard error) were compared using least significant difference (LSD).

ISOLATION AND SCREENING OF POTENTIAL MICROORGANISMS FROM TEA RHIZOSPHERE

4.1.1. Isolation of antagonistic microorganisms from tea rhizosphere

Rhizosphere soil samples were collected from nineteen different tea gardens located at various regions of sub-Himalayan West Bengal and Assam. Serial dilution of the soil samples and spreading onto soil extract agar resulted in the isolation of 212 bacterial and 72 fungal strains (Table 4.1.1a and Table 4.1.1b, Fig. 4.1.1). The microbial isolates were coded according to the place from where the source soil was collected. Each isolated microbial strain was maintained as pure culture in either NA medium (for bacteria) or PDA medium (for fungi).

4.1.2. Screening for *in vitro* antagonism against *Lasiodiplodia theobromae*

All isolated bacterial and fungal strains were screened for the presence of biocontrol activity against an important fungal pathogen *Lasiodiplodia theobromae* known to cause the diplodia disease in tea. Results showed that fifty one bacterial isolates were effective in controlling the advancement of fungal mycelia along the bacterial line of streaking. Another twenty fungal isolates were also found to restrict the radial growth of fungal pathogen (Fig. 4.1.2). Thus, 24% of the total bacterial isolates and 27.8% of the fungal isolates exhibited antifungal activity during initial screening.

4.1.3. Evaluation of *in vitro* antifungal activity of isolated bacteria

Preliminary screening study for antifungal activity was followed by quantitative evaluation of antagonistic potential. Thus, all fifty one bacterial isolates found to be antagonistic during initial screening were further evaluated quantitatively for *in vitro* antifungal activity through dual culture test against six root pathogens (*Lasiodiplodia theobromae*, *Rhizoctonia solani, Sphaerostilbe repens, Poria hypobrunnea, Ustulina zonata* and *Fomes lamaoensis*) and two foliar pathogens (*Pestalotiopsis theae* and *Colletotrichum camelliae*) of tea in both solid (PDA) and broth (PDB) medium.

4.1.3.1. Dual culture assay in PDA

Dual culture test showed that 35 bacterial isolates exhibited significant level of *in vitro* antagonism against all the tested pathogens (Table 4.1.2; Fig. 4.1.3 and Fig. 4.1.4).

Table 4.1.1a: List of bacterial isolates from different rhizospheric soil

Source of isolation	Code assigned	Total No. of isolates	Antagonistic isolates
Bagdogra T.E.	TBD1 - 10	10	TBD1-7
Bagrakote T.E.	BTRL1-11	11	BTRL6, BTRL8, BTRL9, BTRL11
Baradighi T.E.	BTR1-24	24	BTR4, BTR8, BTR18, BTR19, BTR21, BTR22, BTR23
Diana T.E.	D1-10	10	D6, D7,D8
Ellenbarie T.E.	ETR1-24	24	ETR1, ETR17, ETR20, ETR24
Gayaganga T.E.	TGY1 - 17	17	TGY1, TGY2, TGY4, TGY6, TGY7
Good Hope T.E.	GH1-33	33	GH4,GH6,GH12,GH13, GH21, GH22,GH27,GH32
Kharibarie T.E.	KTR1-18	18	KTR6, KTR18
Labak T.E.	TLB1-11	11	TLB3
Matigara T.E.	TMG1 - 15	15	TMG1,TMG2,TMG3,TMG7
Raya T.E.	TR1 - 20	20	TR1,TR5,TR11,TR19,TR20
Red bank T.E.	TRB1 - 19	19	TRB1, TRB2, TRB4, TRB7,TRB12, TRB14, TRB18
Total number of isolates		212	51

T.E.: Tea Estate

Table 4.1.1b: List of fungal isolates from different rhizospheric soil

Source of isolation	Code assigned	Total No. of isolates	Antagonistic isolates
Dewan T.E.	AD1-10	10	AD2, AD3, AD4, AD6, AD7, AD10
Kumbha T.E.	KV1-10	10	KV8
Monachara T.E.	NB1-10	10	NB2.2, NB4
Bidyanagar T.E.	B2.1-2.10	10	B2.4
Margaret Hope T.E.	M1-10	10	M1, M2, M4
Matelli T.E.	MATBD10	10	MAT2-7
Matigara T.E. (NBU)	NBT1.1-1.12	12	NBT-1.2
Total number of isolates		72	20

T.E.: Tea Estate

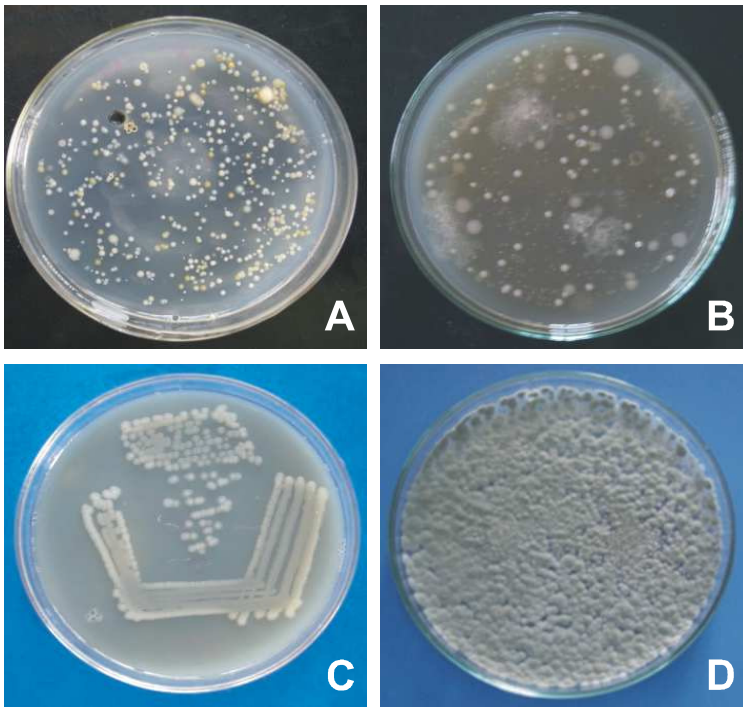


Fig. 4.1.1: Serially diluted sample spread plated on soil extract agar (SEA) plates: (A) Sample at 10^{-1} dilution, (B) Sample at 10^{-3} dilution, (C) Isolated single colony of a bacterial isolate, (D) Isolated fungal culture.

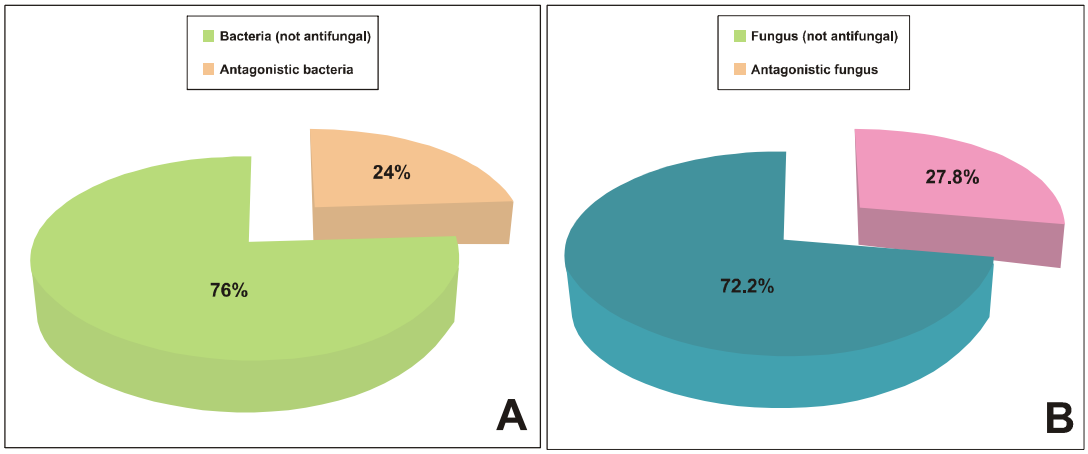


Fig. 4.1.2: Percentage of antagonistic and non-antagonistic bacterial and fungal isolates out of the total number of microbial strains isolated from tea rhizosphere of North-East India.

The isolates ETR17 and KTR6 were found to be most promising as they inhibited the growth of all test pathogens to more that 63% (Table 4.1.2). Among other strains, BTR19, BTR22, BTR21, D7, ETR20, GH13, TMG1, TR5, TRB14, TR1, TBD7 and TLB3 showed high level of inhibition of mycelial growth of all pathogens while the remaining isolates showed moderate to low level of inhibition. Some strains exhibited wide variations in their antifungal activity, for example, D8 showed 71% activity against *L. theobromae* but only 32% activity against *R. solani*. Similarly, T5-2 showed 62% activity against *L. theobromae* but only 13% activity against *U. zonata*. The highest antagonistic activity was exhibited by KTR6 (84%) against *F. lamaoensis* (Table 4.1.2). The strain showed excellent antagonism against *P. hypobrunnea* (83.3%), *P. theae* (82.2%) and *R. solani* (83.3%) also. ETR17 also showed more than 80% activity against four pathogens and more than 70% activity against three others among eight tested. Considering the overall performances of the strains, ETR17 and KTR6 was selected for *in vivo* studies. Maximum growth inhibition was recorded against *L. theobromae* where 44 isolates achieved more than 55% inhibition of pathogen growth. *C. camelliae* was also found to be almost equally susceptible towards the antagonists. *U. zonata*, on the other hand, was the least affected pathogen, where only twenty eight bacterial isolates exhibited low level (<50%) of pathogen growth inhibition (Table 4.1.2). All the 35 strains showing antagonism against all tested pathogens were used for biochemical characterization tests.

4.1.3.2. Dual culture assay in PDB

Based on the results of dual culture test in PDA medium, fourteen promising antagonistic bacteria were selected for evaluation of antifungal activity in broth culture (PDB) against all the test pathogens. The mycelial dry weight recorded in dual culture flasks was much lower than the control flasks which were inoculated only with the pathogen (Table 4.1.3a and Table 4.1.3b). The results of the liquid dual culture assay showed that the strains ETR17, KTR6, D7 and ETR20 inhibited the production of mycelial biomass of all the pathogens studied to the highest degree. The isolate ETR17 was the best antagonist as it showed highest inhibition of mycelial biomass production (>70.0%) against six of the eight tested pathogens followed by KTR6 which demonstrated more than 65.0% reduction of mycelial growth of seven pathogens (Table 4.1.3a). ETR20 and D7 also showed considerable reduction of mycelial growth (>55.0% inhibition) of all the pathogens under study (Table 4.1.3a).

Maximum inhibition of mycelial growth was observed against *P. theae* where all the isolates recorded >50.0% inhibition of mycelial biomass after 7 days of incubation (Table 4.1.3a and 4.1.3b, Fig. 4.1.5). Steady and progressive fungal growth was observed in the control flask until 5th day for *L. theobromae* and *P. theae* beyond which the growth slowed down. In case of *F. lamaoensis*, *R. solani* and *S. repens*, similar observation on progressive increase of mycelial biomass in the control flasks was observed until the 6th day and thereafter, the growth slowed down. However, in the control flasks inoculated with *U. zonata* and *P. hypobrunnea* the progressive increase in mycelial biomass was observed even after 6 days of incubation (Fig. 4.1.5).

4.1.4. Study on *in vitro* antagonism of biocontrol fungal isolates

Twenty different fungal isolates showing antagonistic potential during initial screening against *L. theobromae* were tested for *in vitro* antagonism against the eight pathogens of tea (Table 4.1.4; Fig. 4.1.6). Results showed that the most potential isolates were AD10, AD2, NBT1.2, B2.4, AD6, AD7 and KV8 which exhibited maximum inhibition of mycelial growth of all the pathogens. Three other fungal isolates, AD3, AD4 and NB4 also exhibited considerable antagonistic activity against all the pathogens under *in vitro* conditions. AD10 was the best fungal antagonist as it showed remarkable reduction of mycelial growth and recorded the highest percent inhibition of 95.5% against *F. lamaoensis* (Fig 4.1.6D). Besides, it showed excellent growth inhibition of *P. hypobrunnea* (91.1%) and *R. solani* (82.2%). The fungus overgrew over the pathogens *P. hypobrunnea* (Fig. 4.1.6C) and *F. lamaoensis*. AD7 also showed good inhibition (91.1%) against *L. theobromae* and *P. theae* (94.4%). It also restricted the growth of *P. theae* completely as evident by its vigorous growth in the entire plate (Fig. 4.1.6B). Other isolates such as AD6 (82.2% against *L. theobromae* and 78.9% against *R. solani*), AD2 (68.9% against *R. solani* and 67.8% against *L. theobromae*) and NBT1.2 (81.1% against *C. camelliae* and *F. lamaoensis*) also showed notable antagonistic activity. AD6 exhibited 82.2% inhibition of *L. theobromae* and 78.9% inhibition of *R. solani* but only 48.9% inhibition of *C. camelliae* growth. In other instances, antagonistic isolates considerably restricted the growth of the fungal pathogens like NBT1.2 restricted *R. solani* mycelial growth (Fig. 4.1.6E), AD4 restricted *L. theobromae* (Fig. 4.1.6A), M4 restricted *S. repens* (Fig. 4.1.6G) and KV8 inhibited *U. zonata* (Fig. 4.1.6H).

In other instances, antagonistic isolates considerably restricted the growth of the fungal pathogens like NBT1.2 restricted *R. solani* mycelial growth (Fig. 4.1.6E), AD4 restricted *L. theobromae* (Fig. 4.1.6A), M4 restricted *S. repens* (Fig. 4.1.6G) and KV8 inhibited *U. zonata* (Fig. 4.1.6H). A strong inhibitory activity of NB2.2 against *C. camelliae* (61.1%) was evident from the dual culture plate assay, where the bacterium grew over the advancing region of mycelial growth of *C. camelliae* (Fig. 4.1.6F). The antagonistic activity exhibited by individual isolates against the different pathogens varied widely, for instance, AD3 showed 72.2% activity against *F. lamaoensis* but only 37.8% against *C. camelliae*. M4 showed maximum inhibitory activity of 66.7% against *C. camelliae* followed by 64.4% against *S. repens* (Fig. 4.1.6G) but inhibitory activity against *R. solani* (31.1%) was low. The isolate MAT4 was least effective in inhibiting the growth of fungal pathogens with a minimum inhibition of 15.5% of *C. camelliae* and *R. solani* and maximum inhibition of 33.3% against *F. lamaoensis*. Amongst the pathogens, *L. theobromae* was found to be most susceptible towards the fungal antagonists as its growth was inhibited by AD7 (91.1%), AD6 (82.2%) and AD10 (75.5%) (Table 4.1.4). *P. hypobrunnea* and *F. lamaoensis* were the other most susceptible pathogens. The most resistant pathogen was *U. zonata* which showed a maximum inhibition of only 75.5% inflicted by the isolate AD10 (Table 4.1.4). Considering the overall antagonistic activity of the isolates, ten fungal strains were selected for further *in vitro* characterization studies.

Table 4.1.2: *In vitro* study of antagonistic activity of bacterial isolates against fungal pathogens of tea by dual culture test

ANTAGONISTS	FUNGAL PATHOGENS							
	L. theobromae	***C. camelliae***	***R. solani***	***S. repens***	***F. lamaeonsis***	***P.hypobrunnea***	***P. theae***	***U. zonata***
	% inhibition over control							
BTR4	66.7 ± 1.5	54.4 ± 1.5	60.0 ± 1.4	48.9 ± 1.6	66.7 ± 1.5	65.5 ± 1.3	62.2 ± 1.3	53.3 ± 1.3
BTR8	43.3 ± 1.4	25.5 ± 1.4	46.7 ± 1.2	53.3 ± 1.4	56.7 ± 1.2	55.5 ± 1.4	62.2 ± 0.9	37.8 ± 1.5
BTR18	45.5 ± 1.3	46.7 ± 1.2	33.3 ± 1.2	57.8 ± 1.6	55.5 ± 1.6	61.1 ± 1.4	58.9 ± 1.6	42.2 ± 1.4
BTR19	74.4 ± 1.5	75.5 ± 1.3	65.5 ± 1.5	57.8 ± 1.7	70.0 ± 1.5	67.8 ± 1.4	63.3 ± 1.3	61.1 ± 1.3
BTR21	67.8 ± 1.6	73.3 ± 1.2	62.2 ± 1.4	65.5 ± 1.5	66.7 ± 1.5	67.8 ± 1.6	67.8 ± 1.5	60.0 ± 1.2
BTR22	60.0 ± 1.3	62.2 ± 1.5	64.4 ± 1.5	78.9 ± 1.0	60.0 ± 1.6	54.4 ± 1.4	62.2 ± 1.4	55.5 ± 1.7
BTR23	64.4 ± 1.3	56.7 ± 1.6	52.2 ± 1.2	71.1 ± 1.3	61.1 ± 1.2	48.9 ± 1.1	56.7 ± 1.3	43.3 ± 1.3
BTRL6	68.9 ± 1.4	56.7 ± 1.1	47.8 ± 1.5	51.1 ± 1.6	64.4 ± 1.5	46.7 ± 1.5	52.2 ± 1.7	56.7 ± 1.7
BTRL8	68.9 ± 1.7	60.0 ± 1.2	57.8 ± 1.3	55.5 ± 1.3	54.4 ± 1.1	53.3 ± 1.2	53.3 ± 1.6	44.4 ± 1.2
BTRL9	65.5 ± 1.1	47.8 ± 1.5	61.1 ± 1.1	53.3 ± 1.4	57.8 ± 1.6	54.4 ± 1.2	56.7 ± 1.0	46.7 ± 1.5
BTRL11	68.9 ± 1.7	67.8 ± 1.7	50.0 ± 1.0	61.1 ± 1.5	62.2 ± 1.4	56.7 ± 1.4	55.5 ± 1.4	54.4 ± 1.5
D6	67.8 ± 1.4	68.9 ± 1.4	65.6 ± 1.2	62.2 ± 1.2	60.0 ± 0.9	57.8 ± 1.4	76.7 ± 1.4	45.5 ± 1.3
D7	70.0 ± 1.6	62.2 ± 1.2	58.9 ± 1.4	73.3 ± 0.9	74.4 ± 1.4	73.3 ± 1.1	63.3 ± 1.5	63.3 ± 1.6
D8	71.1 ± 0.9	67.8 ± 1.5	32.2 ± 1.2	66.7 ± 1.3	54.4 ± 1.3	53.3 ± 1.6	66.7 ± 1.5	44.4 ± 1.3
ETR1	77.8 ± 1.7	67.8 ± 1.6	65.5 ± 1.2	71.1 ± 1.1	64.4 ± 1.3	71.1 ± 1.5	63.3 ± 1.4	48.9 ± 1.1
ETR17	63.3 ± 1.4	81.1 ± 1.4	77.8 ± 1.2	80.0 ± 1.5	63.3 ± 1.7	80.0 ± 1.4	71.1 ± 1.1	81.1 ± 1.4
ETR20	72.2 ± 1.5	56.7 ± 1.1	80.0 ± 1.2	62.2 ± 1.5	61.1 ± 1.5	74.4 ± 1.6	64.4 ± 1.8	75.5 ± 1.3
ETR24	62.2 ± 1.3	63.3 ± 1.2	53.3 ± 1.4	62.2 ± 1.2	62.2 ± 1.3	66.7 ± 1.6	58.9 ± 1.4	50.0 ± 1.1
GH4	60.0 ± 1.1	62.2 ± 1.8	37.8 ± 1.6	52.2 ± 1.3	58.9 ± 1.0	55.5 ± 1.2	56.7 ± 1.4	55.5 ± 1.5
GH6	60.0 ± 1.1	62.2 ± 1.3	66.7 ± 1.8	60.0 ± 1.8	57.8 ± 1.3	58.9 ± 1.6	55.5 ± 1.6	51.1 ± 1.2
GH12	61.1 ± 1.3	66.7 ± 1.5	44.4 ± 1.6	56.7 ± 1.5	65.5 ± 1.3	60.0 ± 1.4	54.4 ± 1.4	55.5 ± 1.3
GH13	62.2 ± 1.2	73.3 ± 1.3	66.7 ± 1.8	67.8 ± 1.4	65.5 ± 1.2	64.4 ± 1.3	65.5 ± 1.8	60.0 ± 1.1
GH21	54.4 ± 1.8	66.7 ± 1.3	56.7 ± 1.3	55.6 ± 1.7	67.8 ± 1.3	50.0 ± 1.3	60.0 ± 1.0	46.7 ± 1.4
GH22	61.1 ± 1.0	67.8 ± 1.6	58.9 ± 1.7	52.2 ± 1.2	57.8 ± 1.0	58.9 ± 1.4	61.1 ± 1.1	53.3 ± 1.2
GH27	61.1 ± 1.3	58.9 ± 1.3	60.0 ± 1.3	66.7 ± 1.4	67.8 ± 1.7	65.5 ± 1.4	56.7 ± 1.2	45.5 ± 1.6
GH32	58.9 ± 1.7	64.4 ± 1.7	55.6 ± 1.4	60.0 ± 0.9	55.5 ± 1.7	52.2 ± 1.5	64.4 ± 1.3	48.9 ± 1.8

Data after '±' indicates standard error

Cont....

Table 4.1.2(Cont.): *In vitro* study of antagonistic activity of bacterial isolates against fungal pathogens of tea by dual culture test

	FUNGAL PATHOGENS							
ANTAGONISTS	***L. theobromae***	***C. camelliae***	***R. solani***	***S. repens***	***F. lamaeonsis***	***P.hypobrunnea***	***P. theae***	***U. zonata***
	% inhibition over control							
KTR6	68.9 ± 1.6	76.7 ± 1.3	83.3 ± 1.6	77.8 ± 1.3	84.4 ± 1.1	83.3 ± 1.6	82.2 ± 1.2	68.9 ± 1.2
KTR18	21.1 ± 1.6	12.0 ± 1.3	32.2 ± 1.2	24.4 ± 1.3	33.3 ± 1.1	32.2 ± 1.3	50.0 ± 1.4	23.3 ± 1.4
TLB3	66.7 ± 1.2	62.0 ± 1.3	64.0 ± 1.1	64.4 ± 1.1	58.9 ± 1.7	62.2 ± 1.5	65.5 ± 1.5	61.1 ± 1.5
TBD7	67.8 ± 1.6	71.5 ± 1.3	64.0 ± 1.5	70.0 ± 1.1	67.8 ± 1.6	65.5 ± 1.2	62.2 ± 1.1	60.0 ± 1.1
TMG1	72.2 ± 1.4	66.7 ± 1.4	64.4 ± 1.2	68.9 ± 1.5	62.2 ± 1.5	60.0 ± 1.2	67.8 ± 1.5	57.8 ± 1.7
TMG2	56.7 ± 1.5	55.5 ± 1.3	46.7 ± 1.4	44.4 ± 1.3	34.4 ± 1.5	31.1 ± 1.6	48.9 ± 1.5	11.1 ± 1.2
TMG3	56.7 ± 1.7	37.8 ± 1.7	33.3 ± 1.2	45.6 ± 1.2	44.4 ± 1.6	42.2 ± 1.2	45.5 ± 1.6	33.3 ± 1.2
TMG7	56.0 ± 1.2	43.3 ± 1.8	44.4 ± 1.3	37.8 ± 1.0	34.4 ± 1.5	35.5 ± 1.4	44.4 ± 1.3	20.0 ± 1.1
TRB1	72.2 ± 1.2	67.8 ± 1.7	74.4 ± 1.3	64.4 ± 1.2	65.5 ± 1.3	61.1 ± 1.4	71.1 ± 1.7	60.0 ± 1.0
TRB2	58.9 ± 1.7	47.8 ± 1.7	44.4 ± 1.1	32.2 ± 1.1	31.1 ± 1.2	31.1 ± 1.2	46.7 ± 1.3	35.5 ± 1.4
TRB4	61.1 ± 1.5	54.4 ± 1.7	40.0 ± 1.3	31.1 ± 1.2	32.2 ± 1.8	24.4 ± 1.6	38.9 ± 1.5	31.1 ± 1.2
TRB7	61.1 ± 1.6	52.2 ± 1.3	33.3 ± 1.2	34.4 ± 1.3	33.3 ± 1.1	36.7 ± 1.1	33.3 ± 1.2	30.0 ± 1.1
TRB12	58.9 ± 1.6	56.6 ± 1.6	38.9 ± 1.6	33.3 ± 1.6	31.1 ± 1.1	33.3 ± 1.3	27.8 ± 1.6	20.0 ± 1.3
TRB14	74.4 ± 1.5	70.0 ± 1.4	61.1 ± 1.2	65.5 ± 1.1	66.7 ± 1.3	67.8 ± 1.2	72.2 ± 1.5	62.2 ± 1.3
TRB18	67.8 ± 1.3	70.0 ± 1.1	60.0 ± 1.6	64.4 ± 1.4	57.8 ± 1.7	66.7 ± 1.5	67.8 ± 1.6	61.1 ± 1.2
TR1	70.0 ± 1.3	75.5 ± 1.3	67.8 ± 1.5	67.8 ± 1.6	63.3 ± 1.1	58.9 ± 1.2	66.7 ± 1.8	66.7 ± 1.6
TR5	71.6 ± 1.5	76.7 ± 1.4	66.7 ± 1.2	63.3 ± 1.1	65.5 ± 1.4	64.4 ± 1.4	65.5 ± 1.4	64.4 ± 1.2
TR11	58.9 ± 1.4	48.9 ± 1.2	31.1 ± 1.0	30.0 ± 1.0	34.4 ± 1.5	28.9 ± 1.2	32.2 ± 1.3	24.4 ± 1.6
TR19	58.9 ± 1.4	56.7 ± 1.7	24.4 ± 1.6	35.5 ± 1.2	37.8 ± 1.4	30.0 ± 1.3	34.4 ± 1.6	22.2 ± 1.3
TR20	60.0 ± 1.4	58.9 ± 1.7	34.4 ± 1.5	36.7 ± 1.6	35.5 ± 1.5	34.4 ± 1.4	20.0 ± 1.4	13.3 ± 1.2
TGY1	64.0 ± 0.9	57.8 ± 1.7	66.7 ± 1.5	30.0 ± 1.0	34.4 ± 1.2	33.3 ± 1.2	24.4 ± 1.4	26.7 ± 1.6
TGY2	62.0 ± 1.4	61.1 ± 1.6	25.6 ± 1.4	27.8 ± 1.5	28.8 ± 1.7	26.7 ± 1.5	22.2 ± 1.2	13.3 ± 1.6
TGY4	60.0 ± 1.0	64.4 ± 1.4	33.3 ± 1.4	26.7 ± 1.4	30.0 ± 1.3	25.5 ± 1.2	28.9 ± 1.5	11.1 ± 1.5
TGY6	66.7 ± 1.4	67.8 ± 1.3	32.2 ± 1.3	34.4 ± 1.0	36.7 ± 1.3	31.1 ± 1.2	23.3 ± 1.2	18.9 ± 1.6
TGY7	50.0 ± 1.3	56.7 ± 1.4	30.0 ± 1.1	11.1 ± 0.9	21.1 ± 1.5	16.7 ± 1.5	26.7 ± 1.1	17.8 ± 1.5

Data after '±' indicates standard error

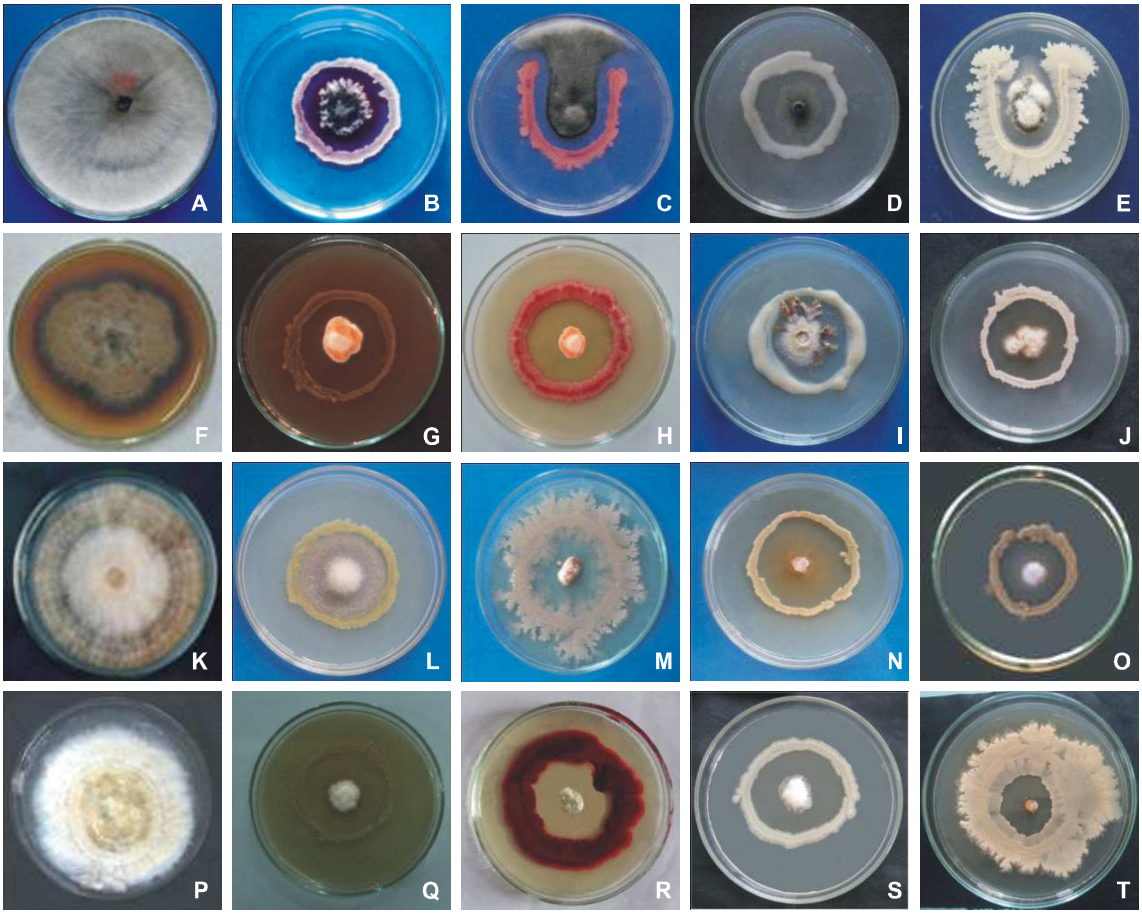


Fig. 4.1.3: *In vitro* inhibition of fungal pathogens by antagonistic bacterial isolates in dual culture plates in PDA:
(A) Culture of *Lasiodiplodia theobromae* (Control); Inhibitory effect of D7 (B), ETR17 (C), ETR20 (D) and KTR6 (E) on *L. theobromae*;
(F) Culture of *Sphaerostilbe repens* (Control); Inhibitory effect of D7 (G), ETR17 (H), ETR20 (I) and KTR6 (J) on *S. repens*;
(K) Culture of *Rhizoctonia solani* (Control); Inhibitory effect of D7 (L), BTR21 (M), ETR20 (N) and KTR6 (O) on *R. solani*;
(P) Culture of *Poria hypobrunnea* (Control); Inhibitory effect of D7 (Q), ETR17 (R), ETR20 (S) and KTR6 (T) on *P. hypobrunnea*.

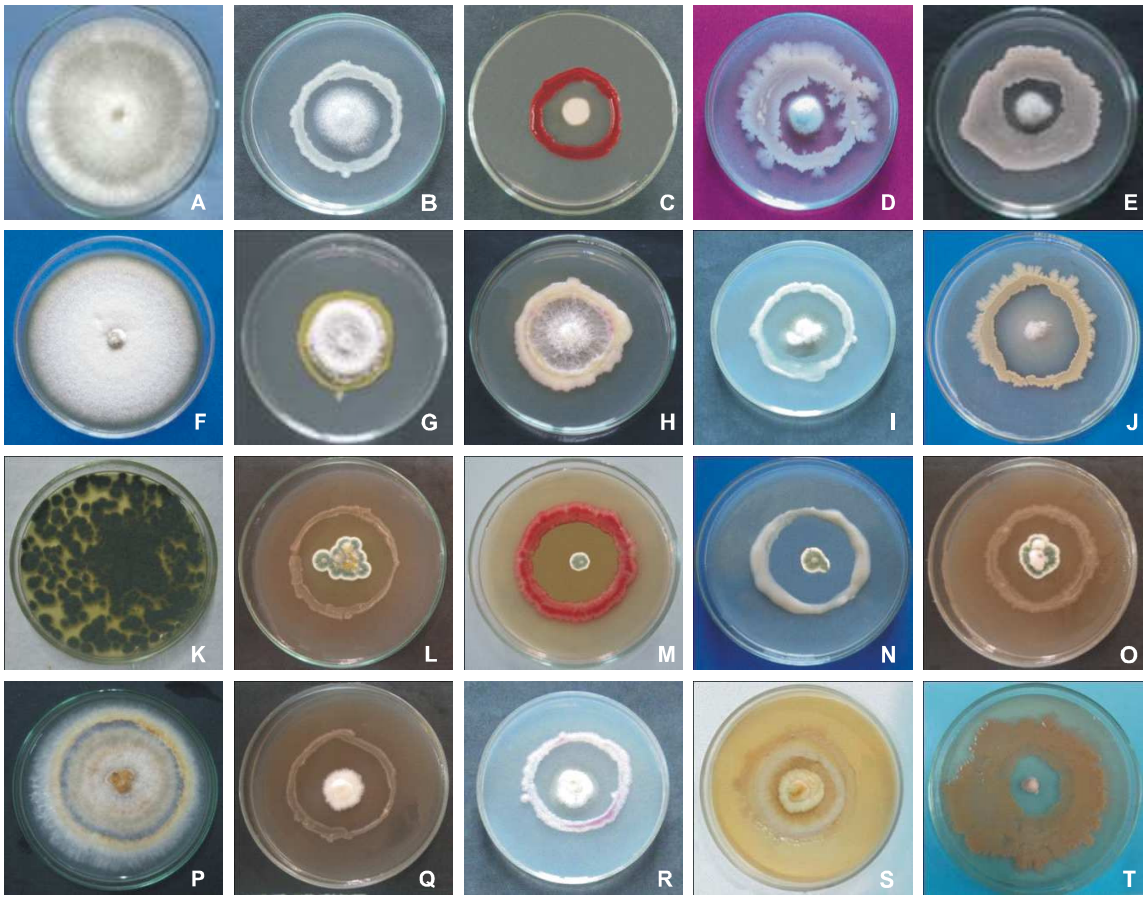


Fig. 4.1.4: *In vitro* inhibition of fungal pathogens by antagonistic bacterial isolates in dual culture plates in PDA:
(A) Culture of *Colletotrichum camelliae* (Control); Inhibitory effect of D7 (B), ETR17 (C), BTR19 (D) and KTR6 (E) on *C. camelliae*;
(F) Culture of *Pestalotiopsis theae* (Control); Inhibitory effect of D7 (G), ETR17 (H), ETR20 (I) and KTR6 (J) on *P. theae*;
(K) Culture of *Ustulina zonata* (Control); Inhibitory effect of D7 (L), BTR21 (M), ETR20 (N) and KTR6 (O) on *U. zonata*;
(P) Culture of *Fomes lamaoensis* (Control); Inhibitory effect of D7 (Q), ETR17 (R), ETR20 (S) and KTR6 (T) on *F. lamaoensis*.

Table 4.1.3a: Reduction in biomass of pathogenic fungi by antagonistic bacterial isolates after 7 days of inoculation in dual culture assay in potato dextrose broth

Fungal Pathogens	% reduction in fungal biomass						
	BTR19	**BTR21**	**BTR22**	**D7**	**ETR17**	**ETR20**	**KTR6**
L. theobromae	47.3±0.9	45.4±1.2	44.3±1.7	57.0±1.3	77.1±0.9	60.8±1.2	76.8±1.1
C. camelliae	55.2±1.3	48.6±1.0	48.8±1.2	61.2±1.0	76.7±1.2	64.8±1.2	75.9±1.1
R. solani	53.9±1.1	52.6±1.0	51.5±1.3	58.8±1.0	75.7±1.2	59.6±1.3	68.2±1.1
P. theae	61.6±1.2	58.7±1.3	59.2±1.1	66.2±1.1	77.0±1.0	63.8±1.0	79.6±1.4
F. lamaoensis	57.8±1.0	59.4±1.2	56.2±1.2	66.1±1.2	68.4±1.0	66.4±0.9	70.6±1.3
U. zonata	50.9±1.2	47.7±1.0	49.1±1.1	58.8±1.4	66.4±1.5	57.6±1.4	65.5±1.1
S. repens	48.5±1.1	44.4±1.1	45.7±1.1	55.0±1.1	75.3±1.3	62.4±1.2	70.4±1.3
P. hypobrunnea	48.8±1.4	46.8±1.1	47.6±1.4	63.2±1.2	68.3±1.1	61.0±1.0	64.6±1.1

Data after '±' indicates standard error

Table 4.1.3b: Reduction in biomass of pathogenic fungi by antagonistic bacterial isolates after 7 days of inoculation in dual culture assay in potato dextrose broth

Fungal Pathogens	% reduction in fungal biomass						
	GH13	**TLB3**	**TBD7**	**TMG1**	**TR1**	**TR5**	**TRB14**
L. theobromae	43.7±1.6	43.9±0.9	51.7±1.0	44.7±1.5	46.4±1.2	45.2±1.4	50.2±0.9
C. camelliae	46.8±1.0	47.5±1.1	49.1±1.3	47.5±1.2	49.6±0.9	42.4±1.1	44.7±1.1
R. solani	49.0±1.0	46.4±1.1	55.0±0.9	53.6±1.1	45.0±1.0	46.4±1.0	47.8±0.9
P. theae	59.4±0.9	56.8±1.2	60.3±1.2	59.6±1.1	55.0±1.1	57.0±1.1	57.0±1.2
F. lamaoensis	50.8±1.2	45.4±1.2	44.0±1.3	53.9±1.4	45.1±1.1	49.2±1.4	47.2±1.3
U. zonata	46.7±1.3	46.1±1.3	46.5±1.1	46.3±0.9	49.3±1.3	46.8±1.2	50.7±1.3
S. repens	44.4±0.9	46.5±1.1	46.5±1.0	55.3±1.2	55.3±1.0	49.9±1.1	43.7±1.4
P. hypobrunnea	44.9±1.2	47.6±1.3	47.3±1.0	49.3±1.3	47.6±1.4	48.8±1.3	50.0±1.4

Data after '±' indicates standard error

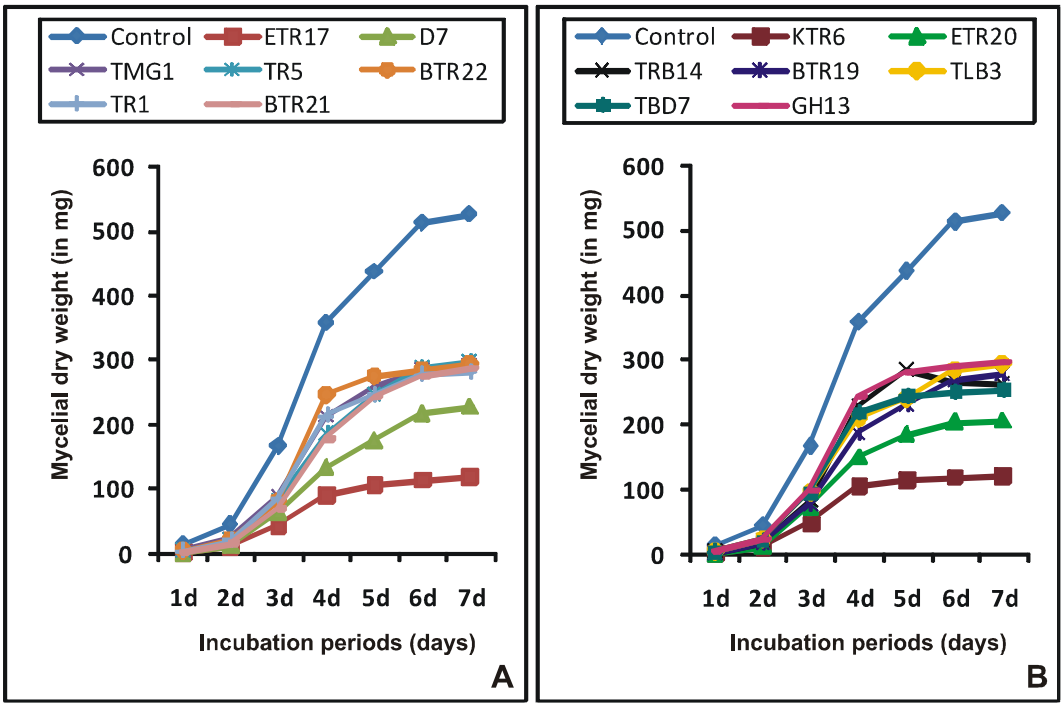


Fig. 4.1.5a: *In vitro* antagonism exhibited by antagonistic bacterial isolates in liquid culture against *L. theobromae*.

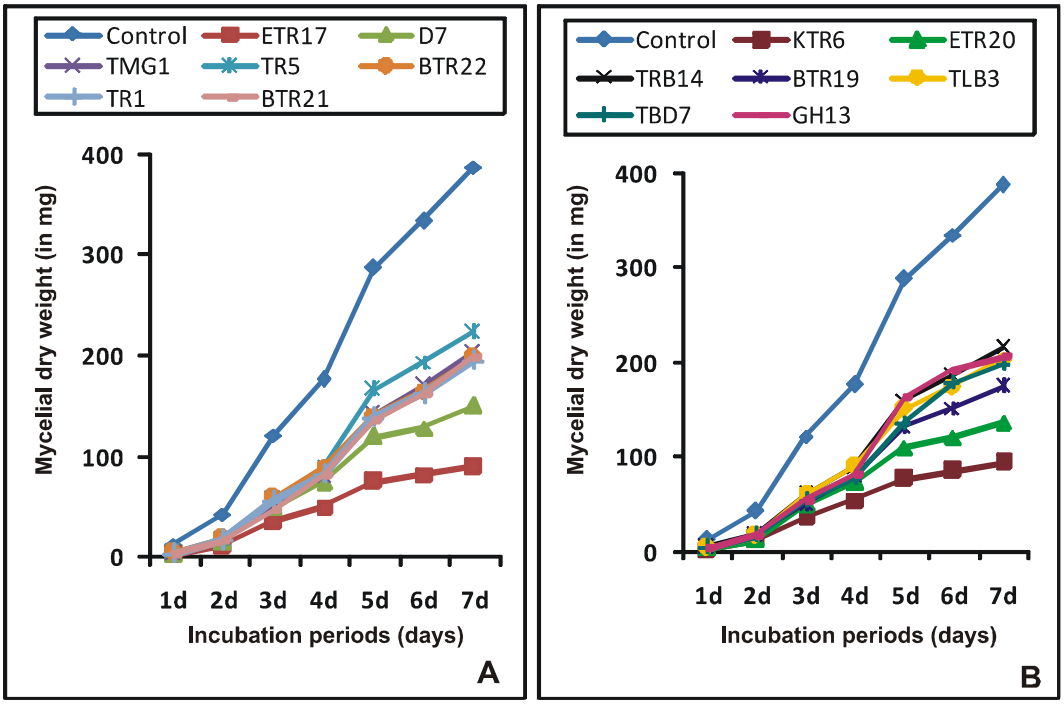


Fig. 4.1.5b: *In vitro* antagonism exhibited by antagonistic bacterial isolates in liquid culture against *C. camelliae*.

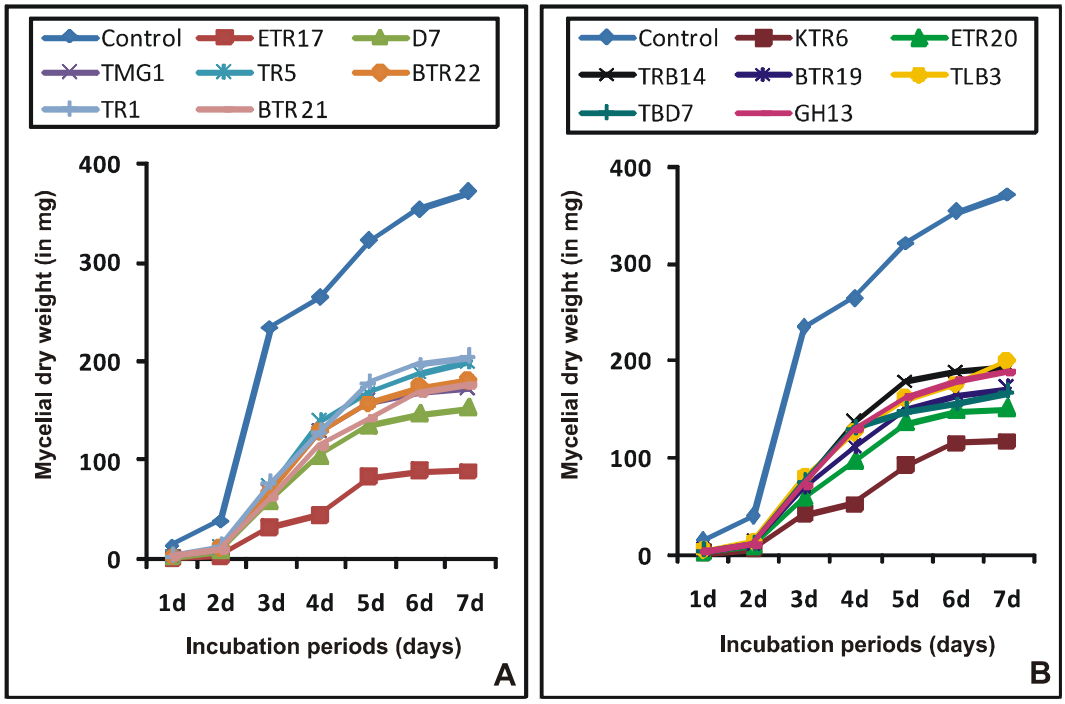


Fig. 4.1.5c: *In vitro* antagonism exhibited by antagonistic bacterial isolates in liquid culture against *R. solani.*

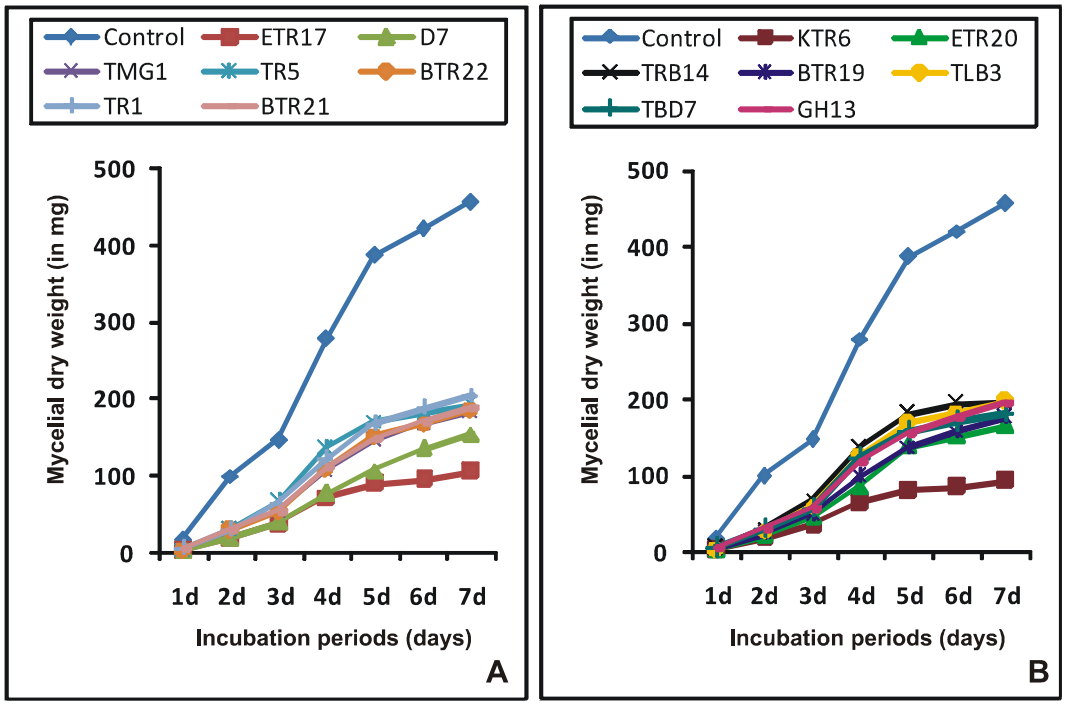


Fig. 4.1.5d: *In vitro* antagonism exhibited by antagonistic bacterial isolates in liquid culture against *P. theae.*

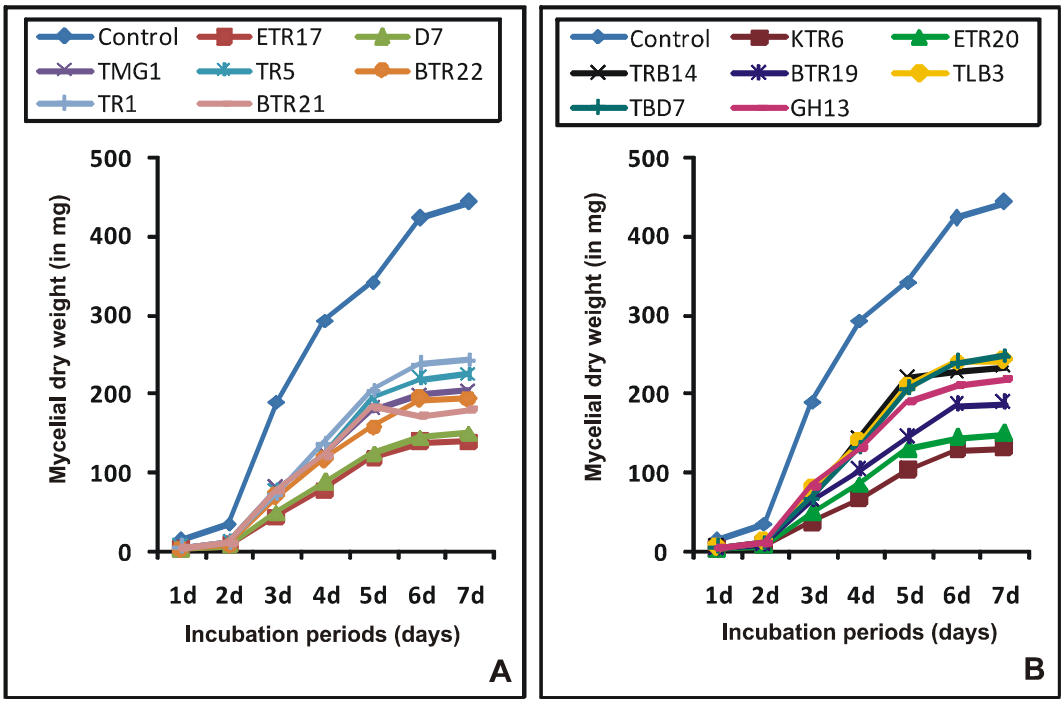


Fig. 4.1.5e: *In vitro* antagonism exhibited by antagonistic bacterial isolates in liquid culture against *F. lamaoensis*.

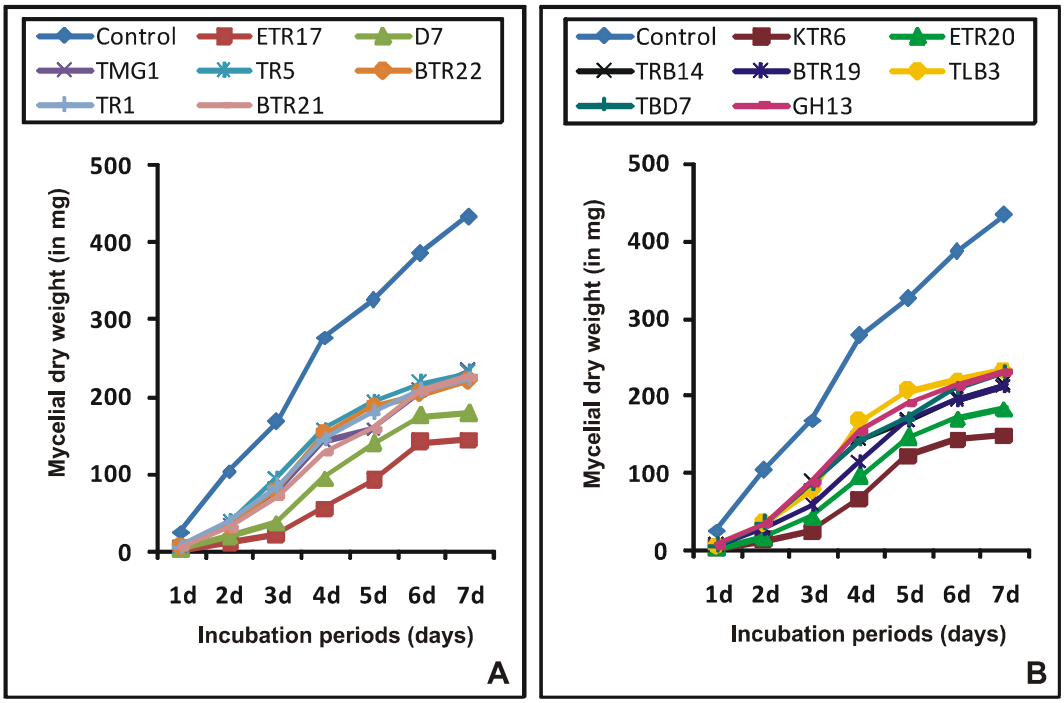


Fig. 4.1.5f: *In vitro* antagonism exhibited by antagonistic bacterial isolates in liquid culture against *U. zonata*.

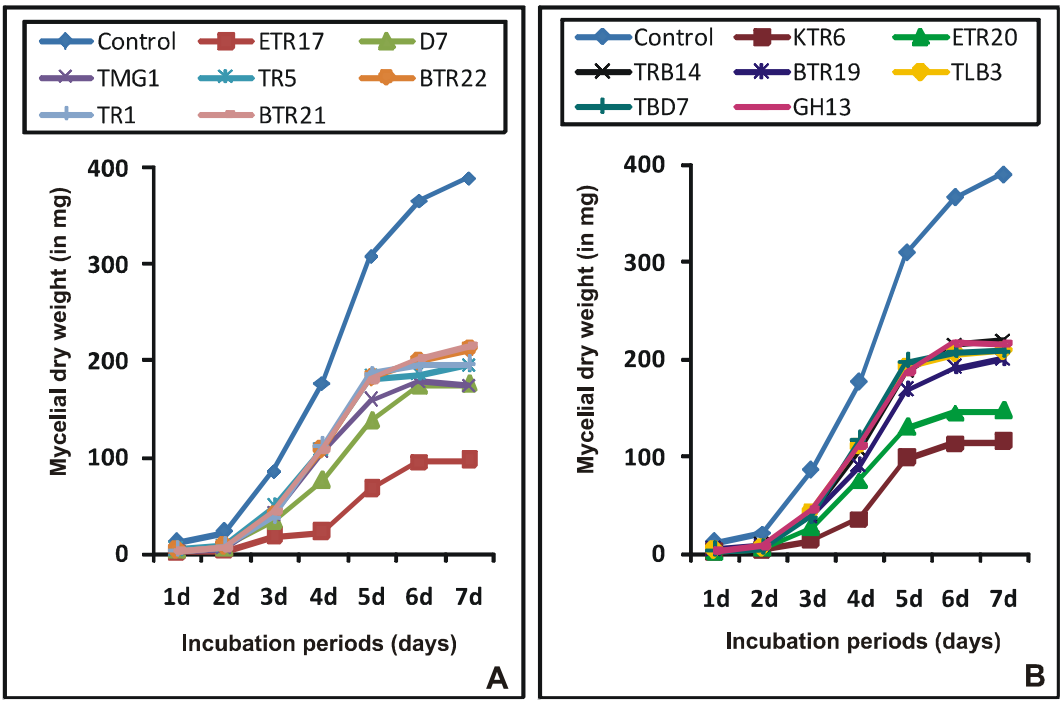


Fig. 4.1.5g: *In vitro* antagonism exhibited by antagonistic bacterial isolates in liquid culture against *S. repens*.

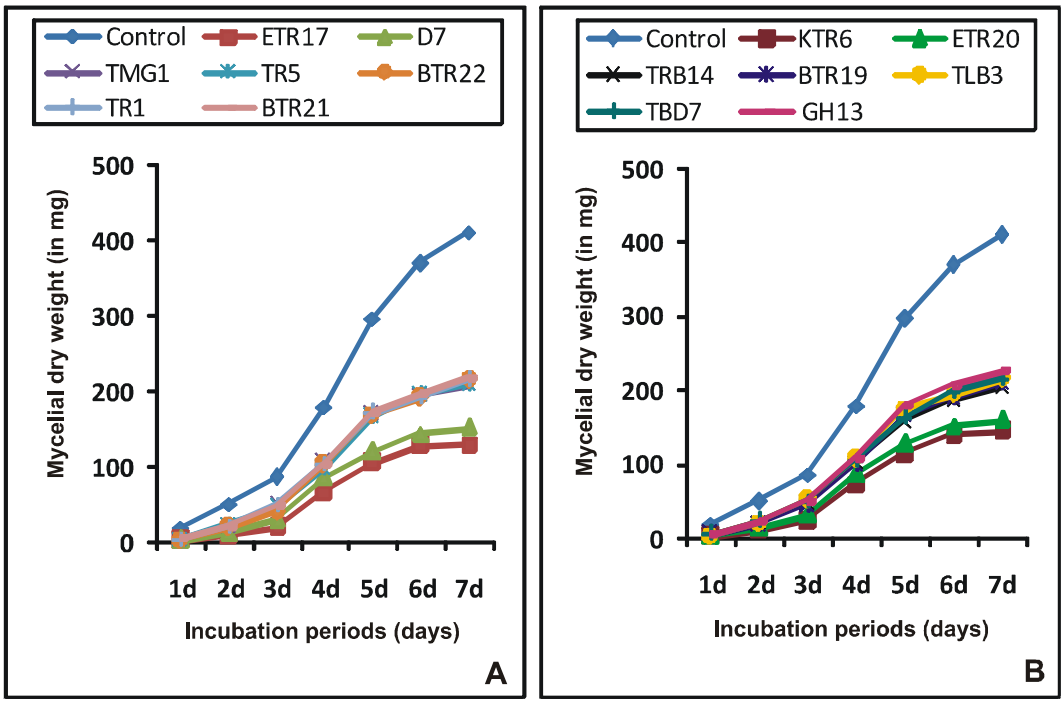


Fig. 4.1.5h: *In vitro* antagonism exhibited by antagonistic bacterial isolates in liquid culture against *P. hypobrunnea*.

Table 4.1.4: *In vitro* study of antagonistic activity of fungal isolates against fungal pathogens of tea by dual culture test

ANTAGONISTS	FUNGAL PATHOGENS							
	L. theobromae	*C. camelliae*	*R. solani*	*S. repens*	*F. lamaeonsis*	*P.hypobrunnea*	*P. theae*	*U. zonata*
	% inhibition over control							
AD2	67.8 ± 0.9	65.5 ± 0.8	68.9 ± 1.1	61.1 ± 1.2	65.5 ± 1.0	65.5 ± 1.7	60.0 ± 1.5	56.7 ± 1.3
AD3	67.8 ± 1.0	37.8 ± 0.6	66.7 ± 1.5	55.5 ± 0.9	72.2 ± 1.4	64.4 ± 1.4	57.8 ± 1.6	53.3 ± 1.3
AD4	63.3 ± 0.9	64.4 ± 0.6	62.2 ± 1.3	51.1 ± 1.3	58.9 ± 1.7	56.7 ± 1.2	54.4 ± 1.6	48.9 ± 1.7
AD6	82.2 ± 1.0	48.9 ± 1.0	78.9 ± 1.5	64.4 ± 1.6	64.4 ± 1.6	68.9 ± 1.7	65.5 ± 1.4	57.8 ± 1.5
AD7	91.1 ± 1.3	57.8 ± 0.9	70.0 ± 1.4	61.1 ± 1.5	63.3 ± 1.4	71.1 ± 1.3	94.4 ± 1.4	61.1 ± 1.1
AD10	67.8 ± 0.7	73.3 ± 0.6	82.2 ± 1.3	72.2 ± 1.7	95.5 ± 1.2	91.1 ± 1.6	70.0 ± 1.1	75.5 ± 1.5
B2.4	75.5 ± 1.0	70.0 ± 1.3	74.4 ± 1.1	62.2 ± 1.1	67.8 ± 1.6	62.2 ± 1.0	64.4 ± 1.1	55.5 ± 1.3
KV8	67.8 ± 1.3	64.4 ± 1.2	61.1 ± 1.0	61.1 ± 1.0	68.9 ± 1.5	65.5 ± 1.2	66.7 ± 1.2	56.7 ± 1.0
M1	43.3 ± 1.3	36.7 ± 0.8	43.3 ± 1.3	48.9 ± 1.6	45.4 ± 1.2	51.1 ± 1.3	43.3 ± 1.3	37.8 ± 1.4
M2	38.9 ± 1.5	33.3 ± 1.4	34.4 ± 1.2	42.2 ± 1.5	51.1 ± 1.6	37.8 ± 1.6	46.7 ± 1.7	25.5 ± 1.3
M4	61.1 ± 1.4	66.7 ± 1.0	31.1 ± 1.0	64.4 ± 1.5	37.8 ± 1.6	43.3 ± 1.4	47.8 ± 1.8	33.3 ± 1.2
MAT2	40.0 ± 1.1	33.3 ± 1.2	45.5 ± 0.8	25.5 ± 1.5	34.4 ± 1.5	33.3 ± 1.7	51.1 ± 1.6	28.9 ± 1.7
MAT3	34.4 ± 1.4	31.1 ± 1.3	32.2 ± 1.2	34.4 ± 1.6	24.4 ± 1.2	21.1 ± 1.4	35.5 ± 1.5	15.5 ± 1.6
MAT 4	21.1 ± 1.7	15.5 ± 1.3	15.5 ± 1.3	24.4 ± 1.2	33.3 ± 1.4	28.9 ± 1.7	21.1 ± 1.6	18.9 ± 1.4
MAT 5	41.1 ± 1.5	40.0 ± 1.1	44.4 ± 1.8	21.1 ± 1.4	21.1 ± 1.6	25.5 ± 1.4	36.7 ± 1.4	24.4 ± 1.2
MAT6	41.1 ± 1.7	35.5 ± 1.0	44.4 ± 1.7	23.3 ± 1.2	28.9 ± 1.7	35.5 ± 1.7	44.4 ± 1.4	34.4 ± 1.2
MAT 7	43.3 ± 0.9	42.2 ± 1.3	34.4 ± 1.6	33.3 ± 1.1	32.2 ± 1.3	32.2 ± 1.6	37.8 ± 1.6	21.1 ± 1.6
NB2.2	45.5 ± 0.8	61.1 ± 1.0	60.0 ± 1.4	55.5 ± 1.3	58.9 ± 1.5	56.7 ± 1.5	57.8 ± 1.6	43.3 ± 1.7
NB4	55.5 ± 1.3	57.8 ± 0.9	51.1 ± 1.5	51.1 ± 1.5	54.4 ± 1.8	55.5 ± 1.7	55.5 ± 1.3	45.5 ± 1.2
NBT1.2	58.9 ± 1.2	81.1 ± 1.5	68.9 ± 1.5	66.7 ± 1.0	81.1 ± 1.8	78.9 ± 1.2	68.9 ± 1.6	68.9 ± 1.8

Data after '±' indicates standard error

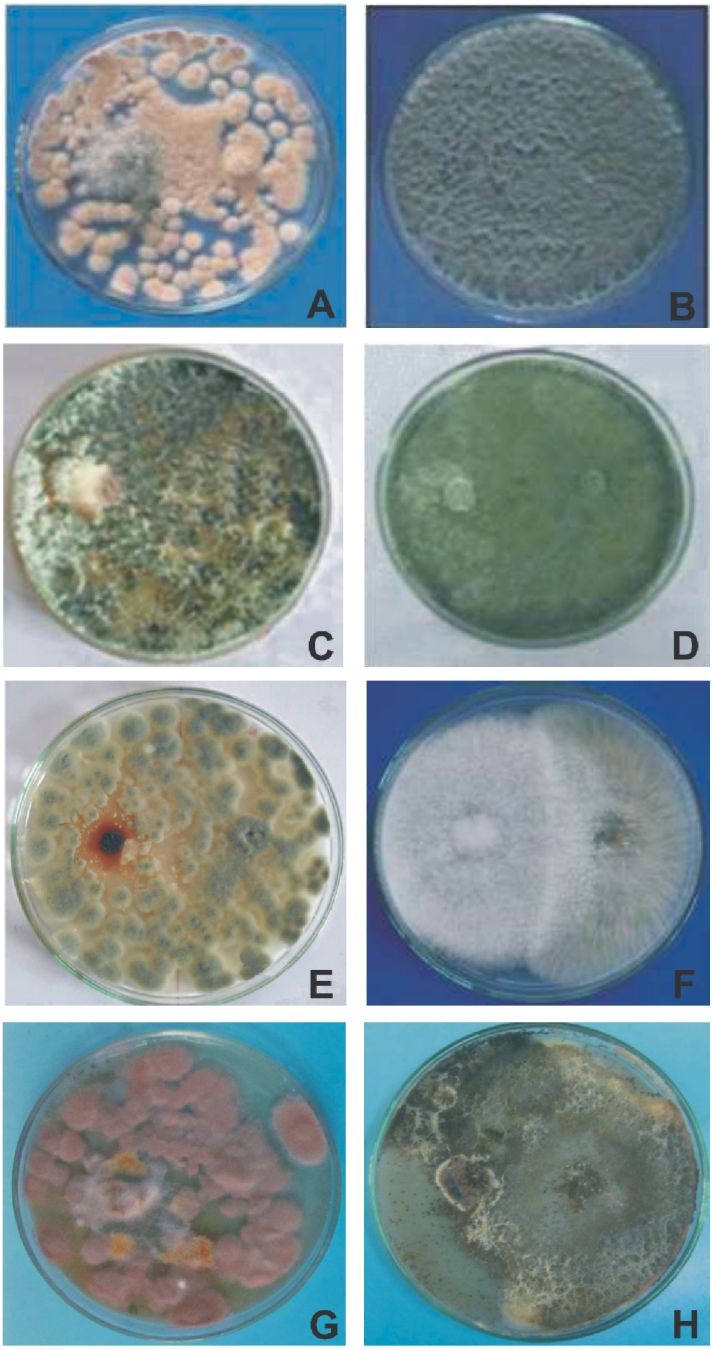


Fig. 4.1.6: *In vitro* inhibition of fungal pathogens by antagonistic fungal isolates in dual culture plates in PDA (the pathogens were inoculated to the left side whereas the fungal antagonists were inoculated to the right side of the PDA plates):
Inhibitory effect of (A) AD4 on *Lasiodiplodia theobromae*, (B) AD7 on *Pestalotiopsis theae*, (C) AD10 on *Poria hypobrunnea*, (D) AD10 on *Fomes lamaoensis*, (E) NBT1.2 on *Rhizoctonia solani*, (F) NB2.2 on *Colletotrichum camelliae*, (G) M4 on *Sphaerostilbe repens*, (H) KV8 on *Ustulina zonata*.

CHARACTERIZATION AND IDENTIFICATION OF POTENTIAL ANTAGONISTS

All bacterial isolates that were found to be antagonistic to the eight fungal pathogens of tea were subject to characterization in a polyphasic approach which included morphological, biochemical and phylogenetic studies.

4.2.1. Morphological characterization

Morphological characterization of thirty five potent biocontrol bacteria revealed different variations in size, shape, color, growth pattern, motility and edge morphology (Table 4.2.1a and 4.2.1b). All isolates except GH12, GH27, GH32 and TR5 were found to be motile. Robust swarming on the surface of agar plates was exhibited by KTR6, ETR17, BTR21, TBD7 and TRB18. Other isolates such as ETR1, ETR17, TMG1, TR1, GH21, BTR22 and BTR23 also showed moderate swarming. Different types of cell shapes were noted amongst the isolates; isolates ETR20 and ETR24 were long rods; ETR17, ETR1 and BTR8 were short rods; GH12 and GH27 were coccobacilli; GH4, GH13, GH32, BTRL8, BTRL11, TRB14 and TRB1 were cocci and the remaining twenty one isolates were standard rod shaped. Endospore formation was noticed in KTR6, BTR21, BTR19, BTR22, BTR23, BTR4, BTRL9, GH21, GH22, TRB18, TLB3, TBD7 (Table 4.2.1a and 4.2.1b). Three isolates (ETR17, ETR1 and TR1) produced red pigment; two isolates, ETR20 and ETR24 produced yellow colored pigment and isolate D8 exhibited orange colored colony on NA medium. Isolate D6 produced yellowish green fluorescent pigment (Fig. 4.2.1a) in *Pseudomonas* agar (for Fluorescein) (Himedia Laboratories, India) plate.

4.2.2. Biochemical characterization

Biochemical characterization studies were carried out with selected bacterial isolates having biocontrol potential against a wide spectrum of fungal pathogens of tea. The results of biochemical tests are tabulated in Table 4.2.2a, 4.2.2b, 4.2.2c, 4.2.2d and 4.2.2e. Among thirty five isolates, sixteen were Gram negative and nineteen were Gram positive. All the isolates except ETR20 and ETR24 were indole negative. Nineteen isolates were positive for VP test whereas only six strains (ETR20, ETR24, BTR18, BTRL8, GH21 and GH22) were positive for MR of which BTR18, GH21 and GH22 were positive for both. Oxidase positive trait was exhibited by fifteen isolates while twenty nine isolates scored positive for catalase test. Twenty strains were fermentative among

which eight (ETR20, ETR24, BTR22, BTR23, BTR4, BTR18, BTRL8, D7) were gas producing. The rest fifteen isolates (KTR6, BTR8, BTR19, BTRL9, GH4, GH6, GH12, GH13, GH27, D6, D8, TRB1, TRB14 and TRB18) were found to be oxidative in nature (Fig. 4.2.1a). All strains except BTRL11, GH12, GH13, GH21 and D8 were capable of utilizing citrate. Only thirteen strains scored positive for gelatin hydrolysis. The isolates mostly reduced nitrate to nitrite although four isolates (BTR23, GH13, TRB1 and TRB14) could further reduce nitrite also. Four strains (TLB3, BTRL8, ETR20 and ETR24) were positive for H_2S production. All the isolates were negative for phenylalanine deaminase and very few were tested positive for 3-ketolactose. Twenty bacterial isolates showed DNase activity while ten exhibited urease activity (Fig. 4.2.1a). Twenty two were positive for â-galactosidase production indicated by an ONPG positive trait. Only the gram negative isolates showed positive growth on MacConkey agar while the gram positive strains failed to grow. The strains GH32, D7, ETR20, ETR24, TMG1 and BTRL11 produced pink colored colonies on MacConkey agar while ETR1, ETR17, TR1 and BTR8 produced colorless colonies and the rest eight gram negative strains produced white colored colonies. Twenty seven isolates scored positive for ornithine decarboxylation, twenty four strains were positive for arginine and twenty five were positive for lysine decarboxylation. The isolates varied in their ability to produce acid from different carbohydrates with an exception to glucose, which was utilized by all the strains. Bergey's Manual of Systematic Bacteriology was studied to determine the identity of the bacterial antagonists at least upto genus level.

4.2.3. Identification of the antagonistic isolates

The results of phylogenetic analyses were consistent with the biochemical and morphological traits of the selected bacterial isolates as reported in Bergey's Manual of Systematic Bacteriology [Palleroni (1984); Claus et al. (1986); Evans (1986); Kocur (1986); Farmer (2005); Frederiksen (2005); Grimont and Grimont (2005b); Grimont and Grimont (2005a); Juni (2005); Olsen and Moller (2005); Priest Fergus (2005); Svec and Devriese (2009)]. The identity of the isolates for which phylogenetic analyses were not executed was confirmed based on the findings from biochemical and morphological characterization studies. The identity of thirty five biocontrol bacteria has been enlisted in Table 4.2.3. It was found that three belonged to the genus *Pseudomonas*; strain D6 was identified as *P. putida*, D8 as *P. vesicularis*. BTR8 was designated as *Pseudomonas* sp. since the species could not be ascertained.

Table 4.2.1a: Morphological and culture characteristics of antagonistic bacterial isolates

Bacterial Strains	Characteristics									
	Shape	Size (length x breadth) (µm)	Motility	Endospore formation[a]	Colony characteristics					
					Growth	Color	Surface	Elevation	Pigment[b]	In broth
BTR4	Rod	2.1 x 0.8	Motile	+	Fast	White	Smooth, opaque	Raised	-	Turbid
BTR8	Small rod	1.2 x 0.7	Motile	-	Moderate	Cream	Smooth shiny, translucent	Flat	Faint Yellow	Turbid and pellicle formed
BTR18	Rod	1.8 x 0.6	Motile	-	Moderate	Cream	Smooth, shiny, entire margin	Convex	-	Turbid
BTR19	Rod	1.2 x 0.6	Motile	+	Moderate	Cream	Dull, opaque, margin undulated	Flat	-	Turbid and pellicle formed
BTR21	Rod	2.2 x 0.9	Motile	+	Fast growing	White	Rough, opaque	Flat	-	Turbid with pellicle
BTR22	Rod	1.7 x 0.8	Motile	+	Fast growing	White	Smooth, wrinkled	Raised	-	Pellicle formed, turbid
BTR23	Rod	1.8 x 0.7	Motile	+	Fast growing	White	Smooth, wrinkled	Raised	-	Pellicle formed, turbid
BTRL6	Rod	1.6 x 0.7	Motile	-	Moderate	White	Smooth, shiny	Convex	-	Turbid with pellicle
BTRL8	Coccus	0.6 diameter	Motile	-	Moderate	Off-white	Smooth, translucent, shiny	Flat	-	Turbid
BTRL9	Rod	2.3 x 0.9	Motile	+	Fast	White	Dull surface, opaque, undulated margin	Raised	-	Turbid, Pellicle formed
BTRL11	Coccus	0.5 diameter	Non-motile	-	Moderate	White	Smooth, entire margin, translucent, shiny	Flat	-	Turbid
D6	Rod	1.8 x 0.8	Motile	-	Moderate	Off white	Smooth shiny	Flat	Yellowish green	Turbid and pellicle formed

[a]: '+' indicates endospore formation, '-' indicates no visible spores; b: '-' indicates absence of pigments

Table 4.2.1b: Morphological and culture characteristics of antagonistic bacterial isolates

Bacterial Strains	Characteristics									
	Shape	Size (length x breadth) (µm)	Motility	Endospore formation[a]	Colony characteristics					
					Growth	Color	Surface	Elevation	Pigment[b]	In broth
D7	Rod	2.0 x 0.9	Motile	-	Moderate	Cream	Smooth	Flat	No	Turbid
D8	Rod	1.8 x 0.8	Motile	-	Slow	Orange	Smooth, circular, glistening, margin entire	Convex	No	Pellicle formed, adheres firmly to wall of culture tube
ETR1	Short rod	1.1 x 0.6	Motile	-	Fast	Red	Opaque, shiny, margin entire	Convex	Red	Reddish turbid
ETR17	Short rod	1.0 x 0.6	Motile	-	Fast	Red	Opaque, Shiny, margin entire	Convex	Red	Reddish turbid
ETR20	Long rod	3.1 x 1.1	Motile	-	Moderate	Pale Yellow	Smooth, Opaque, shiny, entire margin, fetid odour	Convex	Pale Yellow	Turbid, yellowish tint, opaque
ETR24	Long rod	3.0 x 1.0	Motile	-	Moderate	Pale Yellow	Smooth, Opaque, shiny, entire margin, fetid odour	Convex	Pale Yellow	Turbid, opaque with yellowish tint
GH4	Coccus	1.2 diameter	Non-motile	-	Moderate	Cream	Smooth, translucent, margin entire	Slightly raised	-	Turbid
GH6	Coccobacilli	1.0 x 1.5	Non-motile	-	Moderate	Off white	Smooth, translucent, circular, entire margin	Raised	-	Pellicle formed
GH12	Coccobacilli	1.2 x 1.6	Non-motile	-	Moderate	Off white	Smooth, translucent, entire margin, circular	Raised	No	Pellicle formed
GH13	Coccus	1.3 diameter	Non-motile	-	Fast	Cream	Opaque, dull, entire margin, smooth	Low convex	No	Turbid with pellicle
GH21	Rod	2.9 x 0.9	Motile	+	Fast growing	Off white	Dull, opaque	Flat	No	Turbid with pellicle

[a]: '+' indicates endospore formation, '-' indicates no visible spores; b: '-' indicates absence of pigments

Table 4.2.1c: Morphological and culture characteristics of antagonistic bacterial isolates

Bacterial Strains	Characteristics									
	Shape	Size(length x breadth) (µm)	Motility	Endospore formation[a]	Colony characteristics					
					Growth	Color	Surface	Elevation	Pigment[b]	In broth
GH22	Rod	2.7 x 0.8	Motile	+	Fast growing	Off white	Rough, opaque	Flat	No	Turbid with pellicle
GH27	Coccobacilli	1.1 x 1.5	Non-motile	-	Moderate	Off white	Smooth, opaque, circular, entire margin	Raised	No	Pellicle formed
GH32	Coccus	1.1 diameter	Non-motile	-	Moderate	Off white	Smooth, punctiform, entire margin	Convex	No	Turbid and pellicle formed
KTR6	Rod	2.0 x 0.7	Motile	+	Fast	White	Rough, opaque, margin undulate	Flat	-	Pellicle
TLB3	Rod	2.0 x 0.7	Motile	+	Fast growing	White	Rough, opaque	Flat	No	Turbid with pellicle
TBD7	Rod	2.1 x 0.9	Motile	+	Fast growing	Off white	Rough, opaque, irregular margin	Flat	No	Turbid with pellicle
TMG1	Rod	2.1 x 1.0	Motile	-	Moderate	White	Dull, not circular	Flat	No	Turbid
TRB1	Coccus	1.3 diameter	Motile	-	Moderate	Cream	Smooth, margin entire, not circular	Slightly raised	Yellowish	Turbid
TRB14	Coccus	1.2 diameter	Motile	-	Moderate	Cream	Smooth, margin entire	Low convex	No	Turbid
TRB18	Rod	2.0 x 0.7	Motile	+	Fast growing	White	Dull, opaque	Flat	No	Turbid with pellicle
TR1	Rod	1.0 x 0.6	Motile	-	Moderate	Red	Opaque, Shiny, margin entire	Low convex	Red	Reddish turbid
TR5	Rod	1.0 x 0.4	Non-motile	-	Moderate	White	Translucent, margin entire, circular	Slightly raised	No	Turbid

[a]: '+' indicates endospore formation, '-' indicates no visible spores; b: '-' indicates absence of pigments

Table 4.2.2a: Biochemical characters of biocontrol isolates

Biochemical	**Bacterial Antagonists[a]**						
Characteristics	**BTR4**	**BTR8**	**BTR18**	**BTR19**	**BTR21**	**BTR22**	**BTR23**
Gram character	**+**	**-**	**-**	**+**	**+**	**+**	**+**
Indole production	-	-	-	-	-	-	-
Methyl Red	-	-	+	-	-	-	-
VP test	+	-	+	-	-	+	+
Citrate utilization	+	+	+	+	+	+	+
Acid in TSI agar	+	-	+	+	+	+	+
Gelatin hydrolysis	-	-	-	+	-	+	-
Oxidase	-	+	-	+	+	+	+
Catalase	+	+	+	+	+	+	+
O-F	F/g	O	F/g	O	O	F/g	F/g
H_2S in TSI agar	-	-	-	-	-	-	-
Growth in MacConkey medium	-	+/nc	+/w	-	-	-	-
ONPG	+	-	+	-	+	+	-
Urease	-	-	-	-	+	+	+
Nitrate reduction	+	-	+	+	+	+	+
Nitrite reduction	-	-	-	-	-	-	+
Ornithine decarboxylase	+	-	+	+	+	+	+
Arginine decarboxylase	+	+	+	+	+	+	+
Lysine decarboxylase	+	-	+	+	+	+	+
Phenylalanine deamination	-	-	-	-	-	-	-
DNase production	-	+	-	+	+	+	+
3-ketolactose production	-	-	-	-	-	-	-
Carbohydrates acid from:							
D-Glucose	+	+	+	+	+	+	+
D-Mannitol	+	+	+	-	+	+	+
Fructose	+	+	+	+	+	+	+
Cellobiose	+	-	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Adonitol	+	-	-	-	-	+	-
L-Arabinose	+	-	-	-	-	-	-
L-Rhamnose	+	-	-	-	-	+	-
m-Inositol	+	-	-	-	-	+	-
Raffinose	+	-	-	-	-	+	-
Maltose	+	-	+	+	+	+	+
Trehalose	+	-	+	-	+	+	-
D-Sorbitol	+	-	+	-	+	+	-
Lactose	-	-	-	-	-	+	+
D-Xylose	+	+	+	+	+	+	-

[a] F: Fermentative; O: Oxidative; F/g: Fermentative and gas producing; '+': positive; '-': negative; 'w': white colored colony; 'p': pink colored colony; 'nc': colonies not colored

Table 4.2.2b: Biochemical characters of biocontrol isolates

Biochemical	**Bacterial Antagonists**[a]						
Characteristics	**BTRL6**	**BTRL8**	**BTRL9**	**BTRL11**	**D6**	**D7**	**D8**
Gram character	**-**	**+**	**+**	**+**	-	-	-
Indole production	-	-	-	-	-	-	-
Methyl Red	-	+	-	-	-	-	-
VP test	+	-	+	-	-	+	-
Citrate utilization	+	+	+	-	+	+	-
Acid in TSI agar	+	+	+	-	-	+	-
Gelatin hydrolysis	-	-	+	-	-	+	-
Oxidase	-	-	-	-	+	-	+
Catalase	+	-	+	-	+	+	-
O-F	F	F/g	O	F	O	F/g	O
H_2S in TSI agar	-	+	-	-	-	-	-
Growth in MacConkey medium	+/w	-	-	+/p	+/w	+/p	+/w
ONPG	+	-	+	+	-	+	-
Urease	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	+	-
Nitrite reduction	-	-	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+	+	+
Arginine decarboxylase	+	-	+	-	+	+	+
Lysine decarboxylase	+	+	+	+	+	+	+
Phenylalanine deamination	-	-	-	-	-	-	-
DNase production	-	-	+	-	+	-	-
3-ketolactose production	-	-	-	-	-	-	**+**
Carbohydrates acid from:							
D-Glucose	+	+	+	+	**+**	+	+
D-Mannitol	+	+	+	+	**+**	+	-
Fructose	+	-	+	+	**+**	+	-
Cellobiose	+	-	-	-	**-**	+	+
Sucrose	+	+	+	+	**+**	+	-
Adonitol	-	-	-	-	**-**	-	-
L-Arabinose	-	-	-	-	**+**	+	-
L-Rhamnose	-	-	+	+	**-**	+	+
m-Inositol	-	-	+	-	**-**	-	-
Raffinose	-	-	-	-	**-**	+	-
Maltose	+	-	-	+	**-**	+	+
Trehalose	+	-	+	+	**-**	+	-
D-Sorbitol	-	+	-	-	**-**	+	-
Lactose	-	+	-	+	**-**	+	-
D-Xylose	+	+	-	+	**+**	+	-

[a] F: Fermentative; O: Oxidative; F/g: Fermentative and gas producing; '+': positive; '-': negative; 'w': white colored colony; 'p': pink colored colony; 'nc': colonies not colored

Table 4.2.2c: Biochemical characters of biocontrol isolates

Biochemical	**Bacterial Antagonists[a]**						
Characteristics	**ETR1**	**ETR17**	**ETR20**	**ETR24**	**GH4**	**GH6**	**GH12**
Gram character	-	-	-	-	+	-	-
Indole production	-	-	+	+	-	-	-
Methyl Red	-	-	+	+	-	-	-
VP test	+	+	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	-
Acid in TSI agar	+	+	+	+	+	-	-
Gelatin hydrolysis	+	+	-	-	-	-	-
Oxidase	-	-	-	-	+	-	-
Catalase	+	+	+	+	+	+	+
O-F	F	F	F/g	F/g	O	O	O
H_2S in TSI agar	-	-	+	+	-	-	-
Growth in MacConkey medium	+/nc	+/nc	+/p	+/p	-	+/w	+/w
ONPG	+	+	+	+	-	-	-
Urease	-	-	+	+	-	-	-
Nitrate reduction	+	+	+	+	+	-	-
Nitrite reduction	-	-	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	-	-	+
Arginine decarboxylase	-	-	+	+	-	+	+
Lysine decarboxylase	+	+	-	-	-	+	+
Phenylalanine deamination	-	-	-	-	-	-	-
DNase production	+	+	-	-	+	+	-
3-ketolactose production	-	-	-	-	-	-	-
Carbohydrates acid from:							
D-Glucose	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	-	-	-
Fructose	+	+	+	+	+	+	+
Cellobiose	-	-	+	+	-	-	-
Sucrose	+	+	+	+	+	-	-
Adonitol	-	-	+	+	-	-	-
L-Arabinose	+	+	+	+	-	+	-
L-Rhamnose	-	-	+	+	-	+	-
m-Inositol	+	+	-	-	-	-	-
Raffinose	-	-	+	+	+	-	-
Maltose	+	+	+	+	-	-	-
Trehalose	+	+	+	+	-	-	-
D-Sorbitol	+	+	+	+	-	-	-
Lactose	-	-	+	+	-	-	-
D-Xylose	-	-	+	+	-	-	-

[a] F: Fermentative; O: Oxidative; F/g: Fermentative and gas producing; '+': positive; '-': negative; 'w': white colored colony; 'p': pink colored colony; 'nc': colonies not colored

Table 4.2.2d: Biochemical characters of biocontrol isolates

Biochemical Characteristics	**Bacterial Antagonists[a]**						
	GH13	**GH21**	**GH22**	**GH27**	**GH32**	**KTR6**	**TLB3**
Gram character	**+**	**+**	**+**	**-**	**+**	**+**	**+**
Indole production	-	-	-	-	-	-	-
Methyl Red	-	+	+	-	-	-	-
VP test	+	+	+	-	-	+	+
Citrate utilization	+	-	+	+	+	+	+
Acid in TSI agar	+	+	+	-	-	+	+
Gelatin hydrolysis	+	-	-	-	-	+	+
Oxidase	-	+	+	-	-	-	+
Catalase	+	+	+	+	-	+	+
O-F	O	F	F	O	F	O	F
H_2S in TSI agar	-	-	-	-	-	-	+
Growth in MacConkey medium	-	-	-	+/w	+/p	-	-
ONPG	-	-	+	-	-	+	+
Urease	+	-	-	-	-	+	-
Nitrate reduction	+	+	-	-	+	+	+
Nitrite reduction	+	-	-	-	-	-	-
Ornithine decarboxylase	-	+	-	+	+	+	+
Arginine decarboxylase	-	-	-	+	+	+	+
Lysine decarboxylase	-	-	-	+	+	+	+
Phenylalanine deamination	-	-	-	-	-	-	-
DNase production	+	+	+	-	-	+	+
3-ketolactose production	+	-	-	-	-	-	-
Carbohydrates acid from:							
D-Glucose	+	+	+	+	+	+	+
D-Mannitol	-	-	-	-	-	+	-
Fructose	+	+	-	+	+	+	+
Cellobiose	-	-	-	-	+	+	+
Sucrose	-	+	+	-	-	+	+
Adonitol	-	-	-	-	-	-	-
L-Arabinose	+	-	-	-	-	-	-
L-Rhamnose	+	-	-	-	-	-	-
m-Inositol	-	-	-	-	-	+	+
Raffinose	-	-	-	-	-	-	+
Maltose	+	+	+	-	+	+	+
Trehalose	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-
Lactose	-	+	+	-	+	-	-
D-Xylose	-	-	-	-	-	-	-

[a] F: Fermentative; O: Oxidative; F/g: Fermentative and gas producing; '+': positive; '-': negative; 'w': white colored colony; 'p': pink colored colony; 'nc': colonies not colored

Table 4.2.2e: Biochemical characters of biocontrol isolates

Biochemical Characteristics	**Bacterial Antagonists[a]**						
	TBD7	**TMG1**	**TRB1**	**TRB14**	**TRB18**	**TR1**	**TR5**
Gram character	+	-	+	+	+	-	-
Indole production	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-
VP test	+	+	-	-	+	+	+
Citrate utilization	+	+	-	-	+	+	+
Acid in TSI agar	+	+	-	-	+	+	-
Gelatin hydrolysis	+	+	+	-	-	+	-
Oxidase	+	-	+	+	+	-	+
Catalase	+	+	+	+	-	+	+
O-F	F	F/g	O	O	O	F	F
H_2S in TSI agar	-	-	-	-	-	-	-
Growth in MacConkey medium	-	+/p	-	-	-	+/nc	+/w
ONPG	+	+	-	-	+	+	+
Urease	-	-	+	+	-	-	+
Nitrate reduction	+	+	+	+	+	+	+
Nitrite reduction	-	-	+	+	-	-	-
Ornithine decarboxylase	+	+	-	-	+	+	+
Arginine decarboxylase	+	+	-	-	+	-	+
Lysine decarboxylase	+	+	-	-	+	+	-
Phenylalanine deamination	-	-	-	-	-	-	-
DNase production	+	-	-	-	+	+	+
3-ketolactose production	-	-	-	-	-	-	-
Carbohydrates acid from:							
D-Glucose	+	+	+	+	+	+	+
D-Mannitol	+	+	+	-	-	+	+
Fructose	+	+	-	-	+	+	+
Cellobiose	+	+	-	+	+	-	+
Sucrose	+	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	-
L-Arabinose	-	+	-	-	-	-	+
L-Rhamnose	-	+	-	-	-	-	+
m-Inositol	+	-	-	-	+	+	+
Raffinose	-	+	+	+	-	-	+
Maltose	+	+	+	+	+	+	+
Trehalose	-	+	-	+	+	+	-
D-Sorbitol	-	+	-	+	+	+	+
Lactose	-	+	-	-	-	-	-
D-Xylose	-	+	-	-	-	-	+

[a] F: Fermentative; O: Oxidative; F/g: Fermentative and gas producing; '+': positive; '-': negative; 'w': white colored colony; 'p': pink colored colony; 'nc': colonies not colored

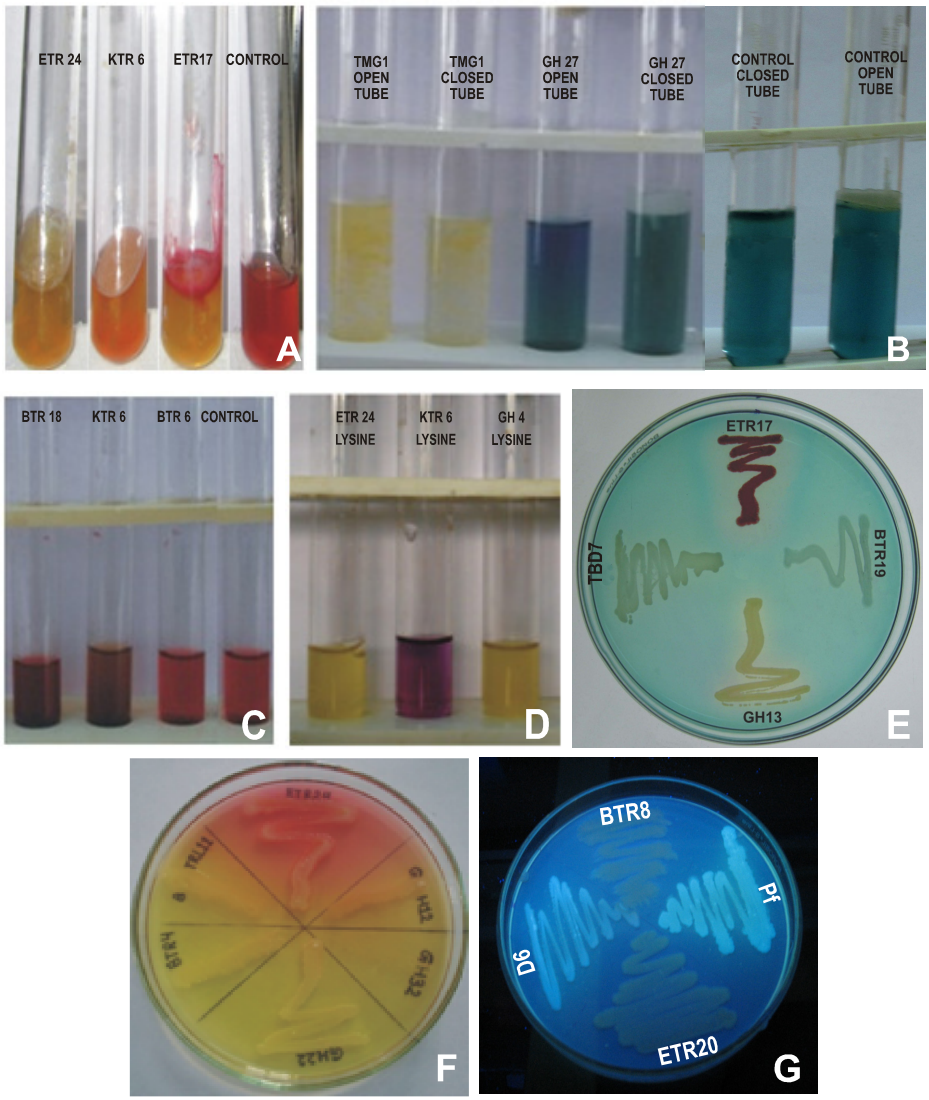


Fig. 4.2.1a: Biochemical characterization of antagonistic bacterial isolates:

(A) Production of acid in TSI agar indicated by color change from red to yellow.

(B) O-F test showing fermentative nature of TMG1 indicated by change in color from blue to yellow in both open and paraffin sealed tube and oxidative nature of GH27 indicated by yellow color only in the open tube but not in the paraffin sealed tube.

(C) Nitrate reduction shown by deep red coloration in nitrate broth by KTR6 and BTR18 while isolate BTR8 tested negative.

(D) Lysine decarboxylation indicated by color change from purple to yellow by ETR24 and GH4.

(E) DNase activity on DNA agar plate exhibited by ETR17 and GH13 indicated by formation of halo around bacterial line of growth. Strains BTR19 and TBD7 tested negative.

(F) Urease production indicated by color change from yellow to pink by ETR24. Negative test was exhibited by strains GH32, GH22, BTR4, GH12 and BTRL11.

(G) Fluorescence shown by strains D6 and positive control *P. fluorescens* (Pf) (isolate no. NRRL B23932) on *Pseudomonas* agar plate (for fluorescein) under UV light source (254 nm). The strains BTR8 and ETR20 does not show fluorescence.

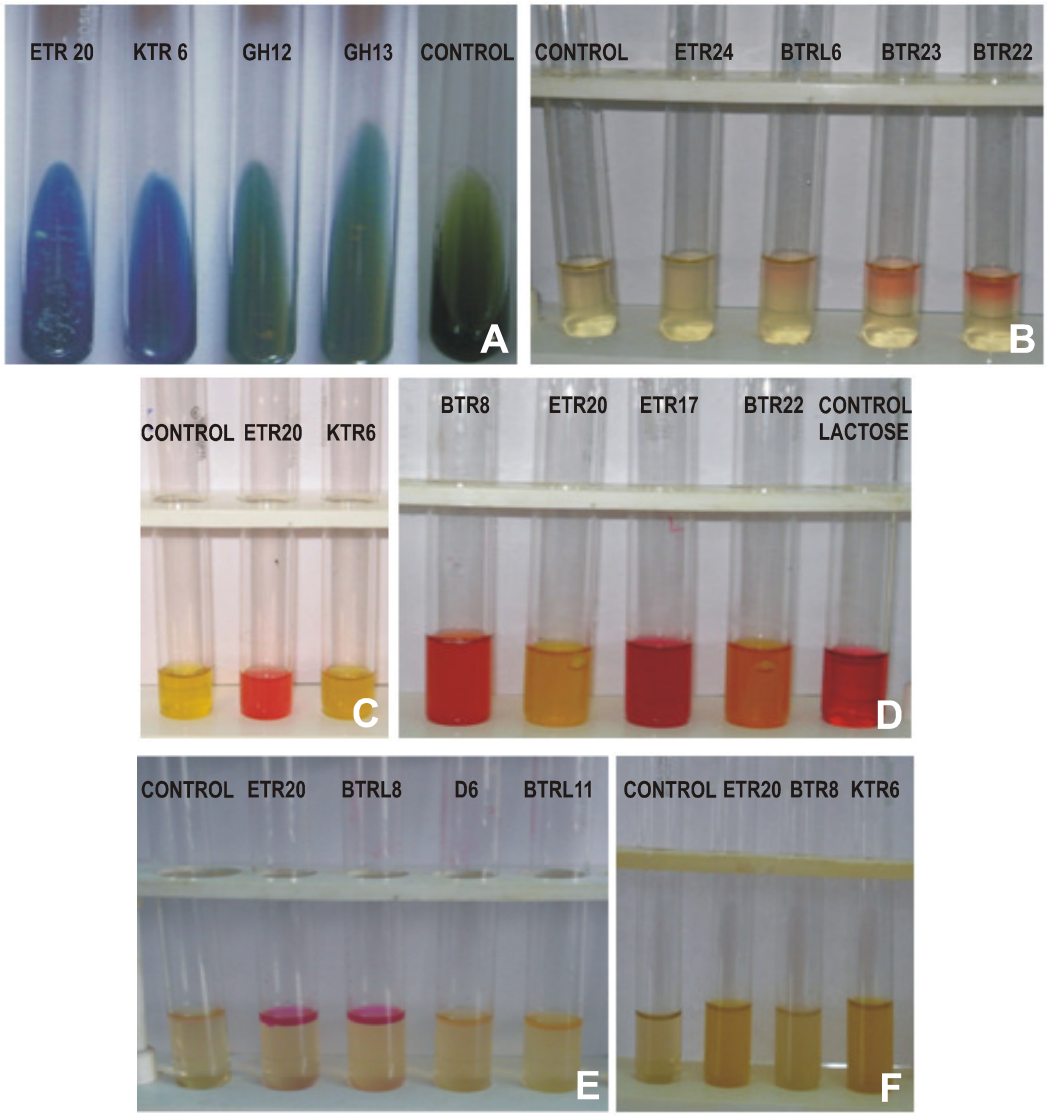


Fig.4.2.1b: Biochemical characterization of antagonistic bacterial isolates:

(A) Citrate utilization by KTR6 and ETR20 indicated by color change of the medium from green to blue. Strains GH12 and GH13 tested negative.

(B) Voges Proskauer (VP) test positive for BTR22 and BTR23 indicated by formation of red colored ring in the medium. On addition of VP reagents ETR24 and BTRL6 exhibited negative result.

(C) Methyl red (MR) test positive exhibited by ETR20 indicated by color change from yellow to red after the addition of MR indicator. KTR6 tested negative.

(D) Lactose fermentation exhibited by strains ETR20 and BTR22 indicated by appearance of yellow color alongwith gas formation. BTR8 and ETR17 tested negative.

(E) Indole production by strains ETR20 and BTRL8 indicated by red colored ring on top of the medium. Strains D6, BTRL11 and BTR19 did not produce indole.

(F) ONPG test positive for ETR20 and KTR6 indicated by golden yellow color of the medium compared to straw colored medium of the control tube. BTR8 tested negative.

Three isolates viz. ETR1, ETR17 and TR1 were presumed to be *Serratia marcescens*, two strains ETR20 and ETR24 were identified as *Citrobacter* sp. and isolates D7 and TMG1 as *Enterobacter* sp. Eleven isolates were identified as *Bacillus* strains of which KTR6 was *B. subtilis* while BTR4, BTR19, BTR21, BTR22, BTR23, BTRL9, GH22, TBD7, TLB3 and TRB18 were identified as *Bacillus* sp. The isolate GH21 was identified as *Paenibacillus* sp. (Priest Fergus, 2005). Two isolates BTRL6 and BTR18 were *Cedecea* sp. while the isolates GH4, TRB1 and TRB14 were identified as *Micrococcus* sp. and GH13 was identified as *M. varians*. Three strains GH12, GH6 and GH27 were identified as *Acinetobacter* sp. while TR5, BTRL11, GH32, BTRL8 bacterial strains were identified as *Actinobacillus* sp. (Olsen and Moller, 2005), *Aerococcus* sp. (Evans, 1986), *Enterococcus* sp. (Svec and Devriese, 2009) and *Gemella* sp. (Collins and Falsen, 2009) respectively.

4.2.4. Phylogenetic characterization

The proteinase and RNase treated genomic DNA isolated from strains D7, ETR17, ETR20 and KTR6 run on agarose gel produced distinct fluorescent bands when viewed in a UV-transilluminator (Fig. 4.2.2A). PCR amplification of 16SrRNA gene using Universal primers generated an amplicon of band size 1500bp when viewed on 1% agarose gel (Fig. 4.2.2B). On obtaining the sequences of 16S rRNA gene amplicons from sequencing service provider, they were deposited in the NCBI GenBank for which accession numbers were assigned. Isolates D7, ETR17, ETR20, and KTR6 were assigned the accession numbers HM150755, JX566992, HM150756 and HM150757 respectively. Similarity search of the 16S rRNA gene sequences using the NCBI BLAST tool resulted in 98% to 99% similarity in case of ETR17, KTR6 and D7 with other related sequences deposited in the GenBank. However, isolate ETR20 exhibited maximum similarity of 95% with other related sequences in the Genbank. The isolate ETR17 showed 99% similarity with *Serratia marcescens*, KTR6 showed 99% similarity with *Bacillus subtilis*, ETR20 showed 95% similarity with *Citrobacter freundii,* other *Citrobacter* and *Klebsiella* species while D7 showed 99% similarity with *Enterobacter* sp. followed by 98% similarity with different species of *Enterobacter* as well as *Leclercia adecarboxylata*, *Cedecea davisae* and *Pantoea* sp. The phylogenetic relationship of the bacterial isolates KTR6, ETR20, D7 and ETR17 with other related bacterial species was established based on the partial 16SrRNA gene sequences obtained from the isolates.

Phylogenetic tree was constructed for each of these isolates based on the genetic relationships between the allied strains in the NCBI database using MEGA version 4.0. It was observed that ETR17 clustered with *S. marcescens*, KTR6 clustered with *B. subtilis*, ETR20 clustered with *Citrobacter* sp. and D7 clustered with *Enterobacter* sp. (Fig. 4.2.3a and Fig. 4.2.3b).

4.2.5. Amplification of fluorescent *Pseudomonas* specific 16S-23S rDNA ITS region amplification

In order to identify the fluorescent pseudomonads among the antagonistic *Pseudomonas* isolates, BTR8, D6 and D8, 16S-23S rRNA intervening region (ITS) was PCR amplified. The antifungal bacterial isolate D6 produced an amplicon of band size around 550bp which was compared to that of a standard *Pseudomonas fluorescens* strain (NRRL B23932), confirming the identity of the isolate being a fluorescent pseudomonad (Fig. 4.2.2 C). The isolates D8 and BTR8 did not produce the specific amplicon and were therefore not considered as fluorescent pseudomonads.

4.2.6. Hemolytic activity of selected bacterial antagonists

The test for hemolytic activity on blood supplemented TSA plates revealed that the isolates ETR17, ETR1, TR1 and D7 lacked the hemolytic activity since no halo formation occurred around the bacterial colonies. However, formation of faint green colored halo around the colonies of ETR20 and ETR24 was observed which indicated that the isolates exhibited α-hemolytic activity.

4.2.7. Sensitivity of isolated bacterial antagonists to chemical fungicides

Fungicide resistance of thirty five antagonistic bacterial isolates was evident against all the tested fungicides viz. thiophanate methyl, bavistin, captan and mancozeb (Table 4.2.4). All the isolates showed resistance towards the fungicides at various concentrations as expected. In general, thiophanate methyl and bavistin were less effective in checking the growth of the bacterial isolates compared to mancozeb and captan. The lowest MIC recorded was 3.9 mg ml^{-1} which was exhibited by captan against 4 isolates. The highest MIC on the other hand was more than 1 g ml^{-1} which was recorded by thiophanate methyl (against 8 isolates) and bavistin (against 10 isolates).

4.2.8. Antibiotic sensitivity profile of selected bacterial antagonists

Selected potential bacterial isolates, D7, ETR17, ETR20 and KTR6 were tested for antibiotic sensitivity against commercially available antibiotics. The antibiotic

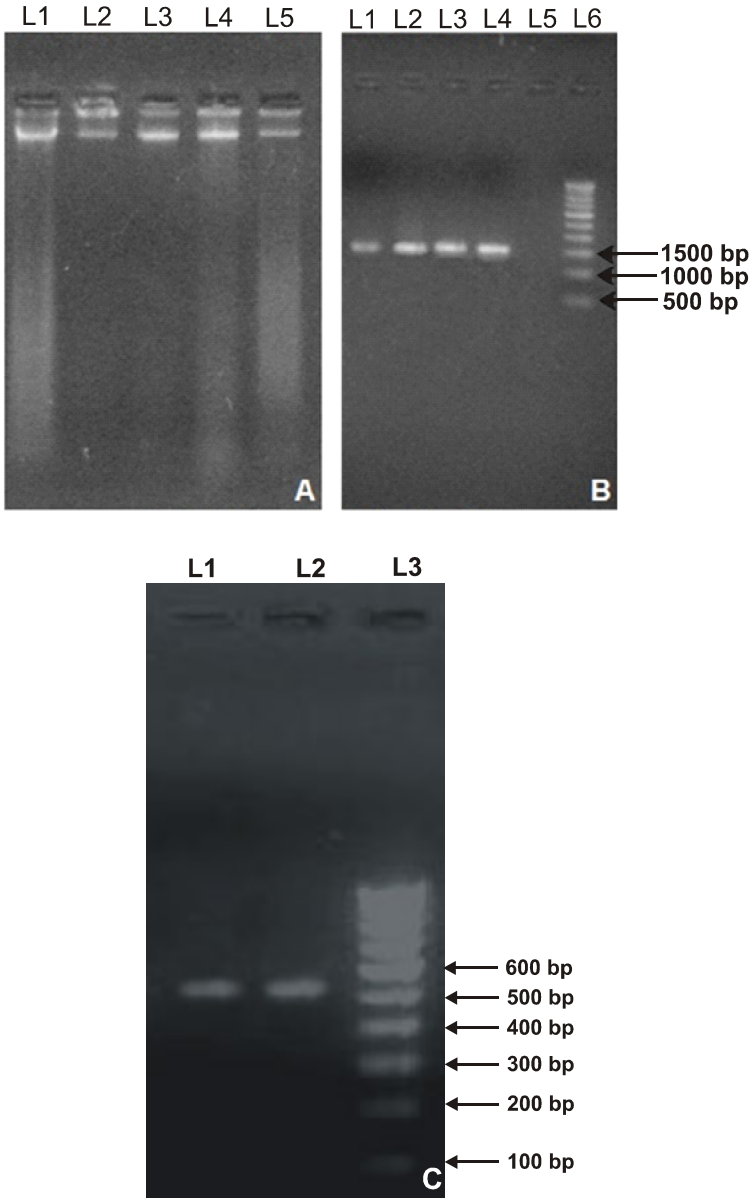


Fig. 4.2.2: Agarose gel electrophoresis of DNA samples viewed under UV light:
(A) Genomic DNA extracted from different bacterial strains.
(B) PCR amplified product (1500bp) obtained by amplification of the 16S rRNA gene of different bacterial isolates using Universal primers fD1 and rp2 primers: L1: ETR20; L2:ETR17; L3: KTR6 and L4: D7 compared to L6: 500bp DNA ladder used as size marker.
(C) PCR amplification of 16S-23S rRNA ITS region of 560bp length from L1: isolate D6 and L2: *P. fluorescens* NRRL B23932 (positive control) compared to L3: 100bp DNA ladder using fluorescent *Pseudomonas* specific primers.

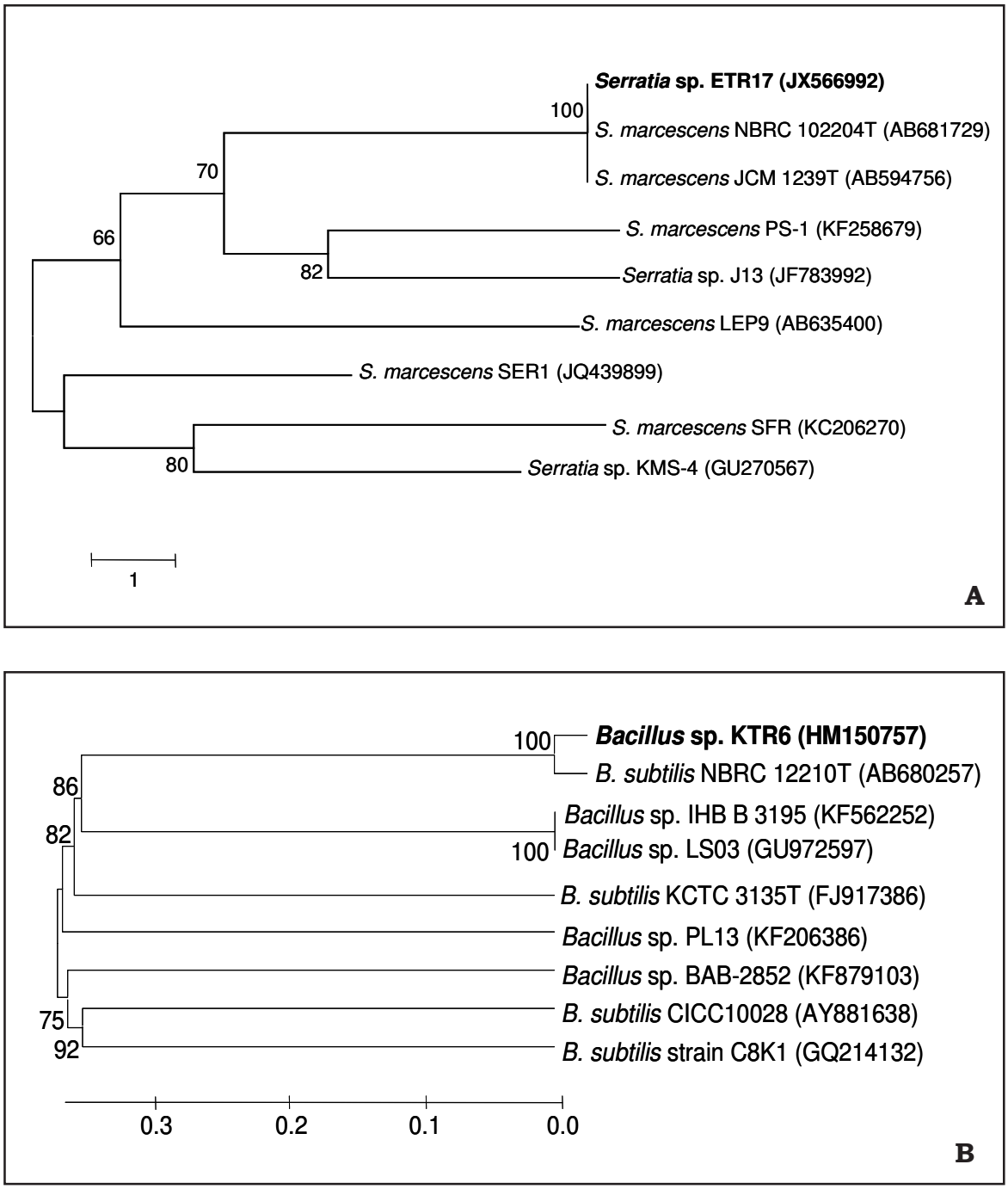
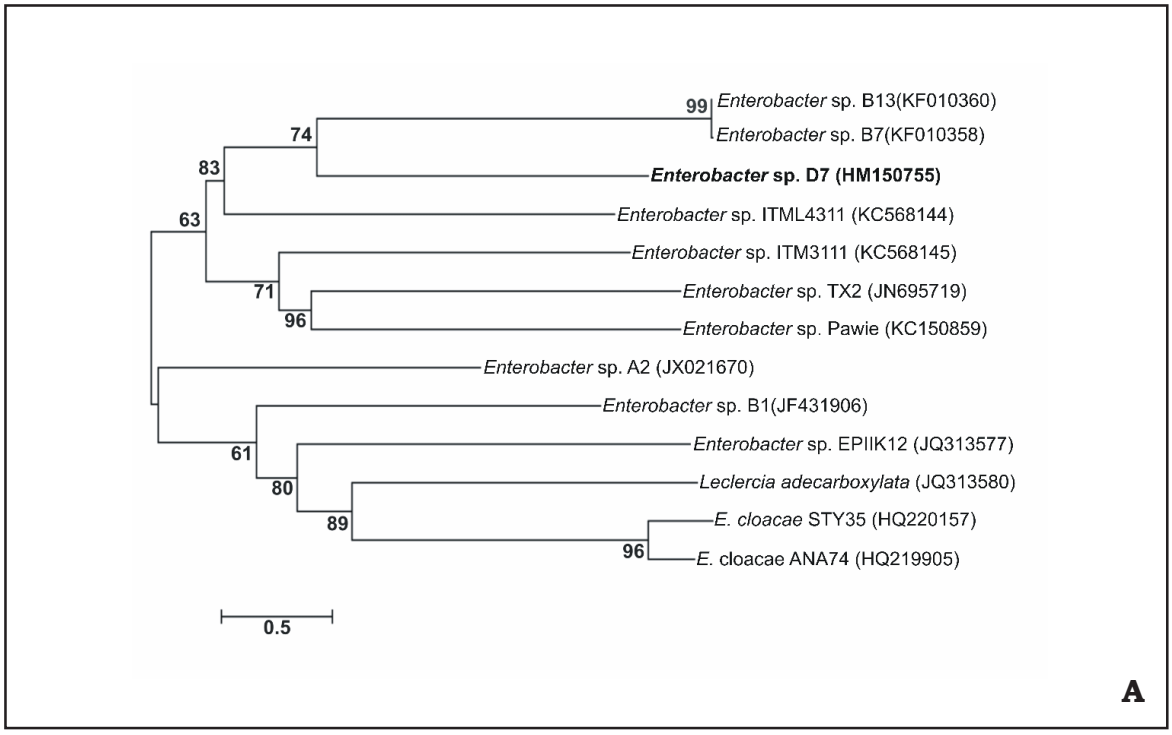


Fig. 4.2.3a: Phylogenetic tree of bacterial strains (A) ETR17 (B) KTR6. The evolutionary history was inferred using the Neighbor-Joining method. The significance of each branch is indicated by a bootstrap for 1000 subsets. The GenBank accession number of the sequences compared with the target sequence is provided within the parentheses. Strain represented in bold was isolated during present study.



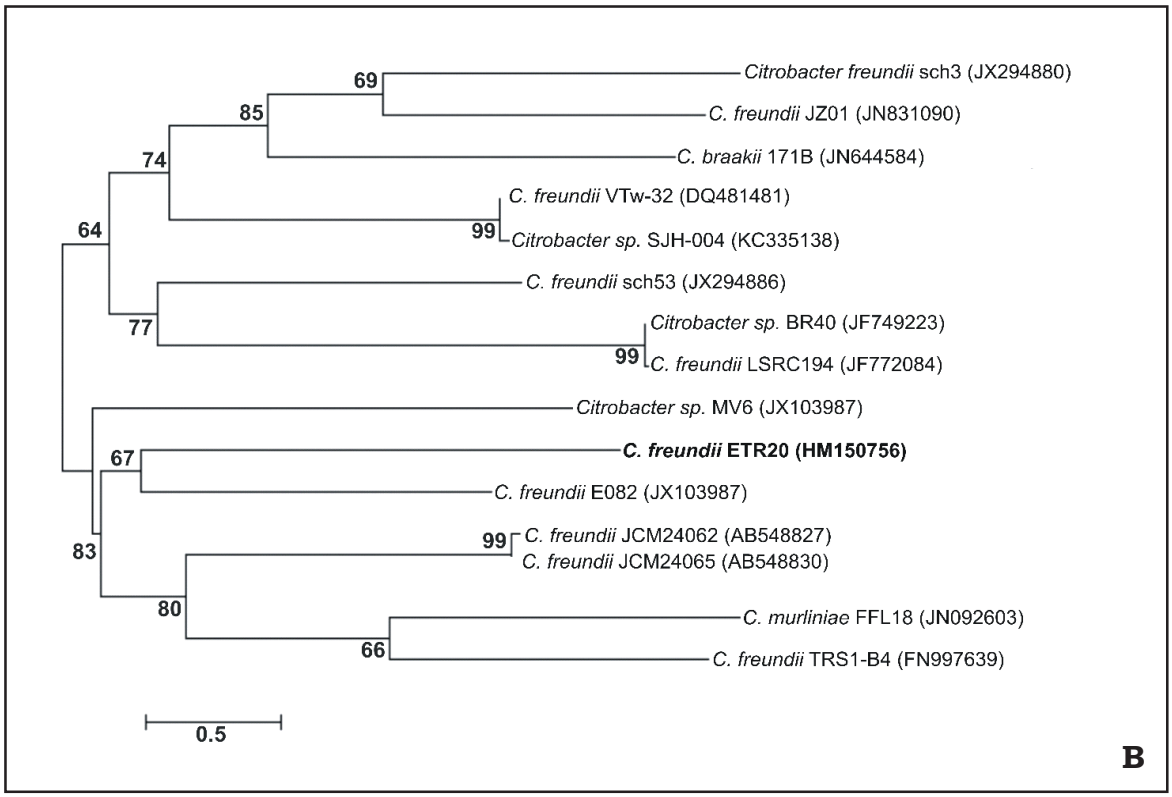


Fig. 4.2.3b: Phylogenetic tree of bacterial strains (A) D7 (B) ETR20. The evolutionary history was inferred using the Neighbor-Joining method. The significance of each branch is indicated by a bootstrap for 1000 subsets. The GenBank accession number of the sequences compared with the target sequence is provided within the parentheses. Strain represented in bold was isolated during present study.

Table 4.2.3: List of antagonistic bacterial isolates identified based on morphological, biochemical and phylogenetic (selected strains) studies

Bacterial Strains	Identity
BTR4	*Bacillus* sp.
BTR8	*Pseudomonas* sp.
BTR18	*Cedecea* sp.
BTR19	*Bacillus* sp.
BTR21	*Bacillus* sp.
BTR22	*Bacillus* sp.
BTR23	*Bacillus* sp.
BTRL6	*Cedecea* sp.
BTRL8	*Gamella* sp.
BTRL9	*Bacillus* sp.
BTRL11	*Aerococcus* sp.
D6	*Pseudomonas putida*
D7	*Enterobacter* sp.
D8	*Pseudomonas vesicularis*
ETR1	*Serratia* sp.
ETR17	*Serratia* sp.
ETR 20	*Citrobacter* sp.
ETR24	*Citrobacter* sp.
GH4	*Micrococcus* sp.
GH6	*Acinetobacter* sp.
GH12	*Acinetobacter* sp.
GH13	*Micrococcus varians*
GH21	*Paenibacillus* sp.
GH22	*Bacillus* sp.
GH27	*Acinetobacter* sp.
GH32	*Enterococcus* sp.
KTR6	*Bacillus subtilis*
TLB3	*Bacillus* sp.
TBD7	*Bacillus* sp.
TMG1	*Enterobacter* sp.
TRB1	*Micrococcus* sp.
TRB14	*Micrococcus* sp.
TRB18	*Bacillus* sp.
TR1	*Serratia* sp.
TR5	*Actinobacillus* sp.

Table 4.2.4: Fungicide susceptibility of the potent biocontrol strains towards four commonly used fungicides

Bacterial isolates	Minimum inhibitory concentration of fungicides			
	Thiophanate methyl (mg ml^{-1})	Mancozeb (mg ml^{-1})	Bavistin (mg ml^{-1})	Captan (mg ml^{-1})
BTR4	1000	31.5	>1000	31.5
BTR8	1000	31.5	>1000	15.6
BTR18	250	62.5	1000	7.8
BTR19	250	62.5	250	15.6
BTR21	500	125	500	250.0
BTR22	250	62.5	250	7.8
BTR23	250	31.5	250	31.5
BTRL6	250	62.5	>1000	125.0
BTRL8	250	31.5	250	15.6
BTRL9	250	31.5	250	31.5
BTRL11	250	31.5	500	31.5
D6	250	31.5	250	31.5
D7	1000	62.5	1000	62.5
D8	250	31.5	>1000	3.9
ETR1	>1000	31.5	>1000	3.9
ETR17	>1000	31.5	>1000	15.6
ETR20	>1000	31.5	>1000	7.8
ETR24	>1000	31.5	>1000	7.8
GH4	250	31.5	250	15.6
GH6	500	31.5	250	500.0
GH12	250	31.5	250	15.6
GH13	250	31.5	250	3.9
GH21	250	31.5	250	15.6
GH22	250	31.5	250	15.6
GH27	>1000	31.5	1000	15.6
GH32	250	62.5	250	500.0
KTR6	250	31.5	250	15.6
TBD7	250	31.5	250	7.8
TLB3	250	31.5	250	3.9
TMG1	>1000	31.5	1000	31.5
TR1	>1000	31.5	>1000	7.8
TR5	250	31.5	500	31.5
TRB1	500	31.5	1000	7.8
TRB14	250	31.5	500	7.8
TRB18	>1000	31.5	>1000	31.5

The antibiotic sensitivity and resistance varied considerably among the isolates since they belonged to different genera (Table 4.2.5). Ampicillin and vancomycin antibiotics were resisted by all the isolates under study. The isolates were highly sensitive to co-trimoxazole, ceftriaxone and ciprofloxacin, followed by amikacin, chloramphenicol, ceftazidime, gentamycin, nalidixic acid, netilmycin and tobramycin. The isolate D7 showed maximum resistance towards eight antibiotics followed by ETR17 and ETR20 which were highly resistant to six antibiotics. The isolate KTR6 was more sensitive and intermediately resistant to the antibiotics except ampicillin.

4.2.9. Bacterial growth kinetics by turbidometry

The growth curve of fourteen most potential bacterial antagonists is depicted in Fig. 4.2.4 (a,b,c,d,e). The isolates exhibited wide variation in the time period of different growth phases notably in the exponential and stationary phases. A sigmoid pattern of growth represented by an initial short lag phase followed by a long exponential phase and finally stationary phase was observed for all strains (Fig. 4.2.4 a,b,c,d,e). Generally, the strains reached the stationary phase within 12-14h. However, strain D7 was found to be fast growing as it reached the stationary phase within 8 h. Isolate BTR19 on the other hand multiplied slowly and took almost 22 h to reach the stationary phase.

4.2.10. Morphological characteristics of fungal antagonistic isolates

The antagonistic fungal isolates exhibited variations in color, growth patterns, and sporulation characters (Fig. 4.2.5a). The culture characteristics and microscopic observations made on the hyphae and spores of ten potential biocontrol isolates are summarized in Table 4.2.6a. Strains AD6 and AD10 grew profusely on PDA and quickly filled up the petriplate. The color of the mycelia was initially white which gradually darkened and changed to green by 5-7 days in both the strains. Spores were noticed after 7 days in AD6 but AD10 culture showed sporulation by the fifth day. Strains AD4, NB4 and AD3 also showed extensive growth. The color of the mycelia which was initially white for all the strains showed differential darkening resulting in variation in final color. Sporulation was noted around the seventh day. Strains NB1.2, KV8, B2.4, AD7 and AD2 showed moderate growth but their color and sporulation characters varied widely. Microscopic observations revealed wide variation in spore size, shape and morphology (Fig. 4.2.5b). The characters of spores of individual strains are listed in detail in Table 4.2.6b.

Table 4.2.5: Antibiotic sensitivity[a] profile of biocontrol bacterial isolates

Antibiotics	Concentration (in mcg)	Bacterial strains			
		ETR17	KTR6	ETR20	D7
Amikacin	30	S	S	I	S
Ampicillin	10	I	R	R	R
Amoxycillin	10	S	I	I	R
Cefadroxil	30	R	S	R	R
Cefoperazone	75	R	S	I	S
Ceftazidime	30	S	I	S	S
Ceftriaxone	30	S	S	S	S
Chloramphenicol	30	S	S	S	I
Ciprofloxacin	5	S	S	S	S
Cloxacillin	1	R	S	R	R
Co-Trimoxazole	25	S	S	S	S
Erythromycin	15	R	S	I	R
Gentamycin	10	S	S	I	S
Nalidixic Acid	10	S	S	I	S
Netilmycin	10	S	S	I	S
Nitrofurantoin	300	I	I	R	R
Norfloxacin	10	S	S	I	I
Penicillin	10	R	S	R	R
Tobramycin	10	S	S	S	I
Vancomycin	30	R	I	R	R

[a] Sensitive (S); Intermediate (I); Resistant (R)

4.2.11. Phylogenetic characterization of fungal antagonistic isolates based on partial 18S rRNA gene sequence

Ten fungal antagonistic strains were used for phylogenetic characterization studies. The isolated genomic DNA treated with proteinase and RNase and electrophoresed on 0.8% agarose gel produced distinct fluorescent bands when viewed in a UV-transilluminator (Fig. 4.2.6A). PCR amplification of the 18S rRNA gene using ITS1 and ITS4 primers generated an amplicon of band size 500-600bp (Fig. 4.2.6B). The gene sequences of all the antagonists were deposited in the NCBI Genbank for which accession numbers were assigned (Table 4.2.7).

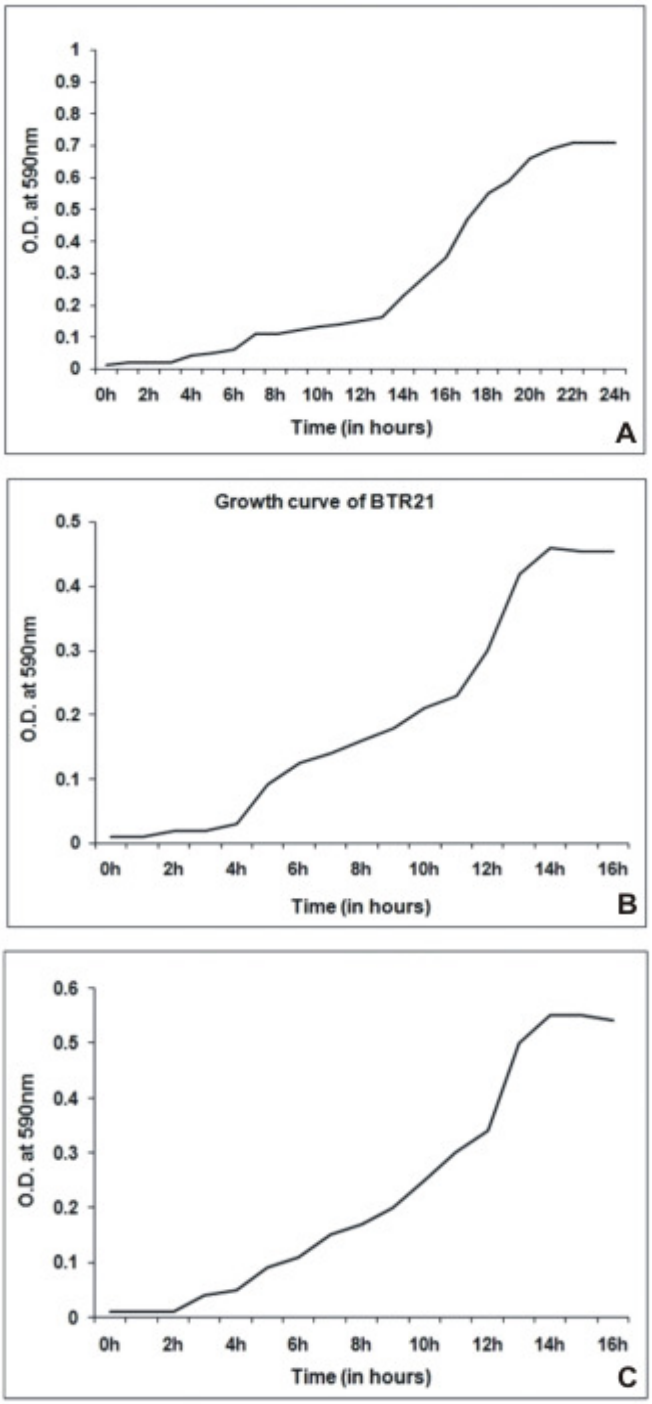


Fig. 4.2.4a: Growth curve of antagonistic bacterial isolates BTR19(A), BTR21(B) and BTR22 (C).

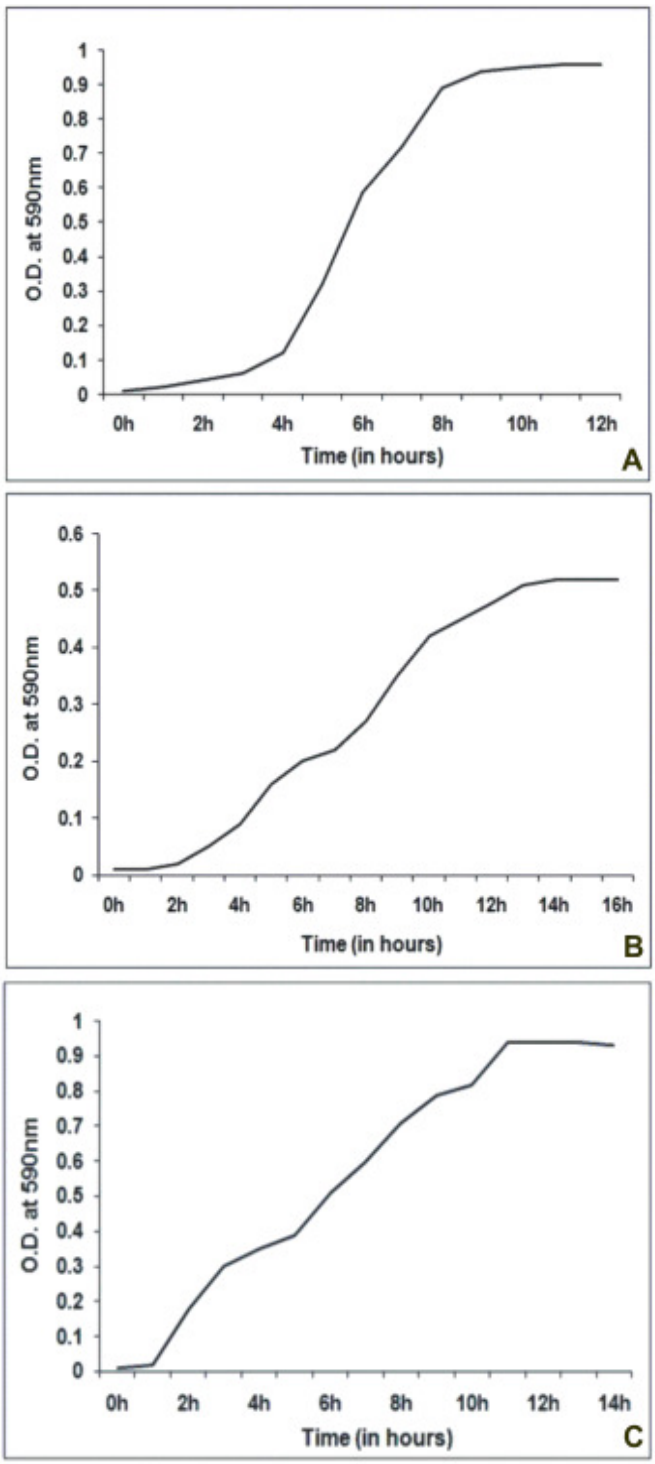


Fig.4.2.4b: Growth curve of antagonistic bacterial isolates D7(A), ETR17(B) and ETR20 (C).

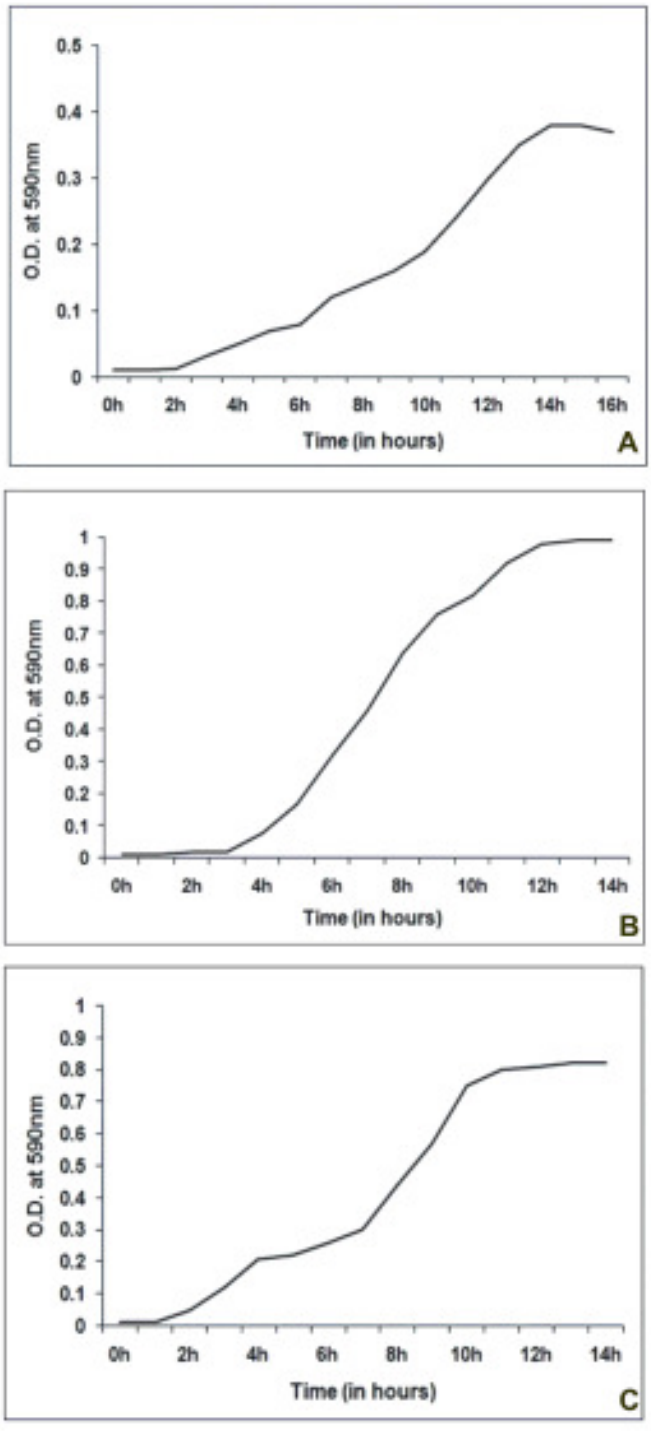


Fig. 4.2.4c: Growth curve of antagonistic bacterial isolates GH13 (A), KTR6 (B) and TBD7 (C).

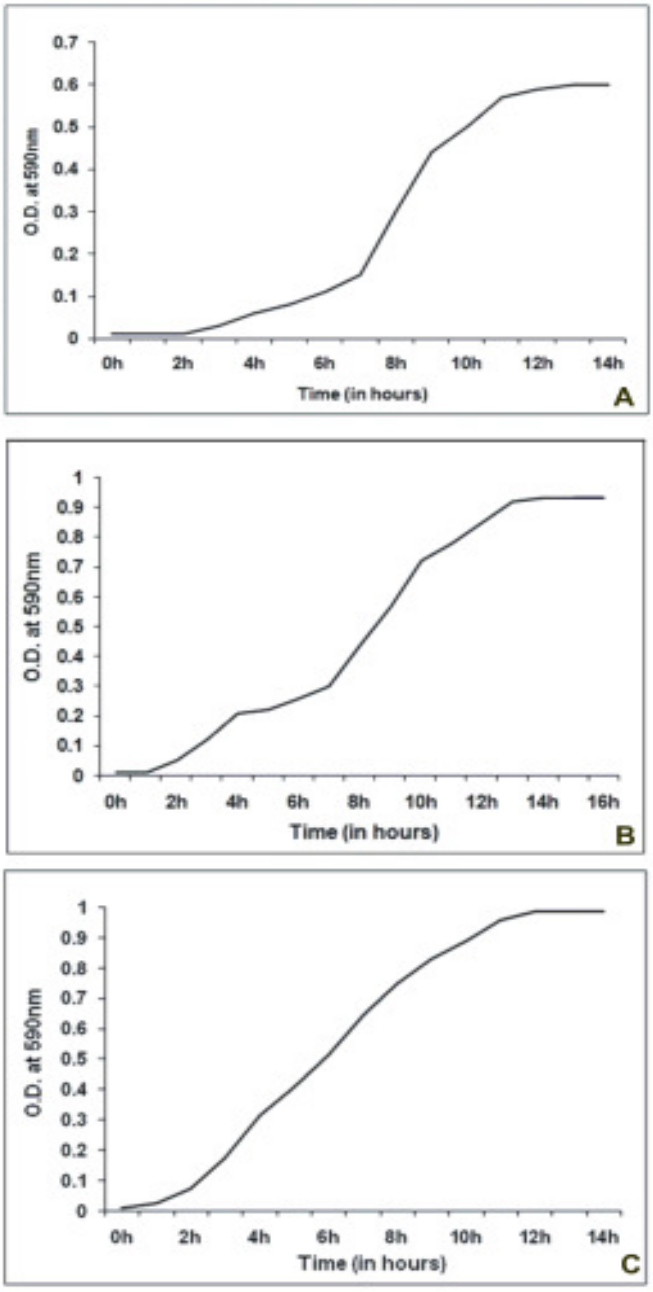


Fig. 4.2.4d: Growth curve of antagonistic bacterial isolates TLB3 (A), TMG1(B) and TR1(C).

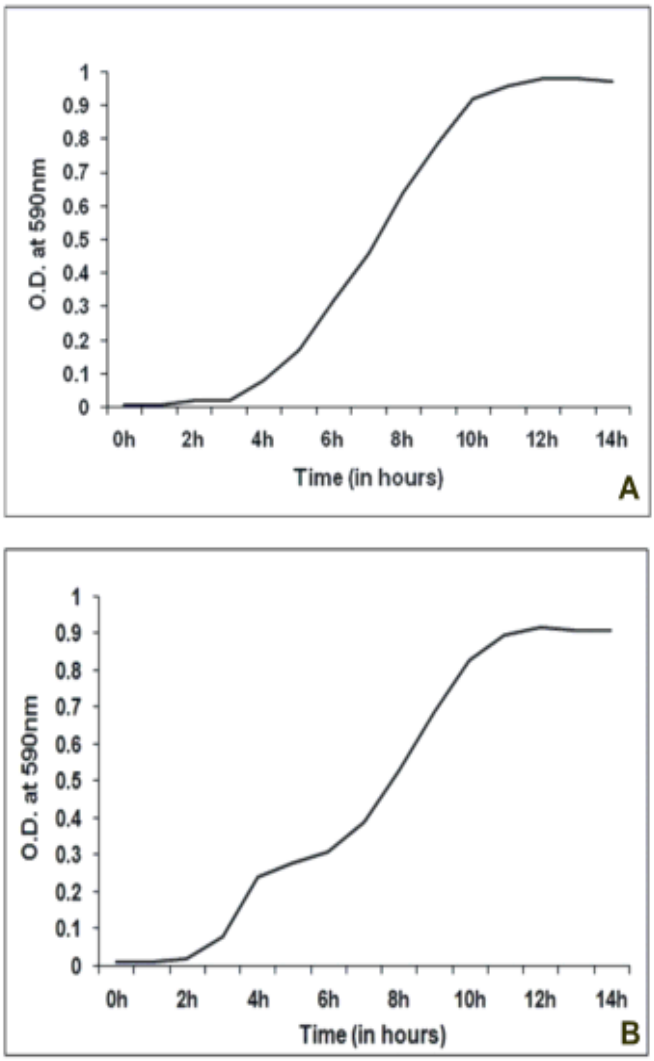


Fig. 4.2.4e: Growth curve of antagonistic bacterial isolates TR5 (A) and TRB14 (B).

Table 4.2.6a: Culture characteristics of biocontrol fungal isolates in potato dextrose agar plates

Fungal strains	**Culture Characteristics**			
	Initial colour	**Final colour**	**Sporulation**	**Growth pattern**
AD2	White	Gray with powdery appearance	Starts within 7 days	Moderate
AD3	White	Dark green color with white margins	Starts between 7-10 days	Extensive
AD4	White	Lilac	Starts within 7 days	Extensive
AD6	White	Green	Starts between 7-10 days	Extensive, profuse
AD7	Green	Blackish green	Starts within 7 days	Moderate
AD10	White	Green	Starts within 5 days	Fast and extensive
B2.4	Gray	Greenish gray	Starts between 7-8 days	Moderate
KV8	White	Gray	Starts between 7-10 days	Moderate
NB4	White	White with pale green-grey centre	Starts within 7 days	Extensive
NBT1.2	White	Dark green, powdery colony	Starts within 7 days	Moderate

Table 4.2.6b: Sporulation and hyphal characteristics of biocontrol fungal isolates grown in potato dextrose agar plates

Fungal strains	Spore characteristics				Hyphal / mycelial characteristics
	Colour	Size (Length x breadth, µm)	Shape	Arrangement	
AD 2	Hyaline	1.9 x 1.9	Round	Conidiophores contain bottle–shaped phialides. Conidia are arranged in chains	Hyphae septate, long (200 x 3.2 µm) and hyaline
AD3	Deep green	3.6 x 3.6	Round	Arranged singly at the end of the conidiophore containing phialides	Hyphae are septate, long, thin (200 x 3.2 µm) and hyaline
AD 4	Hyaline	2.0 x 3.0	Oval	Conidiophores are verticillate and bottle-shaped phialides. Conidia are arranged in chains	Hyphae septate, thin (200 x 3.4 µm), thread like and hyaline
AD 6	Blackish green	1.86 x 1.86	Round	Arranged in chains at the end of flask shaped phialides	Hyphae septate (150 x 3.8 µm) and hyaline
AD 7	Hyaline	3.1 x 3.1	Round	Conidiophores with vesicle at the end, conidia arranged as radial chains	Hyphae septate, and long (200 x 3.5 µm) hyaline
AD-10	Green	3.3 x 3.5	Almost Round, Smooth Walled	Arranged in clusters, phialides are present	Hyaline septate hyphae (66.6 x 2.96 µm)
B 2.4	Hyaline	2.5 x 2.9	Oval	Numerous in numbers and arranged in chains present at the ends of flask shaped phialides	Hyphae are long, very thin (148 x 3.7 µm) and hyaline
KV 8	Hyaline	3.0 x 3.0	Round	Numerous in numbers, vesicle present at the end of conidiophore	Hyphae are long, septate, and hyaline, very thin (200 x 3.6 µm)
NB 4	Bluish-green	3.7 x 3.7	Round	Arranged singly, numerous in numbers	Hyphae septate, long (150 x 3.2 µm) and hyaline with conidiophore
NBT 1.2	Blue-green	4.0 x 3.7	Almost round	Arranged in clumps arising from the phialides, numerous in number	Hyphae septate, hyaline, long (150 x 2.5 µm)

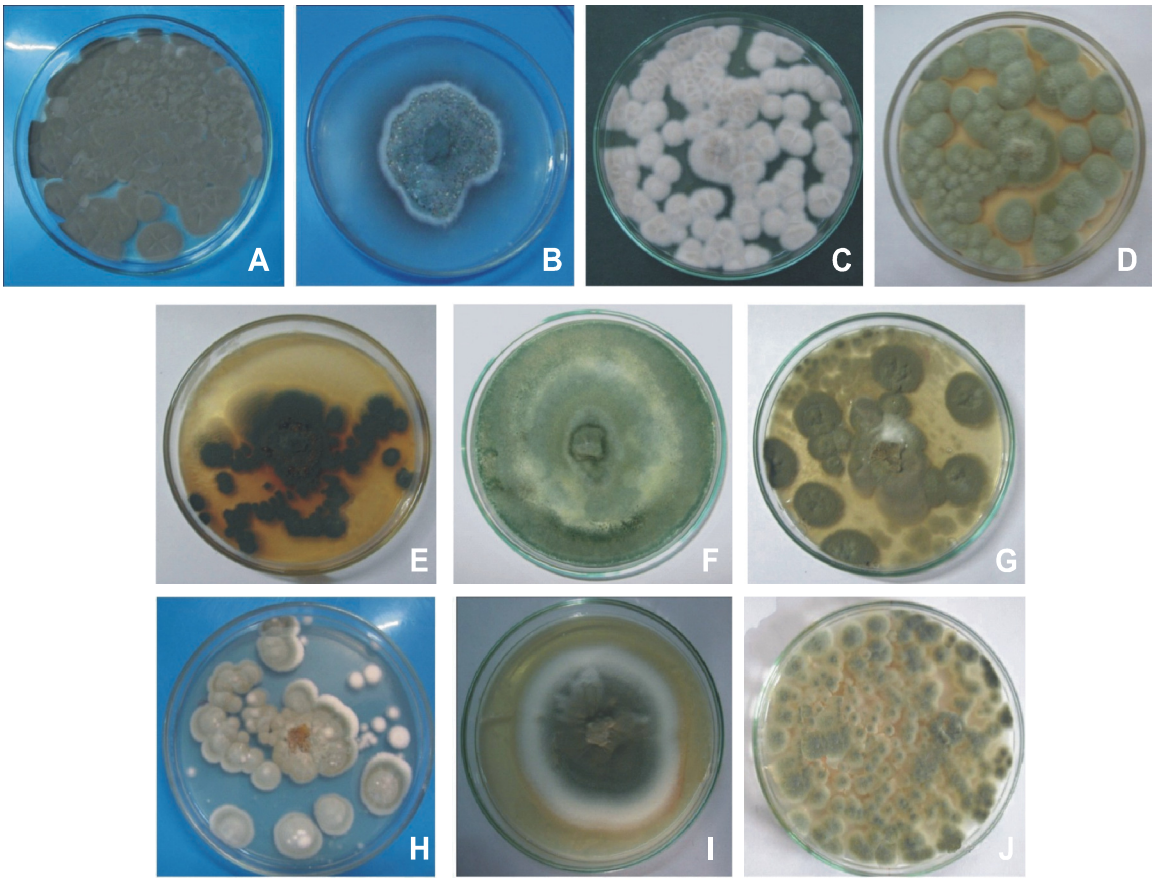


Fig. 4.2.5a: Morphological and culture characteristics of antagonistic fungal isolates cultured on PDA Plates: (A) AD2, (B) AD3, (C) AD4, (D) AD6, (E) AD7, (F) AD10, (G) B2.4, (H) KV8, (I) NB4 and (J) NBT1.2.

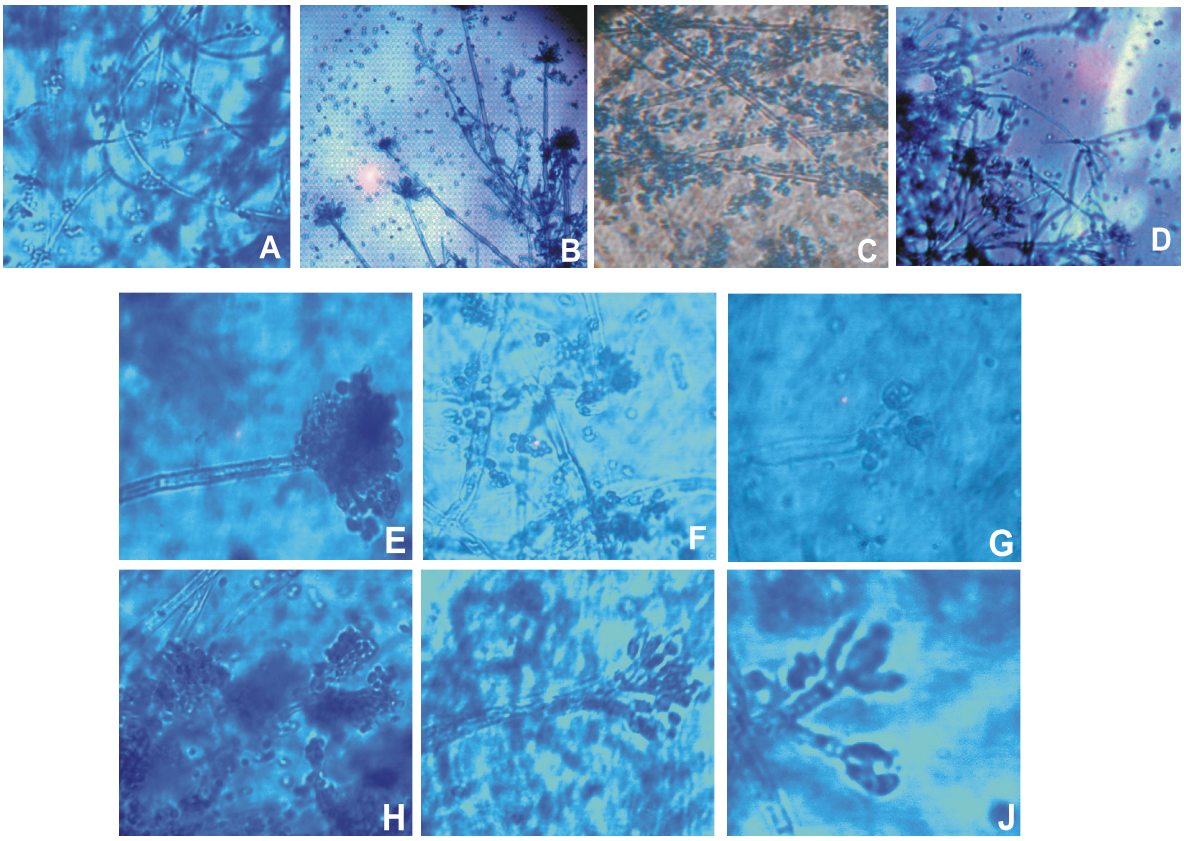


Fig.4.2.5b: Microscopic observations of mycelial and spore characteristics of the antagonistic fungal isolates observed under light microscope: (A) AD2, (B) AD3, (C) AD4, (D) AD6, (E) AD7, (F) AD10, (G) B2.4, (H) KV8, (I) NB4 and (J) NBT1.2.

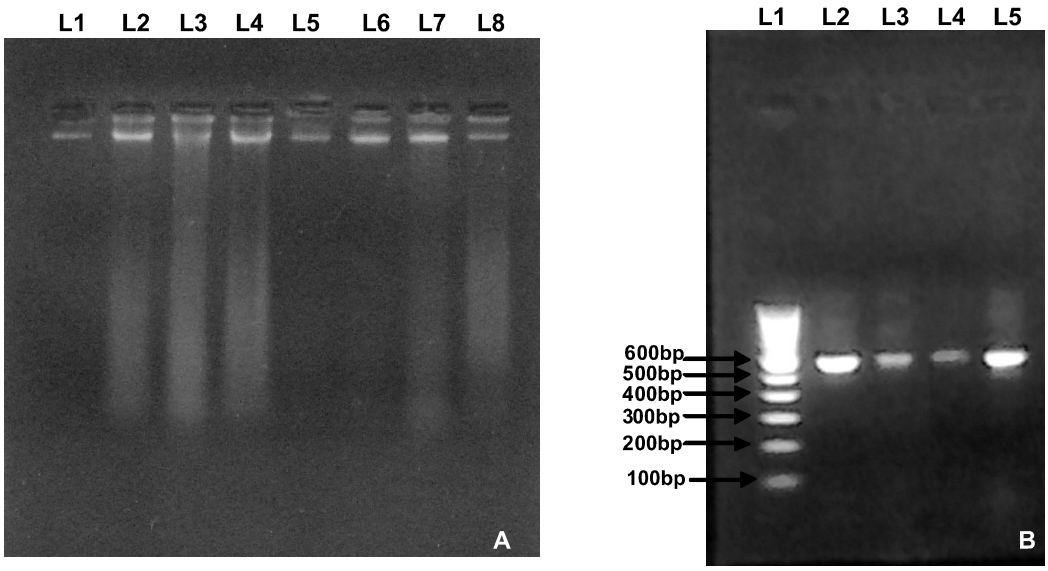


Fig. 4.2.6: Agarose gel electrophoresis showing: (A) Genomic DNA extracted from fungal strains. L1: AD2; L2:AD3; L3: AD4; L4: AD6; L5: AD7; L6: AD10; L7: KV8; L8: B2.4.
(B) PCR amplification of the 18S rRNA gene of 600bp length from fungal antagonists. L1: 100bp ladder; L2: NBT1.2; L3: B2.4; L4: AD6 and L5: AD7.

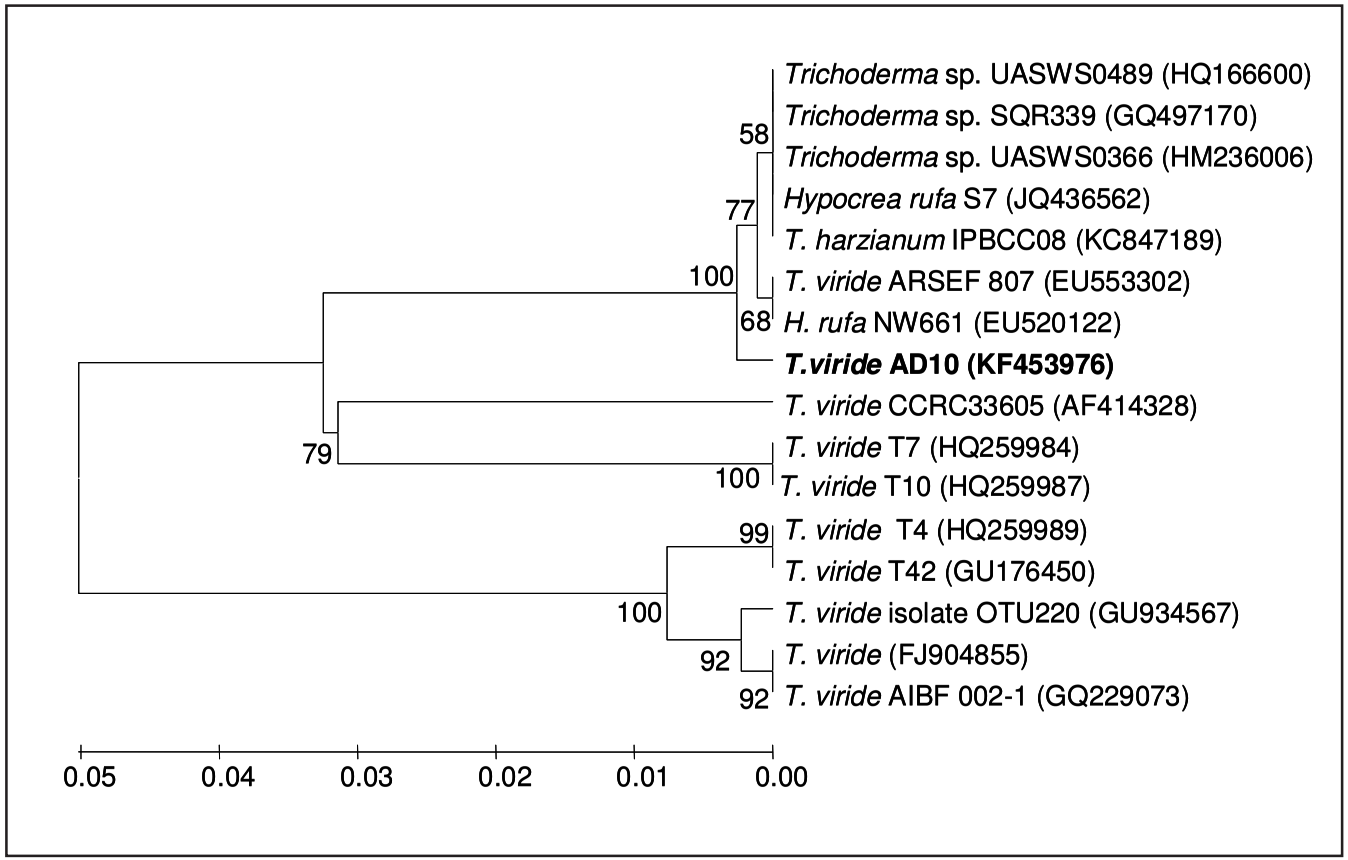


Fig. 4.2.7: Phylogenetic tree of bacterial strains AD10. The evolutionary history was inferred using the Neighbor-Joining method. The significance of each branch is indicated by a bootstrap for 1000 subsets. The GenBank accession number of the sequences compared with the target sequence is provided within the parentheses. Strain represented in bold was isolated during present study.

4.2.12. Identification of fungal antagonistic isolates

A similarity search performed with the 18S rRNA gene partial sequence of the fungal isolates using BLAST tool along with morphological observations and microscopy revealed the identity of the present isolates (Table 4.2.7). It was found that one isolate had close similarity with *Trichoderma* spp., two isolates had close similarity with *Aspergillus* spp., five isolates were similar to *Penicillium* spp., one isolate was similar to *Paecilomyces parvus* while another was similar to *Paecilomyces lilacinus*. The isolate AD10 showed maximum similarity (98%) with *Trichoderma harzianum*, *T. aureoviride*, *T. atroviride*, *Trichoderma* sp. and *T. lixii.* The phylogenetic tree constructed with the gene sequence of AD10 and other *Trichoderma* spp. in the Genbank revealed that AD10 clustered with *T. viride* and other *Trichoderma* sp. with a high bootstrap value (Fig. 4.2.7). However, due to the morphological resemblance of AD10 to *T. viride* and identity confirmation by the ITCC, IARI, New Delhi, the isolate was identified as *T. viride* in the present study. In case of AD2, the morphological and microscopic observations matched with that of *Paecilomyces* spp. and the isolate was also identified as *Paecilomyces parvus* by IARI, New Delhi (Table 4.2.7). Therefore, the 18S rRNA gene sequence was deposited as that of *P. parvus* in the Genbank although the BLAST search showed similarity with *Basidiomycota* sp. Similarly, the BLAST analysis of the 18S rRNA gene sequence of NBT1.2 showed resemblance with *Basidiomycota* sp. but the morphological characters and microscopic observations made in the laboratory identified the isolate as *Penicillium citrinum* which was again confirmed by IARI (Table 4.2.7). BLAST searches with partial 18S rRNA gene sequence of AD7 and AD3 showed 98% similarity with sequences from *Aspergillus* spp. which was in parity with the morphological and microscopic observations. The isolates were therefore, identified as *Aspergillus* sp. Similarly, searches with 18S rRNA gene sequence of AD6 and B2.4 showed 90-95% similarity the 18S rRNA gene sequences of other *Penicillium* spp. and the data were supported by the morphological and microscopic observations (Table 4.2.6b; Fig. 4.2.5b) which enabled us to identify the isolates as *Penicillium* sp. In case of KV8 and NB4, the BLAST searches showed maximum similarity of 80-85% with Ascomycota sp. and *Aspergillus* sp. but it contradicted with the microscopic observations which identified the isolates as *Penicillium* sp. Further, identification of KV8 and NB4 as *Penicillium waskmanii* and *Penicillium implicatum* by IARI, New Delhi confirmed our findings (Table 4.2.7).

Table 4.2.7: List of biocontrol fungal isolates identified based on morphological, microscopic and phylogenetic studies

Bacterial Strains	**GenBank Accession No.**	**IARI Identification Code**	**Identity**
AD2	KF836742	7682.10	*Paecilomyces parvus*
AD3	KF453975	-	*Aspergillus sp.*
AD4	KF836741	9137.13	*Paecilomyces lilacinus*
AD6	KF453974	-	*Penicillium sp.*
AD7	KF453972	-	*Aspergillus sp.*
AD10	KF453976	7683.10	*Trichoderma viride*
B2.4	KF453973	-	*Penicillium sp.*
KV8	KF866294	9135.13	*Penicillium waskmanii*
NB4	KF866295	9136.13	*Penicillium implicatum*
NBT1.2	KF866296	7681.10	*Penicillium citrinum*

MECHANISM OF ACTION OF ANTAGONISTIC MICROORGANISMS

4.3.1. Antifungal metabolite production

Biocontrol microbes are known to produce secondary metabolites like siderophore, cell-wall degrading enzymes and HCN, therefore, all the present antagonistic microbial isolates were tested for production of these antifungal metabolites.

4.3.1.1. Production of hydrolytic enzymes by bacteria

Majority of the antagonistic bacterial isolates were capable of producing several hydrolytic enzymes such as cellulase, chitinase, protease, amylase, and lipase (Table 4.3.1a). Four isolates (ETR1, ETR17, KTR6 and TRB14) were capable of producing chitinase as evident from the clear halo formation under UV light in chitin agar plates (Fig. 4.3.1B). None of the isolates produced pectinase whereas 18 of them exhibited cellulase activity confirmed by the formation of halo around the wells loaded with bacterial culture filtrate (Fig. 4.3.1A). Twenty five isolates scored positive for protease activity as they could hydrolyze casein which was evident from a clear halo production around the line of bacterial growth in opaque casein agar medium (Fig. 4.3.1D). Sixteen isolates were positive for amylase production, indicated by clear halo formation around the bacterial cultures inoculated on starch agar plates flooded with iodine (Fig. 4.3.1C). Lipase production was exhibited by twenty bacterial isolates indicated by translucent halo around the line of bacterial growth. The highest number of enzymes produced by any strain was four which was produced by KTR6, BTR19, BTR21, BTR22, BTR23, TR5, TBD7 and TLB3. Few isolates such as BTRL11, BTRL8, ETR20 and ETR24 did not produce any of the tested enzymes.

4.3.1.2. Production of hydrolytic enzymes by fungal isolates

Test for production of hydrolytic enzymes (cellulase, pectinase, chitinase and DNase) was performed with ten fungal isolates, selected on the basis of higher antifungal activity against the tested fungal pathogens. The results are summarized in Table 4.3.1b. Eight fungal strains (NBT1.2, KV8, B2.4, AD2, AD3, AD6, AD7 and NB4) were found to produce clear non fluorescent zones around culture filtrate bearing wells and thereby scored positive for chitinase test (Fig. 4.3.2C). Cellulase production evident by a clear zone around

Table 4.3.1a: Hydrolytic enzymes produced by the bacterial antagonists

Bacterial Strains	Antifungal enzymes					
	Cellulase	**Chitinase**	**Pectinase**	**Lipase**	**Amylase**	**Protease**
BTR4	-	-	-	-	+	+
BTR8	-	-	-	+	-	+
BTR18	-	-	-	-	-	-
BTR19	+	-	-	+	+	+
BTR21	+	-	-	+	+	+
BTR22	+	-	-	+	+	+
BTR23	+	-	-	+	+	+
BTRL6	-	-	-	+	-	-
BTRL8	+	-	-	-	-	-
BTRL9	+	-	-	-	+	+
BTRL11	-	-	-	-	-	-
D6	-	-	-	+	-	+
D7	+	-	-	+	-	+
D8	+	-	-	-	+	+
ETR1	-	+	-	+	-	+
ETR17	-	+	-	+	-	+
ETR20	-	-	-	-	-	-
ETR24	-	-	-	-	-	-
GH4	-	-	-	+	-	+
GH6	-	-	-	+	-	+
GH12	-	-	-	+	-	+
GH13	+	-	-	-	-	+
GH21	+	-	-	-	+	+
GH22	-	-	-	-	+	+
GH27	+	-	-	-	-	+
GH32	-	-	-	+	-	+
KTR6	+	+	-	-	+	+
TBD7	+	-	-	+	+	+
TR1	-	-	-	+	-	+
TR5	+	-	-	+	+	+
TRB1	+	-	-	-	-	-
TRB14	-	+	-	+	+	-
TRB18	+	-	-	+	-	+
TLB3	+	-	-	+	+	+
TMG1	+	-	-	+	-	+

+: positive; '-': negative

Table 4.3.1b: Hydrolytic enzymes produced by the fungal antagonists

Fungal Strain	Antifungal enzymes			
	Cellulase	**Chitinase**	**Pectinase**	**DNase**
AD2	+	+	-	-
AD3	+	+	-	-
AD4	+	-	-	-
AD6	+	+	-	-
AD7	+	+	-	-
AD10	+	-	-	-
B2.4	+	+	-	-
KV8	+	+	-	-
NB4	+	+	-	-
NBT1.2	+	+	-	-

+: positive; '-': negative

wells containing culture filtrate was exhibited by all the ten fungal strains tested (Fig. 4.3.2B). None of the fungal isolates were capable of exhibiting pectinase and DNase activities.

4.3.1.3. Production of microbial siderophore

Siderophore production is a common antagonistic property observed in many biocontrol strains, thus production of siderophore was tested with all the present microbial antagonistic isolates both qualitatively as well as quantitatively. The chemical nature of the siderophore was also determined in each case.

4.3.1.3.1. Production of siderophore by bacterial antagonists

Twenty five bacterial isolates were found to secrete siderophore in CAS agar plates which was evident by a typical orange halo around the colonies in the bluish green CAS agar medium (Fig. 4.3.1H). For some strains, the colour of the halo around colonies varied from pale yellow to pale red. There were variations in the size of the halo also. Production of all three types of siderophore was recorded when the culture supernatant were tested chemically (Table 4.3.2a). Arnow's test revealed that nineteen bacterial strains produced catecholate type of siderophore. A pink color indicated presence of catecholate in the culture supernatant (Fig. 4.3.1G). Nine strains produced hydroxamate type of siderophore, detected by the formation of deep red coloration when culture supernatant was treated with tetrazolium salt (Fig. 4.3.1I). Spectral

scan in the $FeCl_3$ test showed peaks between 420-460nm which confirmed the presence of hydroxamate siderophore in the culture supernatant. Eight bacterial strains (ETR20, BTR19, BTR21, D7, D8, KTR6, GH13 and BTR22) produced carboxylate type of siderophore, indicated by peak formation between 190-280nm. ETR17 and BTR4 produced both catecholate and hydroxamate siderophore while KTR6, GH13 and TBD7 produced hydroxamate and carboxylate type of siderophores. Some isolates (ETR20, BTR19, BTR21, D7, D8 and TLB3) showed production of two types of siderophores. One bacterial isolate, BTR22, was capable of producing all three types of siderophores.

CAS shuttle assay was used to estimate the amount of siderophore produced by each isolate. Results revealed that BTR19 was the highest producer which recorded 71 percent siderophore units (psu) followed by D8 and GH6 (67 psu) (Table 4.3.2b, Fig. 4.3.3). Other good producers of siderophore were D6 (65 psu), BTR21 (62 psu), BTR4 (61 psu), GH12 (60 psu) and BTR8 (60 psu). Least amount of siderophore production was recorded by TR5 (20 psu) followed by TR1 (22 psu) and TRB1 (22 psu).

4.3.1.3.2. Production of siderophore by fungal antagonists

Amongst the fungal isolates, NBT1.2, AD7 and AD3 produced siderophore on CAS agar plate (Fig. 4.3.2). All the three fungal isolates produced hydroxamate type of siderophore (Table 4.3.2c). The fungal biocontrol isolate AD3 (70 psu) produced maximum amount of siderophore followed by NBT1.2 (61 psu), detected by CAS shuttle assay (Table 4.3.2b). The isolates AD7 (40 psu) was a moderate siderophore producer. A comparative analyses of microbial siderophore production by fungal and bacterial strains (>50% siderophore units) is represented in Fig. 4.3.3.

4.3.1.4. Production of HCN

Three antagonistic bacterial isolates viz. ETR1, GH12, GH21 out of the tested thirty five strains produced hydrogen cyanide which was detected by observing the color change of the filter paper strip attached to the lid of petriplate from yellow to pale red (Fig. 4.3.1E; Table 4.3.3a). All the antagonistic fungal isolates scored negative for HCN production.

Table 4.3.2a: Types of siderophore produced by bacterial isolates[a]

Bacterial strains	Siderophore production	Type of siderophore		
		Catecholate	Hydroxamate	Carboxylate
BTR4	+	+	+	-
BTR8	+	+	-	-
BTR18	-	-	-	-
BTR19	+	+	-	+
BTR21	+	+	-	+
BTR22	+	+	+	+
BTR23	-	-	-	-
BTRL6	-	-	-	-
BTRL8	-	-	-	-
BTRL9	-	-	-	-
BTRL11	-	-	-	-
D6	+	-	+	-
D7	+	+	-	+
D8	+	+	-	+
ETR1	+	+	-	-
ETR17	+	+	+	-
ETR20	+	+	-	+
ETR24	+	+	-	-
GH4	+	+	-	-
GH6	+	+	-	-
GH12	+	-	+	-
GH13	+	-	+	+
GH21	+	-	+	-
GH22	-	-	-	-
GH27	-	-	-	-
GH32	-	-	-	-
TBD7	+	-	+	+
TLB3	+	+	-	+
TMG1	+	+	-	-
TR1	+	+	-	-
TR5	+	+	-	-
TRB1	+	+	-	-
TRB14	-	-	-	-
TRB18	+	+	-	-

+: positive; '-': negative

Table 4.3.2b: Amount of siderophore produced by antagonistic bacterial isolates

Bacterial Strains	**Absorbance at 630 nm (A_s)**	**%Siderophore units $[(A_r^a - A_s)/ A_r^a]x100$ (psu)**
BTR4	0.094	61.0 ± 1.0
BTR8	0.096	60.0 ± 1.5
BTR19	0.070	71.0 ± 1.1
BTR21	0.091	62.0 ± 1.1
BTR22	0.098	59.0 ± 1.2
D6	0.084	65.0 ± 1.1
D7	0.108	55.0 ± 1.2
D8	0.079	67.0 ± 0.8
ETR1	0.101	58.0 ± 1.4
ETR17	0.110	54.0 ± 1.5
ETR20	0.098	59.0 ± 1.0
ETR24	0.139	42.0 ± 1.1
GH4	0.101	58.0 ± 1.1
GH6	0.079	67.0 ± 1.4
GH12	0.096	60.0 ± 1.2
GH13	0.100	58.0 ± 1.6
GH21	0.110	54.0 ± 0.8
TBD7	0.144	40.0 ± 0.9
TLB3	0.139	42.0 ± 1.3
TMG1	0.120	50.0 ± 1.5
TR1	0.187	22.0 ± 1.1
TR5	0.192	20.0 ± 0.7
TRB1	0.187	22.0 ± 1.2
TRB18	0.146	39.0 ± 1.0

[a]: Absorbance of the uninoculated medium at 630nm (Here, Ar =0.240)

Table 4.3.2c: Siderophore characterization and quantitative estimation by siderophore producing antagonistic fungal isolates

Fungal strains	Siderophore[a]	Absorbance at 630nm (A_s)	% Siderophore unit $[(A_r^b - A_s)/A_r^b] \times 100$ (psu)
AD2	-	-	-
AD3	+	0.071	70.0 ± 1.1
AD4	-	-	-
AD6	-	-	-
AD7	+	0.143	40.0 ± 1.0
AD10	-	-	-
B2.4	-	-	-
KV8	-	-	-
NBT1.2	+	0.093	61.0 ± 1.0

[a] '+': positive; '-': negative; only hydroxamate type of siderophore produced; [b]Absorbance of the uninoculated medium at 630nm (Here, Ar =0.240)

4.3.2. Evaluation of PGPR traits

For evaluating PGPR traits, all the antagonistic bacterial and fungal isolates were tested for IAA production and phosphatase activity. Results revealed that, eighteen of the thirty five bacterial isolates were capable of producing IAA; the amount of IAA produced varied from 15µg ml^{-1} to 135µg ml^{-1} under test conditions (Table 4.3.3). The strains ETR20 and GH6 recorded highest production that amounted to 134µg ml^{-1} and 135µg ml^{-1} respectively. Other strains such as BTR18 (90µg ml^{-1}) and ETR24 (86µg ml^{-1}) showed moderate IAA production. Least amount of IAA was produced by TR5 (15µg ml^{-1}) followed by GH13 and GH32 (21µg ml^{-1} each). IAA production was also exhibited by two fungal isolates, AD10 (65µg ml^{-1}) and NB4 (40µg ml^{-1}). A comparative graphical representation of the amount of IAA production by selected microbial antagonists is shown in Fig. 3.4. Phosphatase activity in Pikovskaya's agar was exhibited by the bacterial strains KTR6, ETR20, ETR24, BTR4 and BTR18. This was evident by a clear halo formation around bacterial growth on Pikovskaya's agar plates after 2-5 days of incubation (Fig. 4.3.1F). All the ten tested fungal isolates scored negative for phosphatase activity in the same medium.

4.3.3. Scanning electron microscopy

Scanning electron microscopic study was used to understand the antagonism involved in the interaction between the fungal root pathogen

Table 4.3.3: Test for IAA, phosphatase and HCN produced by bacterial antagonists

Bacterial strains	Phosphatase	IAA (µg ml^{-1})	HCN
BTR4	+	47.0 ± 1.0	-
BTR8	-	28.0 ± 0.9	-
BTR18	+	90.0 ± 1.1	-
BTR19	-	-	-
BTR21	-	60.0 ± 0.9	-
BTR22	-	31.0 ± 1.2	-
BTR23	-	-	-
BTRL6	-	-	-
BTRL8	-	31.0 ± 1.5	-
BTRL9	-	-	-
BTRL11	-	-	-
D6	-	35.0 ± 0.7	-
D7	-	39.0 ± 0.9	-
D8	-	-	-
ETR1	-	39.0 ± 0.8	+
ETR17	-	40.0 ± 1.0	-
ETR20	+	134.0 ± 1.4	-
ETR24	+	86.0 ± 0.8	-
GH4	-	-	-
GH6	-	135.0 ± 0.9	-
GH12	-	58.0 ± 0.9	+
GH13	-	21.0 ± 1.3	-
GH21	-	39.0 ± 1.3	+
GH22	-	-	-
GH27	-	-	-
GH32	-	21.0 ± 0.9	-
KTR6	+	50.0 ± 0.9	-
TBD7	-	-	-
TLB3	-	-	-
TMG1	-	-	-
TR1	-	-	-
TR5	-	15.0 ± 1.1	-
TRB1	-	-	-
TRB14	-	-	-
TRB18	-	-	-

+: positive; '-': negative; '±': standard error, mean of three replicates

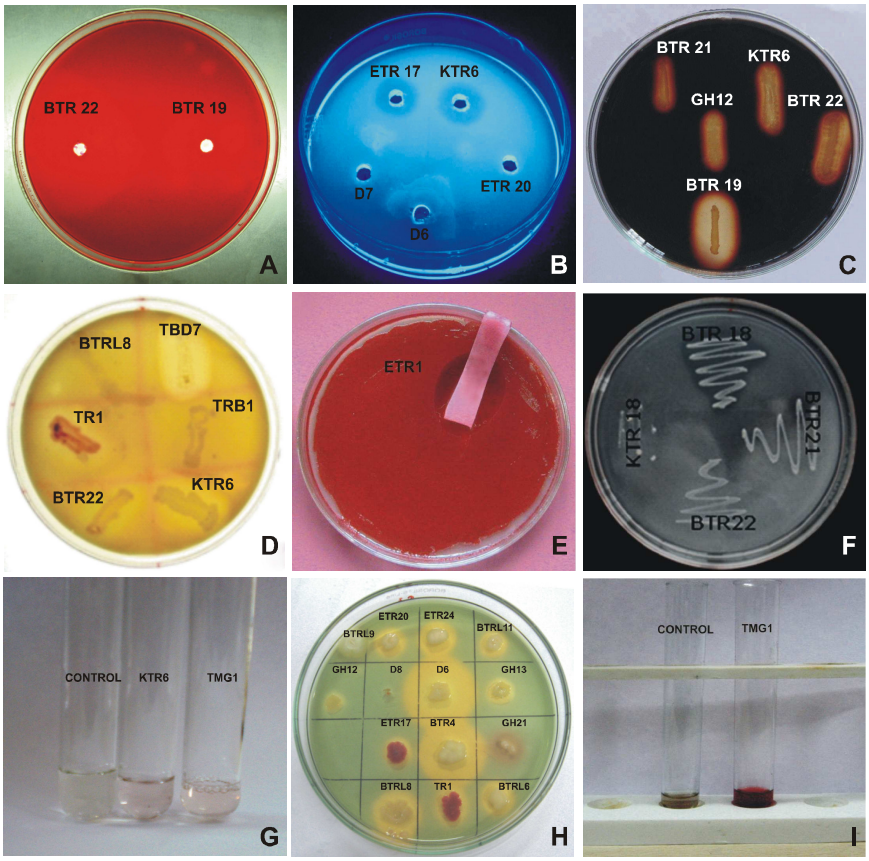


Fig. 4.3.1: Production of antifungal metabolites by antagonistic bacterial isolates:

(A) Cellulase production by BTR22 and BTR19 evident from the halo formation on cellulose agar.

(B) Non-fluorescent lytic zones around the wells under UV light indicating chitinase production by ETR17 and KTR6. Strains D6, D7 and ETR20 tested negative for chitinase activity.

(C) Amylase production indicated by clear zone in deep blue background around the colonies shown by strains BTR21, KTR6, GH12, BTR22 and BTR19.

(D) Clear halo around colonies on casein agar plate indicating protease production by strains TBD7, TR1, BTR22 and KTR6 while BTRL8 and TRB1tested negative.

(E) Red coloration on filter paper strip indicating HCN production by ETR1.

(F) Phosphate solubilization by BTR18 isolate on Pikovskaya's agar medium exhibited by clearing around the bacterial growth. Other strains (BTR21, BTR22, KTR18) tested negative.

(G) Catecholate type of siderophore production by strains KTR6 and TMG1 indicated by pink coloration in Arnow's test.

(H) Production of siderophore by strains ETR20, ETR24, BTRL11, GH12, D6, GH13, ETR17, BTR4, GH21, BTRL8, TR1 and BTRL6 evident by the orange halo around bacterial colonies in CAS agar medium.

(I) Formation of red coloration by TMG1 culture filtrate in tetrazolium salt test confirming hydroxamate type of siderophore production.

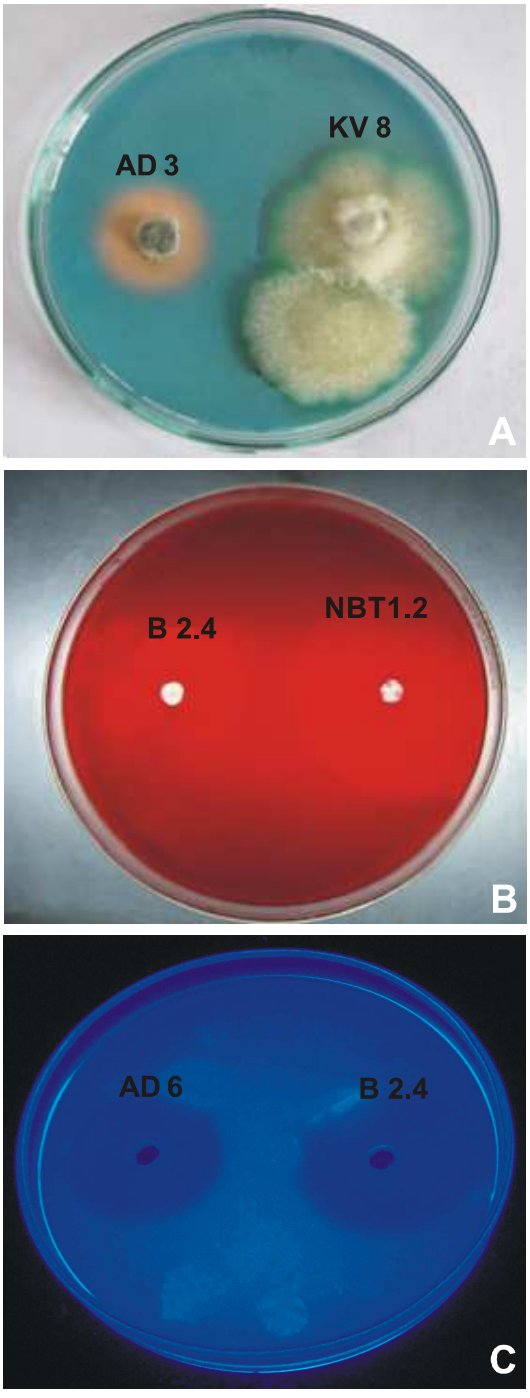


Fig.4.3.2: Production of antifungal metabolites by antagonistic fungal isolates:
(A) Siderophore production by fungal antagonist AD3 indicated by the orange halo around bacterial colonies in CAS agar medium. Strain KV8 tested negative.
(B) Cellulase production by B2.4 and NBT1.2 exhibited by halo formation on cellulose agar medium
(C) Non-fluorescent lytic zones around the wells under UV light indicating chitinase production by AD6 and B2.4.

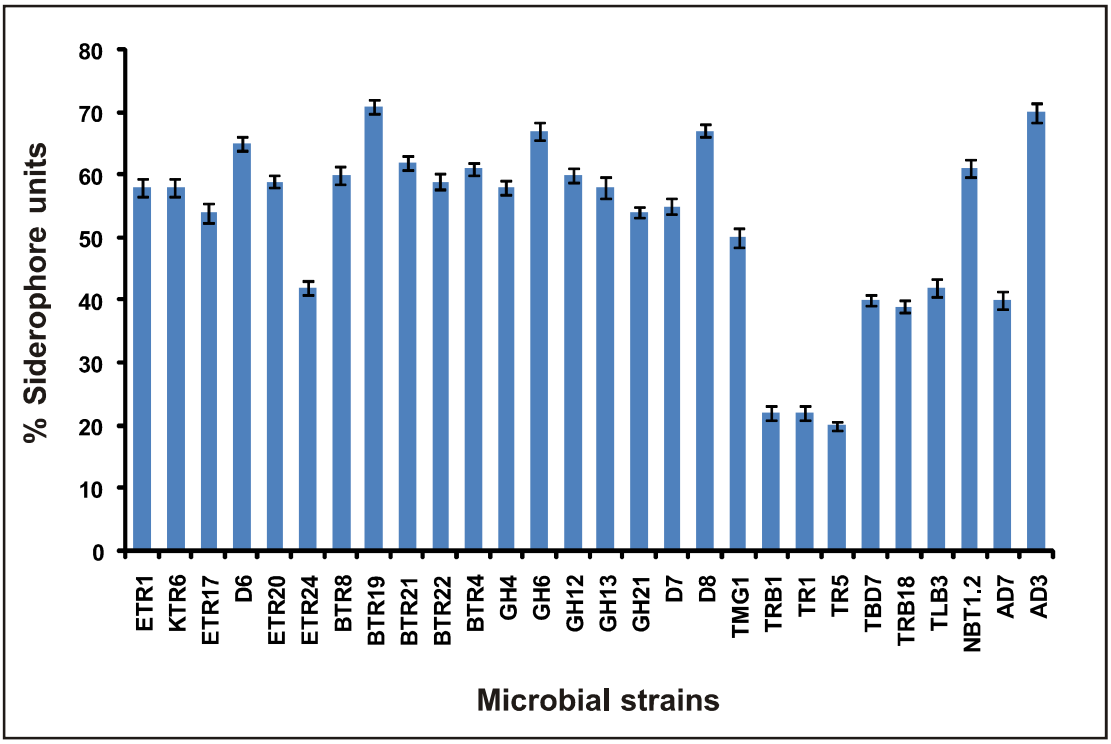


Fig. 4.3.3: Siderophore production by bacterial and fungal antagonists.

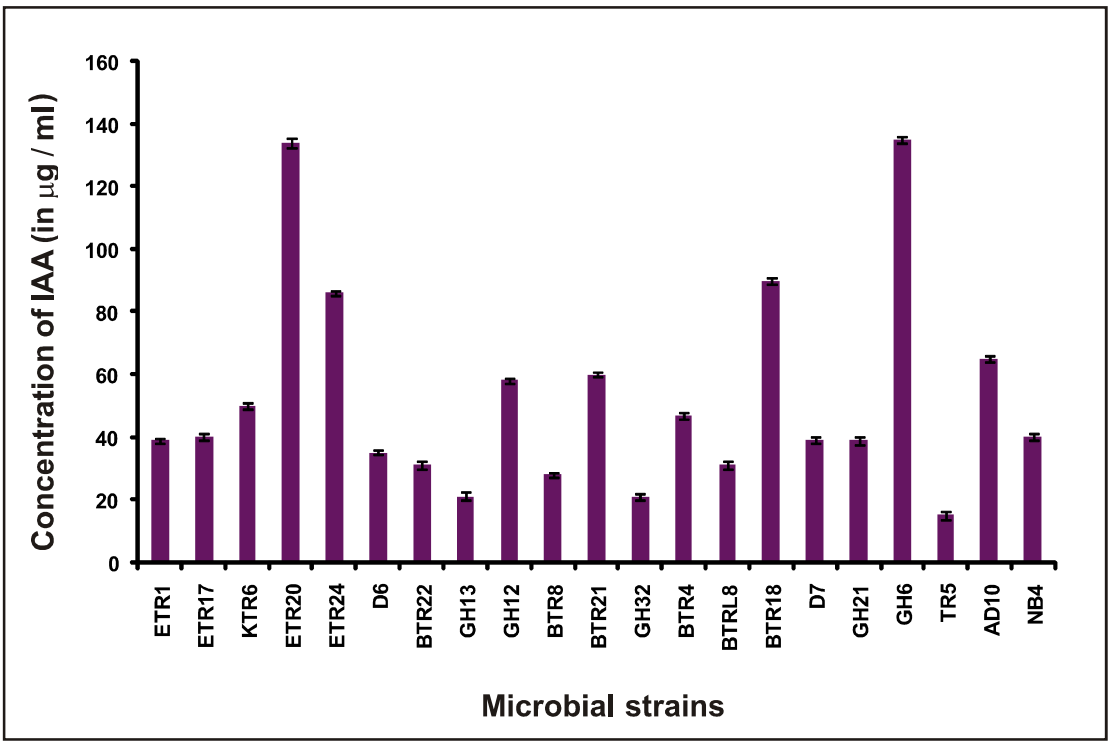


Fig. 4.3.4: Amount of IAA produced by selected microbial antagonists.

Rhizoctonia solani and two potential bacterial strains ETR17 and KTR6. The results of the study are represented in Fig. 4.3.5. From the photographs, severe deformations of the fungal mycelia like surface irregularities, bulging and bursting at specific sites is evident. Bursting of the fungal mycelia resulted in release of the protoplasm from fungal cell (Fig. 4.3.5A,B). KTR6 caused rapid degeneration of fungal cell wall and release of cellular contents of the pathogen (Fig. 4.3.5A). The secondary metabolites and diffusible hydrolytic enzymes produced the bacterial isolates are the possible cause of such degradation of pathogen mycelia. ETR17 caused clearing of hyphal fluid in some areas of the mycelia (Fig. 4.3.5B). The fungal isolate AD10 parasitized the pathogen *R. solani* as evident from the mycelia of AD10 winding around pathogen hyphae in the microscopic field (Fig. 4.3.5C). The results were compared with the control where the mycelium of *R. solani* remained normal (Fig. 4.3.5D).

4.3.4. Detection of antibiotics in culture supernatant

Antibiotic production by all the present bacterial isolates was tested using the concentrated cell free culture supernatants. Antibiotics were detected in twenty strains grown on three different media, CPM-Ca^{2+}, PPM and YEM based on the spectral scan in UV and visible range of light spectrum. The wavelength maxima obtained (Table 4.3.4a) almost matched with the standards which were obtained at 324 nm (pyoluteorin), 225 nm (pyrrolnitrin), 535 nm (prodigiosin), 374 nm (phenazine) and 237 & 313 nm (pyocyanin). Pyrrolnitrin was detected in GH6, ETR20, ETR24, ETR1, ETR17, GH12, BTRL6, BTRL8, BTR8, D6, D7 and TR1 in YEM and PPM media with absorbance maxima between 210-225nm. Pyoluteorin was detected in D6 cultured in both YEM and PPM media at 324nm while 2,4-diacetylphloroglucinol (DAPG) was detected in GH12 grown in PPM medium only at 274nm. Phenazine production was recorded in BTRL6 in both YEM and PPM media at 368nm. The antibiotic prodigiosin was detected in ETR1, ETR17 and TR1 having wavelength maxima of 535/536nm. Lipopeptide/peptide antibiotic production was detected in the sterile culture supernatants of KTR6, BTR4, BTRL11, BTR21, BTR22, BTR19 and BTR23 grown in CPM-Ca^{2+} medium at 270-274nm and 220-230nm.

Table 4.3.4a: Spectrophotometric detection of antibiotics produced by antagonistic gram negative bacterial isolates[a]

Bacterial strains	Media used	Wavelength of Absorbance	Antibiotics produced maxima (nm)
BTR8	YEMB	225	Pyrrolnitrin
	PPM	225	Pyrrolnitrin
BTR18	YEMB	220	Pyrrolnitrin
	PPM	220	Pyrrolnitrin
BTRL6	YEMB	368	Phenazine
	PPM	368	Phenazine
BTRL8	YEMB	210	Pyrrolnitrin
	PPM	224	Pyrrolnitrin
D6	YEMB	220 & 324	Pyrrolnitrin & Pyoluteorin
	PPM	220 & 324	Pyrrolnitrin & Pyoluteorin
D7	YEMB	212	Pyrrolnitrin
	PPM	220	Pyrrolnitrin
ETR1	YEMB	218	Pyrrolnitrin & Prodigiosin
	PPM	221 & 536	Pyrrolnitrin & Prodigiosin
ETR17	YEMB	220 & 536	Pyrrolnitrin & Prodigiosin
	PPM	220 & 536	Pyrrolnitrin & Prodigiosin
ETR20	YEMB	210	Pyrrolnitrin
	PPM	210	Pyrrolnitrin
ETR24	YEMB	212	Pyrrolnitrin
	PPM	221	Pyrrolnitrin
GH6	YEMB	224	Pyrrolnitrin
	PPM	224	Pyrrolnitrin
GH12	YEMB	225	Pyrrolnitrin
	PPM	272	DAPG
TR1	YEMB	220 & 535	Pyrrolnitrin & Prodigiosin
	PPM	220 & 535	Pyrrolnitrin & Prodigiosin

[a] Abbreviations: YEMB: Yeast extract mannitol broth; PPM: Pigment producing medium

Table 4.3.4b: Spectrophotometric detection of antibiotics produced by antagonistic gram positive bacterial isolates[a]

Bacterial strains	Media used	Wavelength of Absorbance maxima (nm)	Antibiotics produced
BTR4	CPM-Ca^{2+}	274	Lipopeptide/ Peptide
BTR19	CPM-Ca^{2+}	224	Lipopeptide/ Peptide
BTR21	CPM-Ca^{2+}	274	Lipopeptide/ Peptide
BTR22	CPM-Ca^{2+}	225 & 270	Lipopeptide/ Peptide
BTR23	CPM-Ca^{2+}	225 & 273	Lipopeptide/ Peptide
BTRL11	CPM-Ca^{2+}	272	Lipopeptide/ Peptide
KTR6	CPM-Ca^{2+}	224 & 274	Lipopeptide/ Peptide

[a] Abbreviations: CPM-Ca^{2+}: Casamino acid peptone mannitol-Ca^{2+}

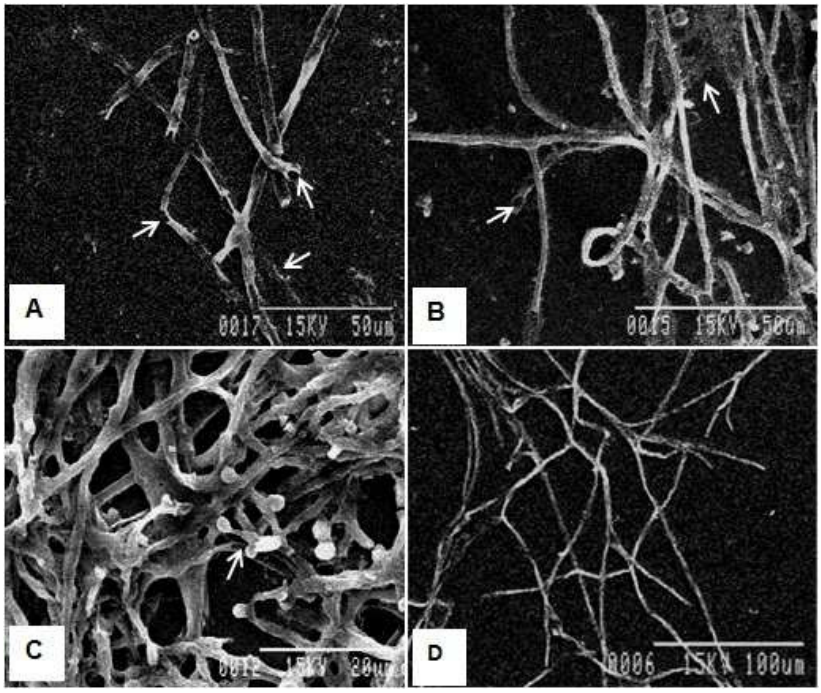


Fig. 4.3.5: Scanning electron microphotographs showing the antagonistic effect of biocontrol microbial isolates on *Rhizoctonia solani*.

(A) Lysis of mycelia caused by *B. subtilis* strain KTR6 (indicated by arrows).

(B) Mycelial distortion caused by *S. marcescens* strain ETR17 (indicated by arrows).

(C) Mycoparasitism exhibited by *T. viride* strain AD10.

(D) Intact mycelia of the pathogen *R. solani*.

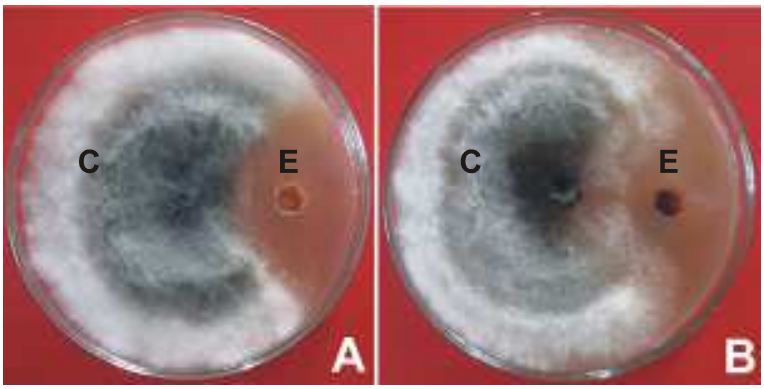


Fig. 4.3.6: Inhibition of mycelial growth of the pathogen *L. theobromae* by crude extracellular culture extract (E) obtained from the antagonistic bacterial isolate *B. subtilis* strain KTR6 (fig.A) and *S. marcescens* strain ETR17 (fig.B). The control well (C) containing acetone did not produce inhibition zone.

4.3.5. Antifungal activity of extracellular culture filtrates

The extracellular culture extract obtained from the bacterial isolates *S. marcescens* ETR17 and *B. subtilis* KTR6 were studied *in vitro* for inhibition of pathogen growth in PDA plates. The extract containing the diffusible antifungal metabolite secreted by the antagonistic bacteria produced clear zones of inhibition around the wells against *L. theobromae* (Fig 4.3.6). The MIC values produced by extracellular metabolites of ETR17 and KTR6 were recorded as 100 μgml^{-1} and 110 μgml^{-1} respectively.

4.3.6. Assessment of biofilm formation by microtitre plate assay

Biofilm formation by all the thirty five antagonistic bacterial isolates was assessed by measuring the intensity of crystal violet stained bacterial cells adhered to the wells of a 96 well polystyrene or microtiter plate. The extent of biofilm formation was tested in two different media, LB and M9YE. Results revealed that biofilm formation increased with the increase in incubation period i.e. higher biofilm formation was observed after 48h of incubation of the bacterial strains in microtiter plates compared to 24h of incubation (Table 4.3.6). The bacterial strains showed better biofilm production in LB medium compared to M9YE medium. Maximum biofilm formation capability in both the media was exhibited by the strain GH6 which recorded an OD value of 1.027 in LB and 0.837 in M9YE medium after 48 h incubation. BTR23 (O.D.= 0.802) and BTR8 (O.D.= 0.770) also showed good biofilm formation after 48 h in LB medium. On the other hand TLB3 recorded minimum OD value in both LB (0.116) and M9YE (0.105) media.

Table 4.3.6: Biofilm formation by antagonistic bacterial isolates in two different media at two different time periods of incubation

Bacterial Strains	C.V. OD (at 595nm)			
	Luria Bertani medium		M9 yeast extract medium	
	24h	48h	24h	48h
BTR4	0.257	0.389	0.072	0.414
BTR8	0.508	0.770	0.506	0.568
BTR18	0.076	0.247	0.063	0.151
BTR19	0.335	0.389	0.265	0.355
BTR21	0.433	0.535	0.261	0.479
BTR22	0.352	0.518	0.099	0.349
BTR23	0.255	0.802	0.150	0.529
BTRL6	0.155	0.332	0.116	0.308
BTRL8	0.113	0.334	0.036	0.321
BTRL9	0.154	0.621	0.118	0.127
BTRL11	0.432	0.499	0.375	0.381
D6	0.261	0.439	0.245	0.399
D7	0.280	0.379	0.229	0.254
D8	0.202	0.707	0.113	0.287
ETR1	0.204	0.281	0.111	0.183
ETR17	0.254	0.448	0.118	0.193
ETR20	0.339	0.349	0.203	0.245
ETR24	0.181	0.248	0.118	0.191
GH4	0.147	0.574	0.094	0.537
GH6	0.593	1.027	0.407	0.837
GH12	0.122	0.219	0.110	0.212
GH13	0.163	0.247	0.134	0.228
GH21	0.129	0.435	0.106	0.298
GH22	0.113	0.298	0.103	0.224
GH27	0.111	0.364	0.105	0.223
GH32	0.133	0.441	0.122	0.390
KTR6	0.180	0.238	0.152	0.228
TBD7	0.336	0.519	0.229	0.415
TLB3	0.116	0.182	0.105	0.132
TMG1	0.330	0.381	0.234	0.312
TR1	0.122	0.381	0.114	0.245
TR5	0.384	0.434	0.238	0.374
TRB1	0.433	0.617	0.312	0.547
TRB14	0.119	0.383	0.108	0.312
TRB18	0.384	0.517	0.312	0.474

OCCURRENCE OF ANTIFUNGAL COMPOUNDS IN CRUDE EXTRACELLULAR EXTRACT OF *S. marcescens* ETR17 CULTURE

4.4.1. Antifungal activity detection by agar cup bioassay

In order to purify and determine the chemical nature of the antifungal principle, the crude and concentrated extracellular culture extracts were fractionated by silica gel column chromatography. Four fractions F2, F4, F9 and F10 were found to exhibit antifungal activity by agar cup bioassay method against all three test pathogens (*R. solani, L. theobromae* and *S. repens*) as evident by the formation of clear zone around the wells cut on pathogen seeded PDA plates. Four other fractions i.e. F1, F3, F5 and F7 were active against *R. solani* and *L. theobromae* whereas the fractions F6 and F8 did not exhibit antagonistic activity against any of the pathogens used in the study.

4.4.2. Antifungal activity detection by bioautography

The crude concentrated acetone extract of obtained from ETR 17 was separated by TLC and the developed plates were sprayed with the spore suspension of *L. theobromae*. A clear zone of inhibition of the mycelial growth was observed at R_f = 0.3 and R_f = 0.6 (Fig. 4.4.1). The column purified fractions F2, F4, F9 and F10 exhibiting maximum antifungal activity were further tested by bioautography technique. It was found that for F2 and F4, a clear zone of inhibition against *L. theobromae* was observed at R_f = 0.6. For the fractions F9 and F10, inhibition of mycelial growth was detected by the formation of a large clear zone with an R_f = 0.8.

4.4.3. Detection of antibiotics in partially purified column fractions by TLC

The antifungal fractions F2, F3, F4, F9 and F10 were further assessed for detection of antifungal compound(s) by TLC. The standard antibiotics pyrrolnitrin and prodigiosin were detected at R_f = 0.80 and R_f = 0.60 respectively in thin layer chromatograms (Fig. 4.4.2). The pyrrolnitrin antibiotic standard produced a deep green colored quenching at 254nm whereas the prodigiosin standard produced a reddish pink colored band under visible light. Comparative analysis of each bioactive fraction with standard antibiotics in TLC plates under UV light revealed that the major compound in fractions 9 and 10 produced spots at migrated to R_f = 0.80 which was similar to the spot produced by standard pyrrolnitrin. Similarly prodigiosin was detected in fractions F2, F3 and F4 (R_f = 0.6).

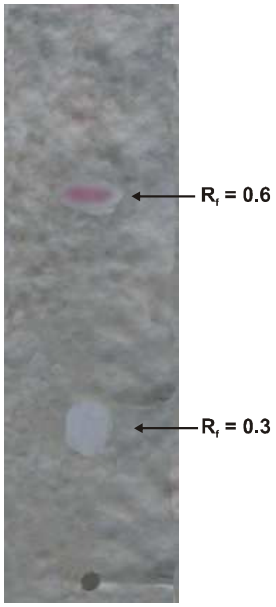


Fig. 4.4.1: Zones of inhibition of fungal growth (*L. theobromae*) produced by extracellular crude extract of *S. marcescens* ETR17 on bioautography plates.

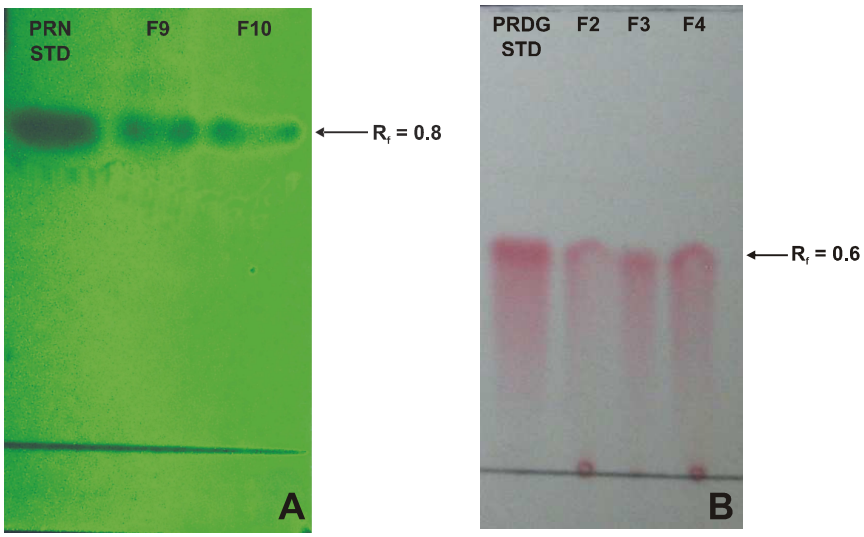


Fig. 4.4.2: Detection of antibiotics in extracellular culture extract of the antagonistic *S. marcescens* strain ETR17 by thin layer chromatography:

(A) Pyrrolnitrin detection under ultra-violet light (254nm) in the partially purified fractions F9 and F10 obtained previously by silica gel column chromatography of the crude culture extract.

(B) Detection of prodigiosin in the partially purified column fractions F2, F3 and F4 under visible light [Standard prodigiosin (PDG) and pyrrolnitrin (PRN) spotted in extreme left in both (A) and (B)].

4.4.3.1. UV-VIS Spectrophotometric analyses of partially purified antibiotics recovered from preparative TLC plates

In order to confirm the occurrence of antibiotics, the compounds at the spot regions detected by analytical TLC were recovered following preparative TLC and further analyzed by UV-VIS spectrophotometry and HPLC. UV-VIS scan revealed that the fractions F9 and F10 produced two sharp peaks at 210nm and 249nm which was similar to the pyrrolnitrin standard (Fig. 4.4.3a). The standard pyrrolnitrin produced two sharp peaks: the first major one at 217nm and second one at 251nm (Fig. 4.4.3a). No peak was observed in the visible range indicating the absence of prodigiosin in the TLC purified fractions F9 and F10. On the contrary, for the fractions F2, F3 and F4 where prodigiosin was detected by TLC, a sharp peak at 536nm (visible region) was produced corresponding to the prodigiosin standard (Fig. 4.4.3b).

4.4.3.2. HPLC analysis of partially purified antibiotics recovered from preparative TLC plates

Comparison of the HPLC profiles obtained for the purified compounds recovered from preparative TLC plates with the profiles of standard antibiotics revealed that a single major peak, which was obtained with the fractions F9 and F10 at retention time (RT) of 3.024 min and 3.022 min respectively was comparable to standard pyrrolnitrin (RT: 3.020 min) (Fig. 4.4.4a). The antibiotic, purified from fractions F2, F3 and F4 by preparative TLC, were combined prior to HPLC analysis. The sample produced two major peaks (RT: 8.268 min and 9.024min) which was similar to that of the standard prodigiosin (RT: 7.813 min; and 9.018) (Fig. 4.4.4b).

4.4.3.3. LC-ESI-MS analysis of partially purified antifungal metabolite

Direct liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis of the combined bioactive fractions F9 and F10 obtained by column chromatography revealed molecular ion peak at m/z 259 $(M + 2H)^+$ (Fig. 4.4.5). This confirmed the presence of pyrrolnitrin in the extracellular culture extract of *S. marcescens* ETR17. The combined column fractions F9 and F10 obtained previously from silica gel column chromatography of the crude culture extract was analysed using a gradient elution program with methanol and ammonium acetate buffer at a flow rate of 0.8 ml min^{-1} at 254 nm to obtain a major peak at retention time 14.85 minutes corresponding to pyrrolnitrin.

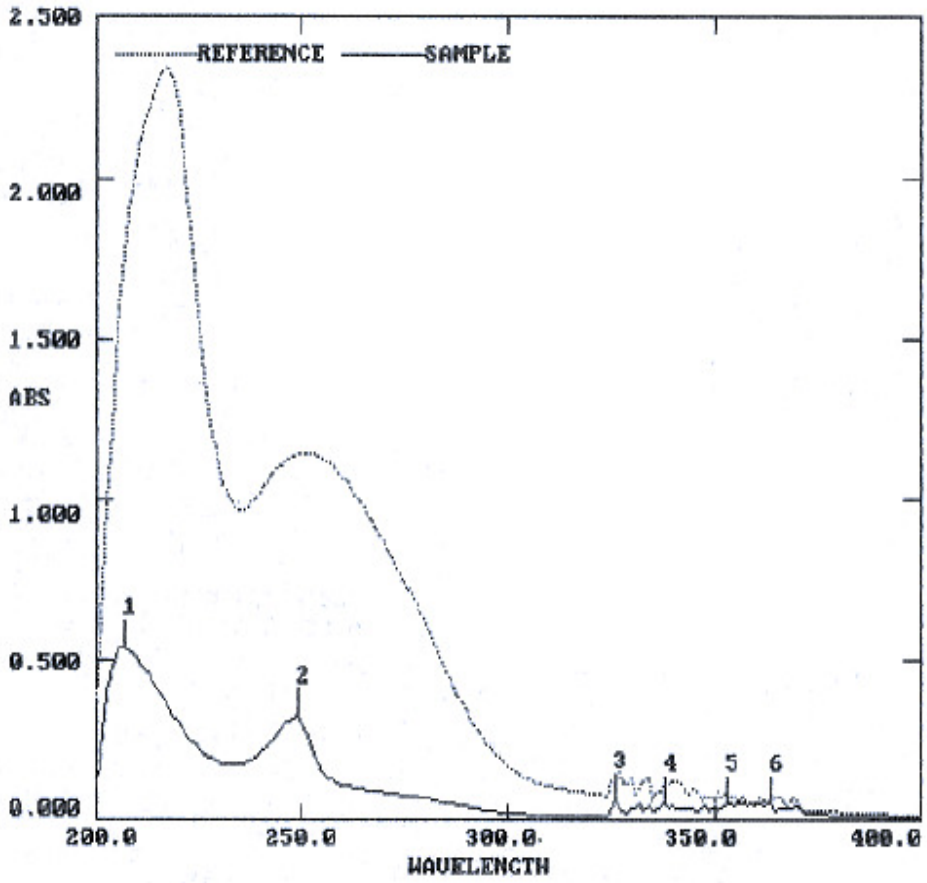


Fig. 4.4.3a: Detection of pyrrolnitrin in extracellular culture extract of the antagonistic *S. marcescens* strain ETR17 by UV-Spectrophotometric analyses (between 200nm to 400nm). Pyrrolnitrin was purified by preparative thin layer chromatography from fraction F9 obtained previously from silica gel column chromatography of the crude culture extract. Two sharp peaks at 210nm and 249nm obtained with the partially purified compound was similar to the pyrrolnitrin standard (217nm and 251nm).

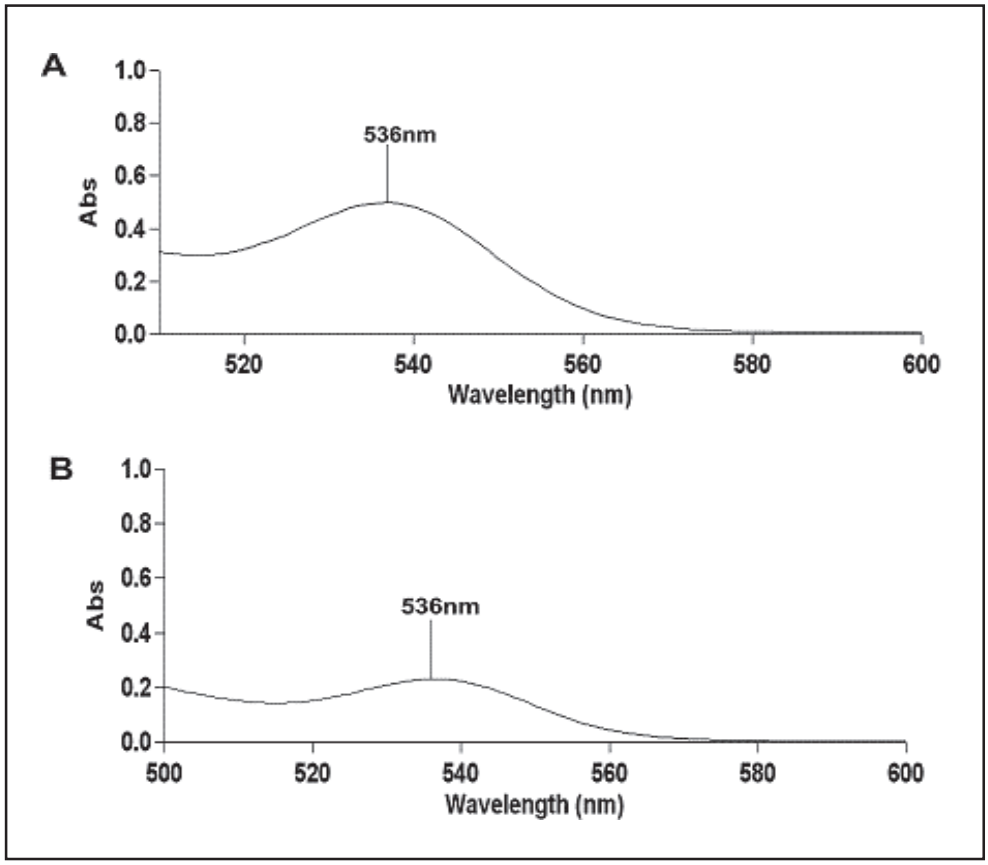


Fig. 4.4.3b: Detection of prodigiosin in extracellular culture extract of the antagonistic *S. marcescens* strain ETR17 by VIS-Spectrophotometric analyses (between 500nm to 600nm). Prodigiosin was purified by preparative thin layer chromatography from fraction F2 obtained previously from silica gel column chromatography of the crude culture extract. (A) Prodigiosin standard having absorbance maxima at 536nm. (B) A peak at 536nm obtained with the partially purified compound.

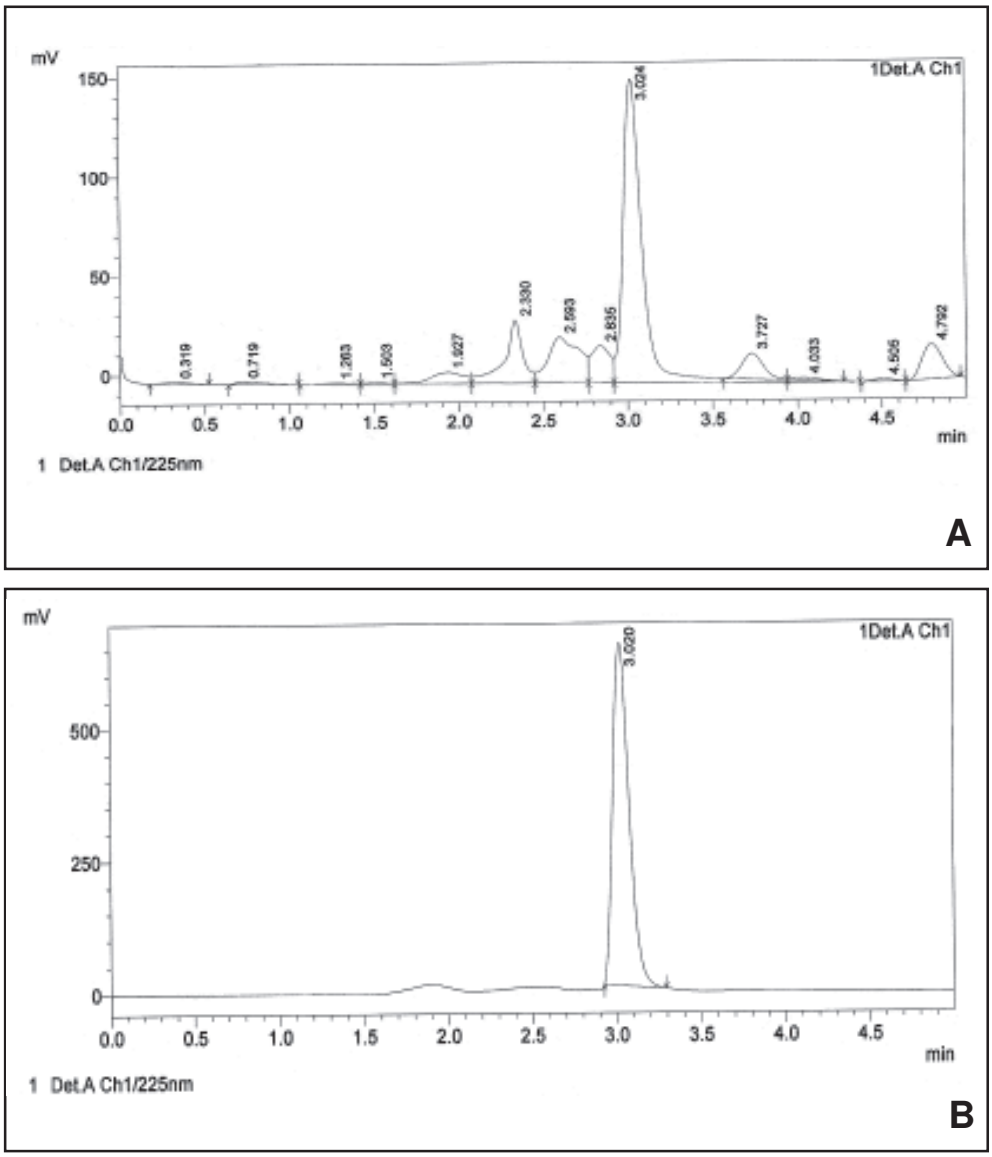


Fig. 4.4.4a: Detection of pyrrolnitrin in extracellular culture extract of the antagonistic *S. marcescens* strain ETR17 by high performance liquid chromatography. (A) Partially purified fraction F9 obtained previously from silica gel column chromatography of the crude culture extract was eluted isocratically in 100% methanol at a flow rate of 1ml min^{-1} at 225 nm to obtain a major peak at retention time = 3.024 min. (B) Chromatogram of standard pyrrolnitrin showing a peak at retention time = 3.020 min.

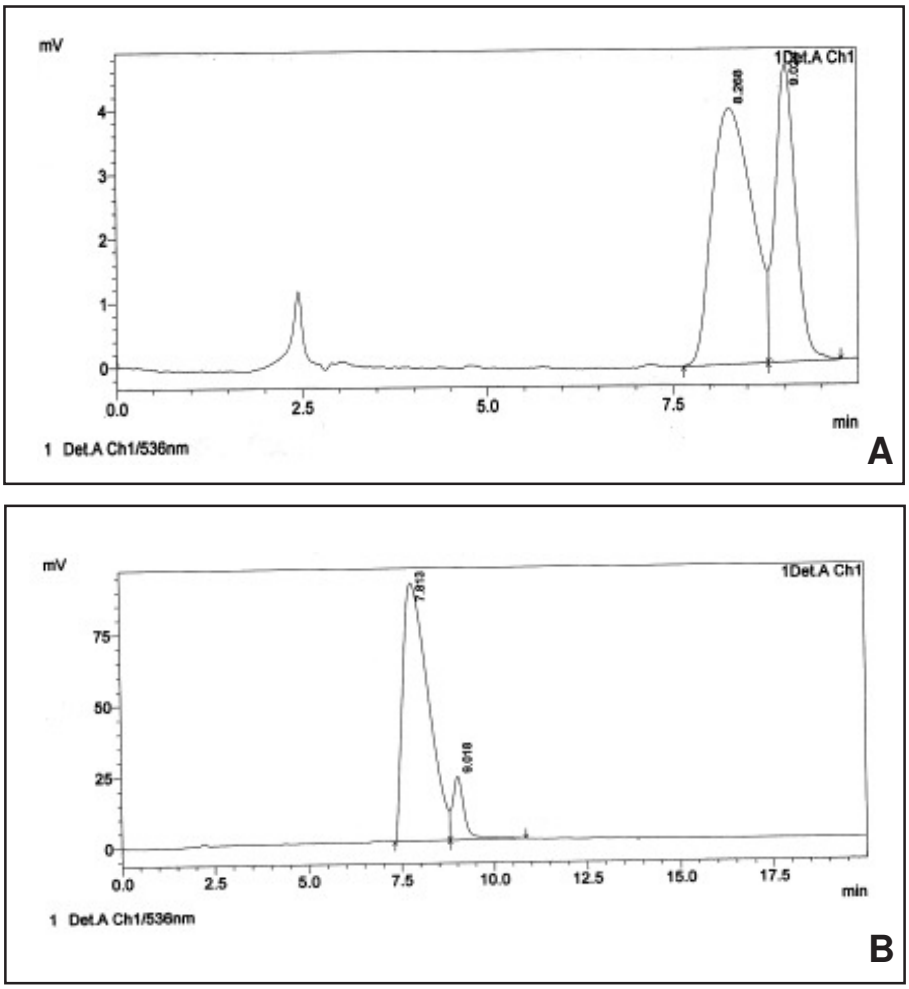


Fig. 4.4.4b: Detection of prodigiosin in extracellular culture extract of the antagonistic *S. marcescens* strain ETR17 by high performance liquid chromatography. (A) Partially purified combined fractions F2, F3 and F4 obtained previously from silica gel column chromatography of the crude culture extract was eluted isocratically in 100% methanol at a flow rate of 1ml min^{-1} at 536 nm to obtain two major peaks at retention time = 8.268 min and 9.024 min. (B) Chromatogram of standard prodigiosin also showing two peaks at retention time = 7.813 min and 9.018 min.

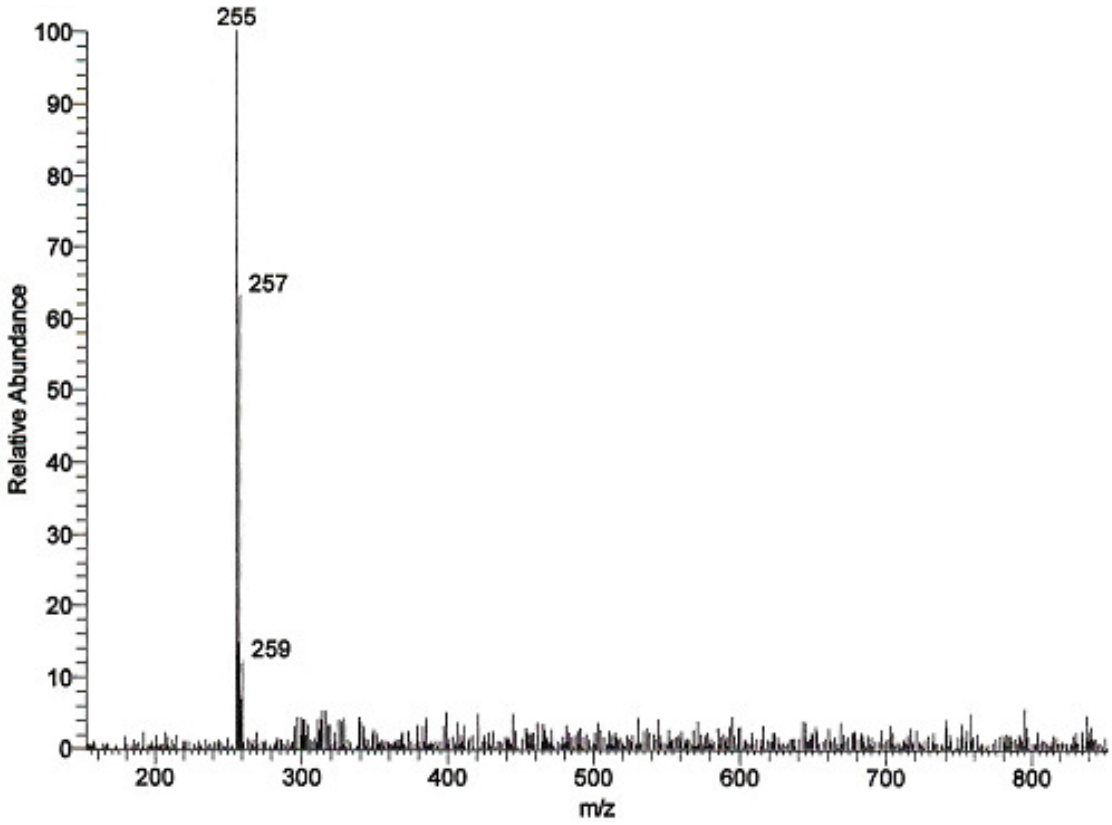


Fig. 4.4.5: Detection of pyrrolnitrin in extracellular culture extract of the antagonistic *S. marcescens* strain ETR17 by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis. The combined column fractions F9 and F10 obtained previously from silica gel column chromatography of the crude culture extract was analysed using a gradient elution program with methanol and ammonium acetate buffer at a flow rate of 0.8 ml min^{-1} at 254 nm to obtain a major peak at retention time 14.85 minutes corresponding to pyrrolnitrin. Mass spectra showing molecular ion peak at m/z 259 $(M + 2H)^+$ indicating pyrrolnitrin.

In vivo EVALUATION OF THE BIOCONTROL EFFICACY OF THE BACTERIAL ISOLATES

4.5.1. Pathogenicity test and verification of Koch's postulates

All inoculated plants of TS-520 variety showed severe blackening along with foul odour of the roots after 7 days of inoculation with the pathogen *R. solani* which is an indicative of root rotting (Fig. 4.5.1). The variety TS-462 also showed disease symptoms after 10 days of pathogen inoculation but the severity was less compared to TS-520 (Fig. 4.5.1). The fungal culture isolated from the excised roots was found to be similar to that of the inoculated pathogen *R. solani* when compared based on microscopic observations. The reisolation of *R. solani* from infected root parts confirmed it as a pathogen of tea and thereby the Koch's postulations were verified.

4.5.2. Biocontrol of root rot disease in tea using bacterial formulations

Talc based formulations of two powerful biocontrol isolates ETR17 and KTR6 (*S. marcescens* and *B. subtilis* respectively) were evaluated for their ability to protect tea seedlings (TS-520 variety) against *Rhizoctonia* root rot caused by *R. solani* (Fig. 4.5.2a and 4.5.2b). TS-520 variety was selected for the study since it was more susceptible towards *R. solani* infection compared to TS-462. The results summarized in Table 4.5.1 revealed that the strains could suppress the disease both individually and in mixed condition regardless of the soil being sterilized or unsterilized. However, protection provided by mixed formulations scored better not only when compared to the individual strains but also in comparison to the fungicide. Of the two strains, ETR17 exhibited higher disease control efficiency than KTR6. The efficacy of disease reduction of ETR17 and KTR6 was found to be 90.5% and 86% respectively when tested individually; but it increased to 93.0% when applied in mixed formulations under sterile soil condition (Table 4.5.1, Fig. 4.5.3b). The symptoms of root rot disease was noticed in the control set after 10 days of inoculation but only after 20 days in the sets treated with biocontrol bacteria. The disease progression was unhindered in the control set which recorded a mean disease index value of 73.3 (unsterile soil) by the 45th day (Fig. 4.5.3a). But, in the treated sets, the mean disease index generally did not rise or rose only slightly at the 35th day and showed prominent signs of recovery by the 45th day (Fig. 4.5.3a). The overall disease control efficiency was slightly lower in unsterile

Table 4.5.1: Inhibition of root-rot caused by *R. solani* in tea seedlings (TS-520) by powdered formulations prepared with antagonistic bacterial isolates ETR17 and KTR6

Treatments	Mean disease index				PEDC[a]	
	15dapi[b]	25dapi	35dapi	45dapi	35dapi	45dapi
Control (Sterilized soil)	26.7 ± 1.6	43.3 ± 1.6	58.3 ± 1.6	71.7 ± 1.7	0	0
Control (Unsterilized soil)	28.3 ± 1.4	46.7 ± 1.7	63.3 ± 1.5	73.3 ± 1.3	0	0
Fungicide (Sterilized soil)	0	18.3 ± 1.4	21.7 ± 1.4	11.7 ± 1.2	62.8 ± 1.4	84.0 ± 1.6
Fungicide (Unsterilized soil)	0	23.3 ± 1.6	23.3 ± 1.6	15.0 ± 1.6	63.2 ± 1.3	79.1 ± 1.7
ETR17 (Sterilized soil)	0	13.3 ± 1.7	13.3 ± 1.7	6.8 ± 1.5	77.2 ± 1.0	90.5 ± 1.5
ETR17 (Unsterilized soil)	0	15.0 ± 1.4	18.3 ± 1.3	8.3 ± 1.0	71.1 ± 1.5	89.1 ± 1.0
KTR6 (Sterilized soil)	0	21.7 ± 1.5	20.0 ± 1.4	10.0 ± 1.3	65.7 ± 1.6	86.0 ± 1.3
KTR6 (Unsterilized soil)	0	23.3 ± 1.4	23.3 ± 1.6	11.3 ± 1.4	63.2 ± 1.2	84.6 ± 1.5
ETR17 + KTR6 (Sterilized soil)	0	6.7 ± 1.6	8.3 ± 1.5	5.0 ± 1.5	85.8 ± 1.7	93.0 ± 1.6
ETR17 + KTR6 (Unsterilized soil)	0	8.3 ± 1.7	13.3 ± 1.7	6.8 ± 1.7	80.0 ± 1.3	90.7 ± 1.8
LSD	2.2	4.7	4.6	4.1	3.8	4.1

[a]PEDC= Percent efficacy of disease control; [b]dapi= days after pathogen inoculation; Data represents the mean '±' standard error LSD = Least significant difference at P = 0.05

soil in comparison to sterile soil, evident from a higher disease index and lower PEDC values in almost all treatments.

4.5.3. Plant growth promotion by bacterial formulations

Plant growth was enhanced to a desirable extent by both the bacterial strains (Fig. 4.5.4). Among the two strains used, KTR6 showed better plant growth promotion trait compared to ETR17 treatment both in sterilized and unsterilized soil sets (Table 4.5.2). ETR17 increased shoot and root length of tea seedlings in sterilized soil set by 6cm and 4.7cm respectively while shoot and root length in unsterilized soil set was increased by 6.4cm and 3.8cm respectively. The isolate KTR6 increased the shoot and root length in sterilized soil set by

Table 4.5.2: Effect of powder formulations of biocontrol bacterial isolates ETR17 and KTR6 on growth of tea plants (TS-520) during *in vivo* study

Treatment	Plant height					
	Shoot length (cm)		Increase	Root length (cm)		Increase
	(0 day)	(45 days)	in shoot length (cm)	(0 day)	(45 days)	in root length (cm)
Control (Sterilized soil)	12.0±1.4	15.0±1.6	3.0±0.9	7.5±1.4	10.0±1.1	2.5±1.1
Control (Unsterilized soil)	11.6±1.8	15.0±1.1	3.4±1.3	10.0±1.0	12.0±1.3	2.0±1.3
ETR17 (Sterilized soil)	12.0±1.3	18.0±0.9	6.0±1.3	8.5±1.3	13.2±1.3	4.7±1.6
ETR17 (Unsterilized soil)	12.8±1.9	19.2±1.2	6.4±1.5	10.0±1.0	13.8±1.6	3.8±1.4
KTR6 (Sterilized soil)	12.5±1.7	20.2±1.7	7.7±1.4	10.2±1.4	13.7±1.5	3.5±1.4
KTR6 (Unsterilized soil)	11.0±1.7	21.0±1.4	10.0±1.2	8.9±1.1	14.2±1.3	5.3±1.6
ETR17 + KTR6 (Sterilized soil)	12.5±1.4	24.2±1.6	11.7±1.1	10.4±1.3	14.5±1.3	4.1±1.2
ETR17 + KTR6 (Unsterilized soil)	12.3±1.8	23.7±1.3	11.4±1.5	10.2±1.4	14.4±1.4	4.2±1.2
LSD	4.9	4.1	3.9	3.8	4.0	4.1

Data represents the mean '±' standard error, LSD= Least significant difference at P = 0.05

7.7cm and 3.5cm respectively and in unsterilized soil treatment KTR6 increased the shoot and root length by 10.0cm and 5.3cm respectively (Table 4.5.2). The mixture of the two strains showed maximum growth promotion of the tea seedlings as evident from the results (Table 4.5.2). Treatment with mixed formulations in sterilized soil led to an increase in shoot and root length by 11.7cm and 4.1cm respectively while application in unsterilized soil set enhanced shoot length by 11.4cm and root length by 4.2cm (Table 4.5.2).

4.5.4. Viability of bacterial cells (*S. marcescens* ETR17) in talc formulation

The bacterial cell population of isolate ETR17 remained static upto six months in the talc formulation when stored at room temperature and gradually decreased from the seventh month (Fig. 4.5.5).

4.5.5. Sustainability of *S. marcescens* strain ETR17 in the rhizosphere

Results of indirect ELISA (Table 4.5.3) showed that the absorbance value (at 492nm) of antigen-antibody reaction conducted with soil antigens prepared from the pots inoculated with ETR17 and ETR17+ KTR6 and ETR17 antiserum

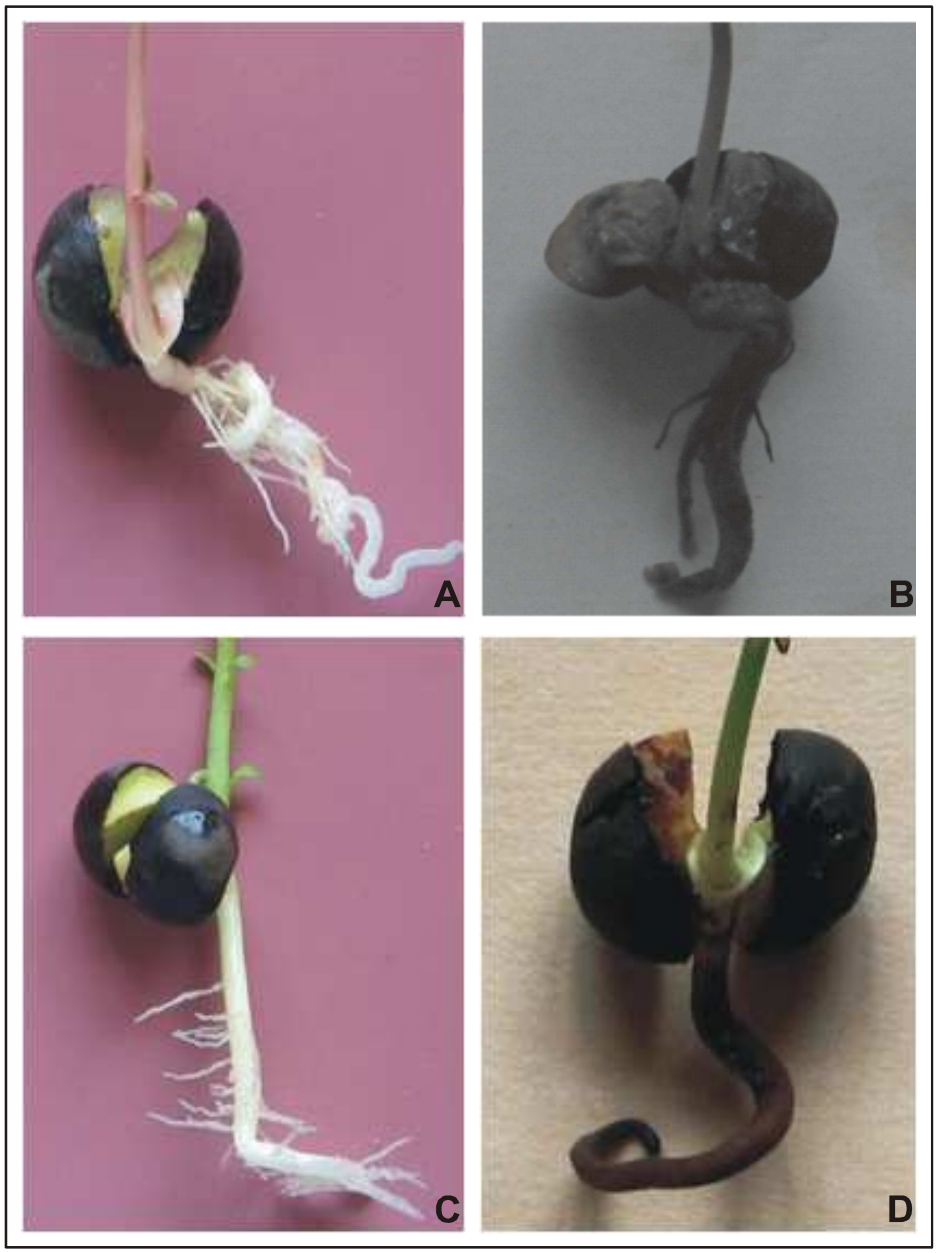


Fig. 4.5.1: Root rot disease in young germinated tea seedlings of TS-520 and TS-462 varieties 15 days after challenge inoculation with the pathogen *R. solani.*

(A) Uninfected healthy root of uninoculated control (TS-520 variety).

(B) Infected root of pathogen inoculated TS-520 seedling.

(C) Uninfected healthy root of uninoculated control (TS-462 variety).

(D) Infected root of pathogen inoculated TS-462 seedling.

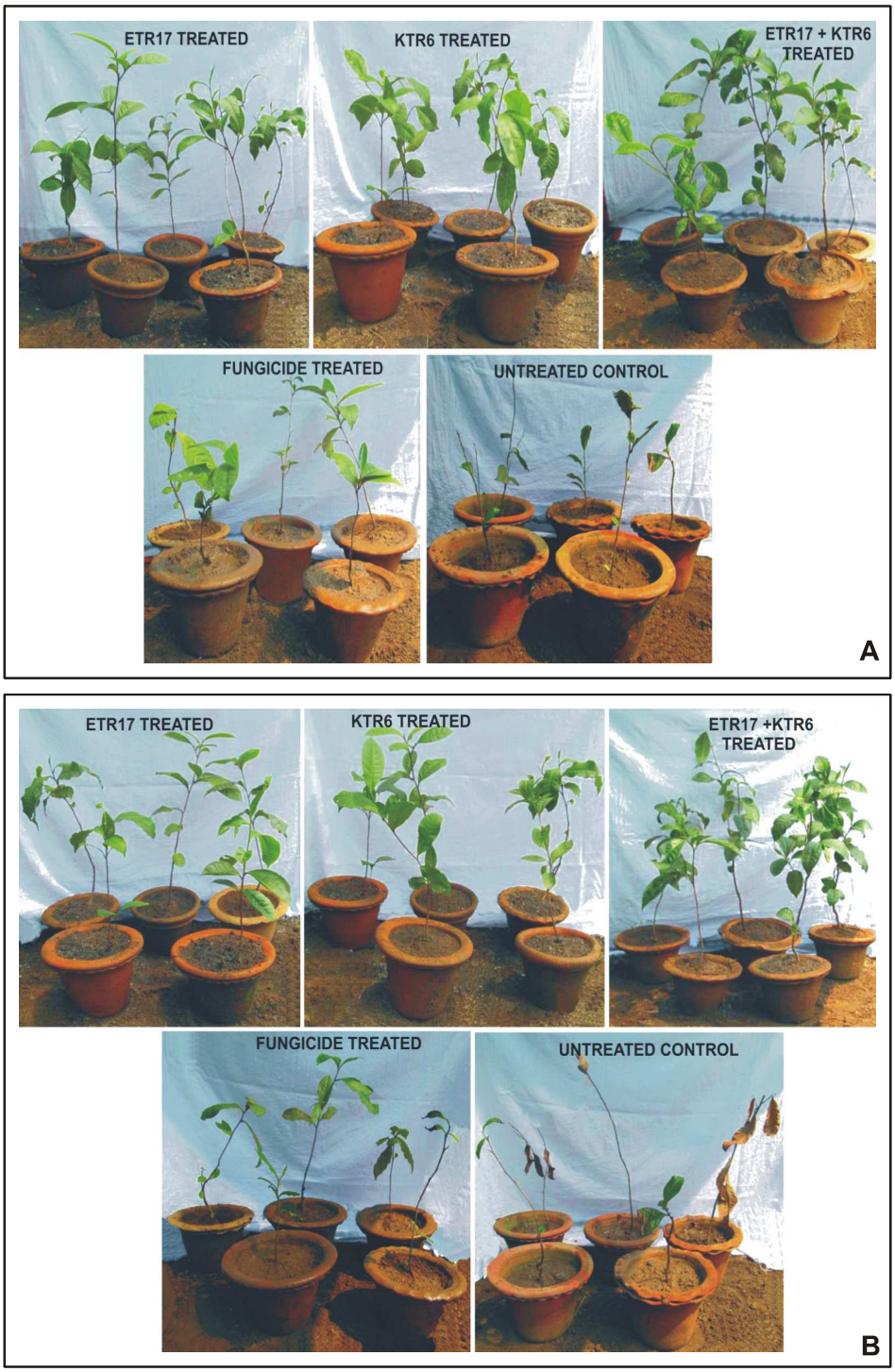


Fig. 4.5.2a: Reduction of root rot disease in tea (TS-520 variety) after 45 days of treatment by talc based formulations prepared individually with ETR17 and KTR6 and as mixture using both KTR6 and ETR17 strains under (A) Sterile and (B) Unsterile conditions.

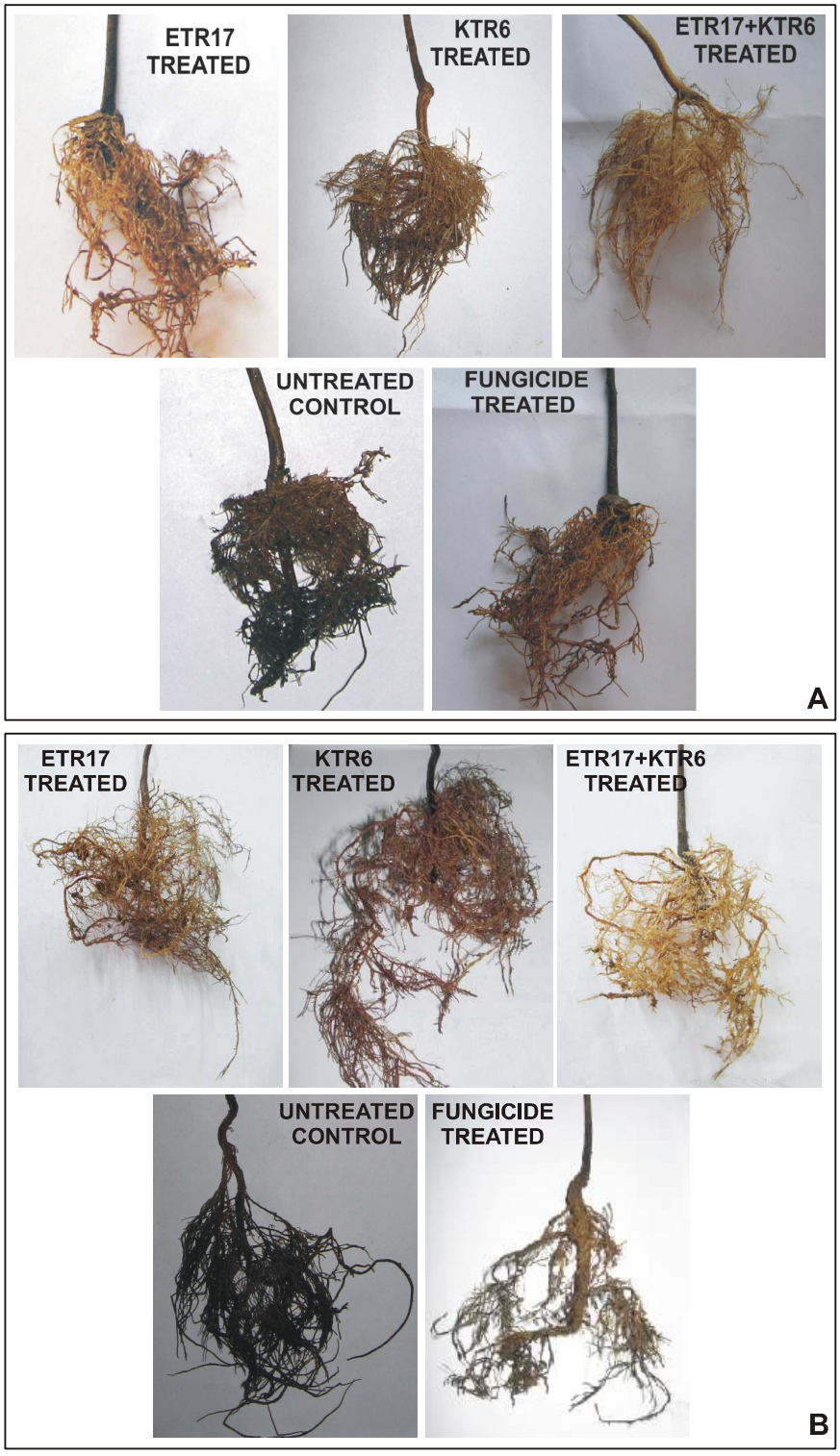


Fig. 4.5.2b: Root rot disease occurrence in tea (TS-520 variety) after 45 days of treatment by talc based formulations prepared individually with ETR17 and KTR6 and as mixture using both KTR6 and ETR17 strains under (A) Sterile and (B) Unsterile conditions.

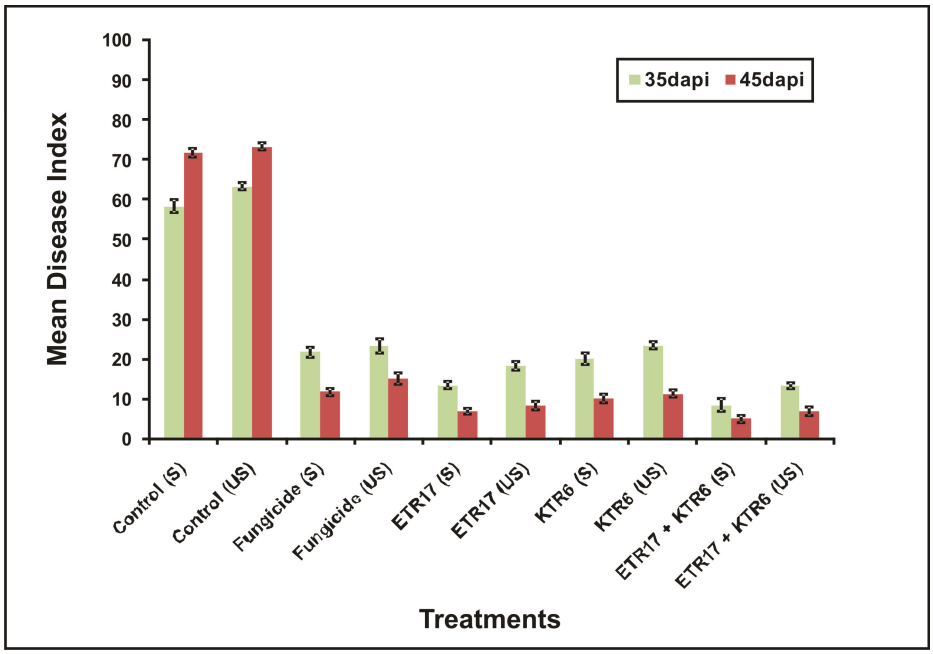


Fig. 4.5.3a: Inhibition of root rot caused by *R. solani* in tea seedlings by biocontrol formulations containing antagonistic bacterial strains ETR17 and KTR6 in individual and mixed conditions under sterile (S) and unsterile (US) conditions 35 days after pathogen inoculation (35dapi) and 45 days after pathogen inoculation (45dapi).

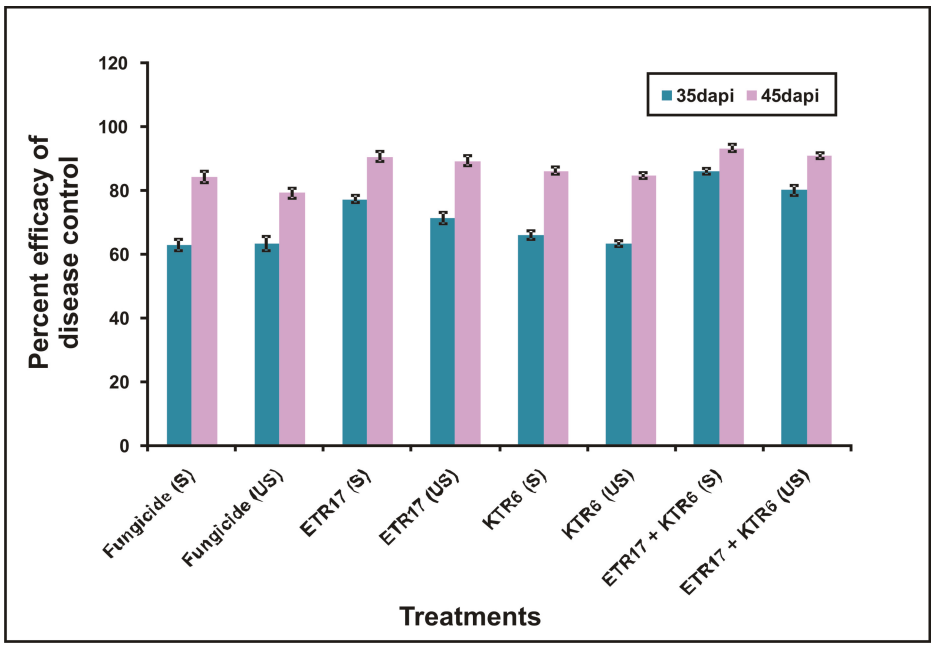


Fig. 4.5.3b: Percent efficacy of disease control (PEDC) of root rot disease in tea seedlings by individual and mixed formulations of the antagonistic bacterial strains ETR17 and KTR6 under sterile (S) and unsterile (US) conditions, 35 days after pathogen inoculation (35dapi) and 45 days after pathogen inoculation (45dapi).

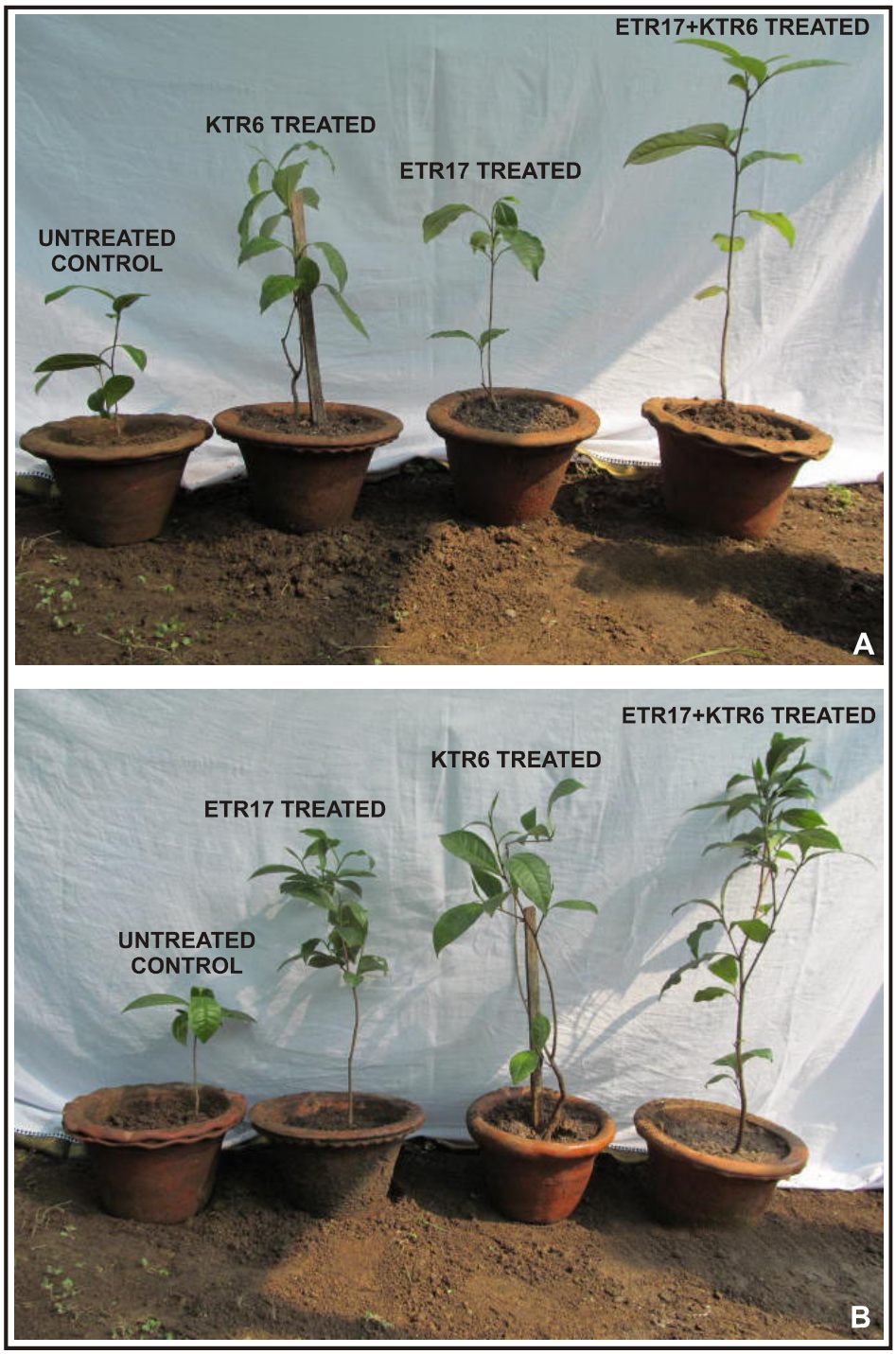


Fig. 4.5.4: Effect of the talc-based formulations prepared with individual strains KTR6 and ETR17 and strain mixture of KTR6 and ETR17 on the growth of tea seedlings (TS-520 variety) under (A) Sterile and (B) Unsterile conditions.

was higher than that of uninoculated soil antigen and antigen prepared from soil inoculated with KTR6 alone (negative control). Positive control was set with ETR17 whole cell antigen which was used to raise the antibody in the rabbit, showing a high absorbance value of 0.744 at 492nm after reacting with the specific homologous antibody. The concentration of the bacterial cell present in the positive control (whole cell antigen of ETR17) was determined to be $1.2x10^9$ cfu/ml (Table 4.5.3) by plate counting on NA medium. A standard curve was plotted with varying concentrations against the corresponding ELISA values of the positive control (data not shown) which was used to determine the concentration of the bacterium *S. marcescens* ETR17 in the soil after *in vivo* study. The polyclonal antibody raised against ETR17 was specific and therefore, no cross reaction was observed when reacted with KTR6 antigen. This study confirmed the presence of ETR17 in tea rhizosphere on application of talc formulation of both the individual strain and also when the strain was used in association with KTR6 both in sterilized soil and unsterilized soil experimental sets. Bacterial concentration was estimated to be $8x10^8$ cfu g^{-1} and $9x10^8$ cfu g^{-1} in the soil which was originally sterilized and unsterilized respectively 45 days after application of the formulation to the soil (Table 4.5.3). In case of mixed formulation treatments $7x10^8$ $cfug^{-1}$ and $8x10^8$ $cfug^{-1}$ concentration of the bacterium was recorded in sterilized and unsterilized soil respectively after 45 days of treatment. This study confirmed the presence of ETR17 in rhizosphere on application of talc formulations of the individual strain and also when the strain was used in association with KTR6.

4.5.6. Colonization of tea roots by bacterial isolate ETR17

Examination of transverse sections of root of tea seedling treated with ETR17 suspension portrayed abundant colonization of some portions of the roots by the bacterium (Fig. 4.5.6).

4.5.7. Biocontrol of root rot disease in tea using fungal antagonist

As evident from the results of dual culture test on PDA medium, the most potent fungal antagonist *Trichoderma viride* AD10 was used to control *R. solani* root rot in tea seedlings *in vivo* (Fig. 4.5.7). It was observed that AD10 could effectively reduce the root rot disease incidence in a susceptible tea variety TS-520 upto 90.8% under sterile conditions and 85.7% under unsterile conditions after 45 days of challenge inoculation of the tea roots by *R. solani*

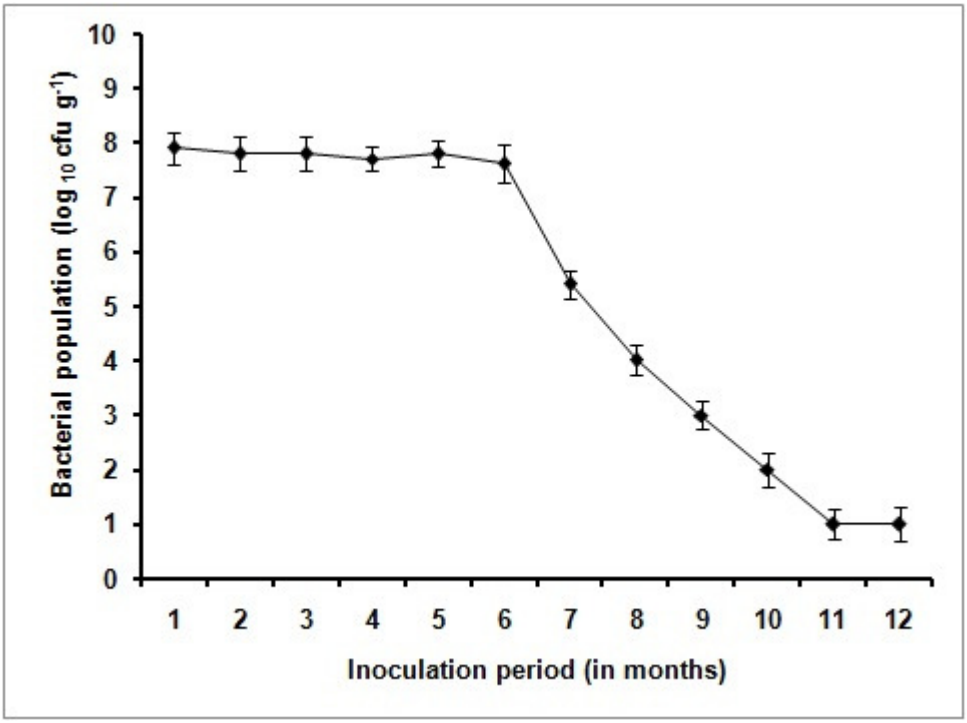


Fig. 4.5.5: Viability of *S. marcescens* ETR17 isolate in talc formulation. The bacterial cell population was measured for 12 months at 1 month time interval.

Table 4.5.3: ELISA detection of *S. marcescens* strain ETR17 by antigen-antisera reaction between soil antigens prepared from tea rhizosphere soil, treated with biocontrol formulations; and ETR17 antiserum after 45 days of soil application

Treatment	**Absorbance values at 492nm**	**Bacterial concentration (cfu/g soil)**
Control (Sterilized soil)	0.010	0
Control (Unsterilized soil)	0.166	1.0×10^8
ETR17 (Sterilized soil)	0.581	8.0×10^8
ETR17 (Unsterilized soil)	0.660	9.0×10^8
ETR17 + KTR6 (Sterilized soil)	0.499	7.0×10^8
ETR17 + KTR6 (Unsterilized soil)	0.589	8.0×10^8
Positive control		**Bacterial concentration (cfu/ml)**
ETR17 (whole cell antigen)	0.744	1.2×10^9
Negative control		
KTR6 (Sterilized soil)	0.087	0
KTR6 (Unsterilized soil)	0.204	0

Antisera dilution: 1:100; goat anti-rabbit IgG HRP conjugate (secondary antibody dilution): 1:10,000

(Table 4.5.4; Fig. 4.5.8a and 4.5.8b). The disease symptoms were observed after 10 days of pathogen inoculation for both the sterile and unsterile treatments using the fungal strain AD10. The fungal antagonist was more effective in comparison to the treatment by chemical fungicide thiophanate methyl (Table 4.5.4; Fig. 4.5.8a and 4.5.8b).

Table 4.5.4: Effect of fungal strain AD10 on biocontrol of root rot disease caused by *R. solani* in tea seedlings (TS-520)

Treatments	**Mean Disease Index**				**PEDC[a]**	
	15dapi[b]	**25dapi**	**35dapi**	**45dapi**	**35dapi**	**45dapi**
Control (Sterilized Soil)	43.3 ± 1.6	63.3 ± 1.5	71.7 ± 1.6	73.3 ± 1.7	0	0
Control (Unsterilized Soil)	58.3 ± 1.7	71.7 ± 1.6	76.7 ± 1.7	81.6 ± 1.4	0	0
Fungicide (Sterilized soil)	8.3 ± 1.5	15.0 ± 1.2	26.7 ± 1.5	16.7 ± 1.5	62.8 ± 1.5	77.2 ± 1.5
Fungicide (Unsterilized soil)	13.3 ± 1.3	21.7 ± 1.0	31.7 ± 1.8	20.0 ± 1.3	58.7 ± 1.6	75.5 ± 1.6
AD10 (Sterilized soil)	6.7 ± 1.3	13.3 ± 1.2	18.3 ± 1.4	6.7 ± 1.2	74.5 ± 1.7	90.8 ± 1.7
AD10 (Unsterilized soil)	10.0 ± 1.4	20.0 ± 1.3	23.3 ± 1.3	11.7 ± 1.0	69.6 ± 1.8	85.7 ± 1.4
LSD	3.8	3.7	4.1	4.0	3.8	4.1

[a]PEDC= Percent efficacy of disease control; [b]dapi= days after pathogen inoculation; Data represents the mean '±' standard error LSD= Least significant difference at P = 0.05

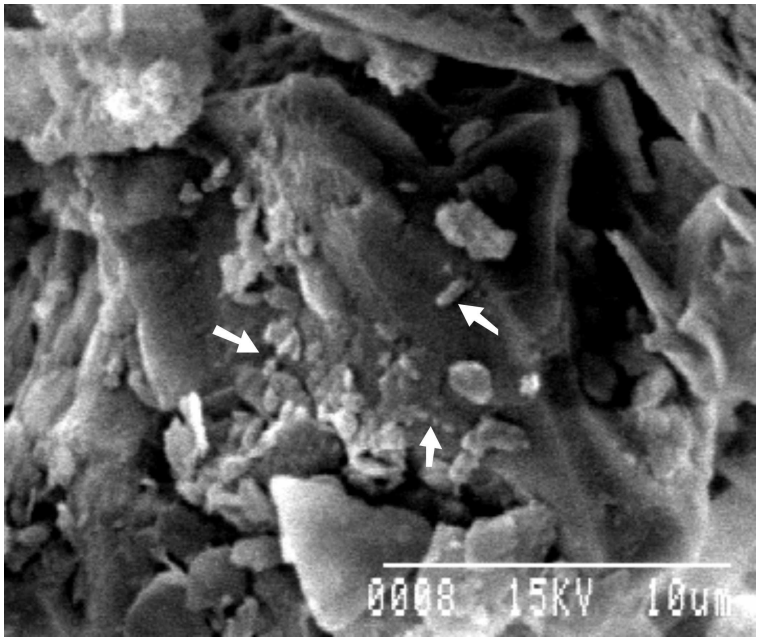


Fig. 4.5.6: Scanning electron micrograph of transverse section of root of tea seedling exhibiting the internal colonization by bacterial strain ETR17 (indicated by arrows) after 24h of inoculation with bacterial suspension ($1x10^8$).

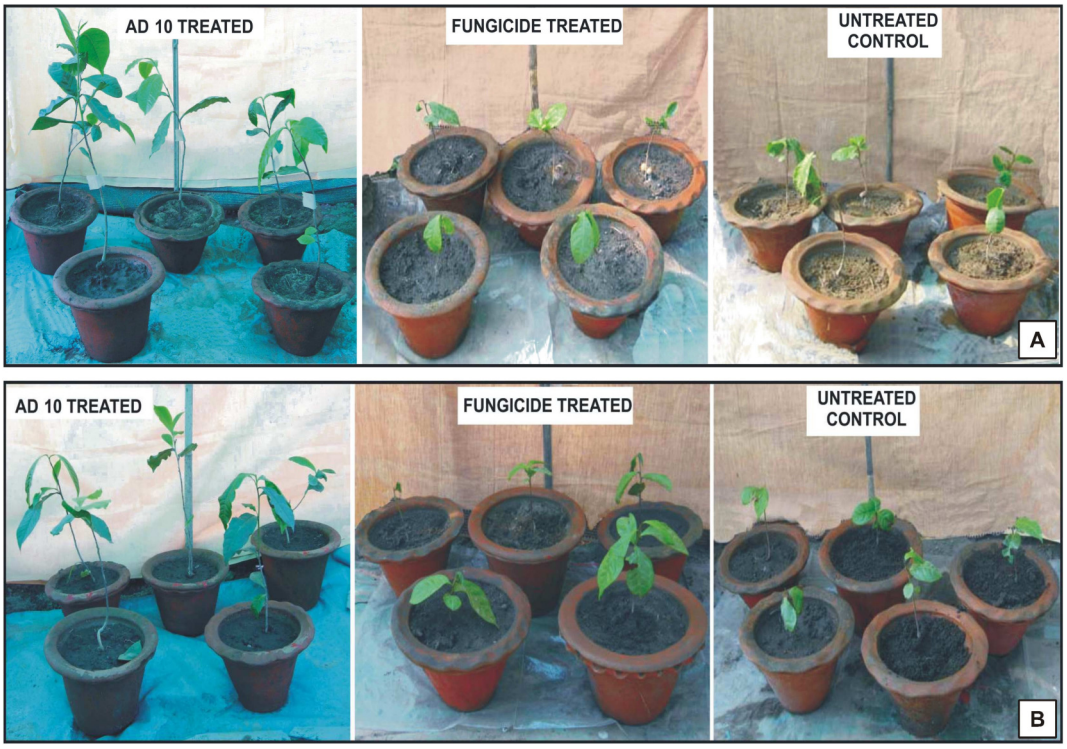


Fig. 4.5.7: Reduction of root rot disease in tea (TS-520 variety) after 45 days of treatment by *Trichoderma viride* AD10 isolate under (A) Sterile and (B) Unsterile conditions.

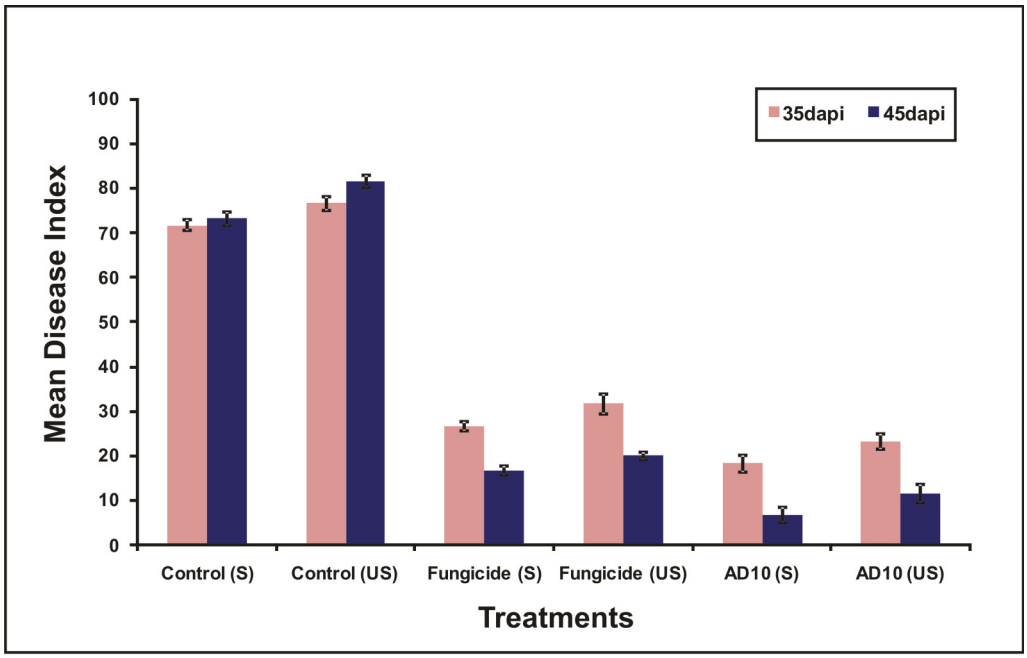


Fig. 4.5.8a: Mean Disease Index of root rot in tea seedlings caused by *R. solani* and treated by antagonistic fungal strain AD10 under sterile (S) and unsterile (US) conditions 35 days after pathogen inoculation (35dapi) and 45 days after pathogen inoculation (45dapi).

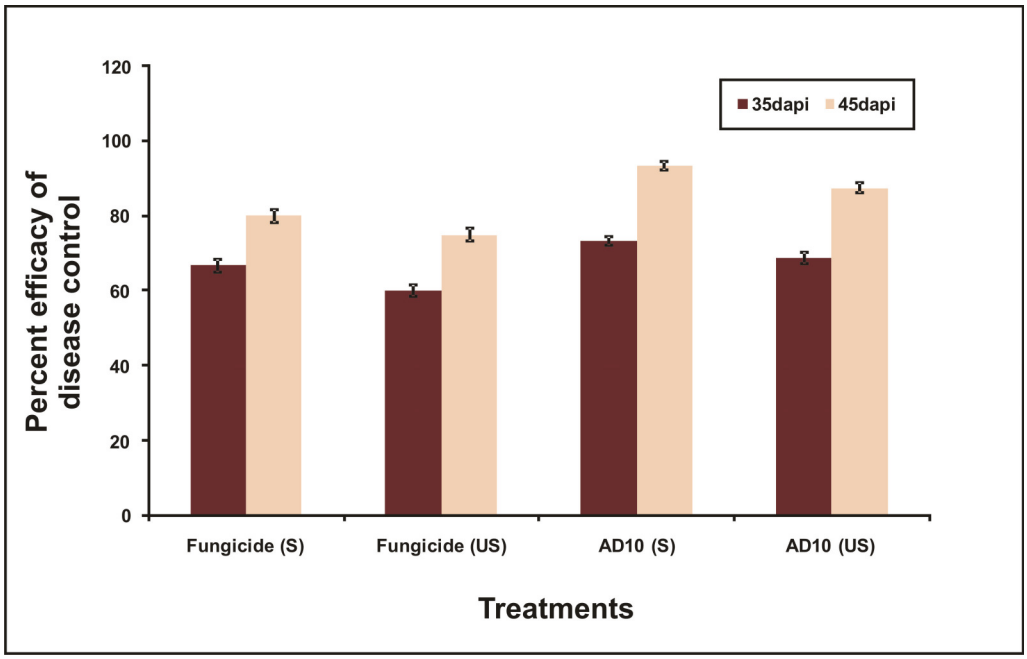


Fig. 4.5.8b: Percent efficacy of disease control (PEDC) of root rot disease in tea seedlings by the antagonistic fungal strain AD10 under sterile (S) and unsterile (US) conditions 35 days after pathogen inoculation (35dapi) and 45 days after pathogen inoculation (45dapi).

Conventional methodologies of disease control such as crop rotation, breeding for resistant varieties and the application of pesticides are often insufficient to manage root diseases of important crop plants (Haas and Defago, 2005). The sustainability of agriculture and horticulture can be enhanced by introducing the concept of "Biocontrol" which includes the use of resident or introduced microflora to decrease the incidence and severity of diseases (Compant et al., 2005; Haas and Defago, 2005; Pliego et al., 2011). The discovery of disease suppressive soils provided the basic idea that microorganisms could be used as biocontrol agents for disease management in agriculture (Ownley and Windham, 2004). Substantial evidences obtained over the past few decades clearly show that microorganisms associated with the plant rhizosphere can play the role of natural antagonists and provide a safe way to protect susceptible plants and maintain them free of diseases despite sufficient exposure to virulent soilborne pathogens (Weller et al., 2002).

Rhizosphere is a rich source for the isolation of diverse group of micro-organisms since the microbial population is affected by the exudates like ions, oxygen, water, secondary metabolites, organic compounds and mucilaginous substances released by the plant roots (Bais et al., 2006; Shukla et al., 2011). The abundance of microorganisms is maximum in the vicinity of plant roots i.e. the rhizosphere since the soil microflora uses the substances released by plant roots as source of nutrition (Badri and Vivanco, 2009; Buee et al., 2009). The microbial communities in the rhizosphere soil are not static and it varies according to the composition of soil such as pH and organic nutrients available and also on the type of plants, nature of plant root exudates, local climate and season of plant growth (Buee et al., 2009; Nihorimbere et al., 2011).

In the present study, tea gardens of sub-Himalayan region of West Bengal and some parts of Assam were chosen as sampling sites for the isolation of biocontrol agents. The tea garden rhizosphere was selected due to the fact that microorganisms isolated from their natural habitat would be more efficient in controlling the disease of the resident plants (Karimi et al., 2012). The rhizosphere harbours the microbial community 500-1000 times more than the bulk soil which includes the soil borne pathogens as well as the beneficial microbes (Rouatt et al., 1960; Ownley and Windham, 2007); therefore, there lies the maximum chance of obtaining potential microbial antagonists for plant disease suppression. Among the microbial groups, rhizosphere is dominated by fungal and bacterial species, where, the abundance of fungi is 10 to 20

times higher and that of bacteria is 2 to 20 times higher compared to the bulk soil (Morgan et al., 2005).

The present study reports the isolation of 212 culturable bacterial strains and 72 fungal strains isolated solely from the tea rhizosphere of different geographical locations. Soil extract agar medium containing the extract from the soil of the same region was used in order to enrich the medium for isolation of maximum number of bacterial strains residing in that habitat. Soil extract agar medium was used by Hamaki et al. (2005) for isolation of novel bacteria belonging to Bradyrhizobiaceae and actinomycetes from soil samples of forest in Japan. Isolation of a rhizosphere inhabiting biocontrol *Streptomyces sp*. strain Ap1 in soil extract agar medium was also reported by Bouizgarne et al. (2006). Biocontrol pseudomonads were isolated in the same medium by Johansson et al. (2003) and used for controlling *Fusarium culmorum* causing wheat seedling blight. The initial screening of the isolated microbial cultures for *in vitro* antagonism was performed against *Lasiodiplodia theobromae*, known to cause diplodia disease in tea. The disease is significant because it can attack any part of the tea plant (Sarmah, 1960; Saha et al., 2008). Survey of the nurseries of Darjeeling district (West Bengal, India) showed that *L. theobromae* was constantly associated with young tea plants (Saha et al., 2008). *In vitro* screening for antagonism against *L. theobromae* showed that fifty one bacterial strains and twenty fungal strains were capable of antagonizing the pathogen in dual culture test on solid medium (PDA). The strains identified as antagonists were further assayed for *in vitro* antagonistic activity against seven (*R. solani*, *C. camelliae*, *P. theae*, *U. zonata*, *F. lamaoensis*, *S. repens* and *P. hypobrunnae*) other foliar and root pathogens of tea. Results revealed that thirty five isolates were capable of antagonizing all the pathogens and were selected for further characterization.

The isolates ETR17 and KTR6 were found to be the most promising bacterial strains as evident from *in-vitro* tests. The inhibition percentage of ETR17 ranged from 63% to 81% and that of KTR6 ranged from 68% to 84.4% against all the test pathogens. Twelve other isolates, D7, ETR20, GH13, BTR19, BTR22, BTR21, T2-1, T4-5, T3-14, T4-1, T1-7 and T1-C also showed considerable degree of antifungal activity against all the pathogens. The inhibition percentage of these isolates ranged from 55% to 80%. The dual culture test in solid media has been used successfully by several researchers to *Discussion*

identify antagonistic microorganisms from soil. Karimi et al. (2012) reported the isolation of twelve bacterial strains antagonistic against *Fusarium oxysporum* including *B. subtilis* and *P. aeruginosa* out of 232 isolates from rhizosphere and root of chickpea. They observed that the *B. subtilis* isolate (B28) showed the highest inhibition percentage (51.16%) in PDA plates compared to other isolates in dual culture test. Nourozian et al. (2006) evaluated the *in vitro* antagonistic activity of *P. fluorescens* and *B. subtilis* isolates by dual culture test against *F. graminearum.* The mycelial growth inhibition was significantly reduced by *P. fluorescens* biov 1 strain 32 compared to *B. subtilis* strain 53 in PDA medium. Similarly, the *in vitro* biocontrol efficacy of 19 indigenous bacteria isolated from rhizosphere soil was evaluated by dual culture against the fungal pathogen *Ganoderma boninense,* causing basal stem rot in oil palm (*Elaeis guineensis*)(Buana et al., 2011). They observed that the bacterial isolate B3.4 showed the highest growth inhibition percentage (58.75%) while B3.3 showed the lowest growth inhibition percentage (12.20%) amongst the isolated bacteria. Berg et al. (2005) used Waksman agar to screen 2648 bacterial isolates for their antagonistic ability to suppress growth of *V. dahlia* and *R. solani* in an *in vitro* dual culture assay. The study revealed that 349 (14.4%) of the bacterial isolates were antagonistic against one or both the fungal pathogens, out of which 301 isolates were antagonistic to *R. solani* while 208 isolates were antagonistic to *V. dahliae*. In another study, the antagonistic activity of the strains CHA0 (*Pseudomonas fluorescens*), IE-6S+ (*P. aeruginosa*) and 569Smr (*Bradyrhizobium japonicum*) were evaluated against the tomato pathogens, *M. phaseolina, F. solani* and *R. solani* by the dual culture test in petriplates containing Czapek Dox agar (Siddiqui and Shaukat, 2002). The *P. aeruginosa* strain IE-6S+ produced zones of inhibition of 3mm and 4 mm respectively against *M. phaseolina* and *R. solani* while in case of *F. solani*, the colonies of both the pathogen and the bacterial antagonist met each other and no further growth of either organism was observed. *Serratia marcescens* R-35 exhibited antifungal potential against *Phytophthora parasitica* in King's B and PDA medium reducing the mycelial growth of the pathogen (de Queiroz et al., 2006). Dual culture using *S. marcescens* in KB medium (40% inhibition) resulted in better antagonistic potential compared to the PDA medium (37% inhibition. In another study on *in vitro* antagonism of five bacterial strains (*Pseudomonas* sp. GRS 175 and *P. aeruginosa* GPS 21, GSE 18, GSE 19 and GSE 30) isolated from groundnut rhizosphere by dual culture test on PDA plates, Kishore et al. (2005) observed

that the strains were highly effective against all the eight test pathogens of groundnut, *A. flavus*, *A. niger*, *C. arachidicola*, *P. personata*, *P. arachidis*, *R. bataticola*, *S. rolfsii* and *R. solani*. GSE 19 produced the maximum inhibition zone of 23.5mm against *R. bataticola* and 23.0mm against *R. solani* while the inhibition zone produced against the rest six pathogens were moderate.

The fourteen bacterial strains with potential antifungal activity were further selected for testing *in vitro* antagonism in liquid (PDB) medium against the eight pathogens used earlier. The results of dual culture assay in liquid medium slightly differed from the antagonistic activity exhibited in PDA medium. However, the isolates ETR17 and KTR6 were again found to be the most promising strains. A maximum reduction of mycelial biomass of *P. theae* was exhibited by ETR17 and KTR6 of upto 76.7% and 79.6% respectively after 7 days of incubation at 28°C. The isolate ETR17 showed maximum reduction of mycelial biomass production (>70.0%) of all the pathogens except *U. zonata* (68.0%) and *P. hypobrunnae* (68.3%) followed by KTR6 exhibiting more than 65.0% reduction of mycelial growth of all the pathogens other than *P. hypobrunnae* (64.6% inhibition). Harish et al. (1998) also studied the effect of *B. subtilis* AF1 on biomass production of a pigeonpea wilt pathogen, *Fusarium udum*. They observed a significant reduction in the biomass of *F. udum* following co-inoculation with AF1 after 0, 12 and 24 h compared to the control flask inoculated with *F. udum* alone. Kim (2007) observed the effect of four antagonistic bacteria, on the growth of *A. parasiticus* ATCC 15517 in all purpose Tween 80 (APT) broth. The mycelial dry weight of *A. parasiticus* in the mixed culture (bacteria and fungus co-inoculated flask) was reduced by the bacterial antagonists *Leuconostoc mesenteroides* (35.5%), *L. plantarum* (20.9%), *L. casei* (21.5%), and *B. subtilis* (86.2%) to a desirable extent in comparison to the control at the end of the incubation period. The highest inhibition of mycelial growth was exhibited when *B. subtilis* was co-inoculated to the APT broth. Mitoi et al. (2012) observed the inhibitory activity of the bacterial strains, *B. licheniformis* and *P. aeruginosa* against the fungal pathogen *A. alternata* on liquid medium. A poorly developed fungal mycelium in comparison to the control was observed. The color of the *Pseudomonas – Alternaria* co-cultivation medium turned yellow and fluoresced under UV illumination, confirming the synthesis of fluorescent pigments by the bacterial strains. Ashwini and Srividya (2013) observed almost 100% reduction in dry weight of the pathogen *C. gloeosporioides* when co-cultured with *B. subtilis* strain BC2 in PDB.

Chakraborty et al. (2009) determined the *in vitro* antagonistic effect of *Ochrobactrum anthropi* against six fungal pathogens, *Sclerotinia sclerotiorum*, *Sphaerostilbe repens*, *Phellinus noxious*, *Sclerotium rolfsii*, *Poria hypolaterita* and *Alternaria alternata* in both solid (NA) and liquid medium (NB). They observed a maximum inhibition of mycelial biomass of *P. noxious* (58.3%) compared to the pathogen growth in the control flask after 10 days. Additionally, the authors found that the mycelial inhibition of the pathogens in solid medium was more significant compared to the liquid medium. However, in the present study, the mycelial growth inhibition of the pathogens was significant in both the solid and the liquid medium.

Among the twenty antagonistic fungal strains selected by initial screening in the present study, ten isolates showed potential antifungal activity *in vitro* against all the pathogens and were selected for further studies. However, only seven isolates, AD2, AD6, AD7, AD10, B2.4, KV8 and NBT1.2 showed most promising broad spectrum antifungal activity with inhibition percentage ranging from 56% to 95%. The isolate AD10 was the most potential fungal isolate recording 67.8% to 95.5% inhibition against the fungal pathogens and therefore was selected for *in vivo* studies. The other four strains showed a wide variation in mycelial inhibition with a highest percentage of 72.2% (by AD3 against *F. lamaoensis*) and a lowest percentage of only 45.5% (by AD4 against *U. zonata*). In the present study, no inhibition zone was produced in the dual culture PDA plates; rather an intermingling of the cultures (i.e. the pathogen and biocontrol fungi) and complete growth of the biocontrol fungi over the pathogen were observed (in dual culture plates). Lack of inhibition zone formation indicated that the biocontrol isolates did not produce any antibiotic and the pathogens were suppressed by the hyperparasitism, secretion of cell-wall degrading enzymes or competitive inhibition (Demirci et al., 2011; Gomathi and Ambikapathy, 2011). Dual culture test was used by several workers to study the *in vitro* antifungal activity of several soil fungi (Abou-Zeid, 2008; Demirci et al., 2011; Alamri et al., 2012). Gomathi and Ambikapathy (2011) observed the *in vitro* antagonistic activity of several fungal strains isolated from soil against the plant pathogen, *Pythium debaryanum* by dual culture test on PDA medium. They found that nine fungal isolates, *A. flavus* (45.8%), *A. fumigates* (43.7%), *A. niger* (45.8%), *A. sulphureus* (58.3%), *A. sydowi* (56.2%), *Penicillium sp.* (52.0%), *T. harzianum* (66.6%), *T. koeningii* (62.5%), *T. viride* (60.4%) showed considerable level of antagonistic activity against the test pathogen.

Adebanjo and Bankole (2004) observed the inhibition of *Colletotrichum lindemuthianum* (causing anthracnose in cowpea plants) growth and production of inhibition zones in PDA plates co-inoculated with the pathogen and the biocontrol fungi, *Aspergillus flavus*, *A. ochraceus*, *Penicillium aurantiogriseum* and *Trichoderma viride*-TH14 and *T.*viride-TH31, which hyperparasitized the pathogen. Forty five different fungal isolates were isolated from tuber-borne sclerotia of *R. solani* in Erzurum province of Turkey by Demirci et al. (2011). Twenty one isolates demonstrated *in vitro* antifungal activity by dual culture test against *R. solani* which included the species of *Acremonium*, *Chaetomium*, *Cladosporium cladosporioides*, *Fusarium*, *Gliocladium*, *Paecilomyces*, *Penicillium*, *Plectosporium*, *Sporothrix*, *Trichoderma* and *Verticillium dahliae*. *T. harzianum* (72%, 69%) and *Paecilomyces* sp. (50%) exhibited highest inhibition under *in vitro* conditions against *R. solani*. Abou-Zeid (2008) observed that four fungal isolates, *Gliocladium* spp. and *Trichoderma* spp. significantly inhibited the radial growth of several pathogenic fungi affecting the medicinal plants in Saudi Arabia, with different percentages in dual culture test. Both overgrowth of the antagonistic fungi over the pathogen mycelium, and inhibition zone in front of the pathogen colony were observed. *G. deliquescens* and *G. virens* showed maximum inhibition against *Penicillium chrysogenum* (63.3% and 65.6% respectively) while *T. viride* and *T. hamatum* showed maximum inhibition against *Alternaria alternata* (62.7% and 67.24% respectively). Alamri et al. (2012) studied the *in vitro* antagonistic activity of *T. harzianum* JF419706 in dual culture plates inhibiting the growth of the phytopathogens, *A. alternata, F. oxysporum, Exserohilum rostratum, M. phaseolina, P. ultimum* and *R. solani*. The biocontrol fungus parasitized the mycelial growth of all the pathogens by producing several cell wall degrading enzymes at significantly high concentrations.

The thirty five effective bacterial isolates and ten potential fungal isolates were selected further for identification (at least up to the genus level) and characterization of the biocontrol mechanism exhibited by them. Since, the aim of present study was to isolate the most promising antagonistic strains active against a broad spectrum of pathogens; therefore, the strains which failed to restrict the growth of all the test pathogens were not considered for further characterization.

The selected 35 antagonistic bacterial isolates were subjected to characterization studies by a polyphasic approach which included morphologicaland physiological characterization by general observations on

colony characteristics and microscopic observations followed by biochemical tests. The phenotypic characterization studies included microscopic observation of the cell size and morphology, and culture characteristics in growth media. Additionally, an array of biochemical tests was conducted to characterize the strains by their phenotypes. Phylogenetic characterization of potential bacterial isolates D7, ETR17, ETR20 and KTR6 selected on the basis of *in vitro* antagonism studies in solid and liquid medium was performed via 16SrRNA gene sequencing followed by sequence similarity searches using multiple sequence alignment and generation of phylogenetic tree using bioinformatics software (MEGA 4.0). The results of phenotypic and genotypic tests (if done) were analysed for proper identification of the antagonistic isolates. 16S rRNA gene analysis has played a crucial role in the accurate identification of bacterial isolates because of the widespread use of PCR and DNA sequencing in the past decade (Clarridge, 2004; Woo et al., 2009; Schlaberg et al., 2012). Although, 16S rRNA gene is an important diagnostic marker for prokaryote identification, but corresponding development in phenotypic description and cell culture characteristics are essential to actually describe the bacterial strains and work with them under laboratory conditions (Bochner, 2009). In the present study, results of phenotypic test, primarily led to the identification of the bacterial isolates; all the isolates were identified to the genus level and six strains were identified upto the species level. A diverse group of bacteria were isolated from the rhizosphere soil which included *Bacillus, Pseudomonas*, *Serratia, Acinetobacter, Citrobacter*, *Enterobacter*, *Gemella, Actinobacillus, Paenibacillus, Enterococcus, Aerococcus, Cedecea* and *Micrococcus.* Phylogenetic analysis of four most potent biocontrol isolates was performed to substantiate the phenotypic tests. Since the 16S rRNA gene is universal in bacteria, therefore, the comparison of the 16S rRNA gene sequences isolated from the organisms of interest allows the discrimination between organisms at the genus level and in some cases even at the species and sub-species level across all major phyla of bacteria (Clarridge, 2004). However, there is a limitation of this type of identification strategy when two different bacterial species possess almost the same 16S rRNA gene sequence. Even the use of public databases such as NCBI GenBank often challenging since many sequences with errors in the nucleotide data are deposited and the similarity search using BLAST function may mislead the user providing erroneous identity of the isolate (Woo et al., 2009). In such cases, for appropriate identification of the bacterial isolates, it is necessary to correlate with accurate phenotypic characterization results.

Among the bacterial antagonists, the most common isolated genus was *Bacillus* since eleven strains belonged to this genus. *Bacillus* strains are well known contributors to the disease suppressiveness of soils or providing protection to the plants from disease(s) caused by soil-borne fungal pathogens (Kumar et al., 2012; Rosenzweig et al., 2012). Literature survey represents a large number of soil bacteria associated with disease suppression potential as *Bacillus* and more commonly as *B. subtilis* (Hernandez-Suarez et al., 2011; Arguelles-Arias et al., 2009). We recognized KTR6 isolate as *B. subtilis* via phenotypic and phylogenetic considerations. The 16S rRNA gene sequence of KTR6 showed 99% similarity with *B. subtilis* gene sequences. The criteria of ≥99% similarity to define a species and ≥95% to ≥99% to define a genus were used by Bosshard et al. (2003). *Bacillus* is widely used as a biocontrol agent and also preferred over other bacteria due to its spore forming capacity which enables comfortable integration into bioformulated products with extensive shelf-life (Haas and Defago, 2005; Domenech et al., 2006; Bouizgarne, 2013). Several strains of *Bacillus* have been used for the commercial development of biofungicides (Emmert and Handelsman, 1999; Restuccia et al., 2006; Domenech et al., 2006). *Bacillus* spp. has been reported to be involved in the suppression of a variety of plant diseases such as 100% reduction of root damage by *B. subtilis* microencapsulates in tomato plants caused by *R. solani* and *F. oxysporum* (Hernandez-Suarez et al., 2011); suppression of anthracnose disease in cowpea caused by *Colletotrichum lindemuthianum* (Adebanjo and Bankole, 2004); biocontrol of *Fusarium* wilt in banana (Zhang et al., 2011) and eggplant (Saha et al., 2012b) by *B. subtilis* bioformulation; *Fusarium* wilt and *Rhizoctonia* damping off disease control in tomato and pepper by formulated product of *B. subtilis* and *B. licheniformis* (Domenech et al., 2006) and suppression of take-all in wheat by endophytic *Bacillus subtilis* E1R-j (Liu et al., 2009).

In the present study, three isolates ETR1, ETR17 and TR1 were found to belong to the genus *Serratia* All three strains showed considerably high degree of antagonism *in vitro*. The 16S rRNA gene sequence of ETR17 showed 99% similarity with *Serratia marcescens* gene sequences. Depending on the phenotypic characters and the 16S rRNA gene sequence, the identity of ETR17 isolate was confirmed as *S. marcescens*. The other two *Serratia* isolates were also identified as *S. marcescens* by comparing the biochemical test results with that of ETR17 and other *Serratia* strains reported in literature. It was

found that *S. marcescens* unlike *S. plymuthica* and *S. liquefaciens* does not produce gas in glucose, shows lysine and ornithine decarboxylation but not arginine decarboxylation and does not ferment L-arabinose and xylose (Grimont and Grimont, 2006). Various species of the genus *Serratia* are well established biocontrol agents and used increasingly as bioinoculants for the inhibition of fungal pathogens (Muller et al., 2009; Saha et al., 2012c). Research on *Serratia* in the past has shown that rhizosphere inhabiting strains of *S. plymuthica*, *S. marcescens* and *S. liquefaciens* possessed appreciably high antifungal activity against a number of fungal pathogens (Kalbe et al., 1996; Someya et al., 2005; Gould et al., 2008; Wang et al., 2013). The diseases inhibited by *Serratia* spp. include *Verticillium* wilt and *Phytophthora* root rot in strawberry (Kurze et al., 2001); *Phytophthora* blight of pepper (Shen et al., 2002); rice sheath blight (Someya et al., 2005); damping-off of cucumber caused by *Pythium ultimum* (Roberts et al., 2007) and dry rot of potato caused by *Fusarium sambucinum* (Gould et al., 2008). The results of *in vitro* haemolytic test using human blood agar performed with the *Serratia* isolates in the present study clearly indicated that the strains were non-pathogenic to human beings and can be used safely for *in vivo* biocontrol studies.

Members of the Pseudomonadacae family are of great interest for the researchers in the field of biocontrol since they play an important role in plant protection (Weller, 2007). Among them several species like *P. fluorescens*, *P. aeruginosa*, *P. putida* extensively colonize the plant roots and rhizosphere, and antagonize several plant pathogens (Lugtenberg et al., 2001; Berg et al., 2002; Couillerot et al., 2009). In the present study, the isolates D6, D8 and BTR8 were identified as pseudomonads but only D6 exhibited yellow-green fluorescence in *Pseudomonas* agar plates. Amplification of 16S-23S rRNA ITS region gene using primers specific for fluorescent pseudomonads was observed only in D6 leading to further corroboration of the results obtained from phenotypic tests. The same fluorescent pseudomonad specific primers as used in this study were utilized to amplify the 16S–23S rRNA ITS region and thereby confirm the identity of the fluorescent pseudomonads isolated from soil by Kumar et al. (2002). The authors isolated the colonies directly in King's B medium in which the fluorescent pseudomonads exhibit characteristic fluorescence. Eighteen fluorescent isolates were selected for PCR test; all of which produced the expected amplicon size of 560bp and were confirmed as fluorescent pseudomonads.

Other commonly occurring rhizobacteria with biocontrol potential such as *Acinetobacter, Paenibacillus*, *Citrobacter*, *Enterobacter* and *Micrococcus* were also isolated from tea rhizosphere in the present study. The isolation of antagonistic *Acinetobacter* strains indigenous to tea garden soil is being reported for the first time. Trotel-Aziz (2008) observed the *in vitro* antagonistic activity of *Acinetobacter lwoffii* PTA-113 and PTA-152 against *B. cinerea*, the causal agent of gray mold. Liu et al. (2007) also reported the isolation of an endophytic *Acinetobacter* strain LCH001 from healthy stems of the plant *Cinnamomum camphora* (L.) Presl. The bacterium inhibited the mycelial growth of several phytopathogenic fungi such as *Cryphonectria parasitica*, *Glomerella glycines*, *Phytophthora capsici*, *F. graminearum*, *B. cinerea*, and *R. solani*.

Phenotypic characterization studies identified one antagonistic bacterial strain in the current study as *Paenibacillus* sp., which is a well known biocontrol agent. Aliye et al. (2008) evaluated the role of *Paenibacillus* strains for bioprotection of potato (*Solanum tuberosum*) against bacterial wilt (*R. solanacearum*). Donmez et al. (2011) reported the antagonistic potential of *Paenibacillus* for the suppression of gray mold caused by *B. cinerea* in strawberry plants. Niu et al. (2013) also demonstrated the biocontrol potential of the rhizobacterium *Paenibacillus polymyxa* M-1 against phytopathogenic *Erwinia spp.*

Four bacterial strains were characterized phenotypically as *Micrococcus* sp. in the present study. Isolate GH13 was identified up to the species level as *Micrococcus varians* because the strain scored positive for nitrate, gelatin, citrate and urease whereas negative for oxidase test which distinguishes it from other members of the genus. *M. varians* has earlier been reported as biocontrol agent. For instance, the biocontrol efficiency of endophytic *M. varians* isolated from the peels of potato tubers was assessed against the soil-borne plant pathogens *Phytophthora infestans*, *Fusarium sambucinum*, *F. avenaceum* and *F. oxysporum* using an *in vitro* bioassay technique. The bacterium was more effective in controlling *P. infestans* compared to the *Fusarium* isolates (Sturz et al., 1999). The biocontrol and plant growth promoting potential of another *Micrococcus* species, *M. luteus* was also reported by several workers (Chakraborty et al., 1998; Yazici et al., 2011; Raza and Faisal, 2013). The role of *M. luteus* for controlling brown blight disease in tea caused by *Glomerulla cingulata* was evaluated by Chakraborty et al. (1998). In another study, Yazici et al. (2011) observed the reduction of early blight disease severity by treating the tomato plants with *M. luteus* IK-81.

Two promising biocontrol bacteria were identified as *Enterobacter sp.* in this study. The 16S rRNA gene sequence of isolate D7 showed 99% similarity with the gene sequences of *Enterobacter* sp. followed by 98% similarity with different species of *Enterobacter* as well as *Leclercia adecarboxylata*, *Cedecea davisae* and *Pantoea* sp. However, the phenotypic traits as evident from the results of morphological and biochemical tests closely resembled the genus *Enterobacter* (Palleroni, 1984) and therefore the identity was confirmed as *Enterobacter* sp. The isolate ETR20 showed 95% similarity with *Citrobacter freundii,* other *Citrobacter* and *Klebsiella* species but the phenotypic characters had close resemblance with the *Citrobacter* genus and therefore the isolate was identified as *Citrobacter* sp. Both *Enterobacter* and *Citrobacter* are reported as biocontrol agents in literature (Patel et al., 2008; Haggag Wafaa, 2010; Patel et al., 2010). Donmez et al. (2011) demonstrated the *in vitro* antagonistic activity of *Enterobacter intermedius* against *B. cinerea,* causing gray mold disease in strawberry. *E. cloacae* EcCT-501 was found to suppress *Pythium* damping-off of cucumber and other plant hosts (van Dijk and Nelson, 2000). Kavroulakis et al. (2010) reported that the inhibitory effects of *Enterobacter* sp. AR1.22 against *F. oxysporum* was most effective among all other rhizobacteria used in the study. Janisiewicz et al. (2012) reported the antagonistic activity of a *Citrobacter freundii* isolate against fruit rot pathogen *Monilinia fructicola*.

Isolation of novel bacterial antagonists indigenous to tea rhizosphere belonging to the *Enterococcus*, *Aerococcus*, *Cedecea*, *Actinobacillus* and *Gemella* genera were also reported in the present study. Bacterial isolates of each of these genera were identified phenotypically. Two efficient antifungal bacteria were recognized as *Cedecea* sp. by biochemical and morphological characters. Earlier studies have shown *Cedecea* as biocontrol agent. For example, Romeiro et al. (2000) reported the isolation of *Cedecea davisae* isolate UFV-54 from the phylloplane of tomato and found that the bacterium was successful in controlling the growth of the pathogen *Pseudomonas syringae* pv. tomato *in vitro* and in green house experiments. Szentes et al. (2013) also demonstrated the antagonistic activity of *Cedecea* sp. isolated from *Sphagnum* sp. (moss) surface against the pathogens *A. alternata* and *F. oxysporum*. Chen et al. (1995) isolated cotton endophytic *Aerococcus* strains with antagonistic potential against *F. oxysporum* f. sp. *vasinfectum* causing *Fusarium* wilt disease. *Actinobacillus spp.* are common soil inhabitants and are often isolated from the rhizosphere soil (Gardener et al., 2001). Biocontrol potential of this bacterium has not been

reported and frequently this genus is found as a human and animal disease causing agent. In our study, a single bacterial isolate with strong antifungal activity closely represented *Actinobacillus*, however, the identity was not confirmed by phylogenetic studies.

The growth kinetics study of the most effective bacterial antagonists was also performed. Growth kinetics is an important characterization test which is of immense importance in bacterial inoculum preparation during *in vivo* biocontrol studies. Moreover, for isolation of the antifungal secondary metabolites, the bacterial cultures are harvested mostly in the late exponential and stationary phases of bacterial growth (Bano and Mussarat, 2003; Brodhagen et al., 2004; Murugappan et al., 2012); therefore, it becomes necessary to determine the different growth phases of bacterial isolates. In the present study, the growth rates of the bacterial antagonists except one *Bacillus* isolate (stationary phase after 20h) were fast since the stationary phase was attained much earlier (between 10-12h). The antibiotic sensitivity of the four most potential antagonistic bacterial isolates towards the commercially available antibiotics was also for a broader characterization of isolates. The antibiotic resistance exhibited by the bacterial antagonists can be used as markers for their identification when applied as bioinoculants in field trials for controlling tea diseases in future. In a similar study, Gopalakrishnan et al. (2012) reported the antibiotic sensitivity and resistance of several biocontrol bacteria towards chloramphenicol, kanamycin, nalidixic acid, streptomycin, tetracycline and ampicillin. In another instance, the antibiotic susceptibility of lactic acid bacteria isolated from rhizosphere of olive and desert truffles having biocontrol potential was also evaluated by Fhoula et al. (2013).

We observed that the present antagonistic bacterial isolates were resistant to the frequently used chemical fungicides in tea gardens. The most potential bacterial antagonists were tolerant to all the fungicides at a concentration higher than that applied in the fields. The MIC data generated in the study can be very useful in developing formulations for an effective integrated disease suppression strategy by involving the chemical fungicides and biocontrol bacteria. Kondoh et al. (2001) have successfully used flutolanil, a chemical pesticide and *B. subtilis* RB14-C for integrated control of damping off caused by *Rhizoctonia solani* in tomato plants. The treatment reduced the use of flutolanil to one-fourth compared to the single use of the pesticide for reduction of disease occurrence with same efficacy. Chakraborty et al. (2013)

also studied the *in vitro* insecticide tolerance of PGPR strains *S. marcescens*, *B. amyloliquefaciens* and *B. pumilis*. They observed that all the three isolates could tolerate the commonly used insecticides in tea gardens viz. Contaf 5E, Calixin, Ethion 50EC, Acephate and Confidor at a concentration 1000 times higher than that applied in the fields.

Identification and classification of fungi is based on two different approaches: the traditional approach which includes study of microscopic features, colony characteristics and certain biochemical reactions and the phylogenetic approach based on ribosomal RNA gene sequencing. The limitations of using traditional methods lies in the inadequate identification of closely related fungal species and this led to the development of phylogenetic methods (Bruns et al., 1991). Ribosomal RNA genes are present in multiple copies in an organism's genome (100-200 copies in the genome) and are universally shared by all organisms (Husniati and Oktarina, 2013). The non-coding ITS regions have higher mutation rates than the coding regions and thus have a higher sequence variation. The ITS regions 1 and 2 located between the highly conserved small (18S) and large (28S) ribosomal subunit genes in the rRNA operon are used frequently for fungal identification upto the species level (Husniati and Oktarina, 2013). Species identification through sequence similarity is based on the following parameters: (i) 99-100% similarity between the query sequence and target sequence indicates that the unknown isolate would be assigned to reference species; (ii) 95-99% similarity indicates that the unknown isolate would be assigned to the genus of the reference and (iii) <95% similarity indicates the unknown isolate could be assigned to a family (Bosshard et al., 2003).

In the present study, we focused on the amplification of ITS region of rRNA gene of selected antagonistic fungal strains using ITS1 and ITS4 universal primers (White et al., 2001). Amplified products of size in the range of 500 to 600bp were produced by the primers. The results are in accordance with other researchers who used phylogenetic method for specific identification of fungal isolates (Chakraborty et al., 2010a; Manikprabhu and Lingappa, 2012; Husniati and Oktarina, 2013). The 18S-28S rRNA gene sequence of the most potential biocontrol fungi AD10 showed 98% similarity with sequences mostly of *Trichoderma atroviride*, *T. harzianum, Hypocrea lixii* and *Trichoderma* sp. The identity assigned to the isolate was *T. viride* since the morphological parameters

closely resembled *T. viride* isolates reported in literature (Gams and Bisset, 2002; Chakraborty et al., 2010a; Lunge and Patil, 2012). Additionally the identity of the isolate was confirmed by IARI, New Delhi. Chakraborty et al. (2010a) observed the genetic variability of the 500 to 600 bp amplicons of rRNA gene of nineteen *Trichoderma* strains isolated from rhizosphere soil and constructed a dendogram which showed 99% similarity of the isolates with other *Trichoderma* sequences obtained from the Genbank.

Besides *Trichoderma*, four other antagonistic fungal isolates were identified as *Aspergillus* sp. based on the morphological and microscopic observations and phylogenetic analysis. The 18S-28S rRNA gene sequences of isolate AD7 of our study showed 99% similarity with that of *Aspergillus sydowii* and *Aspergillus* sp. obtained from Genbank and also morphologically closely resembled *Aspergillus* spp. Thus, the identity of AD7 was confirmed as *Aspergillus* sp. Isolate AD3 matched with *Aspergillus* sp. in terms of morphology, sporulation and culture characteristics (Thom and Raper, 1951) but the rRNA gene sequences showed 92% similarity with other GenBank submissions of the genus *Aspergillus*. Similarly, the isolates KV8 and NB4 also showed close similarity with *Aspergillus* based on the traditional approach. However, the 18S rRNA sequence data showed only 85% (KV8) and 80% (NB4) similarity with other *Aspergillus* rRNA gene sequences.

The antagonistic fungi AD6 and B2.4 reported in this study were assigned to the genus *Penicillium* since the morphological features like spore and hyphal characteristics resembled *Penicillium* spp. (Houbraken et al., 2011). 18S rRNA gene sequences of AD6 exhibited 99% similarity whereas B2.4 showed 92% with that of other *Penicillium* sp. Another isolate, NBT1.2 was more specifically identified as *P. citrinum* by comparing morphological observations with other *Penicillium* isolates and the identity was confirmed further by IARI. The isolate AD2 was identified as *Paecilomyces parvus* by IARI, however, the amplified ITS region showed close similarity with other *Basidiomycota* sp. sequences deposited in the Genbank. The phenotypic observations like hyphal and spore characteristics observed under the microscope was closely similar to that of the genus *Paecilomyces* (Samson, 1974). Therefore, AD2 was designated in the present study as *Paecilomyces parvus*. Another potential biocontrol isolate AD4 was also identified as *Paecilomyces lilacinus* based on the culture, hyphal and sporulation characteristics (http://www.mycobank.org; http://www.pf.chiba-

u.ac.jp/english/gallery.html). Ribosomal gene sequence data further supported the morphological characters.

The present work demonstrates a taxonomically diverse group of microorganisms capable of antagonizing broad spectrum fungal pathogens of tea. But the common determinants of antagonism shared by the isolates are the production of a wide array of antifungal metabolites and aggressive rhizosphere colonization. Literature studies suggests that common biocontrol bacteria like *Bacillus*, *Pseudomonas* and *Serratia* synthesize diverse group of secondary metabolites like antibiotics, siderophores, volatile organic compounds, and hydrolytic and detoxifying enzymes (Bais et al., 2004; Compant et al., 2005). The pivotal role of diffusible antibiotics, siderophores and hydrolytic enzymes produced by the fungal antagonists like *Trichoderma*, *Penicillium* and *Aspergillus* in biocontrol has also been widely studied. The secondary metabolites provide competitive advantage to the biocontrol microbes in colonization in the rhizosphere which is rich in root exudates and therefore harbours a diverse array of microbial population. Additionally, the secreted metabolites may also induce systemic resistance in plants against pathogens.

In the current study, two *S. marcescens* strains (ETR17 and ETR1), one *Bacillus* strain (KTR6) and one *Micrococcus* strain (TRB14) out of 35 antagonistic strains showed chitinase activity. However, ETR17 and KTR6 are the most promising strains of this study that has recorded highest antifungal activity. Among the fungal isolates, all strains except AD4 and AD10 showed chitinase production. The function of chitinase produced by antagonistic microorganisms like *Bacillus*, *Pseudomonas*, *Enterobacter*, *Streptomyces*, *Serratia*, *Trichoderma, Aspergillus and Penicillium* as an antifungal protein has been reported by several workers (Chernin et al., 1995; Someya et al., 2001; Jaiganesh et al., 2007; Prapagdee et al., 2008; Mehmood et al., 2009; Velusamy et al., 2011). Chitin (an unbranched homopolymer of 1, 4-β-linked *N*-acetyl-d-glucosamine) is an important component of several phytopathogenic fungi which is not present in plants or other microbes. The production of chitinases to break down the mycelial cell walls of fungal plant pathogens by antagonistic fungal isolates has been considered as a major factor of biocontrol (Inbar and Chet, 1995). Chitinases, along with other lytic enzymes like proteases and 1,3-β-glucanases, degrade fungal cell walls and inhibit fungal growth at the hyphal tips (Kobayashi et al., 2002; Ovadis et al., 2004). High level of extracellular hydrolytic enzymes, chitinase and β-1,3-glucanase production by *Streptomyces hygroscopicus* strain

SRA14 during the exponential and late exponential phases, respectively was reported by Prapagdee et al. (2008). They observed the growth suppression of *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* by the culture filtrates of *S. hygroscopicus* containing the antifungal enzymes collected from the exponential and stationary phases. Velusamy et al. (2011) observed the role of crude chitinase isolated from *Pseudomonas* sp. on the suppression of *F. oxysporum* A3 during the *in vitro* biocontrol assays. Mehmood et al. (2009) also demonstrated the antifungal activity of purified chitinase (60 kDa) enzyme isolated from *Serratia proteamaculans* 18A1 against the pathogenic fungi *Fusarium oxysporum* and *Aspergillus niger*. Another report suggests that an endochitinase CHIT100 (100 μgml^{-1}) purified from *S. plymuthica* HRO-C48 inhibited spore germination and germ tube elongation of the phytopathogenic fungus *B. cinerea* (Frankowskii et al., 2001). Wang et al. (2013) reported that the *S. marcescens* strain JPP1 isolated from peanut hulls produced chitinase which caused degradation of pathogenic *Aspergillus parasiticus* cell walls and reduced the level of aflatoxin production. Pleban et al. (1997) demonstrated chitinase (36 kDa) production by an endophytic *Bacillus cereus* strain 65 which significantly protected cotton seedlings from root rot disease caused by *R. solani*. The role of endochitinase produced by *Trichoderma harzianum* in inducing disease resistance in transgenic tobacco and potato plants was described by Lorito et al. (1998). The transgenic tobacco and potato plants where endochitinase enzyme was expressed were highly resistant towards *Alternaria alternata*, *A. solani*, *B. cinerea* and *R. solani*. Manjula et al. (2004) also demonstrated the role of extracellular chitinase produced by *Trichoderma viride* pq1 in the parasitism of the pathogen *Sclerotium rolfsii*.

Cellulase production by the microbial antagonists was also demonstrated in the present study on Congo red stained CMC plates. Among the bacterial isolates, cellulase production was exhibited by eight *Bacillus* strains; two *Micrococcus* sp. and one isolate each belonging to *Paenibacillus*, *Actinobacillus*, *Enterobacter*, *Eischerichia* and *Pseudomonas* genera. However, the most potential antagonistic bacterial isolate *S. marcescens* ETR17 lacked the production of cellulase. Emtiazi et al. (2007) reported the production of cellulase by three *Paenibacillus* isolates indicated by reduction of congored color on CMC medium. Ashwini and Srividya (2013) noticed desirable levels of production of cell wall degrading enzymes, chitinase, cellulase and glucanase by antagonistic *B. subtilis* isolate in both solid and liquid cultures. The role of extracellular

proteases in the biocontrol of fungal diseases by soil bacteria was also reported by Ahmadzadeh et al. (2006). In the present study, 27 bacterial strains out of 35 displayed proteolytic activity in casein agar medium. Kumar et al. (2012) observed high level of protease activity produced by *B. subtilis* in the range of 0.34 to 0.79 IU/ml. The synergistic effect of lipases and proteases produced by *Pseudomonas chlororaphis* PA23 in biocontrol was evaluated by Poritsanos et al. (2006). Sharma and Tiwari (2005) studied the production of amylase, protease, lipase and chitinase by *Serratia* strains isolated from the river stations of Narmada and emphasized their possible use in biocontrol and biodegradation. Swain and Ray (2009) observed the cellulase and amylase activity *in vitro* by *B. subtilis* strains. Several other bacterial genera like *Citrobacter*, *Enterobacter* have also been found to produce multiple mycolytic enzymes like proteases, lipases and amylases (Purkayastha et al., 2010). However, pectinase activity was not observed in any of our isolates which can be regarded as an advantageous trait because the production of pectinase is considered as an undesirable feature of plant beneficial bacteria (Cattelan et al., 1999).

Hydrogen cyanide is produced by several antagonistic bacteria which effectively inhibits the phytopathogens in addition to the antibiotics even at picomolar concentrations (Ramette et al., 2003; Kumar et al., 2005; Senthilkumar et al., 2009). We have reported the production of hydrogen cyanide by three bacterial isolates with antifungal activity. However, the most potential bacterial isolates *S. marcescens* ETR17 and *B. subtilis* KTR6 and all the fungal antagonists lacked HCN production. Bakker and Schippers (1987) considered the lack of hydrogen cyanide production as a beneficial trait since HCN inhibits plant growth and yield due to the interference with cytochrome oxidation.

Besides hydrolytic enzymes and HCN, many of the present strains were found to produce siderophores and other plant growth promoting metabolites like IAA and phosphatase. IAA has been termed as the best-known hormone produced by the plant-associated microorganisms (Kim et al., 2011) which includes a large number of root colonizing microorganisms such as *Enterobacter* spp., *Serratia* spp., *Pseudomonas* spp., *Bacillus* spp., *Acinetobacter* spp., *Azospirillium* spp., *Agrobacterium* spp., *Aspergillus* spp. and *Trichoderma* spp. (Patten and Glick, 2002; Faltin et al., 2004; Karuppiah and Rajaram, 2011; Miguel Angel et al., 2011; Masunaka et al., 2011). The role of IAA has been implicated in all aspects of plant growth starting from seed germination to shoot growth and leaf abscission (Long et al., 2008). Production of the phytohormone IAA was very

common among the present bacterial isolates as 19 of the 35 bacterial strains scored positive for this trait with two strains, *Citrobacter* sp. ETR20 (134 µg/ ml) and *Acinetobacter* sp. GH6 (135 µg/ml) recording very high production. However, only two fungal antagonists out of ten produced IAA. In a study, Long et al. (2008) noticed that out of the 28 IAA producing isolates, root growth of *Solanum nigrum* increased significantly by the IAA (at a concentration of 1.1-11 µg ml^{-1}) produced by *Pseudomonas* isolates SSR5-2 and BGCR2-9 compared with the control. Miguel Angel et al. (2011) demonstrated the role of auxins and gibberellins synthesized in liquid cultures by an *Aspergillus ustus* isolate for growth promotion of *Solanum tuberosum* and *A. thaliana.* The production of IAA along with phosphatase and siderophore associated with the growth promotion of tea seedlings by *Bacillus amyloliquefaciens, B. pumilis* and *Serratia marcescens* was also noticed by Chakraborty et al. (2013).

Phosphorus is the second key macronutrient present in soil essential for plant growth and vigour (Patel et al., 2008; Velineni and Brahmaprakash, 2011). Although phosphorus is present in both organic and inorganic forms but its ability to form complexes with other soil constituents limits its uptake by the plants. Certain PGPB and PGPF have the ability of producing phosphatase for solubilizing the native phosphate from either organic or inorganic bound forms and facilitating plant growth (Lugtenberg and Kamilova, 2009). In the present study, only five bacterial strains, including two *Bacillus* sp. (BTR4 and KTR6), two *Citrobacter* sp. (ETR20 and ETR24) and one *Cedecea* sp. (BTR18) produced phosphatase on Pikovskaya's agar medium out of all bacterial and fungal isolates. The role of different organic acids in mineral phosphate solubilization by *Citrobacter* sp. DHRSS isolated from sugarcane rhizosphere was studied by Patel et al. (2008). Solubilization of phosphate by *B. megaterium* isolate was observed by Velineni and Brahmaprakash (2011). Maheswar and Sathiyavani (2012) also reported the efficient phosphate solubilizing activity exhibited by *Bacillus* isolates at pH 7 in both solid and liquid medium. Rathore et al. (2012) observed the phosphate solubilizing ability of *Citrobacter freundii* isolated from soil and recorded maximum activity at 28°C ± 2°C (pH 7-8) under static condition of incubation. Several members of *Proteobacteria* including the genera *Burkholderia*, *Pseudomonas*, *Acinetobacter*, *Enterobacter*, *Pantoea*, *Serratia*, *Klebsiella*, *Leclercia*, *Raoultella* and *Cedecea* alongwith the Firmicutes, *Bacillus* and *Brevibacterium* and three species of the genus *Arthrobacter* were reported as active phosphate solubilizers by Pei-Xiang et al. (2012).

The role of iron-chelating siderophore as an efficient antifungal metabolite involved in biocontrol and plant growth promotion by the rhizobacteria is well documented (Haas and Defago, 2005; Sahu and Sindhu, 2011). Siderophores have been demonstrated to play a major role in plant disease suppression by bacterial biocontrol agents like *Serratia, Bacillus, Pseudomonas* etc. inhibiting the growth or the metabolic activity of plant fungal pathogens by sequestering iron (Press et al., 2001; Sayyed et al., 2005; Shanmugam et al., 2011). In the present study, a majority of the microbial isolates including the promising antagonists, KTR6, ETR17 and AD10 were capable of producing different types of siderophores. Twenty five bacterial and three fungal isolates were detected as siderophore producing by the formation of orange, yellow or pale red colored halo of varying sizes on CAS agar plates. The universal CAS agar assay method is adequately sensitive and globally accepted for detecting siderophore production by microorganisms (Schwyn and Neilands, 1987; Milagres et al., 1999). Several Gram negative and Gram positive bacteria and soil fungi have been screened for siderophore production using this assay method (Press et al., 1997; Mahmoud and Abd-Alla, 2001; Machua and Milagres, 2003; Tian et al., 2009; Koo and Cho, 2009; Qi and Zhao, 2013). The variations in colour of halo produced by siderophorogenic bacterial and fungal strains is related to the differences in the nature and concentration of siderophores produced (Milagres et al., 1999). Yu et al. (2011) isolated siderophore producing bacteria from rhizosphere of pepper, tomato and rubber trees in China using the universal CAS agar plate assay. The bacterium *Bacillus* CAS15 recorded as the highest siderophore producer indicated by a large orange halo formation was selected for further biocontrol and plant growth promoting studies. Siderophore production indicated by yellow-orange colored halo around the bacterial colonies was also noticed in case of rhizobacterial isolates RMP3 and RMP5 by modified CAS agar assay (Arora et al., 2001). The isolate RMP5 produced a larger halo in comparison to RMP3.

The siderophores detected in the bacterial and fungal strains during the present study were chemically characterized which revealed that 19 bacterial strains produced catecholate siderophores (detected by Arnow's test), 9 bacterial and three fungal strains produced hydroxamate siderophores (detected by the tetrazolium salt test) and 8 bacteria produced carboxylate siderophores (detected spectrophotometrically). The protocols followed for the siderophore characterization tests in the present study are universally accepted

(Clark, 2004; Urja and Meenu, 2010; Gull and Hafeez, 2012). *Enterobacter cloacae* EcCT-501 having biocontrol efficacy against *Pythium* damping-off of cucumber produced hydroxamate siderophore aerobactin and catecholate siderophore enterobactin (Costa and Loper, 1994). Chaiharn et al. (2009) demonstrated hydroxamate type of siderophore formation by antagonistic bacterial isolates. Bhattacharya (2010) reported the production of catecholate siderophore by *P. fluorescens* strains isolated from the rhizospheric soil and phyllospheres of ornamental plants. The author confirmed the chemical nature of siderophore by observing pinkish-red color formation in Fiss's low iron medium in presence of excess NaOH and yellow color in presence of nitrous acid in Arnow's test. In the present study the fungal strains were found to produce hydroxamate type of siderophores only. Other authors have also noticed production of mainly hydroxamate siderophores by fungal strains. For instance, Baakza et al. (2004) reported that catecholate siderophore was not produced by any of the 35 siderophorogenic fungal strains included in their study. Instead, the strains showed hydroxamate siderophore production by 30 fungi and carboxylate siderophore production by 5 fungal isolates. Urja and Meenu (2010) screened seven fungal strains isolated from soil for siderophore production and observed that all the isolates including *Aspergillus* sp. SR2 and *Penicillium* sp. S12B produced hydroxamate type of siderophore. Dave et al. (2006) stated that among the siderophores, hydroxamates are produced by bacteria and fungi, catecholates by bacteria and carboxylate by some fungi and few bacteria. Gull and Hafeez (2012) confirmed the role of siderophores produced by *P. fluorescens* in the inhibition of the phytopathogen *R. solani* by ferric chloride experiment. In the experiment, different concentrations of iron were supplemented in King's B medium and the effect of iron deprivation caused by *P. fluorescens* culture on the growth of *R. solani* was observed.

In the present study, the percentage of siderophore production was also estimated in the cell free culture supernatant using the CAS shuttle assay. Among the bacterial isolates, a *Bacillus* sp. produced a maximum of 71 percent siderophore units (psu) while an *Aspergillus* sp. that produced 70 psu was the highest siderophore producer among the fungal antagonists. Several researchers have assayed the amount of siderophore produced by biocontrol bacterial and fungal strains. The CAS shuttle assay used in the present study was also used by Sayyed et al. (2005) for quantifying the siderophore produced by fluorescent pseudomonads. The authors found two isolates, *P. fluorescens* NCIM

5096 and *P. putida* NCIM 2847 producing 87% and 83% units respectively of hydroxamate type of siderophore in modified succinic acid medium. In another study, Sharma et al. (2013) studied the siderophorogenic activity of *Bacillus amyloliquefaciens* strain sks_bnj_1 isolated from rhizosphere of soyabean (*Glycine max*). The strain produced yellow to orange colored halo (15.45 mm) on CAS agar medium and further the CAS-shuttle assay revealed 82.35% units of catecholate type of siderophore production. Shobha and Kumudini (2012) observed the siderophorogenic activity of some *Bacillus* isolates antagonistic to *Fusarium oxysporum*. They recorded maximum siderophore production on the 2nd and 3rd day of bacterial growth by several isolates. Similarly, in another study, siderophore quantification carried out with sugarcane rhizosphere inhabiting bacteria revealed that seven isolates produced more than 85% siderophore units (Tailor and Joshi, 2012). Amongst them *Pseudomonas fluorescens* S-11 was found be the most efficient siderophore producer (96%). Siderophore produced by *Trichoderma asperellum* strains isolated from cucmber rhizosphere were also reported to be quantified by Qi and Zhao (2013) by the CAS-shuttle assay.

For sustaining in the rhizosphere and protecting the root system against phytopathogens, the prerequisite for rhizobacteria and fungi is the competition for nutrients and habitats with other microbial inhabitants (Compant et al., 2005). In this context, siderophore production plays a significant role in rhizosphere competence of soil microbes.

In the present study, scanning electron microscopy of the interacting zones of the antagonistic bacteria (*S. marcescens* ETR17 and *Bacillus* sp. KTR6) and plant pathogenic fungi, *R. solani* revealed clear distortion of fungal mycelia indicated by lysis, bulging and bursting of hyphae was observed at the interaction zone in dual culture plates. Enzymatic lysis of cell walls leading to loss of fungal protoplasm is one of the main antagonistic mechanisms involved in the activity of biocontrol agents (Lim et al., 1991; Kim and Chung, 2004). Ordentlich et al. (1988) also observed severe mycelial deformities like holes in the hyphae at the interaction zone of bacterial antagonist *S. marcescens* and the pathogen *Sclerotium rolfsii*. Several structural deformities like hyphal lysis and bulging of the mycelium of *Rhizoctonia bataticola* caused by *Paenibacillus* sp. HKA-15 has been reported by Senthilkumar et al. (2007). The mycelial and spore deformities of *Aspergillus parasiticus* NFRI-95 caused by *S. marcescens* JPP1 crude culture filtrate was observed under SEM by Wang et al. (2013). The authors observed

severe degradation on fungal cell walls represented by abnormal hyphal structures. The present bacterial strains, ETR17 and KTR6 produced several lytic enzymes including extracellular chitinase which degrades chitin, a fungal cell wall component. Thus, it may be concluded that cell wall degradation is involved as a prominent antagonistic mechanism exhibited by the present isolate.

Formation of biofilm has been thought to play a role in biocontrol of several antagonistic bacterial strains including *Bacillus*, *Serratia*, *Acinetobacter*, *Pseudomonas* and *Citrobacter* (Dorsey et al., 2002; Morris and Monier, 2003; Bais et al., 2004; Muller et al., 2009). One of the important mechanisms involved in the success of BCAs like *Bacillus*, which is considered as the first successful BCA against many plant pathogens (Chen et al., 2012), is the production of biosurfactants which is essential for maintaining the aerial structure of biofilms (Danhorn and Fuqua, 2007; Pang et al., 2009). A study comparing the stability of the biosurfactant, surfactin and the lipopeptide antibiotic iturin A, both produced by *Bacillus* strains, revealed that the half-life of surfactin in soil is longer than that of iturin A which suggested the stable role of surfactin in the rhizosphere (Asaka and Shoda, 1996). Bais *et al.* (2004) reported that colonization of plant roots by *B. subtilis* is associated with surfactin production and biofilm formation. The biofilm formed on the surface of Arabidopsis roots was found to protect the plant against infection by *Pseudomonas syringae*. In another study, Chen et al. (2012) found that *B. subtilis* 3610 (used as a model system to study biofilm formation) is able to form robust biofilms on the root surfaces of tomato plants and that the ability to colonize roots requires regulatory genes and biofilm matrix genes responsible for synthesis of two major matrix components: amyloid-like fibres and an exopolysaccharide. In a further study, the authors observed that both biofilm formation and surfactin production were necessary, but neither one alone was sufficient, for plant protection, and that they act synergistically to enhance biocontrol efficacy (Chen et al., 2012).

Like many other *Serratia* spp., the most potential biocontrol strain *S. marcescens* ETR17 of the present study was also an efficient biofilm producing strain as evident from the results of microtitre plate assay. The bacterial strains showed better biofilm production in LB medium compared to M9YE medium. Agarwal et al. (2011a) also studied the effect of media composition on biofilm formation and found that *Salmonella* isolates cultured in LB medium

produced better biofilm compared to Tryptic Soy broth. In a study on characterization of two AHL-mediated quorum sensing system in *S. plymuthica* G3, Liu et al. (2011) observed that although the quorum sensing network is involved in the global regulation of some biocontrol-related traits, the biofilm formation was strain-specific and linked to the original environment of the isolate.

The production of antifungal antibiotics in the cell free culture supernatant of bacterial antagonists in different media also observed during the present study and a novel method for identification of antibiotics in the culture supernatant by spectrophotometry was developed. The CPM-Ca^{2+} medium was selected for detecting antibiotic production by the gram positive antagonistic bacterial strains based on earlier findings by Bernal et al. (2002) who observed five-fold increase in antibiotic (lipopeptide) production by a gram positive antagonistic *Bacillus* sp. in this medium. The authors suggested that the increase in antibiotic concentration was due to the increase in cell wall permeability of the *Bacillus* isolate promoted by Ca^{2+}. A similar observation on the significant increase in antibiotic production by *Lactococcus lactis* IO-1 after addition of Ca^{2+} to the medium was made by Matsusaki et al. (1996). Our results also provide clear evidence of the presence of antibiotics in the culture supernatant of antagonistic bacterial isolates. The use of PPM medium for antibiotic production has been reported mainly in gram negative *Pseudomonas* strains (Whistler and Pierson, 2003; Morello et al., 2004). Rosales et al. (1995) observed large inhibition zones against *R. solani* formed by *P. aeruginosa* and *P. cepacia* due to the production of pyrrolnitrin and phenazine-1-carboxylic acid in PPM medium and suggested that the main component peptone, along with the temperature, aeration and carbon source enhanced the antibiotic yield. Whistler and Pierson (2003) also used PPM medium for culturing *P. aureofaciens* strain 30-84 and its mutants for the extraction of phenazine antibiotic. Another medium used in this study for detection of antibiotics produced by gram negative bacteria was YEMA medium. Edulamudi et al. (2011) observed the production of bacteriocin antibiotic by *Rhizobium* isolates on YEMA medium. Mannitol which is the main carbon source of the medium is reported to increase antibiotic production compared to glucose (Bernal et al., 2002).

Spectral scan of the bacterial antibiotics with immense biocontrol potential either in purified or semi-purified conditions has been performed increasingly for identifying the nature of the compound (Shanahan et al.,

1992; Rodriguez and Pfender, 1997; Duffy and Defago, 1999; Fuente et al., 2004). Whistler and Pierson (2003) detected purified phenazine obtained from *Pseudomonas aureofaciens* strain 30-84 spectrophotometrically having an absorbance maxima of 367nm which is closely similar to the crude secondary metabolite (antibiotic) detected in the culture supernatant of the antagonistic *Cedecea* sp. BTRL6 (peak with absorbance maxima of 368nm) in the present study. Although *Cedecea* sp. has been found to be closely associated with plant surface and the rhizosphere very few report suggests its role as BCA and our study reports the isolation of phenazine producing *Cedecea* sp. possibly for the first time.

The role of pyrrolnitrin as a major factor of biological control of fungal diseases of plants has been well documented (Hill et al., 1994; Burkhead et al., 1994; Parke and Gurian-Sherman, 2001; Hwang et al., 2002; Zhang et al., 2006). Rodriguez and Pfender (1997) obtained the chromatograms of antibiotics, pyrrolnitrin, 2,4-diacetylphloroglucinol and pyoluteorin produced by *P. fluorescens* with absorption maxima of 225 nm, 272 nm and 325 nm respectively which were similar to the antibiotic standards. In this study, peaks occurring in the range of wavelength between 210-225nm was considered as pyrrolnitrin based on earlier findings by Chernin et al. (1996) who observed the absorbance maximum of the major peak of pyrrolnitrin at 210nm. Altogether twelve gram negative strains in this study were capable of producing pyrrolnitrin of which three *Serratia* strains, an *Acinetobacter* sp. and a *Pseudomonas* sp. produced other antibiotics as well. As pyrrolnitrin is known to be involved in biocontrol of antagonistic strains, therefore, the indication of the presence of this antibiotic in the culture supernatant of several bacterial antagonists in the present study may be regarded as a significant finding.

Production of DAPG and pyoluteorin by several antagonistic *Pseudomonas* isolates with biocontrol properties has been widely reported (Fuente et al., 2004; Jousset et al., 2006; Bakthavatchalu et al., 2013). The current study reports the presence of pyoluteorin and DAPG in extracellular secondary metabolites produced by one fluorescent *Pseudomonas* sp. and an *Acinetobacter* sp. respectively. The possible presence of DAPG in the crude culture supernatant of *Acinetobacter* sp. in this study is reported for the first time. Another antibiotic prodigiosin, known to have antifungal, antibacterial, anti-proliferative and immunosuppressive activity is produced mainly by *S. marcescens*. Its role as an effective biocontrol metabolite of *S. marcescens* has been assessed by several

workers (Someya et al., 2003; Someya and Akutsu, 2009). All three antagonistic *Serratia* isolates reported in this study were capable of producing the pigmented antibiotic prodigiosin.

Bacillus spp. with biocontrol activity are known to produce a wide variety of antifungal antibiotics like zwittermycin, kanosamine and lipopeptides of iturin, surfactin and fenzycin families (Ongena and Jaques, 2007; Kumar et al., 2009). In the present study, 6 *Bacillus* isolates and one *Aerococcus* strain BTRL11 was found to produce lipopeptide or peptide antibiotics. It has been reported that most of the peptide antibiotics produced by gram positive *Bacillus* spp. have absorbance maxima between 210-230nm and 270-280nm (Kumar et al., 2009; Gordillo and Maldonado, 2012). The results of this study were in agreement to these findings. The cyclic lipopeptides (CLPs) produced by both gram-positive and gram-negative bacteria are also capable of inducing swarming and biosurfactant properties in the producer strains (Nielsen et al., 2000; Nielsen et al., 2002; Fernando et al., 2005). Interestingly, many of the present *Bacillus* isolates were capable of producing potent swarming motility and biofilm forming potential which may be due to the action of lipopeptides.

Antifungal metabolites especially the antibiotics have been isolated and purified from several biocontrol bacteria in the past (Chernin et al., 1996; Duffy and Defago, 1999; Arguelles-Arias et al., 2009; Selin et al., 2010). In the current study, crude antifungal metabolites were extracted from two most potential bacterial isolates, *S. marcescens* strain ETR17 and *B. subtilis* strain KTR6 by solvent extraction. The crude antifungal compounds were found to possess antagonistic activity against *L. theobromae* of tea as evident from the results of agar cup bioassay. The considerably low MIC value of both KTR6 and ETR17 crude extracts required for inhibiting the growth of *L. theobromae* (110ìg/ ml of KTR6 and 100ìg/ml of ETR17) was due to the high yield of the antibiotic compound. Other authors have also observed antifungal activity of crude culture supernatant in agar plates. The crude concentrated culture supernatant of *P. fluorescens* showed inhibition of pathogenic fungal growth in PDA plates against *P. ultimum*, *M. phaseolina* and *P. oryzae* (Goud et al., 2009). Islam et al. (2012) observed that the ethyl acetate fractions of crude extracellular metabolite produced by *B. subtilis* C9 inhibited the radial growth of tested phytopathogens (concentrations 0.5, 1, and 2 mg/well). The maximum mycelial growth inhibition was recorded at 2mg/ml concentration of the crude metabolite with varying percentages of inhibition against a wide range of plant pathogens including *R.*

solani and *F. oxysporum*. Mageshwaran et al. (2011) determined the MIC of the crude extract of an antibacterial metabolite obtained from *Paenibacillus polymyxa* HKA-15 against *Xanthomonas campestris* pv. phaseoli strains CP-1-1 and M-5 to be 1.7 mg/ml and 1.52 mg/ml respectively. The formation of large zones of inhibition in PDA plates against fungal pathogen by culture supernatant extracts obtained from the current strains strongly suggest the involvement of extracellular antibiotics as one of the possible mechanisms of antagonism.

The crude antifungal metabolite extracted from the most potential bacterial antagonist *S. marcescens* ETR17 was further purified by silica gel column chromatography. Preliminary identification of the antibiotics was done on TLC plates which were further confirmed by UV-spectrophotometry and HPLC analysis. Finally LC-ESI-MS was used to authenticate antibiotic production. In the column purified fractions F9 and F10, pyrrolnitrin antibiotic were detected as one of the major compound with an R_f of 0.80 with respect to the synthetic pyrrolnitrin standard under UV light source (254nm). Upadhyay and Srivastava (2008) detected pyrrolnitrin in the acetone extracted cell pellets of the antagonistic *P. fluorescens* strain Psd cultured in PPM after TLC separation and spraying with diazotized sulphanilic acid. In the current study, the ESI mass spectrum of the partially purified antifungal compound present in the fractions F9 and F10 indicated a molecular ion peak at m/z 259 where two extra hydrogen atoms were linked to the pyrrolnitrin structure (mass 257). The TLC purified pyrrolnitrin antibiotic exhibited absorbance maxima at 210nm and 249nm which was consistent with the UV spectrum of pyrrolnitrin produced by *E. agglomerans* (Chernin et al., 1996). Further, HPLC analysis of the TLC recovered pyrrolnitrin also produced a chromatogram similar to the pyrrolnitrin standard (retention time of 3.02min, 100% methanol elution at 225nm). Liu et al. (2007) detected pyrrolnitrin production by *S. plymuthica* C48 (wild type and mutant strains) by isocratic elution with water (45%), acetonitrile (30%) and methanol (25%) at 225nm having retention time of 31 min by HPLC. Several other workers also detected pyrrolnitrin with different retention time at 225nm using different solvents for elution from *Serratia* and other bacteria by HPLC (Ovadis et al., 2004; Selin et al., 2010; Mozes-Koch et al., 2012). Pyrrolnitrin has been reported to be involved in the protective action of *Serratia* against *Verticillium* wilt and its production has been correlated with concomitant production of AHL, the quorum sensing signal molecule (Liu et al., 2007). Several other workers also detected pyrrolnitrin with different retention time

at 225nm using different solvents for elution from *Serratia* and other bacteria by HPLC (Ovadis et al., 2004; Selin et al., 2010; Mozes-Koch et al., 2012; Selin et al., 2012).

Prodigiosin is also a potent antifungal antibiotic produced mainly by *Serratia marcescens* and its role as an effective biocontrol metabolite has been assessed by many workers (Okamoto et al., 1998; Liu et al., 2007; Someya and Akutsu, 2009). The production of prodigiosin antibiotic by ETR17 isolate could be confirmed by TLC and spectroscopic data. The relative front (R_f) of prodigiosin standard (Sigma) was similar to one of the major bands (0.6) produced by the column eluted fractions F2, F3 and F4 on TLC plate developed in benzene: acetic acid (9:1). An absorbance maximum of 536nm which is characteristic to the prodigiosin antibiotic was similar to the peaks generated by the TLC recovered antibiotic obtained from F2, F3 and F4 fractions. Patil et al. (2011) also reported the detection of prodigiosin produced by *S. marcescens* NMCC46 by UV-VIS spectrophotometry at 536nm and on TLC plate developed in butanol: hexane (2:1) where pink colored pigment was observed at an R_f of 0.9 with respect to the standard. They further analyzed the TLC purified prodigiosin by HPLC and detected the antibiotic as a single peak with retention time of 4.523 min. Prodigiosin and its analogues isolated from a marine bacterium *Zooshikella rubidus* S1-1 were also analyzed by HPLC (Lee et al., 2011). The pigmented antibiotic prodigiosin and its analogue cycloprodigiosin were detected at retention time of 7.86 min and 7.08 min respectively. In the present study, TLC recovered prodigiosin produced two peaks with retention time 8.268 min and 9.024min which were closely similar to the standard (retention time 7.013 min and 9.018 min).

The crude extract and the fractions producing the pyrrolnitrin and prodigiosin antibiotics were also tested for antifungal activity by agar cup and TLC plate bioassay. The bioautography plates of crude antifungal metabolite of ETR17 produced two significant zones of inhibition of *L. theobromae* growth at R_f 0.3 (possibly of pyrrolnitrin) having 1.1 cm zone diameter and at R_f 0.6 (prodigiosin) having 0.5cm zone diameter. The partially purified antibiotics also showed clear zone of inhibition of mycelial growth of the tested phytopathogen formed at R_f positions similar to pyrrolnitrin and prodigiosin. Burkhead et al. (1994) studied the inhibitory effect of pyrrolnitrin against *Fusarium sambucinum* by bioautography and found that the inhibition zone was formed at the R_f of pyrrolnitrin.

Microorganisms belonging to the genera *Bacillus*, *Serratia* or *Trichoderma* are ubiquitous in agricultural soils, and are well adapted to the rhizosphere environment. A number of favourable characters including widespread distribution in soil, colonizing ability in the rhizospheres of host plants, and ability to produce antagonistic secondary metabolites active against several plant pathogens have made several strains of these microorganisms a subject of intense research as biocontrol agents at the genetic and biochemical level (Harman, 2004; Kumar et al., 2011; Saha et al., 2012a and 2012c). A large number of these strains have been utilized as inoculums in plant health management practices to control or inhibit plant pathogens and stimulate plant growth (de Queiroz et al., 2006; Hermosa et al., 2012; Kaki et al., 2013; Sunar et al., 2013). Biological control of deleterious microbes especially fungi by introducing antagonistic microorganisms onto plant surfaces has been the focus of considerable research (Pal and Gardener, 2006; Heydari and Pessarakli, 2010; Saharan and Nehra, 2011; Bhattacharya and Jha, 2012).

A formulated microbial product, is defined as a product composed of biomass of a biocontrol agent and ingredients to improve the survival and effectiveness of the product. The production of a high quality inoculum with a uniformly high biological potential is necessary for successful implementation of biological control (Schisler et al., 2004). In the present study, bioformulations were prepared from the most promising isolates *S. marcescens* strain ETR17 and *B. subtilis* strain KTR6 and applied against induced *Rhizoctonia* root rot in tea in controlled green house experiments. Before the *in vivo* study, the pathogenicity of the *R. solani* strain was confirmed through the verification of Koch's postulates in two seed varieties of tea, TS-462 and TS-520. The pathogenicity of *R. solani* was confirmed through the verification of Koch's postulates. Results showed that TS-520 was more susceptible compared to TS-462 and therefore, TS-520 was used further for *in vivo* biocontrol studies.

In the present study the talc formulations of the bacterial strains effectively reduced the root rot disease incidence in tea plantlets. Results showed 90.5% (ETR17) and 86% (KTR6) reduction in disease incidence in TS-520 variety on soil application of bioformulation under sterile conditions. Method of application is a major aspect that contributes significantly towards achieving a good biocontrol efficiency and plant growth promotion (Xue et al., 2009). The soil application method was used as this method has been shown to produce better levels of colonization and biocontrol efficiency than other methods like

root dipping (Xue et al., 2009) or seed inoculation (Gotz et al., 2006). There are reports on the antagonistic activity of *S. marcescens* against *Pyricularia oryzae* causing blast disease in rice (Jaiganesh et al., 2007). Use of talc-based formulation containing *S. marcescens* was successful in reducing the incidence of rice blast caused by *Pyricularia oryzae* when applied by foliar spray method. Chakraborty et al. (2010b) also observed varying degree of *in vitro* and *in vivo* antagonism using bioformulations of *S. marcescens* against *Fomes lamaoensis* in tea. Kishore et al. (2005) found that chitin-supplemented bioformulation of *Serratia marcescens* GPS 5 and *Bacillus circulans* GRS 243 suppressed the late leaf spot (LLS) disease of groundnut or peanut (*Arachis hypogaea*) caused by *Phaeoisariopsis personata* significantly. Chitin-supplemented (1% wt/vol) application of *B. circulans* GRS 243 and *S. marcescens* GPS 5 resulted in improved biological control of LLS disease (60% reduction) under greenhouse conditions. Chitin supplemented application of GRS 243 and GPS 5 also resulted in improved control of LLS disease in repeated field experiment and increased the pod yields by 62% and 75%, respectively, compared to the control. Bioformulations prepared with talc, saw dust and rice husk using *S. marcescens*, *B. amyloliquefaciens* and *B. pumilis* increased the growth of tea plants under glasshouse and field conditions and also induced systemic resistance (Chakraborty et al., 2013). Other authors have reported *Serratia* strains to be effective against *Phytophthora* blight of pepper (Shen et al., 2002) and *Fusarium* wilt disease incidence in cucumber seedlings (Ahmed, 2010). Jorjani et al. (2011) demonstrated that bioformulation prepared with *Bacillus coagulans* (B2) and *P. fluorescens* (B1) using talc and bentonite powders as inorganic carriers and peat and rice bran as organic carriers significantly promoted the growth of sugar beet plants. Rasu et al. (2013) used farmyard manure based bioformulation of *B. subtilis* strain alongwith *P. fluorescens* and *Trichoderma asperellum* for controlling sugarbeet root rot caused by *Sclerotium rolfsii*. The individual formulation of *B. subtilis* significantly reduced the disease incidence (40%) compared to the fungicide treatment. However, the combined formulation was more effective in disease control (37.5%) than individual formulation. Rajendran and Samiyappan (2008) studied the biocontrol efficacy of talc-based formulation prepared with *Bacillus* sp. EPCO102 and EPCO106 alongwith *P. fluorescens* for inducing disease resistance and controlling damping off disease in cotton caused by *R. solani*. The disease incidence was significantly reduced when the biocontrol strains were used as chitin amended bioformulated

products. Further, the level of defense enzymes like glucanase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase also increased.

In the present study, root rot disease incidence in tea was remarkably reduced (above 80%) in the current study when mixed formulation of the bacterial isolates ETR17 and KTR6 was applied. This could be due to the synergistic effect of two different species involving different mechanisms of biocontrol. The results were consistent for both sterilized and unsterilized soil treatments. Domenech et al. (2006) reported successful use of a bioformulated product LS213 containing *Bacillus*, *Pseudomonas* and *Chryseobacterium* for the control of damping off and wilt diseases in tomato and pepper. The use of multiple strains also improved plant growth by producing several growth inducing factors like plant hormones and siderophore. Similar observation on the control of sheath blight incidence and increase in rice yield by multiple *Pseudomonas fluorescens* strain talc formulation was made by Nandakumar et al. (2001).

Greenhouse studies on the biocontrol potential of the most effective fungal antagonist in the present study were performed. It was observed that the *T. viride* AD10 isolate was capable of reducing the *R. solani* root rot disease incidence in tea plants to a significant level in both sterile (90.8%) and unsterile (85.7%) conditions. Khalili et al. (2012) studied the significant control of brown spot disease in rice caused by *Bipolaris oryzae* under glasshouse conditions using *T. harzianum* and *T. atroviride* isolates. Gveroska and Ziveroski (2011) evaluated the biocontrol potential of *Trichoderma* spp. in controlling root rot disease in tobacco seedlings caused by *R. solani*. *Trichoderma* reduced the disease incidence both as individual treatment and in combination with commercial fungicide. The biocontrol of *F. oxysporum* foot and root rot of Lentil plants mediated by *T. harzianum* isolates was studied by Kashem et al. (2011). They observed only 6.9% root rot incidence in the plants after treating with *T. harzianum* isolates. *Verticillium* wilt in tomato was reduced by 60% two months after treatment with *Trichoderma* isolates, *T. harzianum*, *T. viride* and *T. virens* (Jabnoun-Khiareddine et al., 2009).

Several workers also reported that *Serratia* spp. and *Bacillus* spp. act as promising plant growth promoters (Someya et al., 2005; Muller et al., 2009; Kumar et al., 2011; Mohamed and Gomma, 2012). In the greenhouse trials using the bioformulated products of bacterial antagonists in our study, the *B.*

subtilis isolate KTR6 was more effective in promoting the root and shoot length of the tea seedlings under both sterile and unsterile conditions to a desirable extent compared to *S. marcescens* ETR17 treated and the untreated control plants. However, the mixture of *B. subtilis* and *S. marcescens* strains was most effective in enhancing the growth rate of tea seedlings. Significant increase of root and shoot growth of plants is often attributed to the synthesis of growth promoting factors like siderophores, IAA, gibberellins, cytokinins, nitrogen fixation and solubilization of phosphates (Patel et al., 2008; 2010). The present isolates were capable of producing siderophore and IAA while phosphate was solubilized by *B. subtilis* which attributed to its increased growth promoting capacity. Our results are in parity with the observations of other workers on PGPR strains. Kumar et al. (2011) evaluated the role of a commercial formulation of *Bacillus subtilis* MBI 600 (Integral) for growth promotion in rice cultivars. Seed treatment with Integral increased shoot and root lengths of rice plants significantly. *B. subtilis* harboring multiple plant growth promoting traits such as IAA, siderophore and phosphatase production induced increase in the biomass of white radish plants (Mohamed and Gomma, 2012). Chakraborty et al. (2010b) evaluated the role of *S. marcescens* strain TRS-1 in growth promotion of tea plants. The phosphate solubilizing bacterial strain applied as formulated product enhanced the plant height, leaf biomass, phosphate content of root and leaf and reduced the soil phosphate content. George et al. (2013) studied the PGP activity of *S. marcescens* and *Enterobacter* sp. on coconut, paddy and cowpea. Both the strains were IAA, 1-aminocyclopropane-1-carboxylate deaminase and phosphatase producers; additionally, *S. marcescens* was capable of fixing atmospheric N_2 and producing siderophore.

Sustainability of the most potential bacterium *S. marcescens* ETR17 was tested serologically by indirect ELISA where the absorbance provided an indication of the population of bacteria in the soil, since the antibody raised was specific for the bacterial antigen. The population of the bacterium ETR17 in both sterilized and unsterilized soil treatments (upto 7-8 x 10^8 cfu/g) was abundant in the soil upto a considerable time period. ELISA was used to study colonization of rice plants by nitrogen fixing *Serratia* strain by Gyaneshwar et al. (2001). The authors found that the method was more reliable compared to other techniques because the polyclonal antisera raised in the study exhibited no non-specific reactions with other endophytic bacterial species. Detection of *Enterobacter asburiae* JM22 population, a cotton endophyte by using polyclonal

antisera through ELISA had been reported by Quadt-Hallmann and Kloepper (1996). The method was very specific with detection limit of 10×1.0^4 cfu/ml. Chakraborty et al. (2009) also reported the use of ELISA in monitoring the population of the bacterium *O. anthropi* in the soil and found that it survived well upto six months of application. The sustenance of PGPR strains of *S. marcescens, B. amyloliquefaciens and B. pumilis* in tea rhizosphere was observed 3 months post inoculation (Chakraborty et al., 2010b; 2013). Literature studies suggest that although ELISA was used for several bacterial population studies in soil, plant root associations but the use of this technique for detection and assessment of the population of an efficient biocontrol strain of *S. marcescens* in soil was done for the first time in our study. The bacterium *S. marcescens* could effectively colonize the tea roots as evident from the scanning electron microphotographs showing the localization of the bacterial cells inside the root tissue. Kim and Kremmer (2005) observed colonization of the PGPR isolates; *Bacillus megaterium* GP4 and *Pseudomonas putida* GD4 on the root surface and localization of *Bradyrhizobium japonicum* isolate GD3 in the root surface furrows by scanning electron microscopy.

Assessment of viability of bacterial strains in talc formulations under laboratory conditions is essential for estimating the shelf-life of the strain for long term storage during commercialization. The viability of the bacterium ETR17 in talc formulation upto six months at room temperature suggested that the formulation can be easily stored and used for a long duration. Viability of antagonistic *P. fluorescens* UTPF61 strain in talc formulation at its optimum pH was recorded upto 90 days when stored at 4°C and 26°C (Sadi and Masoud, 2012). Chakraborty et al. (2009) estimated a long-term viability of *O. anthropi* in talc-based formulation under laboratory conditions upto 9 months. Bioformulation of a phosphate solubilizing bacterium *Bacillus megaterium* in talc was assessed for the survivability and 6months of viability was recorded at room temperature (Omer, 2010).

Several studies have been conducted in the past to find ways of suppression of plant diseases which is effective as well as environment-friendly. The present study showed that two specific bacterial biocontrol isolates *S. marcescens* strain ETR17 and *B. subtilis* strain KTR6 with strong antibiotic producing and plant growth promoting capacity was able to reduce the root rot disease incidence in tea plantlets effectively. Similarly a particular *T. viride* isolate was also effective in reducing the disease. The bacteria were used as

bioformulations which imparted long term survivability to the strains thereby strongly indicating that effective commercial formulations can be developed in future and used in tea industry after appropriate field trials. Additionally, the strains showed multiple antagonistic and plant growth promoting characters such as production of antibiotics like pyrrolnitrin and hydrolytic enzymes like chitinase, protease, lipase and phosphatase; and the plant growth hormone IAA. But how far these properties are involved in disease suppression in plants in the soil environment remained unknown. Future studies in determining the exact role of the antibiotics and enzymes in disease suppression is warranted.

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APPENDIX A

CHEMICALS AND REAGENTS

A number of reagents and chemicals were used in the present study out of which the major chemicals are enlisted below. All other common salts, acids and solvents used were purchased from SRL Pvt. Ltd., Mumbai, India and Merck Specialities Pvt. Ltd., Mumbai, India.

CHEMICALS	COMPANY
10 X Taq Polymerase buffer A without $MgCl_2$	Bangalore Genei (India) Pvt. Ltd., India
10 X Taq Polymerase buffer E with 15mM $MgCl_2$	Bangalore Genei (India) Pvt. Ltd., India
16S rRNA gene forward primer	Sigma Aldrich Chemicals Pvt. Ltd., India
16S rRNA gene reverse primer	Sigma Aldrich Chemicals Pvt. Ltd., India
16S-23S ITS region specific forward primer	Sigma Aldrich Chemicals Pvt. Ltd., India
16S-23S ITS region specific reverse primer	Sigma Aldrich Chemicals Pvt. Ltd., India
ITS1	Sigma Aldrich Chemicals Pvt. Ltd., India
ITS4	Sigma Aldrich Chemicals Pvt. Ltd., India
2,3,5-Triphenyl tetrazolium chloride, A.R. salt	HiMedia Laboratories Ltd, India
500bp DNA ladder	Bangalore Genei (India) Pvt. Ltd., India
Acetic acid (glacial)	SRL Pvt. Ltd., India
Acetone	SRL Pvt. Ltd., India
Adonitol	HiMedia Laboratories Ltd., India
Agar powder, Certified	HiMedia Laboratories Ltd, India
Agarose	SRL Pvt. Ltd., India
L-Arabinose	HiMedia Laboratories Ltd., India
L-Arginine hydrochloride	HiMedia Laboratories Ltd, India
L-Asparagine monohydrate	SRL Pvt. Ltd., India
Barbituric acid	HiMedia Laboratories Ltd, India
Benzene	SRL Pvt. Ltd., India

Appendix A

CHEMICALS	COMPANY
Bovine albumin fraction-V	HiMedia Laboratories Ltd, India
Bromothymol blue	SRL Pvt. Ltd., India
Calcium chloride, dehydrate, A.R.	HiMedia Laboratories Ltd., India
Calcium phosphate, dibasic, Extrapure	HiMedia Laboratories Ltd., India
Carboxymethylcellulose sodium salt	HiMedia Laboratories Ltd, India
Casein Acid Hydrolysate, certified	HiMedia Laboratories Ltd, India
Catechol	HiMedia Laboratories Ltd, India
Cellobiose	HiMedia Laboratories Ltd., India
Cetyltrimethylammonium bromide, Molecular Biology Grade	Calbiochem, EMD Biosciences, Inc. La Jolla, CA. Mktd. by: Merck Specialities Pvt. Ltd., India
Chloroform	SRL Pvt. Ltd., India
Chromazurol S	HiMedia Laboratories Ltd, India
Congo red, certified	HiMedia Laboratories Ltd, India
dATP solution (10mM)	Bangalore Genei (India) Pvt. Ltd., India
dCTP solution (10mM)	Bangalore Genei (India) Pvt. Ltd., India
Decarboxylase Test Medium Base (Falkow)	HiMedia Laboratories Ltd, India
Dextrose	HiMedia Laboratories Ltd., India
dGTP solution (10mM)	Bangalore Genei (India) Pvt. Ltd., India
Diethyl ether	SRL Pvt. Ltd., India
p-Dimethylaminobenzaldehyde extrapure A.R.	SRL Pvt. Ltd., India
DNase Test agar w/Toluidine blue	HiMedia Laboratories Ltd, India
dNTP mix (2.5mM each)	Bangalore Genei (India) Pvt. Ltd., India
dTTP solution (10mM)	Bangalore Genei (India) Pvt. Ltd., India
Ethidium bromide	Bangalore Genei (India) Pvt. Ltd., India
Ethyl acetate	SRL Pvt. Ltd., India

CHEMICALS	COMPANY
Ethylenediaminetetra acetic acid disodium salt (EDTA Na_2 salt) extrapure A.R.	SRL Pvt. Ltd., India
Ferric chloride	HiMedia Laboratories Ltd., India
Fluorescent brightener 28	Sigma Aldrich Chemicals Pvt. Ltd., USA
Formalin/Formaldehyde	SRL Pvt. Ltd., India
Freund's complete adjuvant	Bangalore Genei (India) Pvt. Ltd., India
Freund's incomplete adjuvant	Bangalore Genei (India) Pvt. Ltd., India
D-Fructose	SRL Pvt. Ltd., India
Gel loading buffer (6X)	Bangalore Genei (India) Pvt. Ltd., India
Gelatine powder Bacto	s.d. fine-chem Ltd., India
Glutaraldehyde solution	SRL Pvt. Ltd., India
Glycol chitosan	Sigma Aldrich Chemicals Pvt. Ltd.,USA
Goat anti-rabbit IgG-HRP conjugate	Bangalore Genei (India) Pvt. Ltd., India
Gram's crystal violet solution	Merck Specialities Pvt. Ltd., India
Gram's safranin solution	Merck Specialities Pvt. Ltd., India
Hexane	SRL Pvt. Ltd., India
Hexadecyltrimethyl ammoniumbromide	Merck Specialities Pvt. Ltd., India
Hydrogen peroxide	Merck Specialities Pvt. Ltd., India
Indole-3-acetic acid	Merck Specialities Pvt. Ltd., India
m-Inositol	HiMedia Laboratories Ltd., India
Iodine	HiMedia Laboratories Ltd, India
Lactophenol cotton blue	HiMedia Laboratories Ltd, India
Lactose	SRL Pvt. Ltd., India
Lactose broth	SRL Pvt. Ltd., India
L-Lysine hydrochloride	HiMedia Laboratories Ltd., India
Lugol's iodine	Micromaster Laboratories Pvt. Ltd., India
Luria Broth	HiMedia Laboratories Ltd., India

Appendix A

CHEMICALS	COMPANY
Mac Conkey agar	Micromaster Laboratories Pvt. Ltd., India
$MgCl_2$ (25mM)	Bangalore Genei (India) Pvt. Ltd., India
Malachite green	HiMedia Laboratories Ltd, India
D(+)-Maltose	SRL Pvt. Ltd., India
D-Mannitol A.R.	HiMedia Laboratories Ltd., India
Methanol A.R.	SRL Pvt. Ltd., India
Methanol for HPLC	SRL Pvt. Ltd., India
Methanol for UV spectroscopy	SRL Pvt. Ltd., India
Methyl red	Micromaster Laboratories Pvt. Ltd., India
Methylene blue	SRL Pvt. Ltd., India
α-Naphthol	SRL Pvt. Ltd., India
Nitrate reagent-I	Micromaster Laboratories Pvt. Ltd., India
Nitrate reagent-II	Micromaster Laboratories Pvt. Ltd., India
L-Ornithine monohydrate	HiMedia Laboratories Ltd, India
Orthophosphoric acid	Fisher Scientific, Qualigens Fine Chemicals, India
PCR purification kit	Bangalore Genei (India) Pvt. Ltd., India
Pectin	HiMedia Laboratories Ltd, Mumbai, India
Perchloric acid	Merck Specialities Pvt. Ltd., India
Petroleum ether	SRL Pvt. Ltd., India
Phenazine	Sigma Aldrich Chemicals Pvt. Ltd., USA
Phenol red	HiMedia Laboratories Ltd, India
L-Phenylalanine	SRL Pvt. Ltd., India
Picric acid	Merck Specialities Pvt. Ltd., India
Pikovskaya's broth (Medium)	HiMedia Laboratories Ltd, India
PIPES buffer	HiMedia Laboratories Ltd, India; SRL Pvt. Ltd., India
Prodigiosin	Sigma Aldrich Chemicals Pvt. Ltd., USA

Appendix A

CHEMICALS	COMPANY
Proteinase K	Bangalore Genei (India) Pvt. Ltd., India
Pseudomonas agar (for fluorescein)	HiMedia Laboratories Ltd, India
Pyocyanin	Sigma Aldrich Chemicals Pvt. Ltd., USA
Pyrrolnitrin from *Pseudomonas cepacia*	Sigma Aldrich Chemicals Pvt. Ltd., USA
D(+)-Raffinose	HiMedia Laboratories Ltd., India
L(+)-Rhamnose	HiMedia Laboratories Ltd., India
RNase A	Bangalore Genei (India) Pvt. Ltd., India
Seakem ® LE Agarose	Lonza, Rockland, ME, USA
Silica gel (60-120 mesh) for column chromatography	SRL Pvt. Ltd., India
Simmon's citrate agar	SRL Pvt. Ltd., India
Skimmed milk	Amul, India
Sodium dodecyl sulphate	SRL Pvt. Ltd., India
Sodium thiosulphate, extrapure A.R.	SRL Pvt. Ltd., India
D-Sorbitol	SRL Pvt. Ltd., India
Starch soluble extrapure A.R.	SRL Pvt. Ltd., India
Step Up 100bp DNA ladder	Bangalore Genei (India) Pvt. Ltd., India
Sucrose	SRL Pvt. Ltd., India
Soyabean casein digest medium (Tryptone soya broth)	HiMedia Laboratories Ltd, India
Talcum powder Pract	s.d. fine-chem Ltd., India
Taq DNA Polymerase 3U/µl	Bangalore Genei (India) Pvt. Ltd., India
N,N,N',N'-Tetramethyl-p-phenylene diamine dihydrochloride	HiMedia Laboratories Ltd., India
TLC Silica gel 60 F_{254} aluminium sheets	Merck, Germany
TMB/H_2O_2 Substrate for ELISA	Bangalore Genei (India) Pvt. Ltd., India
Trehalose	HiMedia Laboratories Ltd., India
Tris (Hydroxylmethyl) Aminomethane (Tris buffer) pure A.R.	SRL Pvt. Ltd., India

Appendix A

CHEMICALS	**COMPANY**
Tris (Hydroxylmethyl) Aminomethane (Tris Buffer, Tris Base) for molecular biology	SRL Pvt. Ltd., India
Tryptone	HiMedia Laboratories Ltd., India
L-Tryptophan	HiMedia Laboratories Ltd, India
Tween 20	HiMedia Laboratories Ltd., India
Tween 80	HiMedia Laboratories Ltd., India
Urea, ACS	HiMedia Laboratories Ltd, India
D-Xylose	HiMedia Laboratories Ltd., India

APPENDIX B

BUFFERS AND REAGENTS

1. Acetate buffer (pH 4.0)

Solution A (0.2M Acetic acid solution)

Acetic acid	11.5 ml
Distilled water	988.5 ml

Solution B (0.2M Sodium acetate solution)

Sodium acetate	22.2 g
Distilled water	1000.0 ml

For preparing, 100ml of the acetate buffer, 82ml of solution A was mixed with 18ml of solution B and the pH was adjusted to 4.0.

2. Ammonium acetate buffer (500ml; pH 6.5)

Ammonium acetate	38.5 g
Distilled water	400.0 ml

Ammonium acetate was dissolved vigorously in water and glacial acetic acid was added dropwise till the pH reaches upto 6.5. The final volume was adjusted to 500ml with distilled water.

3. Carbonate/bicarbonate buffer (0.2M; pH 9.6)

Solution A (0.2M Sodium carbonate solution)

Sodium carbonate (anhydrous)	21.2 g
Distilled water	1000.0 ml

Solution B (0.2M Sodium bicarbonate solution)

Sodium bicarbonate	16.8 g
Distilled water	1000.0 ml

16 ml of solution A was mixed with 34ml of solution B and the mixture was diluted to 200ml with distilled water to obtain carbonate/bicarbonate buffer of pH 9.6.

4. CTAB DNA extraction buffer (for 1g of fungal mycelia)

Tris-Cl (1M; pH 8.0)	500.00 µl
NaCl (5M)	1.40 ml
EDTA (0.5M; pH 8.0)	200.00 µl
β-mercaptoethanol	10.00 µl
CTAB	100.00 mg
Double distilled water (sterile)	2.89 ml

All the ingredients were mixed in appropriate ratio and warmed at 65°C in order to dissolve the CTAB powder for 20 minutes.

5. Phosphate buffer 0.1 M (pH 7.0)

Solution A (0.2M Disodium hydrogen phosphate solution)

Disodium hydrogen phosphate (Na_2HPO_4)	35.61 g
Distilled water	1000.00 ml

Solution B (0.2M Sodium dihydrogen phosphate)

Sodium dihydrogen phosphate (NaH_2PO_4)	31.21 g
Distilled water	1000.00 ml

61 ml of solution A was mixed with 39ml of solution B and the mixture was diluted to 200ml with distilled water to obtain phosphate buffer of pH 7.0.

6. Phosphate buffer (0.15M; pH 7.2)

Solution A

Sodium dihydrogen phosphate (NaH_2PO_4)	23.4 g
Distilled water	1000.0 ml

Solution B

Disodium hydrogen phosphate (Na_2HPO_4)	21.3 g
Distilled water	1000.0 ml

280 ml of solution A was mixed with 720 ml of solution B and the pH was adjusted to 7.2.

7. Phosphate buffered saline (PBS) (0.15M; pH 7.2)

NaCl (0.8%) and KCl (0.2%) was added to the phosphate buffer (0.15M) and the final pH was adjusted to 7.2.

8. Tris-Cl (1M; pH 8.0)

Tris base	121.1 g
HCl	40-45 ml

Tris base was dissolved in 900ml of distilled water and the pH was adjusted to 8 by adding HCl.

9. Tris-EDTA (TE) buffer (pH 8.0)

Tris buffer (0.5 M)	200.0 µl
EDTA (0.5 M)	20.0 µl

Final volume was made up to 10 ml, autoclaved and stored at room temperature.

10. 50X TAE buffer (pH 8.0; 1000ml)

0.5M EDTA stock (500ml, pH 8.0)

93.0g of EDTA was dissolved in 400 ml of H_2O by adjusting the pH to 8.0 using 1N NaOH and the final volume was made upto 500ml using double distilled water.

TAE buffer

Tris base	242.0 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M)	100.0 ml

Tris base was mixed under stirrer on a magnetic stirrer in 600 ml double distilled water. 0.5M EDTA was then added to the above mixture followed by glacial acetic acid, the pH was adjusted to 8.0 and the final volume was adjusted upto 1000ml using double distilled water. The buffer was stored at room temperature and diluted to 1x when required using double distilled water.

11. Benedict's solution

Sodium citrate	17.30 g
Sodium carbonate (Na_2CO_3)	10.00 g
Copper sulphate ($CuSO_4$)	1.73 g
Distilled water	100.00 ml

Sodium citrate and sodium carbonate were dissolved in 60ml of water. Copper sulphate was dissolved in 20ml of water and added to the citrate-carbonate mixture with constant stirring. The volume was then adjusted to 100ml with distilled water.

12. Kovac's reagent for Indole test

p-dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Conc. HCl	25.0 ml

The aldehyde was dissolved in alcohol by gentle warming in a water bath (about 50-55°C), cooled and then hydrochloric acid was added with care. The solution was stored at 4°C in dark.

13. Methyl Red indicator

Methyl red	0.1 g
Ethyl alcohol (95%)	300.0 ml
Distilled water	200.0 ml

Methyl red was dissolved in alcohol to which distilled water was added and then the mixture was filtered.

14. Nitrate test reagents

Prepared Nitrate reagent I and Nitrate reagent II were used for nitrate and nitrite reduction tests.

15. Nitrite-molybdate reagent

Sodium nitrite	10.0 g
Sodium molybdate	10.0 g
Double distilled water	100.0 ml

Sodium nitrite and sodium molybdate were added in appropriate ratio to double distilled water and stirred well to mix the ingredients.

16. Oxidase test reagent (Wurster's reagent)

N,N,N',N'-Tetramethyl-p-phenylene diamine dihydrochloride	5.0 g
Distilled water	50.0 ml

The solution was prepared under dark conditions immediately before use and the reagent was discarded if it was darkened.

17. Phenol Red indicator

Phenol red	0.2 g
Ethyl alcohol (95%)	500.0 ml
Distilled water	500.0 ml

For preparation of the indicator solution, phenol red was dissolved in alcohol, distilled water was added and then the mixture was filtered.

18. Preparation of CAS Indicator solution

Initially, 60.5 mg of chrome azurol S was dissolved in 50 ml of double distilled water. Then, 10 ml of Fe III solution (27 mg $FeCl_3.6H_2O$ and 83.3 µl concentrated HCl in 100 ml double distilled water) was added, along with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml double distilled water. The HDTMA solution was added slowly while stirring, resulting in a dark blue colored solution (100 ml total volume), which was then sterilized by autoclaving at 121°C for 15 minutes.

19. Reagent for Catalase activity

H_2O_2 solution	10%

20. Reagents for endospore staining

Malachite green stain

Malachite green	5.0 g
Distilled water	100.0 ml

Safranin stain

Safranin (2.5% solution in 95% ethanol)	10.0 ml
Distilled water	100.0 ml

21. Reagents for Gram Staining

Gram's crystal violet, Lugol's iodine and Gram's safranin solutions (ready stock purchased) were used for gram staining.

22. Reagents for VP test

VP reagent-I

α-naphthol	5.0 g
Ethanol (absolute)	95.0 ml

VP reagent-II

Potassium hydroxide (KOH)	40.0 g
Distilled water	100.0 ml

23. Salkowski's reagent

Ferric chloride ($FeCl_3$)	0.05M
Perchloric acid	35%

$FeCl_3$ was added to perchloric acid and mixed till the salt dissolves completely to produce the Salkowski's reagent.

24. 70% ethanol for DNA isolation

Absolute ethanol	70.0 ml
Distilled water	30.0 ml

25. 10% SDS solution for DNA isolation

SDS	10.0 g
Sterile distilled water	100.0 ml

SDS was added to sterile distilled water and heated in water bath to dissolve completely at 65°C.

26. 1% CTAB in 1M NaCl (100ml) for DNA isolation

CTAB	1.0 g
1M NaCl	100.0 ml

Measured amount of CTAB was added to sterile 1M NaCl and heated in water bath to dissolve completely at 60°C.

27. 5M NaCl for DNA isolation

NaCl	292.0 g
Distilled water	1000.0 ml

Measured amount of NaCl was added to distilled water, mixed properly till the salt dissolves and thereafter sterilized by autoclaving at 15 lbs p.s.i pressure for 15 min at 121°C.

APPENDIX C

MEDIA

The compositions of different media used in the present study are described below.

1. Carbohydrate fermentation (utilization) medium

Peptone	10.0 g
Carbohydrate*	5.0 g
NaCl	15.0 g
Phenol red	0.018 g
Distilled water	1000.0 ml
pH	7.3

The above ingredients were mixed by stirring and a specific carbohydrate (*: arabinose, adonitol, cellobiose, glucose, fructose, inositol, sucrose, lactose, maltose, mannitol, raffinose, rhamnose, sorbitol, trehalose and xylose) was added and dispensed into test tubes containing Durham's tube. The tubes were thereafter sterilized at 15 lbs p.s.i at 121°C for 15 minutes.

2. Cellulose agar medium

Carboxy methyl cellulose	10.0g
$FeCl_3$	0.004g
$(NH_4)_2SO_4$	1.0g
NaCl	0.6g
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.5g
KH_2PO_4	0.5g
$CaCl_2.2H_2O$	0.002g
Distilled water	1000.0 ml

All the ingredients were dissolved in distilled water and mixed by stirring and pH was adjusted to 7.0. Agar was added at a concentration of 1.8% to the medium and sterilized at 15 lbs p.s.i. at 121°C for 15 minutes and finally dispensed into petriplates.

3. CPM-Ca^{2+} broth medium (Bernal et al., 2002)

Mannitol	10.0g
Casamino acid/	1.0g
Casein acid hydrolysate	

Peptone	10.0 g
$CaCl_2.2H_2O$	5.0 g
Distilled water	1000.0 ml

The ingredients were dissolved in distilled water by mixing properly and heating if necessary. The pH of the medium was adjusted to 7.0 and thereafter, the medium was sterilized at 15 lbs p.s.i. at 121°C for 15 minutes.

4. CPM-Ca^{2+} agar medium

0.35% agar was added to the CPM-Ca^{2+} broth and dissolved by heating. The medium was then sterilized at 15 lbs p.s.i. at 121°C for 15 minutes and dispensed into petriplates.

5. Decarboxylase medium (Falkow's)

9.0g of decarboxylase test medium base (Falkow) was dissolved in 1000ml of distilled water according to the manufacturer's protocol. Heated and dissolved properly and then distributed to 4 equal parts in test tubes (4ml each). One part without amino acid and remaining three with three amino acids (L-lysine, L-ornithine and L-arginine) at a final concentration of 0.5% were added separately to three tubes. The tubes were then sterilized at 15 lbs p.s.i. at 121°C for 15 minutes.

6. DNase test medium

DNase Test Agar	42.1 g
Distilled Water	1000.0 ml

The ingredients were dissolved; pH was adjusted to 7.3, sterilized at 15 lbs p.s.i at 121°C for 15 minutes and distributed aseptically to sterile petriplates.

7. Gelatin Medium

Gelatin	40.0 g
Tryptone	17.0 g
Peptone	3.0 g
NaCl	5.0 g
K_2HPO_4	2.5 g
Distilled water	1000.0 ml

All the constituents were dissolved excepting gelatin in 500ml of distilled water. The gelatin was added to 400ml of distilled water, boiled and mixed well with the other constituents dissolved earlier and the final volume was adjusted to 1000ml. The medium was dispensed in 5ml volume to the test tubes and sterilized by autoclaving at 121°C for 15 minutes.

8. Hugh and Leifson's media (pH 7.1)

Peptone	2.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Bromothymol blue (0.2%)	15.0 ml
Agar	3.0 g
Distilled water	1000.0 ml

The solids except agar were dissolved by heating in water and pH was adjusted to 7.1, filtered, and the indicator bromothymol blue was added. Then agar was added to the mixture, heated to melt and sterilized at 15 lbs p.s.i. at 121°C for 15 minutes.

9. 3-ketolactose production medium

Medium I

Yeast extract	10.0 g
Dextrose	20.0 g
$CaCO_3$	20.0 g
Agar	11.0 g
Distilled water	1000.0 ml

The above ingredients were mixed well to produce an even suspension and the pH was adjusted to 7.0-7.2. It was then sterilized at 15 lbs p.s.i at 121°C for 15 minutes and distributed aseptically into sterile petriplates.

Medium II

Yeast extract	1.0 g
Lactose	10.0 g
Agar	11.0 g
Distilled water	1000.0 ml

The ingredients were dissolved; pH was adjusted to 7.0-7.2 and then sterilized at 15 lbs p.s.i at 121°C for 15 minutes and distributed aseptically to sterile petriplates.

10. Luria Bertani broth medium

20g of Luria broth was suspended in 1000ml of distilled water and the medium was heated to dissolve the components completely. The medium was then sterilized by autoclaving at 15 lbs p.s.i. pressure and 121°C for 15 minutes and used as required.

11. M9YE medium

M9 salt solution

Na_2HPO_4. $7H_2O$	64.0 g
KH_2PO_4	15.0 g
NaCl	2.5 g
NH_4Cl	5.0 g
Distilled water	1000.0 ml

All the ingredients were dissolved in distilled water and mixed by stirring. Thereafter, to 200ml of M9 salt solution, 700ml of distilled water, 0.3% yeast extract powder, 1% of casein acid hydrolysate, 2ml of 1M $MgSO_4$, 20ml of 20% dextrose and 100ìl of 1M $CaCl_2$ were added and the volume was adjusted upto 1000ml with distilled water. The entire content was then sterilized at 15 lbs p.s.i. at 121°C for 15 minutes.

12. Mac Conkey Agar medium

Mac Conkey Agar	51.55 g
Distilled Water	1000.0 ml

The prepared Mac Conkey agar medium was heated to dissolve the media completely; pH was adjusted to 7.1, sterilized at 15 lbs p.s.i at 121°C for 15 minutes and distributed aseptically to sterile petriplates.

13. MR-VP broth

Peptone	7.0g
Potassium phosphate	5.0g
Dextrose	5.0g
Distilled water	1000.0ml

The above ingredients were heated until the solids were dissolved, filtered, and pH was adjusted to 7.5. Dextrose was then added, mixed properly and dispensed into tubes at the rate of 1.5 ml/tube. The media was sterilized at 15 lbs p.s.i. at 121°C for 15 minutes. Media was used for both MR and VP tests.

14. Motility agar

Peptone	10.0g
NaCl	5.0g
Agar	3.5g
Distilled water	1000.0ml

All the ingredients were mixed by heating and then distributed as 5ml content in test tubes and sterilized by autoclaving at 121°C for 15 minutes.

15. Nitrate Broth

Potassium nitrate (KNO_3)	1.0 g
Nutrient Broth	1000.0 ml

Potassium nitrate was dissolved in the nutrient broth and distributed into tubes containing inverted Durham tubes and sterilized at 15 lbs p.s.i. at 121°C for 15minutes.

16. Nitrite Broth

Sodium nitrite ($NaNO_2$)	0.01g
Nutrient Broth	1000.0 ml

The nitrite was dissolved in the nutrient broth and distributed into tubes and sterilized at 121°C for 15minutes.

17. Nutrient Broth (NB) (Barrow and Feltham, 1993)

Peptone	5.0 g
NaCl	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Distilled water	1000.0 ml

The ingredients were dissolved in distilled water by proper mixing and heating when necessary. The pH of the medium was adjusted to 7.2 with 1N NaOH. Finally the medium was sterilized at 121°C for 15 minutes.

18. Nutrient Agar

To prepare nutrient agar, 1.8% bacteriological agar powder was added to the final nutrient broth solution and heated to melt prior to sterilization.

19. ONPG Broth

ONPG	6.0 g
0.01M Na_2HPO_4	1000.0 ml

ONPG was dissolved in the phosphate solution (pH 7.5) at room temperature and sterilized by filtration through cellulose acetate filter paper (pore diameter 0.2µm).

ONPG solution	250 ml
Peptone water	750 ml

ONPG solution was aseptically added to the Peptone water and distributed in 2.5ml volumes in sterile test tubes.

20. Pectin agar medium

1000ml M9 medium (section 7.3.10.) was amended with 10g pectin, 1.2g yeast extract and 1.8% agar. The ingredients were mixed by heating and sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the medium was dispensed into sterile petriplates and allowed to solidify.

21. Pigment producing medium (PPM) broth (Levitch and Stadtman, 1964)

Peptone	2.0 g
Glycerol	1.0 ml
Sodium chloride	0.5 g
Potassium nitrate (KNO_3)	0.1 g
Tap water	100.0 ml

The ingredients were dissolved in tap water by proper mixing and heating when necessary. The pH of the medium was adjusted to 7.2 with 1N NaOH. Finally the medium was sterilized at 121°C for 15 minutes.

22. Pigment producing medium (PPM) agar

0.35% agar was added to the PPM broth and dissolved by heating. The medium was then sterilized at 15 lbs p.s.i. pressure and 121°C for 15 minutes and dispensed into petriplates.

23. Potato Dextrose Broth

Peeled potato	40.0 g
Dextrose	2.0 g
Distilled water	100.0 ml

Peeled potato was cut into small pieces and boiled in required amount of tap water. The potato broth was strained through muslin cloth and mixed with dextrose. The medium was then sterilized at 15 lbs p.s.i. pressure and 121°C for 15 minutes.

24. Potato Dextrose Agar

2% agar powder was added to the potato dextrose broth to prepare potato dextrose agar. The agar was melted by heating the media before sterilization.

25. *Pseudomonas* Agar (For Fluorescein)

37.3g of the dehydrated medium was dissolved in 1000ml of distilled water according to the manufacturer's protocol; the media was then heated and sterilized at 15 lbs p.s.i pressure and 121°C for 15 minutes and dispensed into sterile petriplates.

26. Simmons' Citrate agar (pH 6.9)

Ammonium dihydrogen phosphate [$(NH_4)_2PO_4$]	1.0 g
K_2HPO_4	1.0 g
NaCl	5.0 g
Sodium citrate	2.0 g
$MgSO_4$	0.2 g
Bromothymol blue	0.08 g
Agar	15.0 g
Distilled water	1000.0 ml

The solids except agar were dissolved by heating in water and pH was adjusted to 7.1, filtered, and the indicator bromothymol blue was added. Then agar was added to the mixture, heated to melt and distributed into test tubes. The media was finally sterilized at 15 lbs p.s.i. pressure and 121°C for 15 minutes and slants were prepared.

27. Soil extract agar (Barrow and Feltham, 1993)

Peptone	5.0 g
Beef extract	3.0 g
Agar	20.0 g
Soil extract	100.0 ml

The above mixture was heated to dissolve; pH was adjusted according to the pH of the soil and sterilized at 121°C for 20 min.

28. Starch agar (pH 7.0)

Starch (soluble)	20.0 g
Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Distilled water	1000.0 ml

The above ingredients were heated to dissolve completely and sterilized at 121°C for 15 minutes.

29. Skimmed milk agar for protease test

Skimmed milk powder	100.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Skimmed milk and 500ml of distilled water were taken in a corked bottle and agar was mixed with rest of 500ml distilled water in a separate bottle. Then both were separately autoclaved at 15 lbs p.s.i. pressure and 121°C for 15 minutes. The two mixtures were added to each other, mixed well and poured into sterile petriplates.

30. Triple Sugar Iron (TSI) Agar

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Dextrose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g
Ferrous sulphate ($FeSO_4.7H_2O$)	0.2 g
NaCl	5.0 g
Sodium thiosulphate ($Na_2S_2O_3.5H_2O$)	0.3 g
Agar	20.0 g
Distilled water	1000.0 ml
Phenol red (0.2% aqueous solution)	12.0 ml

The mixture was heated to dissolve the solids in water and the phenol red indicator solution was added, mixed and dispensed into tubes. The media was sterilized for 15minutes at 121°C and then tubes were cooled and kept in slanting condition to form slopes with deep buts, about 3cm long.

31. Tryptone broth

Tryptone	10.0 g
NaCl	5.0 g
1 M $CaCl_2$	1.0 g
Distilled water	1000.0 ml

The above ingredients were heated until the solids were dissolved and dispensed into tubes. The media was sterilized at 121°C for 15 minutes.

32. Tween 80 hydrolysis (Lipase activity) medium

Peptone	10.0 g
NaCl	5.0 g
$CaCl_2.2H_2O$	0.1 g
Agar	20.0 g
Distilled water	1000.0 ml

The ingredients were dissolved by heating and pH was adjusted to 7.4. Volumes of 500 ml were sterilized in flasks at 15 lbs p.s.i. pressure and 121°C for 15 minutes and then cooled at 40-50°C. Tween 80 was sterilized separately and 5ml of it was added aseptically to each flask to give a final concentration of 1% and then dispensed into sterilized petriplates.

33. Urea media

Peptone	1.0 g
NaCl	5.0 g
KH_2PO_4	2.0 g
Agar	20.0 g
Distilled water	1000.0 ml

The solids were dissolved by heating and pH adjusted to 6.8, filtered and sterilized at 15 lbs p.s.i. pressure and 121°C for 15minutes.

Dextrose	1.0 g
Phenol red (0.2% aqueous solution)	6.0 ml

The above ingredients were added to the molten base, steamed for 1h and cooled to 50°C.

Urea (20% aqueous solution)	100 ml

Urea was sterilized by filtration and added aseptically to the base cooled at 50°C. The medium was aseptically distributed into sterile petriplates.

34. Yeast extract mannitol agar

Yeast extract	2.0 g
Mannitol	10.0 g
KH_2PO_4	0.5 g
$MgSO_4$,7H, O	0.2 g
NaCl	0.1 g
Agar	20.0 g

All the ingredients except agar were dissolved in distilled water. Finally, agar was added and dissolved by boiling before the medium was sterilized at 15 lbs p.s.i pressure for 15 minutes at 121°C.

35. Chemical fungicides used

Trade name	**Chemical name**
Bavistin	Carbendazim [2-(methoxycarbamoyl)-benzimidazole]
Captan	Cis N-trichloromethylthio-4-cyclohexen-1, 2-dicarboximide
Indofil	Mancozeb
Roko	Thiophanate methyl (70% WP)

APPENDIX D

NUCLEOTIDE SEQUENCES

1. 16S rRNA gene partial sequences obtained from potential bacterial antagonists

1.1. *Bacillus subtilis* isolate KTR6 (HM150757):

CTGCGACGCTGGCGGCGTGCCTATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAG
CGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGA
CCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGT
TAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAAC
TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA
GTGGCGAATGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGAGGAGCGATCAGGATTA
GATACCCTGGTAGT

1.2. *Citrobacter freundii* isolate ETR20 (HM150756):

CTCTCGCTCCTTCCATTAACCGGACCTACTTTCCTTCTTTACCATCCAGGGAATTTAGCAGAGATGCTTT
GTTTCTTTGGGAACCCTGCTCTCAGGTGCTGCATGGCCGTCGTCAGTTGTGTTGTGAAATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCA
GTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTG
CTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCC
GGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGGTAGTAATCGTGGATCAGAATTACACGGTG
AATACGTTCCCGGGCCTAGTACACACCGCCCGTCACACCATGGGAGTGGGGCGCAAAAGAAGTAGGTAGC
TAACCATCGGGAGGGCTAGAGCCACCAGGATCCAGCACTATGC

1.3. *Enterobacter sp.* isolate D7 (HM150755):

CTGGTAGTCCCCCCCCGTAAACGATGTCGATTGGAGGTTGTTTCCTTGAGGAGTGGCTTCCGGAGCTAAC
GCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACTTTACCTACTCTTGACATCCAGAGAAC
TTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTT
GTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAA
CTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA
GTAGGGCTTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCA
TAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTA
GATCAGAATGTTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTT
GCAACAAGAAGTAGGTAGCTAACCTTCGGGAGGGCTAGAACCGACCATGGATCCATCACATTTGC

1.4. *Serratia marcescens* isolate ETR17 (JX566992):

TGAGCTAATACNTCATCAATTGACGTACTCGCAGAAGAAGCACCGCTAACTCCGTCAGCAGCCGCGGTAA
TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGAT
GTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTA
GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA
CGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGT
AAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCC
TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTG
GTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCA
GTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTG
CTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCC
GGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGC
TTAACCTTCGGTAGGCA

2. 18S rRNA gene partial sequences obtained from potential fungal antagonists

2.1. *Aspergillus* sp. AD7 (KF453972):

GGGTTACGGCGTAGAGGCTGCATCAGGCGCCACCTCCCACCCGTGAATACCTAACACTGTTGCTTCGGCG
GGGAACCCCCTCGGGGCGAGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGTCTGAGTCT
GAATATAAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAA
CTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTG
GCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCCCGGCTTGTGTGTTGGGTCGTC
GTCCCCCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTT
GTCACCCGCTCGACTAGGGCCGGCCGGGCGCCAGCCGACGTCTCCAACAATTTTTCTTCGGGTGGCCCCC
GGATCAGGCAGGATTACCCGTTGAACTTAAGCATAGTAAACGGGGGGAAAA

2.2. *Aspergillus* sp. AD3 (KF453975):

ATGGCCTCTGAAGCAGGCGGCTGCATAAGGGCGCCAACCTCCCACCCGTGAATACCTAACACTGTTGCTT
CGGCGGGGAACCCCCTCGGGGGCGAGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGTCT
GAGTCTGAATATAAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGC
AGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGC
CCCCTGGCATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCCCGGCTTGTGTGTTGG
GTCGTCGTCCCCCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGG
GGCTTTGTCACCCGCTCGACTAGGGCCGGCCGGGCGCCAGCCGACGTCTCCAACCATTTTTCTTCAGGTT
GACCTCGGATCAGGTAGGGATACCCGCTGAACTTAACCATATGTTTAACCCGCGAGGAA

2.3. *Penicillium waskmanii* KV8 (KF866294):

TCATATCTCAGGACTAGTATGATTATCTCCCTTCAGCCTTGTCATTTGCGCTCTTCGTCGTTTCGTGCGC
GGGTTCCCTGGCCACCAGGACCACCCTATAAACTGCTTTGTTAATGCAGTCAGCTTCAGCAAAAAGTAAT
AATTATTTTACAACTTTCAACAACGGATCGATTGGTTCTGGCATCGATGAAGAACGCGGCTAAATGAGAT
ACGTAGAGTGAATTGAATAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGCATTCG
AAAGTGCATGCCTGTTCGAGCGTCATTTGTACCCGCAAGCTTTGTTTGTGGTTGGGGGTTGTTTTTTTTT
GCCCTTGACCAACGACTCACCAAAAAACAATTGGCTGCCTGCTTGCTTGTTGCGGAGGGCATAACTTTTT
GGCGCTAGAAATAGGAAAAAGAGGTCAGCCATGCATAAAAACTACATATGTACGTTGGACCGCCAACAAA
TCAGCATTCCCCGAAGAACTCAAACAAATCAAGAAAGGGGAAGAAA

2.4. *Penicillium implicatum* NB4(KF866295):

CCAGCGACTGGATTATTATTCCCTTGCACCCTTGTCTCTTGCTCGCTTCGTGTTTCGGGCGCGGGTTCGC
TGGCCACCAGGACCACACTATAAACTTTTTTTTTAATGAGGGCAGGGTTAACAAAAAGAAATAATTAATT
TACAACTTTCAACAACGGATCTCTTGGTTCAGGCATGAATAAAGAACGCAAATGAATGAGGTACGTAGAG
TGAATTGAATAATTGAGTGAATCATTGAATCTTTGAACGCACATTGCGCTGTTTGGTATTCCAAAGGGCA
TGCCTGAGCGAGCGTTGTTTGTTCCCTCTTGCTTTGCTGTTGGGTGTGGGTTGTTTTTTTTTGTCCTTGA
CTAACGATTCAAAAAATAACAATTGGCCGCCTGGTTACTGGGTGCGGGGCGGAGAACCTTTTTGGGCAAG
CAAAAGAGAGAGGAAGACAATCATGAATAAAAACTACCTCTCTACCTTCGACCGCCAGTCAGTGATGCTT
GCCCGATGAACTCAAACAAACAAAGAAAAGGGAAGAA

2.5. *Paecilomyces lilacinus* AD4 (KF836741):

TTTCCGCATGAGCTATCCGGTATCTTCCGACCAGTAACTAAACTTGCTTTAGAGAACGCTCCGTCTCGTG
AGGTGTTGTCCCAGGCCAGGGACTACCCCAAATCTCTTTCTTTTGCGGGGGGCGGAAGCCCCCCCAATTC
CCAAGCCCCAGAGAGGTCACAGATTTTATAAAGCCCGTAACTCTGGTTTCGCCAAAATGCACTAGCGCGC
AAAAATCGCTCTAAAATTTGAAGAGGTCACAGATTTTATAAAGCCCGTAACTCTGGTTTCGCCAAAATGC
ACTAGCGCGCAAAAATCGCTCTAAAATTTGAATATTCACATAAATTTGCGAATACGCATTTCTTGAAGGC
ATATTTGCGCCGCTCTCTTTTTTAGGCGAGATACCCAATAGACGCTTATGCAGGCTGAGCCACGTTTCCTC
GTGATCAGACAGCACACCAGCCCATGCGATATGCAGTGCGACCCCGCGCACCTCGCTGCGTAGTAGCACA
CAGCTCGCGCCGGCAGCGCAAGCGGTCAGTTCGTAGCAGAGCCCAACGTAGCGTCAGACCTA

2.6. *Paecilomyces parvus* AD2 (KF836742):

GTGAGCTGGTTCCTCTGGAACATGTGCTCGCCTTCTGCGCTTTCTACTTATCCACCTGTGCACTTATGTA
GACGGTAGATCAATCTCTCCGCAAGGAGGATGGGAGACTCGAGTTAGCAATAGCTAGTTGACTCTAAAGA
TCTATCCGTCTATGTTACACATACACTTTTAAAAACTTTGAATGTCTGTATTATTGGTCTTTGACCTTTA
AACATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCTGTGAATCATCTGTTCTTTGAACACACCTTGTTCTCTTTGGTATTCCTC
AGAGAATGCCTGTTCGAGAGAGAATTAATTTTCACGCACGACATGTGGACATATCGGTCATTGACGAGAA
TACCCTTCAGGACCTCTATTTAGGTTGGTCGCACACTTCGCCGCACAAATAAAAAAGTTCAAAACAACAA
GGCTTTAATCAGTTTGGACATTGGTTGCAAACTCAACCCATTTCCTTCTAAAAAAAGGTCCCCCGGTGAC

2.7. *Penicillium* sp. B2.4 (KF453973):

TCTTTCCCACCCGTGTTTATTGTACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGGGGGGACTTG
CCCCCGGCCCCGCGCCCGGGAAGACACCATTGAACTCTGTCTGAAGATTGCAGTCTGAGTAGATTAGCTA
AATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCCGCCATCGATGAAGAACGCACCGAATTGCGAT
ACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTTGAACGCACATTGCGCCCCCTGGTATTC
CGGGGGGCATGCCTGTCCAAGCGTCATTGCTGCCCTCAAGCACGAATTGTTTTGTTGGGTTTCGCCCCCC
GTTCTGCGGGGGGCGGTCCCGAAAAGATTCGGCGGCACCTCGTCCGGTCCTGGAGCGTATGGGGTTTTCC
CCCCCGCTCTGTAGGCCGGCCGCCCCCAGCCACAACCCCAAATAAATCTACGGGGTGGACGCCGATCAGG
CGGGATCCGGTTGAATTAAGCATATTATAAGGCGGGGAAAA

2.8. *Trichoderma viride* (= *Hypocrea rufa*) AD10 (KF453976):

GGGCGCTCGCAAACTAGCGGTCGCACGCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCTG
CCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCAAAACTCTTATTGTATACCC
CCTCGCGGGTTTTTTTATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAAC
TTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTG
TCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGCCCTGCCTCTTGGCG
GCGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTATTTTGCACACTCGCAT
CGGGAGCGCGGCGCGTCCACAGCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGAAGAAGTAAAGA
ATCCCCGCAAAACTTCCGCAGGTCCCCTAACCCAGAAAAAAA

2.9. *Penicillium* sp. AD6 (KF453974):

GGGGTTACTGAGCTAGAGTCCTCATGAGGAACACCTCCCACCCGTGTTTATTTACCTTGTTGCTTCGGCG
GGCCCGCCTCACGGCCGCCGGGGGGCACCCGCCCCCGGGCCCGCGCCCGCCGAAGACACCAATGAACTCT
GTCTGAAGATTGCTGTCTGAGCGATTAGCTAAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCG
GCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCT
TTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAATC
ACGGCTTGTGTGTTGGGCCCCGCCCCCCGGCCCCGGGGGGCATCCCCAAAAGGAGTTGGCTGCTCCCCGC
CCGGGCCCCGGGCCCGGGGGCCTCCGCCCGCCGGGGGGCGGGACGGGCGAACCCCCAAGGGACATAAAAA
CTAATTGGGAGGTGGGAACCTGAAAGGGGGGATTCGCACTTTTTAATTTAGGTTACCCTACGAGGGAAAA
CAAA

2.10. *Penicillium citrinum* NBT1.2 (KF866296):

GGCCAGTGTTGGACATAAAAGTGATGCATAAGTTGAGCTGGTTCCTCTGGAACATGTGCTCGCCTTCTGC
GCTTTCTACTTATCCACCTGTGCACTTATGTAGACAGGTAGATCAATCTCTCCGCAAGGAGGATGGGAGA
CTCGAGTTAGCAATAGCTCTTGACTCTAAAGATCTATCCGTCTATGTTACACATACACTTTTAAAAACTT
TGAATGTCTGTATTATTGGTCTTTGACCTTTAAACATATACAACTTTCAGCAACGGATCTCTTGGCTCTC
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT
TTGAACGCACCTTGCGCTCTTTGGTATTCCGAAGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAACCT
ACACTTGGTTTTCAATCGAGTTGTTAGGATTGGACTTGGGAGCTGCTGGCGAGTTTTATTACGAGTCAGC
TCTTCTTAAATGCATTAGCGGTGACTTTAACGACCGTTACTTGGTGTGATAATTATCTACGCCTTGTATA
TGGGAGTTATAATAATGGTCGCAGCTTCTAACTGTCTGTTCATTCAGACTGTTTGCTTGCAAACACTTTT
TGATCTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAAAACCGAAAGAGAAA

APPENDIX E

LIST OF PUBLICATIONS

1) D. Saha, **G. D. Purkayastha**, A. Ghosh, M. Isha and A. Saha (2012). Isolation and characterization of two new *Bacillus subtilis* strains from rhizosphere of eggplant as potential biocontrol agents. *Journal of Plant Pathology.* **94(1)**: 109-118.

2) D. Saha, **G.D. Purkayastha**, A. Saha (2012). Biological control of plant diseases by *Serratia* species: A review or a case study. *Frontiers on Recent Developments in Plant Science*. **1:**99-115.

3) **G. D. Purkayastha**, A. Saha and D. Saha (2010). Characterization of antagonistic bacteria isolated from tea rhizosphere in sub-Himalayan West Bengal as potential biocontrol agents in tea. *Journal of Mycology and Plant Pathology.* **40(1)**: 27-37.

4) D. Saha, **G. D. Purkayastha** and A. Saha (2008). Degradation of mancozeb and thiophanate-methyl by bacteria isolated from tea garden soil. *Environment and Ecology*. **26(4C)**: 2231-2235.