Integrated Management of Branch Canker Disease in Tea

A thesis submitted to the University of Calicut, Malappuram-673 635, Kerala

In partial fulfillment for the award of the degree of

DOCTOR OF PHILOSOPHY IN BOTANY

Submitted by
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June 2017

CERTIFICATE

This is to certify that the thesis, entitled "**Integrated Management of Branch Canker Disease in Tea**" is a record of original research work done by **Mr J. Mareeswaran**, in the Plant Pathology Division, United Planters' Association of Southern India Tea Research Foundation (UPASI-TRF,TRI), Tea Research Institute, Valparai–642127, Coimbatore District, India as a full – time Research Scholar during the period of study from 2013to2017 under my guidance and supervision for the award of the Degree of Doctor of Philosophy in Botany. I further certify that this research work has not previously formed the basis for the award of any other Degree or Diploma or Associate ship or Fellowship or other similar title no any candidate of this or any other University.

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DECLARATION

I **J. Mareeswaran** hereby declare that the thesis, entitled **"Integrated Management of Branch Canker Disease in Tea"** submitted to University of Calicut, Malappuram - 673 635, Kerala, for the Degree of Doctor in Philosophy in Botany is a record of original and independent research work done by me during 2013 to 2017 under the supervision and guidance of **Dr R. Premkumar Samuel Asir,** Head of the Division and Senior Plant Pathologist, Plant Pathology Division of United Planters' Association of Southern India Tea Research Foundation (UPASI-TRF, TRI), Tea Research Institute, Valparai-642 127, Coimbatore Dist, India and it has not previously formed the basis for the award of any other Degree, Diploma, Associateship, Fellowship or any other similar title to any candidate of any University.

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Dedicated To My loving Parents and Teachers

ACKNOWLEDGEMENTS

First and foremost of all, I submit my humble praises and prayers at the feet of Lord Almighty for his showers of blessings.

I deem it great pleasure to place on record my deep sense of gratitude and profound thanks to **Dr R. Premkumar Samuel Asir,** Deputy Director and Head, Division of Plant Pathology and Microbiology, UPASI Tea Research Institute, Valparai, under whose guidance the work was carried out by me and for his invaluable suggestions and criticism throughout the course of this study.

I place my deep sense of reverence to **Dr B. Radhakrishnan**, Director, UPASI Tea Research Foundation, Tea Research Institute, Valaparai, for having given suitable instruction, support and critical suggestions for this work.

My thanks are due to my beloved former Director of UPASI Tea Research Institute, **Dr P. Mohan Kumar,** He permitting me for registering in Ph. D. and his cooperation.

Thanks are also due to **Dr R. Victor J Ilango**, Asst. Director, Botany Division. **Mr N. Palani**, Asst. Director, Chemistry division and **all scientists** of UPASI-TRF, TRI, Valparai, for fulfilling my research programme is thankfully acknowledged.

I also extend my great sense of gratitude to our **UPASI Tea Research Foundation**, **Tea Research Institute**, Valparai for having provided all the needed provisions for preceding my research work.

With great pleasure I express my sincere thanks to **Dr A. Balamurugan**, Manager, T-Stanes and Company Ltd, Coimbatore, for his valuable suggestions, which gave me tools that turned out to be essential in my Ph.D. research.

My sincere thanks to **Dr P. Nepolean,** Plant Pathologist, for the interest and valuable suggestions given during the preparation of this work.

My sincere thanks goes to my well-wishers ***Mr R. Parthibaraj, Mr K. Ranjith, Mr B. Suresh Kumar, Mr D. Kirubakaran, Mr N. Jagadeesh, Mr R. Sooraj, Mr CH. Nagapavan Kumar, Mr E. Edwin Raj, Mrs R. Jayanthi, Dr T. Kuberan, and Dr R. Boopathy Raj,*** in UPASI-TRF, TRI, Valparai, for their help, patience and support in the early years of my research without them help I will never become this stage.

I would like to acknowledge the financial support provided *by* ***NTRF*** *and* ***Tea Board of India***, Kolkata for JRF fellowships for this research work.

"I greatly thanks to the comments of the anonymous reviewers and editors of the articles published with contributions of this work".

The help rendered by ***Mr R. Iyyappan, Mr P. Prakash*** and ***UPASI Farm workers*** in carrying out all the experiments and trail is gratefully acknowledged.

I feel immensely grateful to the "God Almighty" for having endowed me with necessary energy, courage and hope to carry out this task

J.MAREESWARAN

CONTENTS

Abbreviations used in this thesis

ANOVA	Analysis of Variance
β	beta
>	more than
<	Less than
%	Percentage
ºC	Degree Celsius
CD	Critical difference
CV	Coefficient of variation
cm	Centimeter
CFU	Colony Forming Unit
DPS	Diseases Protection Score
EC	Emulsifiable concentrate
Fig. / Figs.	Figure(s)
Fr. Wt.	Fresh weight
G	Grade
g	Gram
ha	Hectare
h / hrs.	Hour(s)
ITS	Internal Transcribed Spacer
kg	Kilogram
L_1	Canker length in cm (Pre-treatment)
L_2	Canker length in cm (Post-treatment)
l / lit	Litre
m	Metre
µg	Microgram
mg	Milligram
mL	Millilitre
min.	Minute(s)
µM	Micro molar
M	Molar

MSL	Mean Sea Level
OD	Optical density
PDI	Percent Disease Intex
PI	Percentage of Inhibition
PDA	Potato Dextrose Agar
ppm	Parts per million
Rpm	Revolutions per minute
RH	Relative humidity
SE	Standard error
SD	Standard Deviations
sp.	species (singular)
spp.	species (plural)
TS	Transverse section
UV	Ultraviolet
v/v	Volume/Volume
w/v	Weight / Volume
WH	Wound Healing
W_1	Canker size in width cm (Pre-treatment)
W_2	Canker size in width cm (Post-treatment)
HCl	Hydrochloric acid
KOH	Potassium hydroxide
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
WP	Wettable Powder

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Chapter-I

INTRODUCTION

Tea is one of the most popular non-alcoholic beverages consumed by nearly half of the world's population. It is produced from the young shoots of the commercially cultivated tea plants (*Camellia* spp.). India produces about 1191.1 million kgs of tea from 5,79,353 ha and in south India tea is grown in 1,19,740 ha in the western ghats.

In 2015-16 it is estimated that Out of the world's total production of 5200.3 million kg of tea, 42.9 % was produced from China then followed by India (22.9 %), Kenya (7.7 %), Sri Lanka (6.3%), Vietnam (3.2 %), Turkey (4.4 %), Indonesia (2.5 %), Argentina (1.6%), Japan (1.6 %), Bangladesh (1.3 %), Uganda (1.0 %), Malawi (0.7 %), Tanzania (0.6 %) and Zimbabwe (0.2 %) (Anonymous, 2016). Although tea production in India was more than 1000 million kg, due to high demand of domestic consumption, it is only 13.0 % was exported (Anonymous, 2016). The first three tea exporting countries are Kenya (25.2 %), Sri Lanka (17.2 %) and China (18.5 %) (Anonymous, 2016).

All parts of the tea bush *viz*., foliage, stem and roots are susceptible to diseases. On an average, the crop loss due to pests and diseases is around 10-15% (Chen and Chen 1990). Tea is grown in a wide range of soil types that are acidic in nature and tea soils are latosols in south India (Baura, 1989).

In India, tea is cultivated extensively in Assam and West Bengal, and also in certain parts of Himachal Pradesh and Uttar Pradesh. Tea gardens in south India are spread over the Western Ghats in Karnataka, Kerala and Tamil Nadu. Tea is grown in 0.58 million ha and it was estimated nearly 88,115 tea plantations are in India which includes Assam (55.4 %), West Bengal (24 %), Tamil Nadu (9.3 %), Kerala (8.3 %), Tripura (1.4 %) and Karnataka (0.49 %). In Tamil Nadu, tea grown areas are in the Nilgiris and the Anamallais.

Regions with well distributed rainfall ranging from 200 to 600 cm and ambient temperatures between 20 °C and 25 °C are ideally suitable for tea cultivation. India produces many types of tea such as CTC, Orthodox, Green tea, Organic tea, Instant tea and other specialty tea to suit the tastes and preferences of domestic and international consumers. However, nearly 90 % of the tea produced in the country is of Crush, Tear, and Curl (CTC)

type because of its preference in the domestic market (Prafulla Chandra Bora and Aniruddha Deka, 1999 and Nalaini Ganapragasam, 1999).

Tea is attacked by a number of pests and diseases which are the major limiting factors in crop productivity. The first comprehensive account on the pests and diseases of tea was given by Watt (1898). Watt and Mann (1903) listed the pests and blights of tea plants. Majority of tea pathogens are fungal origin and more than 300 species of fungi have been reported to affect different parts of the tea plant (Chen and Chen, 1989). Mann and Hutchinson (1904) recorded various tea diseases that were later published by Petch (1923).

Many stem diseases are also recorded in tea which inflicting severe damage to tea plants. Among them, branch canker in tea was first noticed in southern India in 1899, but in Srilanka the diseases was recorded in 1904 (Petch, 1923). Stem diseases like wood rot (*Hypoxylon serpens*), collar canker (*Phomopsis theae*), branch canker (*Macrophoma theicola*) and thorny stem blight (*Tunstallia aculeata*) are predominant in southern India. Among leaf diseases blister blight, grey blight and brown blight are common in south India. Stem diseases are important as they stagnate crop production and sometimes kill the tea bushes.

Pruning operation carried out in tea bushes once in four years increases the risk of stem diseases since it exposes the wood tissues to parasitic and saprophytic fungi. The fungal pathogen can easily enter through prune cuts or tissues damaged by sun-scorch. The pruning cuts also provide an ideal surface for germination of spores (Otieno, 1997). *Macrophoma theicola* has also been observed to cause twig die-back disease of mature tea plants in Taiwan (Arul pragasam, 1992). *Macrophoma* sp. fructifications occur in large numbers all along the dead branches and cause very minute cracks on the surface of the bark (Shanmuganathan and Bopearatchy, 1972). Branch canker is a secondary disease generally attacks the main trunk of the tea plants. It is a wound pathogen and gains its entry through the wounds into the tea plant. The large area of stem is an affected and enlarged due to branch canker pathogen (Plate 1 and 2).

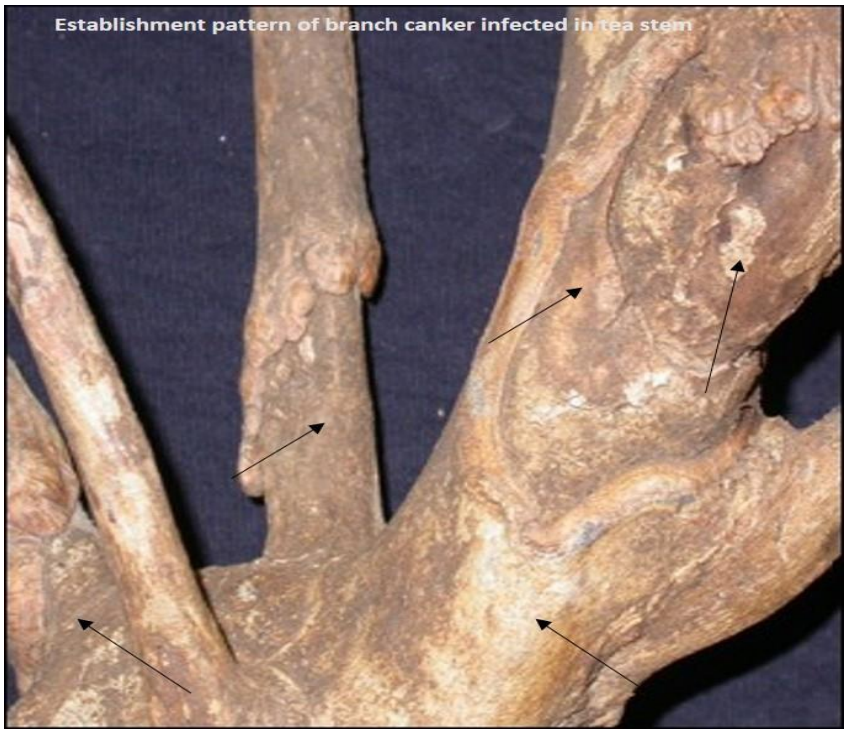
Plate.1 (a, b & c) Occurrence of branch canker disease symptoms in tea plants

Plate.1 (a) Branch canker symptom in tea stem

Plate.1 (b) Severely infected stage of branch canker

Plate.1 (c) Branch canker infected in total bush

Plate.2 Establishment pattern of branch canker infection in tea stem (Line arrow)



I.1. Scope of the present investigation

The survey was conducted in different tea growing areas of southern India for the isolation of branch canker pathogen and biological control. Among the stem diseases, branch canker caused by *Macrophoma* sp. is most widespread and serious stem disease of tea plants. At present, branch canker disease is controlled by removing the affected stems by adopting rejuvenation pruning and applying Copper oxychloride (COC) paste at the cut ends. Even though time to time recommendation on canker diseases are released with respect to cultural operations and chemical control measures, no full-fledged integrated disease management schedule is available. In this concern, the present study on "Integrated management of branch canker disease in tea" gained importance.

Branch canker is one of the important stem diseases of tea. The disease is caused by the fungus *Macrophoma* sp. belongs to the family *Botryosphaeriaceae*. In tea plantation, this disease causes crop loss and it is the limiting factor for yield stagnation. Very few researches have been carried out on the management of this particular stem disease and biological control measures have not been explored so far. The objective of this work is to study the etiology and histopathology of this disease, to develop a germplasm which consists of biocontrol agents *viz.*, *Trichoderma* spp. *Pseudomonas* sp. and *Bacillus* spp. and to find out their antagonistic potential *in vitro* and *in vivo*, field evaluation of new fungicide molecules integrated with certain botanical fungicides and biological control.

Predisposing factors responsible for disease attack, environmental factors that influence the pathogen activity and mode of infection in host tissues will be studied in detail. Histopathological studies will help to understand the mode of infection in the internal parts of tea bushes in order to study the clonal/resistance/susceptibility. The biological control of the tea disease would considerably reduce the fungicide usage in the tea fields and harmful effects of the chemical residues. Finally an integrated disease management package of practices will be developed for the control of branch canker disease in tea.

Chapter-II

REVIEW OF LITERATURE

II.1.Taxonomy of Tea

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Ericales
Family	:	Theaceae
Genus	:	*Camellia*
Species	:	*sinensis*

Tea is believed to be originated from China and discovered in 2737 BC. Since 5th century, the habit of drinking tea has spread from China to other countries. Now, tea is the most widely accepted nonalcoholic beverage consumed mainly for its refreshing, mild stimulant effects, medicinal and cosmetic properties. Tea plant of commerce belongs to the family Theaceae and genus *Camellia*, which consists of approximately 82 species (Ellis and Nyirendra, 1995). It is highly heterogenous, belongs to three distinct races namely "China" type (*Camellia sinensis* (L) O. Kuntze), "Assam" type (*C. assamica* (Masters) Wight) and "Cambod" type (*C. assamica* sp. *lasiocalyx* (Planchon ex. watt) Wight). However, the practice in over the world is to put all the taxa under *C. sinensis* (L.) O. Kuntze irrespective of their taxonomic variation (Hajra, 2001).

Tea is a perennial woody plant having a single main stem from which numerous branches develop to a crown of leaves to get a bushy appearance. Free growing tea plants are shrubs/trees attaining heights varying from one to fifteen meters. The lifespan of the tea bush extends over 100 years. The natural home of the tea plant is considered to be within the fan shaped area included between the Naga, Manipuri and Lushai Hills along the Assam-Burma frontier in the west, through to China.

Tea is grown in various climatic conditions and in acidic soils, however, it also thrives in extreme conditions as well. Tea is grown in laterite soil in southern India and grows well in warm, humid climate with well distributed rainfall and long sunshine hours. Tea leaves are 4-15 cm long and 2.5 cm broad. Fresh leaves contain about 4 % caffeine. The buds and two leaves of the mature plant are called flushes. The plant grows a new flush every 10 to 12 days. Thus, hand plucking is done once in every two weeks.

In ancient days, people consumed tea leaves and its infusion as a medicine. Later on they used it as a luxury drink/beverage. Black tea (fermented), Green tea (non-fermented) and Oolong tea (semi-fermented) are the three types of manufactured tea consumed by various sections of people according to their tastes and cultures (Chu, 1997).

II. 2. Branch canker

'Branch Canker' as the name denotes it is a stem disease that has been recorded from Ceylon where it is fairly distributed. Its action on the bush is variable in some cases or under some conditions it merely causes branch canker; in others it kills back the younger shoots. In a few instances it caused considerable damage in new clearings (about 3 or 4 years old) and a large number of bushes have been killed.

2.1. Symptoms

Branch canker pathogen observed to cause twig die-back of mature tea in Taiwan (Arul Pragasam, 1992). The branch canker pathogen *Macrophoma theicola* was noticed for the first time in Taiwan (Thseng *et al.,* 2004). The diseased patches on the branches appear as slightly sunken lesions surrounded by a ring of callus growth (Sarmah, 1960). The bark of the infected branches was characterized with the presence of pycnidia. Infected branches showed dieback symptom with fruiting bodies on the cankered branches of tea (Thseng *et al*., 2004).

The initial indications of the disease usually appear in the rainy season, when small slightly sunken dark patches may be found on branches (about a centimeter in diameter) i.e. on the red wood. These patches are oval running longitudinally on the stem for about 2 or 3 cm with a breadth up to 1 cm. The bark over these patches is blackened internally, soft and rotten and separated from the wood easily. In very wet weather, it sometimes bears small white or pinkish heaps of spores which are extruded through minute cracks, but this stage of

the fungus usually may not occur. In a later stage, if the diseased bark is lightly shaved and then examined with a lens white circular patches each surrounded by a black ring can be observed, either isolated or united in groups. These are, in general, sections of the fructification of the fungus which causes the disease. The fructification is a minute black spherical body (pycnidia), immersed in the bark, containing spores which appear white in mass. Hence, when it was cut across, it appears as a white circular area, boarded by a black line. As a rule, this is a *Macrophoma* which has been named as *Macrophoma theicola* (Petch, 1923).

Canker affected branches are killed slowly by the invading fungus until the disease spreads to the collar when upper portion of the plant dies. In mild infestations, the canker is callused over completely within a few months, but the fungus may renew its growth forming concentric cankers under adverse conditions. Fructifications are produced on the dead bark during wet weather.

During a recent survey of the inoculum-source of twig die-back of tea plants caused by *M. theicola*, symptoms of canker were observed. It was found that most cankered areas contained minute black pycnidia of *M. theicola* which ooze white conidial masses in strands when incubated under moist conditions. Isolates derived from conidia of *M. theicola* also caused dieback on healthy tea twigs, indicating that fruiting bodies on the cankered branches are the main inoculum sources of twig dieback of tea (Thseng *et al.*, 2004). To control the disease, the affected branches should be cut out to clean healthy wood. Plants should be protected from sun-scorch by pruning during dry weather. As mentioned earlier, though cultural and chemical control methods are recommended to control the canker in tea, biological control measures integrating the conventional methods are lacking.

Many stem diseases like wood rot (*Hypoxylon serpens*), collar canker (*Phomopsis theae*), branch canker (*Macrophoma theicola*) and thorny stem blight (*Tunstallia aculeata*) are predominant in southern India. Leaf diseases such as blister blight (*Exobasidium vexans*), grey blight (*Pestalotiopsis theae*), brown blight (*Glomerella cingulata*) and black rot (*Corticium theae*) are common in India. Among the stem diseases, branch canker is the most serious widespread stem disease. In generally tea plantation, this disease causes yield stagnation and crop loss.

2.2. Crop loss

The crop loss due to canker diseases depends upon severity of pathogen infection and the geographical area (Hajra, 2001). In Taiwan, around 40 % of the tea bushes were killed by twig dieback and in south-east Asian countries, (Muraleedharan and Chen, 1997). Low yield due to incidence of collar and branch canker caused by *Phomopsis theae* and *Macrophoma* sp. was reported from Central Africa (Rattan and Sobrack, 1976). In Sri Lanka, the greenhouse and field observations on 24 clones showed that 16 clones exhibited low resistance and 6 clones recorded intermediate resistance to collar and branch canker diseases. Wang (1983) reported that branch canker disease is most serious stem pathogen during dry conditions and caused the death of tea bushes more than 40 %.

II. 3. Molecular studies

The molecular characterization has been proposed as a complementary tool for the identification of microbial strains. The molecular technique shows high sensitivity and specificity for identification of microorganisms which can be used for classifying microbial strains at taxonomic levels (Sette *et al.,* 2006).

All 16s rRNA genes in a sample are amplified using a universally conserved primer pair for bacteria documentation which is an efficient phylogenic marker for bacteria identification and microbial community analyses. Moreover, the multiple pitfalls of PCR-based analyses, including sample collection, cell lysis and PCR amplification can move the estimation of community composition in the mixed microbial samples (Farrelly *et al.,* 1995 and Von *et al.,* 1997).

In general, phylogenetic model is examined based on the universal ITS sequences and described for compared with another genome species and similarity level. Chowdappa *et al*. (2013) reported that 16S rDNA, PCR amplification used for identification of bacterial strains. Moreover, PCR amplification of 16S rRNA gene was attained for bacterial identification and microbial community analysis (Rainey *et al.,* 1996).

The ITS region is the most extensively used as world wide sequence (Peay, 2008) and Schoch (2012 a,b) reported that ITS region recommended as the universal fungal barcode sequence which has been typically most useful for the molecular systematics at the species level. The majority of fungal ecology research level ranging, next-generation sequencing

methods involves of PCR amplification of conserved regions of DNA. Therefore, the marker genes are used to identify and describe the distribution of taxonomic groups in the fungal community. Although, more recent research work have focused on the sequencing of the functional gene (Baldrian *et al.*, 2012).

Johannesson and Stenlid (1999) reported that mitochondrial rDNA used for fungal identification at diverse taxonomic level. Moreover, ITSI and ITSII are used for identification of higher taxonomic level (Bruns and Shefferson, 2004). Many scientists reported that amplification of ribosomal DNA with worldwide fungal primers followed by restriction endonucleases (RE) RFLP (Restriction Fragments Length Polymorphisms) which have demonstrated to suitable identification of taxa from decay fungi (Adair *et al.*, 2002; Johannesson and Stenlid, 1999; Fischer and Wagner, 1999 and Harrington and Wingfield, 1995).

Several workers reported the detection and identification of fungal from direct decay plants by using Taxon- specific primers (Bahnweg *et al.*, 2002; Moreth and Schmidt, 2001 and Gonthier *et al.*, 2003). For wood rotting fungi the taxon specific primers was developed by Guglielmo *et al.* (2007). The group of *Botryosphaeria australis* and *B. lutea* clade has been identified by using parsimony based on ITS-rDNA (Bernard *et al.*, 2004). Many scientists reported that worldwide oligonucleotide primers precise to fungi has obtained from easy access to nucleotide sequences (Van Belkum *et al.*, 1993; Sherriff *et al.*, 1994 and Sandu *et al.*,1995) and these technique have been used for the identification of fungal taxonomy.

II. 4. Nutritional factors

4.1. Growth characterization on different medium

Jee-song *et al.* (1987) reported that maximum radial growth of *Macrophoma theicola* in wheat-oat medium but not grown well in Potato dextrose medium. Ekbote (1994) identified that maximum radial growth of *C. gloeosporioides* was observed in Richard's agar. Ekbote *et al.* (1997) attained that maximum radial growth of *C. gloeosporioides* was noted in Richard's, Brown and PDA followed by Czapek's agar. The highest radial growth of *C. gloeosporioides* was found in potato source was also noted by Akthar (2000).

4.2. Utilization of carbon sources

Perveen *et al*. (2010) stated that maximum vegetative growth of *Phytophthora capsici* was recorded in maltose. Moreover, the compound of maltose induced the highest vegetative growth of *Gloeosporium* (*Colletotrichum*) reported by Chen and Hun-Yung Hsu (1967). In general, the fungal pathogen utilized the primary metabolism of plants such as carbohydrates, glucose and sugar. In commonly, higher amount of sucrose is required for better radial growth but only little amount of sucrose is used for better sporulation, especially for *C. gloeosporioides* and *C. atrameniarium*. Naik (1985) stated that carbon source of sucrose showed the highest growth of *C. gloeosporioides* followed by glucose, dextrose, citric acid and mannitol.

4.3. Utilization of nitrogen sources

The nitrogen compound of sodium nitrate showed the highest growth of *Pestalotia gracilis* was reported by Yusef and Allam (1967). *Verticillium dahliae* is well sporulated in sodium and potassium nitrates (Selvaraj, 1971). The superior growth of *Hypoxylon serpens* was noted in ammonical nitrogen and the nitrate nitrogen (Venkata Ram, 1973). The maximum sclerotial production of *Botrytis aquamosa* was obtained in the nitrate source compound (Page, 1986). Rajak (1983) reported that maximum growth was obtained in potassium nitrate for *Colletotrichum gloeosporioides and Fusarium oxysporum* also utilized nitrate nitrogen source more efficiently.

II. 5. Effect of abiotic factors

5.1. Temperature

The highest growth of the *Macrophoma theicola* pathogen has been reported in high temperature and lowest water potential, the optimum temperature for the growth of *Macrophoma theicola* ranges between 28 °C to 34 °C. This pathogen did not grow below 12 °C (Jee-song *et al.*, 1987). Jee-song *et al.* (1987) reported that the optimum water potent for the growth of this fungus ranges between -1 bar at 32 C and -9 bar at 24 C.

Satter and Malik (1939) observed that optimum temperature for the growth of *G. cingulata* was at 25 °C to 30 °C. This pathogen even at 25 °C to 30 °C and the minimum and maximum range of temperature were at 10 °C to 15 °C and 35 °C to 40 °C respectively.

Abe and Kono (1956) perceived that optimum temperature ranges for *G. cingulata* was at 24 °C to 28 °C and thermal death point at 55 °C. Verma (1969) and Mancini *et al.* (1973) reported that optimum temperature between 25 °C to 27 °C for the growth of *G. cingulata*. The growth of *Pestalotiopsis mangiferae* is grown well at 27 °C to 30 °C (Sawant and Raut, 1995).

5.2. pH

The optimum pH ranges between 6.0-6.5 which favors the growth of *C. gloeosporioides* under *in vitro* level (Naik, 1986 and Hegde, 1986). Verma (1969) observed that maximum growth of *C. gloeosporioides* at pH 6.0. Naik (1986) and Hegde (1986) reported that maximum growth of *C. gloeosporioides* was found at pH 6.0 and 6.5 respectively.

Ekbote (1994) obtained that highest growth of *G. cingulata* was at pH 6.5 followed by 6.0. Rajak (1983) observed that pH of 7.0 was optimum for *C. gloeosporioides.* Singh and Shankar (1971) observed that maximum growth was at a pH level of 5 followed by 4, 6, 7, 8, 9 and 10. Ashoka (2005) reported that maximum dry weight at a pH 6.0 and Kamanna (1996) observation made that maximum growth of *C. gloeosporioides* was at an optimum pH of 6.0.

5.3. Light

Ashoka (2005) stated that an exposure of the *C. gloeosporioides* to alternate cycles of 12 h of light condition and 12 h of darkness for twelve days results found that maximum dry weight of mycelial and maximum radial growth of this fungus. Kamanna (1996) described that exposure of *C. gloeosporioides* to alternate cycles of 12 h light and 12 h darkness recorded in maximum growth and sporulation.

5.4. RH

Generally the plant pathogen prefers humidity, cool and low temperature for their growth development and spore formations. Reitsma and Van Emden (1950) reported that basidiospore formation was higher which ranges about 80 % of relative humidity. The relative humidity normally ranges from 60 to 80 % are favored for disease development in tea plants (Premkumar, 1996).The maximum spore penetration was occurred at RH above 90 % (Huysmans, 1952).

II. 6. Fungal toxins

Fungal toxins are secondary metabolites developed by fungi which are capable of causing diseases and death in plants (Schmelz *et al.,* 2003 and Trucksess, 2004). The several genera of *Aspergillus*, *Stachybotrys*, *Claviceps*, *Fusarium*, *Diplodia*, *Alternaria* and *Phoma* can be produced several mycotoxins (Betina, 1990). McCormick *et al.* (1998) found that a sum of fungi produced aflatoxins which are dangerous for plant system (example, *Aspergillus parasiticus, Fusarium*.spp and *Penicillium*).

Mycotoxins constituents can be categorized by several properties: Chemical compounds namely terpenoid, glycoside, phenols and polypeptide, these are all produced by fungus or bacteria (Nedelink and Repkova, 1998). Aducci *et al.* (1997) reported that phytotoxin are produced by plant pathogenic fungi or bacteria which are harmful to plant system. Metabolites of Tichothecenes are produced by *Fusarium* spp. which are very important chemical group of mycotoxins, these compounds are mostly toxic to plants (McCormick *et al.,* 1998 and Zemankova and Lebeda, 2001).

II. 7. GC-MS studies

Many scientists reported that bacterial culture produces volatile compounds and can be identified with help of GC-MS. Moreover, Gas Chromatography has been used to identification of volatile compounds produced by *Pseudomonas aeruginosa* and ketones, alkenes and aliphatic compounds from some other bacterial culture filtrate have been confirmed using GC-MS (Labows *et al.,* 1980; Scholler *et al.,* 1997 and Zechman *et al.,* 1986).

The new methods of biocontrol agents culture filtrates, mineral oils and plant extract are used for plant diseases (Wilson *et al.,* 1987; Hegazi and El-Kot, 2008 and Dayyf *et al.,* 1997). Several workers suggested that bacterial culture filtrate compounds of palmitinic acid, 2-heptenal and octadecanoic acid were identified through GC-MS methods, therefore the antifungal activity of bacterial culture filtrate compounds is controlled for the powdery mildew pathogen (Jay-Ran *et al.,* 1998; Dale *et al.,* 2004; Daniele *et al.,* 2006 and Prittee *et al.,* 2007). Furthermore, *Bacillus* sp. culture filtrate compounds of hexadecanoic acid and 9 octadecanoic acid has been identified as biocontrol for the powdery mildew fungus (Pamela, 2002).

The antifungal compounds identified by GC-MS methods from various bacterial and fungal culture filtrate which contains alcohol, aldehyde, esters, alkenes and fatty acids, these compounds have been used as biocontrol for the plant pathogen (Wei *et al.,* 2008). However, the antifungal protein isolated from *Bacillus licheniformis* through HPLC analysis, therefore protein showed satisfactory control of *Rhizoctonia solani*. An antifungal activity of volatile compounds was extracted from culture filtrate of *Bacillus amyloliquefaciens* by using GC-MS analysis which is controlled for the fungal pathogen of *Fusarium oxysporum* f. sp *cubense* (Yuan *et al.,* 2011).

II. 8. Histopathological studies

8.1. Clonal screening

The infected branches were killed slowly by the fungus and the disease spread to the upper part of the plant and eventually the plant dies. So far, very little effort has been directed towards the evaluation of cultivars for susceptibility/tolerance to branch canker disease. Therefore screening of the tea clones for tolerance to branch canker disease is essential for choosing a right planting material for disease prone areas. An earlier study reported that in faba bean the histopathological studies could help in understanding tolerance/resistance mechanism to *Uromyces viciae-fabae* (Sillero and Rubiales, 2002). The susceptible and resistant varieties are discussed through histopathological characterization in *Dianthus caryophyllus* to *Fusarium oxysporum* f.sp.*dianthi* (Baayen and Elgersma, 1985).

Pennypacker and Nelson (1972) reported that susceptible cultivar is examined with histopathological methods (White sim) to *Fusarium* wilt and Harling *et al.* (1984) and Harling and Taylor (1985) with susceptible variety (RedBaron) and resistant variety (Carrier 929) by histopathological studies.

Several scientists reported that mostly plant pathogen infects an intercellular region of vascular tissue (Castesson *et al.,* 1976; Harling and Taylor, 1985 and Pennypacker and Nelson, 1972). In generally, plant cell wall swelling or lysis induced by the plant pathogen (Chambers and Corden, 1963). Von Ramm (1962) reported that germ tube of *Alternaria longipes* penetrated through epidermis layer of tobacco leaves and formation of appressoria. Allen *et al.* (1983) found that production of sporulation and appressoria of *A. cassiae* infected on sunflower plant. Many scientist reported that *Stemphylium botryosum* infection on alfalfa

plant and their germ tube directly penetrates through the epidermal cells but not found in appressoria formation (Borges *et al.,* 1976 and Fallon *et al.,* 1987).

II. 9. Host pathogen interaction studies

An alteration in biochemical composition during host pathogen interaction studies, pathogen entry in to host tissue and its enhancement inside the host. During infection of pathogen changes the metabolism of the host plant. The first findings of Hutchinson (1913) expressed that's evidence of altered permeability in Rangpur tobacco wilt caused by *Bacillus solanacearum*. Many scientists have reported that the host pathogen interaction studies between asparagus and *Stemphylium* sp. (Sutherland *et al.,* 1990 and Fallon *et al.,* 1987). Verma and Singh (1994) studied the biochemical changes in mango leaves infected by *Pestalotiopsis mangiferae*.

Sutton (1996) reported that many canker diseases can be eradicated by choosing resistant varieties of cultivars. The host pathogen resistance contributed the maximum in managing rice blast pathogen caused by *Drechslera oryzae* (Kapoor *et al.,* 2000). Introduction of resistant cultivars shows against scab disease (*Venturia inaequalis*) in apple. *Phytophthora* sp. has been identified to tolerant black pepper by Sharma (2000).

Parasitism absorbed a nutritional interrelationship with host from where it derives its whole nourishment (Starr, 1945). Maximum fungal have a preference hexose sugars than pentose sugars from host plant (Bilgrami and Verma, 1992). Several reviewers reported that many fungi are utilized the glucose and sucrose (Grover and Chona, 1960; Tandon, 1967 and Onsando, 1987). Verma and singh (1994) reported that biochemical reaction changes due to infect of *P. mangiferae* on mango leaves. Sanjay observed that grey blight disease infected on tea leaves by *P. theae* reducing, non-reducing and total sugars reduced in the infected leaves compared to healthy plants.

Total biochemical parameters were changed due to infection of blister blight disease on tea leaves, therefore increases of polyphenol, catechin and protein on initial stages of blister infection leaves, but decreased the all the biochemical parameters on successive stages of blister blight (Baby *et al.,* 1998). Ponmurgan *et al.* (2000) reported that a decrease of physiological parameters *viz*., total chlorophyll and photosynthetic rate has been reported in collar canker caused by *Phomopsis theae*.

II. 10. Biological control measures

The work on biological control of tea diseases is very limited. *In vitro* antagonisms of *Trichodema* sp. and *Gliocladium virens* against the root rot pathogens has been reported by Baby and Chandramouli (1996). *Trichodema viride* and *T. harzianum* showed inhibitory effect against *Poria hypobrunnea* causing stem canker (Das and Barua, 1990). Barthakur and Dutta (1992) observations were made to control thorny stem blight using tea wood pellets colonized by *T. viride* and *T. harzianum*.

Most of the plant pathogens have been controlled by bacterial and fungal antagonists (Cook and Baker, 1983; Campbell, 1989 and Sharma *et al.,* 1999). For example, stem rot disease caused by *Botrytis cinerea* in tomato plants, collar rot of lentil caused by *S. rolfsii* and damping disease of bean seedlings were effectively controlled with biological control like *Trichoderma* sp. *Gliocladium* sp. *and Pseudomonas* sp. (Agarwal *et al.,* 1977; Elad *et al.,* 1982 and Sharma *et al.,* 1999).

The apart from *Trichoderma* sp. *Gliocladium* sp. and *Pseudomonas* sp. another species of *Aspergillus, Penicillium* and *Actinomycetes* have been controlled for the plant diseases. *Trichoderma* sp. and *Gliocladium* sp. are known to produce antibiotic substances. The mechanisms by which disease control is achieved are competition, parasitism and antibiosis (Elad *et al.,* 1980; Baker, 1988 and Lynch, 1990). Mycoparasitic action of different isolates of *Trichoderma* sp. and *G. virens* against to *B. theobromae* has been reported wherein wrinkling bursting and collapsing of pathogen mycelium due to the production of cell wall lytic enzymes were observed (Gupta *et al.,* 1999).

Bacterial biocontrol of *Bacillus* sp. isolated from tea phylloplane efficient controlled black rot disease of tea plants caused by *Corticium invisum* (Barthakur *et al.,* 1993). *Micrococcus luteus* isolated from the phyllosphere of tea was found to show good results against *Glomerella cingulata* (Chakraborty *et al.,* 1998). *Bacillus* sp. isolated from tea phylloplane of blister blight affected leaves were very effective in controlling blister blight (*E. vexans*) in tea (Baby *et al.,* 2004). In another study, it was found that *Pseudomonas* sp. isolated from phyllosphere was very effective in controlling grey blight (*Pestalotia* sp.) in the same crop (Premkumar *et al.,* 2005).

Entomopathogen like *Paecilomyces lilacinus* has been reported as biological control against plant roots pathogens (Wright *et al.,* 2003). Perveen *et al*. (1998) described that *Paecilomyces lilacinus* act as biocontrol for root infecting fungi *viz.*, *Rhizoctonia solani*, *Fusarium* spp. and *Macrophomina Phaseolina*. *Beauveria bassiana* and *Lecanicillium* spp. have been identified as biocontrol for the plant disease (Goettel *et al.,* 2008 and Ownely *et al.,* 2008). Moreover, *Beauveria bassiana* and *Lecanicillium* spp have been reported to against soil plant pathogen such as *Rhizoctonia* sp. *Fusarium* sp. and *Pythium* sp. (Bonnie *et al.,* 2010). *Lecanicillium* spp. has been controlled to against numerous plant pathogenic fungi including powdery mildew (Miller *et al.,* 2004). However, *Lecanicillium* spp have antibiosis, mycoparasitism properties and showed highest antagonist potential against powdery mildew (Kiss, 2003).

II. 11. Chemical control measures

Nene and Thapliyal (1993) reported that an important group of systemic fungicides triazole inhibits the growth of fungal pathogens by interfering with the biosynthesis of sterol which are effective against an extensive range of plant fungal disease caused by Deutromycetes, Ascomycetes and Basidiomycetes. Moreover, Tridemorph successfully controlled the pink disease of *Hevea* sp. caused by *Corticium salmonicolor* (Wastie, 1976). Bioefficacy of propiconazole is controlled the pink disease of rubber through trunk injection by Jacob and Edathil (1983). Helton and Kochan (1968) reported the efficacy of triazoles as chemical paints in managing the canker disease of *Cytospora* in *Prunnus domestica*.

The systemic uptake and translocation of Panoram and Vitavax have been reported in sorghum plants by Shah and Mariappan (1989). Systemic action of tridemorph and several triazoles against the *Exobasidium vexans* of tea was also documented by Venkataram (1974) and Premkumar *et al*. (1998).

An improvement of yield after fungicide treatment is well known in many plant fungal pathogen interactions which is mainly attributed to reduce plant disease severity both as preventive and curative effects. The significant yield was increased after treating wood rot disease of tea with Benomyl by Otieno (1996). Wherever, Rajib *et al*. (2000) observed that an increased seed yield with systemic fungicide of carbendazim soil application to control *Fusarium* wilt of lentils.

Many scientists reported that chemical control of disease occurring on the foliage, green stems and roots are found positive trends in many cases (Campbell, 1989 and Hofte, 1999). Soil borne pathogens and woody stem diseases require large amounts of fungicides since of restricted translocation, degradation by a variety of microorganisms and sometimes by the host plant itself.

To protect the crop from different plant pathogens and pests, several fungicides and pesticides of synthetic chemicals used in tea plantations (Premkumar and Baby, 2005). However, synthetic chemicals proposal better advantages in the form of superior control of pests, plant diseases and high yield which have serious disadvantages such as development of resistance to pesticides and fungicides, the resurgence of pests, outbreak of secondary pests, adverse effects on human health and the environment and the presence of undesirable residue. Biological control holds a lot of promise in such situations, but planters rely on chemical fertilizers and pesticides to control the disease and pests.

II. 12. Plant extract studies

At present, branch canker disease is controlled by removing the affected stems by adopting rejuvenation pruning and applying Copper oxychloride (COC) paste at cut ends (Premkumar and Baby, 2005). Nowadays, plants extracts are being used for seed treatment against bacterial leaf spot of tomato (Ernest *et al.,* 2012). Secondary metabolites of plants produce more phytochemical antifungal and microbial activity and can be used for control of plant diseases (Amadioha, 2003; Prakash and Rao, 1997; Kumar and Parmar, 1996 and Opara and Wokocha, 2008). Several plant extracts are also used to effectively control diseases and pests in tea.

It is reported that approximately 2400 plant species have pest control properties (Grainge and Ahmed, 1988). Neem has been reported to have antifeedant, repellent, toxicant, growth inhibitor effects on pests (Hajra, 2001). *Artemisia vulgaris, Urtica diocia, Polygonum runcinatum, Eupatorium glandulosum* have also shown antifeedant action against bunch caterpillar (Bisen and Kumar, 1997). Even some common weeds in tea fields like *Lantana camara* (Lantana) and *Parthenium hysterophorus* (Congress grass) have shown antifungal activities against *Alternaria* sp. (Srivastava and Singh, 2011). It is desirable to use plant

extracts in controlling tea pests and diseases because they do not leave harmful residues in plant, soil and the practice is found to be highly ecofriendly and economical.

II. 13. Compatibility of biological agents

The plant disease management is obtained through the ecofriendly manner by the usage of biological control method which is adopted to fight against several disease causing plant pathogens. Especially, *Trichoderma* spp. have been tried as biological control against several root, stem and leaf diseases of tea which fight against various plant pathogens, specifically against the pathogenic fungi *Rhizoctonia solani* and *Pythium* sp. The combination of organic and chemicals have been introduced against soil-borne pathogens (Locke *et al.*, 1985) as an ecofriendly approach which reduce the continuous usage of chemical against the plants (Bagwan, 2010). Various reports have been suggested that the IDM package of natural product could be used as different custom of agriculture fields. Dubey (2000) reported that the recommended dosages of fungicides with biological control against web blight of *Rhizoctonia solani* in groundnut plants.

The advancements in the usage of fungicides with biological for the control of plant pathogen as well as the introduction of desired beneficial microbes to the agriculture field in an ecofriendly manner (Papavizas and Lewis, 1981). The present study was aimed to find out the possible combination of bacterial and fungal biocontrol agent with agrochemicals of certain recommended dosages of agrochemicals such as pesticides (Propargite and Deltamethrin), fungicides (Benomyl, Hexaconazole, Companion, Copper oxychloride and Propiconazole) and weedicides (Glyphosate and Ammonium salt of glyphosate), which are useful in the management of pest, diseases and weeds control in tea plantations.

II. 14. Shelf life studies of biocontrol

The biocontrol agents have been identified as potential agents for the management of plant diseases. Commercially, successful use of biocontrol agents like *Trichoderma* spp. has been done for the control of soil borne plant pathogens (Cook and Baker, 1983). Biological control has been used for the control of plant diseases to replace the usage of harmful chemicals in agriculture field. The mass production of biocontrol agents is an active process and reasonable inexpensive. The liquid formulation of *Trichoderma* sp. has been identified as

better an extended shelf-life (Kolombet *et al.,* 2008). In liquid formulation of *Trichoderma* sp. showed good activity against a widespread of plant pathogens such as *Helminthosporium*, *Fusarium*, *Pythium*, *Alternaria*, *Rhizoctonia* and *Verticillium* (Kolombet *et al.,* 2001). The different nutrient substrates on the development of *Trichoderma* sp. have been testified (San Martin *et al.,* 1997).

In general, several commercial formulations of fungal biocontrol agent, *Trichoderma* spp. have been developed in European market for the control of plant pathogens (Whips, 1992). Talc based formulation of *Pseudomonas* sp. and *Trichoderma* sp. has been reported as potential biocontrol agents for *Fusarium* sp. wilt pathogen (Lingan Rajendran *et al.,* 2014). Krishnamurthy and Gnanamanikam (1998) reported that the talc based formulation of *Pseudomonas fluorescens* controlled the rice blast pathogen caused by *Pyricularia grisea*. Commercial makers' point of view, short time for developed formulations, requirement of less labour, small space and control the contamination of products (Deshpande, 2006). The combination product of PY15, TDK1 and *Pseudomonas* sp. was found to be satisfactory control of sheath rot disease in rice (Saravanakumar *et al.,* 2009).

II. 15. Integrated Disease Management

The treatment of antagonistic microbes to control diseases is an additional important component of IDM. *In vitro* screening of biocontrol agents for their antagonistic potential against pathogens, their mode of actions, tolerant with agrochemicals and other inputs are important for their survival in the environmental conditions. There are several reports on integrated disease management in many crops. Chemical control, biocontrol and host resistance have been found to be very effective to against *Phytophthora* diseases of several plantation crops (Sarma, 2000). Nofal and Haggag (2006) reported that controlled powdery mildew of mango plants by application of combination biocontrol agents. Meena and Jeyalakshmi (2006) gave an integrated disease management planned to control the basal stem rot of coconut by soil application of *T. viride*. Ponmurugan *et al.* (2002) reported that collar canker of tea was successfully controlled by combination of various components like chemicals, biocontrol agents, and avoidance of predisposing factors.

White root rot disease (*Dematophora necatrix*) has been highlighted as a key unit in an integrated disease management (Gupta, 2000). Integrated disease management of

Sclerotium root rot of groundnut with *Trichoderma harzianum*, *Rhizobium* and carbendazim has been reported by Muthamilan and Jeyarajan (1996). The phytosanitation, chemical control, biological control and host resistance have been found to be very effective in combination to manage *Phytophthora* diseases of several crops (Sharma, 2000). Foliar, stem and root diseases of apple can be managed by using forecasting tools, host resistance, fungicides and biocontrol agents (Gupta, 2000). Sutton (1996) highlighted the role of cultural, biological, resistant genotypes and fungicides application in attaining economic control of the plant diseases of deciduous fruits.

Several disease management approaches, an addition of fungicides at reduced rates in combination with biocontrol agents has significantly improved disease control and when compared to treatments with biocontrol agent alone (Frances *et al.*, 2002 and Buck, 2004). Integrated practice of biocontrol agents *Fusarium* crown and root rot of tomato plants (Omar *et al.*, 2006), late leaf spot of groundnut (Kishore *et al.*, 2005), *Rhizoctonia* root rot of spring wheat (Duffy, 2000) and postharvest diseases of fruits (Chand Goyal and Spotts, 1996) compared with an individual components of disease management. These findings are indicated that an integrated disease management which will helpful for branch canker disease.

With the above background information, the present study is proposed with following objectives.

Major objectives:

- Random surveying of south Indian tea plantations in order to collect the branch canker pathogen isolates and biocontrol agents.
- To develop a germplasm consists of biocontrol agents to find out their antagonistic potential against branch canker disease under *in vitro* and *in vivo* level.
- Screening of biocontrol agents against branch canker pathogen *in vitro* and *in vivo* and identifying efficient biocontrol agents against pathogen *Macrophoma*. sp.
- To study the etiology and histopathology of the disease
- Life cycle pattern of *Macrophoma*. sp
- Study the biochemical changes during host - pathogen interaction
- Screening of various fungicides against branch canker disease
- Standardize the integrated schedule for controlling the branch canker disease

Chapter-III

MATERIALS AND METHODS

III.1 Systematic classification:

Kingdom	:	Fungi
Phylum	:	Ascomycota
Class	:	Dothideomycetes
Subclass	:	Incertaesedis
Order	:	Botryosphaeriales
Family	:	Botryosphaeriaceae
Genus	:	*Macrophoma*
Species	:	*theicola*

Chemicals, glassware's and media used **(See Appendix-I)**

III.2 Survey

The field surveys were conducted in different tea growing areas of south India including Tamil Nadu, Kerala and Karnataka. The disease specimens were collected from the Anamallais and the Nilgiris of Tamil Nadu, High Ranges, Central Travancore, Wayanad of Kerala and Koppa of Karnataka in order to isolate the efficient bacterial, fungal biocontrol agents and respective fungal pathogen.

III.3 Isolation of branch canker pathogen

The infected stem portions were washed with sterile water before incubating it in a moist chamber. The samples were taken from the moist chamber and surface sterilized with 0.1 % mercuric chloride for 10-20 sec. Then the specimens were washed with sterile distilled water and briefly dried on sterile filter paper (under filtered air in a laminar flow). Infected stem portions were cut into small pieces (2 × 2 mm) and then plated on water agar medium and the same was transferred to PDA agar medium.

III.4 Pathogenicity

Pathogenicity of the isolate was confirmed by Koch's postulates. Two years old, potted plants of BSS-1 (Biclonal Seed Stock -1) seedling were inoculated with the *Macrophoma* sp. after making wounds on the stem portion with a sterile scalpel. Mycelial discs were kept on the wound portion, covered with moist cotton and wrapped with polythene sheet. The plants were incubated in glasshouse to develop the symptom of black color tome pycnidium on the surface of stem portion. The pathogen was re-isolated from the artificially infected stem portions.

III.5 Moisture technique

Under moisture technique, branch canker specimens were kept in moist chamber. Further black color tome shaped pycnidium production on the stem, the study was carried out under laboratory conditions.

III.6 Culture characteristics

The culture characteristics of branch canker isolates were studied in solid and liquid media. Radial growth of the pathogen, colony morphology and production of pycnidia were observed in solid media. Spores were collected from the pycnidium and their morphometric characters were observed. In liquid media mycelial mat was harvested at 7^{th} day interval, dried in oven at 80 °C for overnight and mycelial dry weight was recorded to assess the biomass production.

III.7 Morphological characterization

A preliminary morphological characterization of the isolates was carried out in 9 to 15 days old cultures based on colony color, the ability to produce black tome pycnidium and the distribution pattern of tome shaped pycnidium within the colonies.

III.8. Fungal identification by 18S rRNA

8.1. Extraction of DNA

An indigenous isolates of the branch canker pathogen was inoculated in liquid medium and incubated at 28 °C for 24 h at 180 rpm. According to Azevedo *et al.* (2000) the

genomic DNA was extracted from overnight grown culture of all the branch canker isolates and quantified by fluorimetric method (Dyna Quant, Pharmarcia).

8.2. Sequence of ITS

The 18S rRNA gene, the primers ITS1.58S and ITS2 were amplified by PCR sequenced (Sanger *et al.,* 1977). The final volume of reaction was 10 µL, containing 2.0 µL (Invitrogen 3.0 µ mL^{-1}), 4.0 µL of the rDNA (100 ng μL^{-1}) and 4.0 µL of premix of dyenamic ET terminator. The reaction was analyzed using the Mastercycler thermocycler gradient.

An each programme was carried out with an initial denaturation at 95 °C for 2 min followed by 35 cycles. The each amplification consists of three steps: (denaturation at 95 °C for 1 min), (annealing at 55 °C for 1 min), (extension at 72 °C for 1 min) and (final extension at 72 °C for 5 min). The sequence was performed using the MegaBACE™ 1000 sequencer (Amersham Bioscience). The PCR products were analyzed on electrophoresis condition (2Kv/60 and 6Kv/230 min).

8.3. Phylogenetic analysis

The nucleotide sequence was compared against the sequences available from Gen Bank of the NCBI using the BLAST search tool (Altschul *et al.,* 1990).

8.4. Molecular identification of branch canker pathogen

The molecular identification of indigenous isolates of the branch canker pathogen (NBCHE-6, VPM, UPA-61, UPA-62 and NBCC-2) was carried out by DNA sequencing. The technique employed in DNA isolation, PCR amplification, Sanger sequencing and phylogenetic analysis. PCR was performed with universal primer pairs targeted to the 18s rRNA gene. The PCR positive samples were identified by DNA sequencing of the internal transcribed spacer (ITS) region of the rRNA gene. These isolates of nucleotide sequence were deposited in NCBI and EMBL (Japan) repository. Among the 5 isolates, only three isolates were selected for entire study parameters.

III.9 Standardization of media for the growth of branch canker pathogen

Selection of suitable nutrient media for the growth of *Macrophoma* sp. was carried out *in vitro* using various liquid and solid media such as Cephadox Dextrose Broth/Agar(CDB/A), Potato Dextrose Broth/Agar(PDB/A), Yeast Extract Malt

Broth/Agar(YEMB/A), Sabouraud's Dextrose Agar/Broth(SDA/B), Fungi Indian Standard Broth/Agar(FISB/A), Mineral Salt Medium Broth/Agar(MSMB/A), Rose Bengal Broth/Agar(RBB/A), RICHARD Medium Broth/Agar and Soil Media Broth/Agar. The pathogen was inoculated in the culture media and incubated at room temperature (25 to 30 °C) for 7 days. The growth of the pathogens was calculated on the basis of radial growth (cm) and dry weight (mg)

III.10 Optimization of abiotic factors on the growth of the pathogen

Influence of abiotic factors such as pH (4.0 to 7.0), temperature (20 °C to 50 °C) and light (UV, fluorescent, dark and day light) on the growth of pathogen was studied in PDA and PDB medium.

10.1. pH

The effect of pH on the growth of fungal isolates was studied in both PDA and PDB at the range of pH 4 to 7. The pH was adjusted with the help of acids and alkaline solutions. After 7 days of incubation, the growth of branch canker pathogen was calculated on the basis of dry weight (mg) of fungal mycelial mat and radial growth (cm).

10.2. Temperature

The three selected indigenous isolates of branch canker pathogen were tested under different temperatures ranging from 20 °C to 50 °C using temperature gradient chamber. These isolates were transferred to the PDA medium and incubated at different temperatures such as 20, 25, 30, 35, 40, 45 and 50 °C. After seven days of incubation, colony diameter and mycelia dry weight were measured for assessing the pathogen growth.

10.3. Light

The influence of different light condition on the growth of branch canker pathogen was studied in PDA and PDB. After 7 days of incubation, the radial growth of pathogen (cm) and mycelial dry weight (mg) was measured.

10.4. Relative humidity

The branch canker isolates were kept at below 20,30,40,50, 60, 70, 80 and above 90% RH levels maintained in a growth chamber for 24 h. After 7 days of incubation, the radial growth of branch canker pathogen was calculated on the basis of radial growth (cm).

III.11. Nutritional studies

Growth of the pathogen in different nutritional media of synthetic, semi-synthetic and nutritional requirements was studied in the mineral salts basal medium (MSBM) by replacing the basal medium with various nutrient sources.

11.1. Influence of different carbon and nitrogen sources

Six different carbon compounds *viz*, glucose and fructose (monosaccharides), sucrose (Oligosaccharides), carbohydrate, pectin and starch (polysaccharides) and eight different nitrogenous compounds *viz*., ammonium nitrate, ammonium sulphate, ammonium chloride (ammoniacal nitrogen), sodium nitrate and potassium nitrate (inorganic nitrogen), glycine (organic nitrogen), MSM (Nitrogen compound present) and MSM (Nitrogen compound absent) were used as different sources to assess the radial growth and mycelial dry weight in PDA and PDB.

III.12. GC-MS analysis of extracellular metabolites of branch canker pathogen

The fungal culture supernatant was collected after centrifugation at 10,000 rpm for 15 min in 125 mL separating funnel. The culture supernatant of 50 mL was mixed with equal volume of 50 mL ethyl acetate. After partitioning, the extract was evaporated to near dryness at 60 °C to 65 °C using rotary vacuum evaporator and analyzed in GC-MS as per the following conditions: Column: HP-5MS (5 % Diphenyl/ 95 % Dimethyl poly siloxane), 30 × 0.25 mm × 0.25 µm df, Equipment: GC - 7890A (Agilent), Carrier gas: 1mL/min-splitless, Detector: Mass detector – 5975C (Agilent), Software: Chem station, Injection volume:1µL.

III.13. Biochemical Changes during Host-Pathogen Interaction

13.1. Estimation of stem carbohydrate reserves

Total carbohydrate level was estimated by anthrone reagent method (Mc Cready *et al.,* 1950). The stem powder (100 mg) was taken in a test tube and 15 mL of 52 % perchloric acid was added to it. Then the test tube was incubated at 4 °C for 24 h. The content was filtered through Whatman no. 1 filter paper and volume was made up to 100 mL with distilled water in a volumetric flask. One mL of the extract was pipette out into a test tube and the volume was made up to 5 mL. To this diluted extract, 10 mL of cold anthrone reagent (200 mg of anthrone in 100 mL of conc. Sulphuric acid) was added and the test tube was

heated at 100 °C in a water bath for 10 min and cooled rapidly on ice cubes. Color development was read at 630 nm in a spectrophotometer. For each set of sample, a blank with distilled water instead of extract was kept. Standard graph was prepared with 10, 20, 30, 40 and 50 ppm solution of dextrose and total carbohydrate was determined from the standard graph.

$$\text{Wood carbohydrate level (\%)} = \frac{\text{Graph value} \times 100 \times 5 \times 100 \times 100}{\text{Weight of sample (mg)}}$$

13.2. Reducing sugar (Hedge and Hofreiter, 1962)

About 1.0 g of infected stem portion was taken in a mortar and ground well with 10 mL of double distilled alcohol. The filtrates were collected in 50 mL standard measuring flask. Repeat the process with further two 10 mL of DD alcohol. The filtrate was made up to the mark with DD alcohol. Dilute 5 mL with distilled water and used for the analysis. 1 mL of diluted extract was taken in a test tube and kept under ice cold condition. Take 1, 2, 3, 4 and 5 mL of working standard solution in test tubes. Zero mL serves as blank. Add 4 mL of anthrone regent (200 mg of anthrone in 95 % sulphuric acid) very slowly along the sides of the test tube and shaken well. Keep the test tubes with stand in a boiling water bath for eight minutes. Then cool and read the green colour formed at 630 nm. Draw standard graphs by plotting concentration of glucose in X axis and absorbance at Y axis. From the graph we can calculate the amount of reducing sugar present in the sample.

Calculation

$$\text{Reducing sugars (\%)} = \frac{GR*50*50*100*100}{Wt*5*10^{6*}\ DMC}$$

Where,

GR - Graph reading

Wt - Weight of the sample taken

DMC - Dry matter content of the sample under investigation

III.14. Nutrient analysis (Nitrogen, Phosphorous and Potassium)

The infected stem samples were dried in oven and finely powdered and then used for estimation of nitrogen, potassium and phosphorus contents. The dried stem samples from the branch canker infected stem and healthy stem (without inoculation) samples were used for nutrient analysis.

14.1. Estimation of Nitrogen

About 0.5 to 1 g of ground sample was taken in a Kjeldahl tube and the analysis was carried out using Kjeldahl method (Page *et al.,* 1982). The principle involves the conversion of all forms of nitrogen into ammonium form by digesting with sulphuric acid, potassium sulphate, copper sulphate and sodium thiosulphate at 450 °C. Distillation was carried out in Gerhardt - Kjeltec instruments. The distillate was collected in a conical flask which contains a known quantity of 0.05 N HCL. The excess hydrochloric acid was then titrated against standard sodium hydroxide solution using digital burette.

14.2. Estimation of potassium (Hanway and Heidal 1952)

The digested stem samples were directly fed into the flame photometer (Sherwood 410) and the potassium content was estimated using direct reading.

14.3. Estimation of phosphorus

Five mL of wet digested extract from stock solution was taken in a 25 mL standard measuring flask, to which 10 mL of distilled water, 5 mL of freshly prepared ascorbic acid along with 4 mL of ammonium molybdate solution. The contents were diluted to 25 mL with distilled water, shaken well and allowed to stand for 30 minutes for completion of the reaction. Absorbance of the developed blue color was measured at 882 nm against the reagent blank solution using UV-Visible spectrophotometer (Bray and Kurtz, 1945). The amount of phosphorus present in the sample was calculated using the calibration curve of known concentrations of phosphorus and the results were expressed in mg per kg.

III.15. Histopathological studies

15.1. Screening of different tea cultivars against branch canker

Glass house experiment was conducted in UPASI-TRF, Farm, Valparai-642127, Coimbatore-Dt, Tamil Nadu, India. One year old nursery plants of the tea clones like TRF-1 (yield), Yabukita (Hardy), UPASI-3 (Yield and Quality), UPASI-6 (Hardy), TRF-2

(Quality), CR-6017 (Quality), UPASI-9 (Hardy and Yield), UPASI-17 (Yield), UPASI-26 (Hardy), TRF-4 (Yield and Quality) and TRI-2025 (Hardy and Yield) were evaluated for their susceptibility/tolerance to branch canker disease. The stem portion of the healthy plants was cracked by using sterile blade to remove the outer layer and mycelial disc (2 mm) was inoculated on the cracked portion and covered with sterilized cotton then tied with parafilm. The inoculated plants were kept in glass house conditions at temperature 25 °C and humidity 76.8 %. Healthy plants of same age served as control. The samples were collected after life cycle pattern of pathogen 43rd days from inoculated plants to study the infection levels canker size and number of spores' production. The canker size was measured by the ruler scale (cm) and number of spores was counted by cyclometer through microscopic view condition.

15.2. Preparation of samples

The collected the samples were fixed, immersed with wax content and sectioned with cryostat microtome (Leica Microsystems Nussloch GmbH, Heidelberger Strasse17-9, D-69226 Nussloch (Deutschland), Model-Leica CM 1100-1) were used for histopathological studies. The sections were stained according to the methods outlined by Sass (1958) and Johansen (1940). The stained sections were observed through light microscope (Olympus Microscope Corporation, Tokyo and Japan – Model BX51TRF). The critical observations of host and pathogen interaction were measured by histopathological technique.

III.16. *In vitro* screening of chemical fungicides

Fungicides were screened by poisoned food technique (Adams and Wong, 1991) using PDA medium. Contact, systemic and combination of both were used for the study. Stock solutions of fungicides were prepared in sterile distilled water. Appropriate quantity of the fungicide solution was added to molten PDA medium so as to get the required concentration. The medium was poured into Petri plates and inoculated with 5 mm mycelial discs of the pathogen from a 7th day old culture. The plate containing PDA without fungicides were maintained as control and all treatments were replicated thrice. After seventh day of incubation, the treatment plates were measured along with fully grown control plate and per cent of inhibition (PI) was calculated by Bell'scale method (Bell, 1982).

III.17. *In vitro* screening of botanical fungicides

The commercially available botanical fungicides, Expel (combination of Canolar extract & Tea tree oil), Fungfinish (5 % copper formulation), Tari (Organic Plus Tea special), Nimbicidine (0.03 % Azadiractin EC), Tricure (0.03 % Azadiractin EC), Ecocare, Autospy and Enroot was tested at various concentrations by using food poisoned technique against branch canker pathogen under *in vitro*. After seventh day of incubation, growth of fungal mycelium was recorded and tabulated.

III.18. *In vitro* screening of plant aqueous extracts

Total fourteen plants were locally collected from the same areas of tea field at same time at Valparai-642127. The extracts were prepared from leaves which are having antifungal properties. These plant samples were washed thoroughly under tap water followed by sterilized water. The plant samples were air dried and grinded with the help of pestle and mortar. Stock solution of all the plant extracts were prepared by soaking the grinded plant materials in sterilized water for 2 h at room temperature (27 to 32 °C). Each plant extract was filtered through muslin cloth and finally filtered in whatman paper N0.1. The plant extracts were poured in the conical flasks plugged with cotton and heated at 100 °C for 10 minutes to avoid microbial contamination (Madavi and Singh, 2005). The food poisoned technique was applied for antagonistic level (Nene and Thapilyal, 2000). Different concentrations of plant extract were prepared (5 %, 10 % and 15 % W/V) by adding appropriate quantity of sterilized water in to a stock solution. The leaf extracts were poured in to the petriplate in appropriate concentration, the extract was the mixed with 20 mL/plate containing sterilized PDA medium and as such without addition of plant extracts were served as control plates. The isolated pathogen was grown on PDA medium which was placed at the center of petriplate containing different concentration. For each treatment four replications were maintained at 27±2 °C for 7 days. Radial fungal growth (cm) inhibition was calculated in two directions. The percentage of inhibitions were calculated by the formula (Dissanayake, 2014)

Inhibition (%) = (C-T)/C) × 100

Where, C = growth in control plate, T = growth in treatment plate.

III.19. Evaluation of fungal biocontrol agents

19.1. *In vitro* Screening of fungal antagonistic (Dual culture method)

The fungal biocontrol agents were screened against branch canker pathogen by dual culture method described by Rajendiran *et al*. (2010). The fungal biocontrol agents were isolated from different tea gardens of southern India. The fungal biocontrol isolates were identified as *Trichoderma* spp. through visual, microscopic observation methods and colony, spore forming morphological characters of *Trichoderma* spp. were identified by Rifai (1969) and Bissett (1991) whereas *Gliocladium* sp. was identified as described by Christian and Gary (1998), namely like *G. virens, T. harzianum, T. viride and T. atroviride.* Mycelial discs measuring 6 mm diameter from seven days old culture of both test pathogen and fungal antagonist were inoculated in PDA plates on diametrically opposite points. The Petri plates were incubated at 27 ± 2 ºC and five replications were maintained in each treatment. In control plates, a sterile agar disc without fungal antagonistic was maintained. Antagonistic activity was measured using Bell'scale method (Bell, 1982). The percentage of inhibitions was calculated by the formula of PI=C-T/C×100.

PI - Percentage of Inhibition, C - Radial growth of the pathogen in control, T - Radial growth of the pathogen in dual culture.

19.2. Culture metabolite technique

The effect of fungal biocontrol agents from the culture filtrates were studied by Dennis and Webster (1971) method. The fungal biocontrol agents were inoculated in 100 mL of sterilized Potato Dextrose Broth in 250 mL conical flasks and incubated at 27 ± 2 ºC. The liquid culture medium was filtered through Whatman No.1 filter paper and centrifuged at 10,000 rpm for 15 mins. Then the supernatant was filtered by using Millipore membrane filter paper (0.22 µm). The fungal culture filtrate was added to molten of 100 mL PDA medium to obtain final concentration of 2 % (v/v). This medium was poured into Petri plates (20 mL/plate) and plates were inoculated with 6 mm disc of test pathogen and then amended and in which sterile distilled water served as control. The plates were incubated at 27 ± 2 ºC for seven days. The percentage of inhibitions was calculated by the above formula.

19.3. Hyphal interactions

The fungal antagonistic plates were incubated for 7-9 days and interaction between the opposing cultures including hyphal contact or coiling and lysis were observed under

microscopic level. Hyphal interaction was gently identified from the zone of interaction in dual culture plates with the help of a needle and placed in a drop of lactophenol cotton blue on a microscopic slide (Elad *et al*., 1983).

III.20. Evaluation of entomopathogens against branch canker

20.1. *In vitro* screening of entomophathogens antagonists (Dual culture method)

The potential of entomopathogens were screened against (*Macrophoma* sp.) branch canker pathogen by dual culture method as described by Rajendiran *et al.* (2010). In entomopathogens were procured from Microbial Type Culture Collection and Genebank (MTCC) Chandigarh, namely like *Beauveria bassiana, Paecilomyces fumosoroseus, Lecanicillium lecanii* and *Paecilomyces lilacinus.* The pathogen and antagonist were inoculated in PDA plates on diametrically opposite points. Since the entomopathogens were slow growing in nature, the antagonists were inoculated only before the pathogen colony grew considerably therefore, after 2 days. Linear growth of the biocontrol agents colonizing either over or meet each other the pathogens growth was measured after 9 days of incubation. The testing antagonistic entomopathogens of *Beauveria bassiana, Paecilomyces fumosoroseus, Lecanicillium lecanii* and *Paecilomyces lilacinus* 6 mm discs of antagonist and *Macrophoma* sp. cut from the edge of 7 days old culture were placed 3 cm apart on potato dextrose agar (PDA) plate. The Petri plates were incubated at 27 ± 1 ºC and periodical observations on the growth of the antagonist to colonize the pathogen were recorded. The experimental design used was completely randomized with four dishes for each isolates and control plate (without entomopathogen) a sterilized agar disc plates. Antagonistic activity was measured using Bell'scale method (Bell, 1982). The percentage of inhibitions was calculated by the formula, PI=C-T/C×100. PI- percentage of inhibition, C- radial growth of the pathogen in control, T- radial growth of the pathogen in dual culture.

20.2. Culture metabolite technique

The effect of culture filtrate of entomopathogen was studied following the method of Dennis and Webster (1971). The entomopathogens were inoculated in 100 mL sterilized Potato dextrose broth in 250 mL conical flasks and incubated at 27 ± 1 ºC .The liquid culture medium was filtered through What man No.1. The filtrate was centrifuged at 10,000 rpm for 15 min. The supernatant was filtered using millipore membrane filter paper (0.22 µm).The

entomopathogen filtrate was added to molten PDA to obtain final concentration of 2 % (v/v). The medium was poured into petri plates (20 mL/plate) and plates were inoculated with 6mm disc of test pathogens. PDA plates inoculated with *Macrophoma* sp. but amended with sterile distilled water served as control. The plates were incubated at 27 ± 1ºC for 6 days. The percentage of inhibitions was calculated by above the formula.

20.3. Hyphal interactions of entomopathogens and branch canker pathogen

The entomopathogen and test pathogen at the opposite edges and were incubated for 7-9 days and interaction between the opposing cultures including hyphal contact or coiling and lysis, which was observed under the microscope. Hyphal interaction gently from the zone of interaction in dual culture plates with the help of a needle and placed in a drop of lactophenol cotton blue on a microscopic slide (Elad *et al*., 1983).

III.21. Isolation and identification of bacterial strains

Soil samples collected from different tea growing areas of southern India were serially diluted and plated on Luria Bertani Agar and Kings B medium. Four different bacterial strains were identified using standard biochemical tests described by Bergey's Manual of systematic Bacteriology (Claus and Berkeley, 1986 and Stolp and Gadkari, 1981) (**See Appendix-II**). The phenotypic characters were identified by using the methods described by Gordon *et al.* (1973). Isolation of genomic DNA, PCR amplification of the 16S rRNA gene was carried out as per the method described by Sambrook *et al.* (1989).

21.1. Bacterial identification by 16S rRNA

21. 2. Extraction of DNA

The genomic DNA was extracted from overnight grown cultures of all the four selected bacterial strains using the QIAGEN DNA isolation kits (Qiagen, Valencia, CA). The final volume was suspended in 100 µl of elution buffer (10 mM/L Tris-HCl, pH 8.5) and the OD was measured at 260 nm. The PCR amplification was achieved using a 20 µL reaction mixture containing 100 ng of template DNA, 20 µmol of 16S rRNA primers, 200 µM of dNTPs,1.5 mM of $MgCl_2$, 1U of *Taq* DNA polymerase (MBI Fermentas) and 2 µL of 10x *Taq* polymerase buffer. Amplification was carried out with an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 56 °C for 45 sec, extension at 72 °C for 1 min and final extension at 72 °C for 5 min using a thermocycler

(iCycler; Bio-Rad Laboratories, CA). The PCR products were analyzed on 1 % agarose gel for 16S rRNA amplicons in 1x TBE buffer at 100 V according to Sambrook *et al.* (1989).

21.3. Sequence of PCR Products

The 16S rRNA genes amplified fragments were purified by using QIAquick gel extraction kits (Qiagen, Valencia, CA) from the agarose gel and ligated into the pGEM®-T Easy vector (Promega Corporation, Madison, USA) as per the manufacturer's instructions, then they are transformed into *E. coli* strain DH5α and plated on Luria Bertaini agar medium contained ampicillin (50 µg/mL), X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; 20 µg/mL) and IPTG (isopropyl-β-D-thiogalacto pyranoside; 0.1 mM/mL) (Sambrook *et al.*, 1989).

21.4. Phylogenetic analysis:

These sequences of bacterial strains 16S rRNA genes were compared with those already deposited in the data base using the BLASTN programme which were aligned using CLUSTAL W software (Thompson *et al.*, 1994). Distances were calculated according to Kimura's two-parameter correction (Kimura, 1980). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987).

21.5. Bacterial nucleotide accession numbers

The four bacterial strains were given the code name as WP104, CS-2, TRB and AWRH-40B and their sequence data determined and deposited in the NCBI gene bank data base. The four bacterial strains were given the accession numbers as KM853034 for WP104, KM527836 for CS2, KM527837 for TRB and KM527838 for AWRH-40B by NCBI.

III.22. *In vitro* screening of bacterial antagonistic

22.1. Dual plate technique

The branch canker pathogen inhibition capacity with bacterial biocontrol strains antagonistic were determined following the method out lined by Huang and Hoes (1976). One 6 mm disc of seven days old culture of the pathogen was placed at the centre of a Petri plate containing PDA medium. The bacterial strains were streaked at two opposing side and plates were incubated for 6 days at 30 ºC to 35 ºC and growth diameter of the pathogen was measured and compared to control growth, where the bacterial strains were replaced by sterile Luria Bertani Medium. The antagonistic activity was measured using Bell' scale

method (Bell, 1982). The results were expressed as the mean of the percentage of inhibitions as per the formula PI = C-T/C×100, where, P I – is percentage of inhibition, C- is fungal radial growth of the pathogen in control, T- is radial growth of the pathogen and bacterial strain in dual culture.

22.2. Effect of cell free culture filtrates

The bacterial culture was studied following the method of Dennis and Webster (1971) and inoculated in 100 mL sterilized Luria Bertani Broth in 250 mL conical flasks and incubated at 30 ºC to 35 ºC . The filtrate was centrifuged at 10,000 rpm for 15 min. The supernatant was filtered using by Millipore membrane filter paper (0.22 µm). The bacterial culture filtrate was added to 100 mL molten LBA to obtain final concentration of 4 % (v/v) and poured into Petri plates (20 mL/plate) and plates were inoculated with 6 mm disc of test pathogen. LBA plates were inoculated with test pathogen and then amended with sterile distilled water to serve as control. The plates were incubated for 24, 48 and 72 h at 30 ºC to 35 ºC for 6 days. The percentage of inhibitions was calculated as per the formula given in Bell scale method (Bell, 1982).

22.3. GC-MS analysis in bacterial culture

Bacterial culture supernatant was collected after centrifugation in 125 mL separating funnel and extracted with 50 mL of ethyl acetate. After partitioning, the extract was evaporated to near dryness at 60 ºC - 65 ºC using rotary vacuum evaporator and analyzed in GC-MS as per the following conditions: Column: HP-5MS (5 % Diphenyl/ 95 % Dimethyl poly siloxane), 30 × 0.25 mm × 0.25 µm df, Equipment: GC - 7890A (Agilent), Carrier gas: 1mL/min-splitless, Detector: Mass detector – 5975C (Agilent), Software: Chem station, Injection volume:1µL.

III.23. Compatibility studies

In all agrochemicals like fungicides (Benomyl 50 % WP, Hexaconazole 5 EC, Combination product of Companion [carbendazim 12 % + mancozeb 63 % WP], Copper oxychloride 50 % WP and Propiconazole 25 EC), pesticides (Propargite 57 EC and Deltamethrin 2.8 EC) and weedicides (Glyphosate 41 % SL and Ammonium salt of glyphosate 71 % SG) were determined by the compatibility test against the biological agents using poisoned food technique (Dhingra and Sinclair, 1985). All the agrochemicals at 0.4 %

concentration were added with the bacterial biocontrol agents for compatibility test. Similarly fungicides of 10 ppm concentration and pesticides and weedicides of 50 ppm concentration were mixed with the fungal biocontrol agents to test their compatibility.

The bacterial strains were mixed with sterilized 100 mL LB medium and simultaneously 0.4 % concentration of agrochemicals we added. The culture medium was incubated at 25 ± 2 ºC for 2 days. After incubation, the culture was serially diluted to obtain (10^4, 10^5 and 10^6) dilution by adopting pour plate technique. The counting method of Colony Forming Unit of pour plate was derived by Schmidt and Coldwell (1967). The colony counting was measured the following formula; Number of CFU of bacteria per mL = Number of CFU × Dilution factor / × Aliquot taken.

Stock solutions (1000 ppm) of the agrochemicals were mixed with 100 mL PDA molten medium at respective dosages. After solidification of PDA contains respective agrochemicals with respective dosages, 5 mm disc was cut from 7 days old culture of fungal biocontrol agent was placed in the centre of petri dishes. Control plates without any agrochemicals were maintained simultaneously for comparison. The treatment plates were inoculated at 25 ± 2 ºC for seven days. The percent inhibition of growth colony diameter was measured after 7^{th} day of incubation. The percent inhibition of growth formula I = X-Y/X × 100 (Sundar *et al.*, 1995). Where, I – Percentage of growth inhibition, X – Radial growth (cm) in control (without agrochemical), Y – Radial growth (cm) in treated plates (with agrochemical).

The above mentioned same dosages were followed in the liquid media, the sterilized 100 mL PDB medium contains agrochemicals were inoculated with 7 days old culture of fungal biocontrol agent. Finally liquid media mycelial mat was harvested at 7^{th} days of incubation, dried in oven at 80 ºC for overnight and calculated on the basis of biomass dry weight (mg).

III.24. Shelf-life studies

24.1. Mass production of bacterial bio control agents

Bacterial biocontrol agents were isolated from different districts of tea growing regions and then used for the shelf-life studies. The bacterial strains were confirmed by using 16S rRNA molecular method and their nucleotide sequences were submitted to NCBI gene

bank. The molecular accession number were given by NCBI data base for respective isolates such as *Bacillus subtilis* (Accession No: KM527836), *Pseudomonas fluorescens* (Accession No: KM527837), *Bacillus amyloliquefaciens* (Accession No: KM853034) and *Bacillus licheniformis* (Accession No: KM527838) were grown on the respective medium of Nutrient Broth and Luria Bertani (NB and LB). The talc and coir pith formulations of bio control agents were prepared based on the technique of Jeyarajan *et al.* (1994). A loopful of inoculum of bacterial strains was transferred into conical flask of 250 mL and 500 mL LB medium to be grown for 3 days. Then 250 mL of culture was mixed with sterilized talc powder (1 kg) and 500 mL of culture was mixed with sterilized carrier material of coir pith (1 kg) [Talc based formulation in the 1:4 ratio and Coir pith formulation in 1:2 ratio]. These isolates are being maintained in the repository of UPASI Tea Research Institute, Valparai-642127.

24.2. Mass production of fungal bio control agents

The fungal biocontrol agents were isolated from different tea growing districts of southern India. These fungal biocontrol agents were maintained on PDA media for further studies. Fungal biocontrol agents such as *Gliocladium virens*, *Trichoderma viride*, *Trichoderma harzianum* and *Trichoderma atroviride* were grown on potato dextrose broth medium. The medium is inoculated with 1 disk of 5 mm of 6 days old culture of fungal biocontrol agents and incubated at room temperature (25 ºC to 27 ºC) for 7 days. The formulation of fungal biocontrol was prepared by following the procedure as described earlier.

The formulations were packed in polythene bags and stored at 25 ºC to 28 ºC for different interval. The formulations were serially diluted at respective intervals (30, 60, 90 and 120 days) to obtain 10^6 dilutions by adopting pour plate method. The colony forming (CFU) unit of biocontrol formulation was described by the formula derived by Schmidt and Coldwell (1967).

The formula is given below:

Number of CFU of biocontrol per gram of soil = Number of CFU × Dilution factor / Dry wt of sample × Aliquot taken.

III.25. Integrated Disease Management (Glasshouse conditions)

25.1. Evaluation of chemicals and biocontrol agents

The effect of different fungicides like Copper oxychloride 3 g/L (Fytolon 50 % WP), Benomyl 0.5 g/L (Benofit 50 % WP), Tebuconazole 0.5 g/L (Folicur 250 % EC) and Tridemorph 0.5 g/L (Calixin 80 % EC) were evaluated under glasshouse conditions against the branch disease and biocontrol agent such as *Bacillus* sp. *Pseudomonas* sp. *Trichoderma harzianum* and *Trichoderma viride* at 3 g/L concentration.

25.2. Integrated management of branch canker disease

Systemic fungicides such as Hexaconazole 1mL/L (Contof 5 % EC), Propiconazole 0.7 mL/L (Tilt 25 % EC), Companion 2 g/L (Carbendazim 12 % + Mancozeb 63 % WP) and botanical fungicide of Tricure 3 mL/L (0.03 % Azadiractin EC) were assessed and screened against branch canker disease under glasshouse conditions. Biocontrol strains such as *Bacillus subtilis, Bacillus amyloliquefaciens* and *Gliocladium virens* were critically studied at equal concentrations of 3 g/L. One round of chemical and another round of biocontrol agent were applied alternatively (Alternated schedule).

25.3. Glass house experiment method

The biocontrol agents and chemical fungicides were tested in BSS-1 (Biclonal Seed Stock -1) seedlings kept in glasshouse. The treatments were replicated thrice and each treatments carried out ten plants. Healthy semi-mature stem portions were artificially wounded with a sterile scalpel and inoculated with active spore suspension (1×10^5 spore's mL^{-1}) of the *Macrophoma* sp. in gently cracked stem. The inoculated seedlings were regularly monitored for disease symptoms and the biocontrol agents and fungicides were sprayed on infected plants at seven days interval. The application dosages were followed as per UPASI, TRF – Farm recommendations (Good Agriculture Practice).

The branch canker disease protection in percentage level and calculated by using following formula

$$\text{Wound Healing (WH)} = (L_1 - L_2)$$

Where, $\mathbf{L_1}$ **-** (Pre-treatment) canker length in cm; $\mathbf{L_2}$ **-** (Post-treatment) canker length in cm,

III.26. Integrated Disease Management (Field conditions)

26.1. Biological with chemicals against branch canker

Field experiment was conducted at UPASI Tea Research Foundation, Tea Research Institute, Valparai located at 10° 23' North and 77° 0' East and about 1050 m above Mean Sea Level. The bushes pruned at 60 cm height from ground level and treatments imposed. There were 15 treatments with three replications. All the treatments laid out in randomized block design. The branch canker disease protection calculated by using following formula (Mareeswaran and Premkumar Samuel Asir, 2017), Wound Healing (WH) = (L_1-L_2) + (W_1-W_2), Where L_1- (Pre-treatment) canker length in cm, L_2- (Post treatment) canker length in cm, W_1- (Pre-treatment) canker size in width (cm), W_2- (Post treatment) canker size in width (cm). The canker size values are measured by before application of pre-treatment (length and width in cm) and after application of six months measured by post-treatment (length and width in cm).

The efficacy of three systemic fungicides, Benomyl 0.5 g (Benofit 50 % WP), Tebuconazole 0.5 g (Folicur 250 EC), Tridemorph 0.5 g (Calixin 80 % EC), Contact fungicide of Copper oxychloride 0.5 g (Fytolon 50 % WP) and Botanical extract of Expel (5 % Combination of Canolar extract and tea tree oil, procured from M/s Advance pesticides Pvt. Ltd, Nasik, Maharashtra) were selected and applied as wound paste. In this experiment, biocontrol agents' application (99.5 g) and chemical fungicides (0.5 g) were mixed with help of painting brush (Total 100 g / bush) for making wound pasting after pruning. The bioformulation (1:1 Ratio), Talc (1.0 kg) + biocontrol agent (1 litre liquid culture), chemical fungicides: 0.5 g + 99.5 g of biocontrol talc formulation to make it 100 g / bush. The chemical fungicides along with bioformulations (100 g) were applied. The adjuvant, linseed oil was used to make pasting and wound healing properties. For wound dressing biofungicides application was made into a paste with linseed oil and applied on the pruning cut with help of painting brush. The talc preparations were made in to paste with distilled water (1:2 ratio) and applied on the pruned cuts and canker portion (Ponmurugan *et al.*, 2002).

26.2. Integrated management of branch canker disease

Field experiment for an integrated management of branch canker disease was conducted at UPASI Tea Research Foundation, Tea Research Institute, Valparai. Susceptible

of seedling planted in 1961 area at style of planting (1.2×1.2 m) and bush population (6800 plants/ha) was conducted. An experiment was carried out in naturally infected pruned tea fields and there was 11 treatments with three replications. Each replication consider as 25 bushes. All the treatments were laid out in randomized block design. The incidence of branch canker disease was assessed at before and after treatment imposed. The two times of applications per year during February-March and October-November (2014-2015) was studied to the trail plots. The complete applications dosages were measured as per the recommendation schedule of UPASI Tea Research Institute, Valparai, Tamil Nadu, India.

The selected fungicides of Copper oxychloride (Fytolan 50 % WP) [Bayer Crop Science Ltd.] at 0.2 g and Companion (Carbendazim 12 % + Mancozeb 63 % WP) [Bayer Crop Science Ltd.] at 0.08g were explored at 729 mL water volume/per plot (25 bush dosage). An indigenous biological control such as *Trichoderma harzianum*, *Gliocladium virens*, *Beauveria bassiana* and *Bacillus amyloliquefaciens* were obtained from repository of Division Plant Pathology, UPASI Tea Research Institute, Valparai, Tamil Nadu, India.

Biological control agent was mixed with sterilized talc powder at concentration level of 250 mL culture broth per kg of talc and kept for 2 days of incubation at 25 ± 2 ºC. Tricure (0.03% Azadirachtin) at 4.2 mL, Tari (Organic Tea Special) at 4.2 mL and biological agents at 20.8 g were evaluated against the branch canker disease. Standard cultural practices spray was done by using a Knapsack low volume sprayer at 5 days interval. The commercial botanical fungicide Tricure (0.03 % Azadirachtin) was procured from Margo Biocontrol Pvt, Ltd, Nashik, Maharashtra and Tari (Organic Tea Special) was obtained from TARI BIO TECH, Tanjavur. Branch canker disease protection was calculated by using following formula (Mareeswaran and Premkumar Samuel Asir, 2017). Wound Healing (WH) = (L_1-L_2) + (W_1-W_2). Where **L_1- (**Pre-treatment) canker length in cm, **L_2- (**Post treatment) canker length in cm, **W_1-** (Pre-treatment) canker size in width (cm), **W_2-** (Post treatment) canker size in width (cm). Treatments and their application details are given below.

Treatment details	Dose/ha	Dose/plot	Spraying interval
Copper oxychloride (50 % WP)	50g in 10 liters of water	0.2g/729 mLwater volume	5 days
Companion [Carbendazim 12 % + Mancozeb 63 % WP]	20 g/10 L	0.08g/729 mLwater volume	5 days
Bacillus amyloliquefaciens	5kg/ha	20.8g/729 mLwater volume	5 days
Companion / *Bacillus amyloliquefaciens*	20g/10L+5kg/ha	0.08g/20.8g/729 mL water volume	5 days
Copper oxychloride / *Bacillus amyloliquefaciens*	50g in 10 litres of water +5kg/ha	0.2g/20.8g/729 mL water volume	5 days
Tichoderma harzianum	5kg/ha	20.8g/729 mLwater volume	5 days
Gliocladium virens	5kg/ha	20.8g/729 mLwater volume	5 days
Beauveria bassiana	5kg/ha	20.8g/729 mL water volume	5 days
TARI (Organic Tea Special)	1000 mL/ha	4.2 mL/729 mLwater volume	5 days
Tricure (0.03% Azadirachtin)	1000 mL/ha	4.2 mL/729 mL water volume	5 days
Control	-	Water	5 days

III.27. Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) and the significant means were segregated by critical difference (CD@P=0.05) at various levels of significance using the formulae given by Gomez and Gomez (1984). Analysis of DMRT and were analyzed statistically using special purpose statistical software (SPSS ver. 16.1).

Chapter-IV

Results

IV. 1. Field Survey and Isolation of Branch Canker Pathogen

1.1. Pathogen isolated from different tea regions

Field surveys were conducted in different tea growing regions of south India (The Nilgiris, The Anamallais, Vandiperiyar, and Koppa) for the isolation of branch canker pathogen. Then the infected specimens were collected from different tea growing regions of south India. The pathogen morphology was identified based on their mycelial and spore culture characters followed on standard mycological book by "*Illustrated Genera of Imperfect Fungi*" (Barnett and Hunter, 1972 and Barnett and Hunter, 1998). A total of 23 isolates were collected and four of them showing distinct colony characteristics and spore morphology (Table.1 and Plate.3). Only three isolates were selected for the entire studies. The chosen isolates were desired as based on the different location of tea growing areas. Two isolates were collected from Nilgiris another two isolates from Anamallais and one from Vandiperiyar. The spore is characterized by an oval shaped and non-septum, the pycnidiospores are arranged uniformly inside the oval shape pycnidia (Plate. 4). The morphology of pycnidiospore in under water mounting stains (Plate.5) and lacto phenol cotton blue mounting showed the oval shape of pycnidiospore very clearly (Plate. 6).

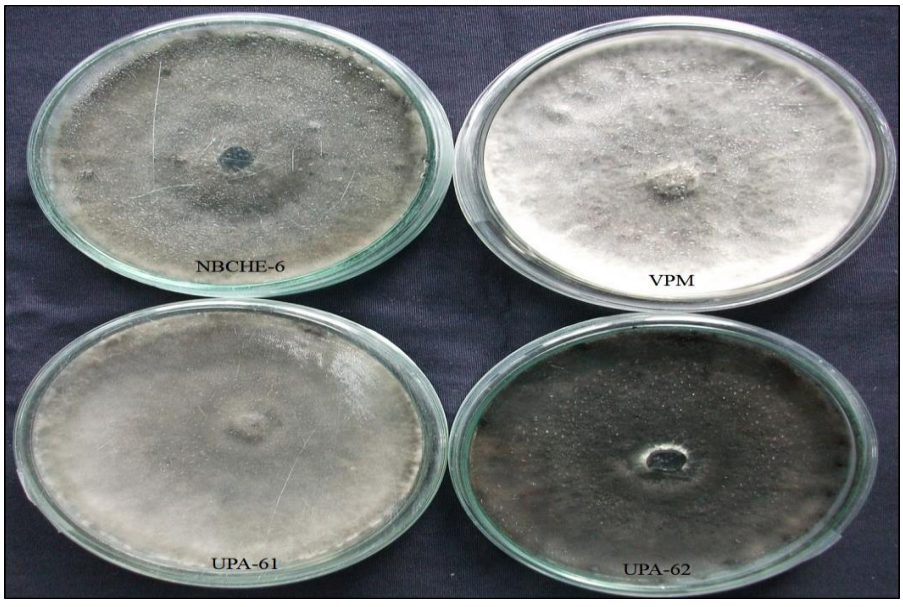
1.2. Molecular identification of branch canker pathogen

These isolates were identified based on their genomic DNA sequencing and amplification by Polymerase Chain Reaction was performed with primer pairs targeted to the 18S rRNA gene of the fungus. A total of five strains were isolated and they are coded as NBCHE-6, NBCC-2, VPM, UPA-61 and UPA-62 (Table.2). These fungal pathogens were confirmed as *Macrophoma* spp. and *Macrophoma theicola* through their molecular identifications and submitted to NCBI (Accession No: JQ234977 for NBCHE-6, Accession No: JQ362417 for NBCC-2, Accession No: KP004441 for VPM, Accession No: KP179221 for UPA-61 and Accession No: KP179222 for UPA-62).

Table.1 Isolation of branch canker pathogen from different tea growing areas of south India

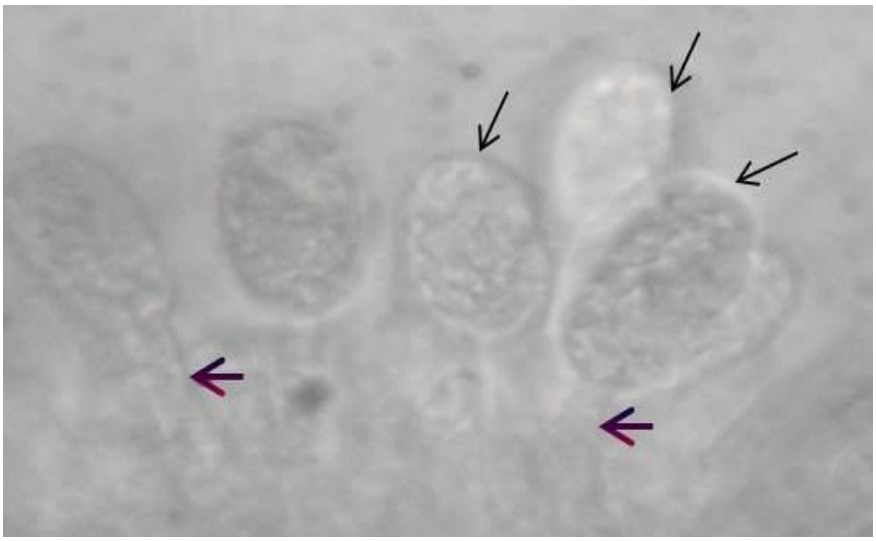
Different tea growing areas	Number of branch canker samples isolates	Identified as
The Anamallais	6	*Macrophoma theicola*
The Nilgiris	8	*Macrophoma* sp.
Vandiperiyar	4	*Macrophoma* sp.
Koppa	5	-

Plate.3 Morphologically distinct colonies of *Macrophoma* spp. isolated from different tea growing areas of South India



NBCHE-6: *Macrophoma* sp. **VPM:** *Macrophoma* sp. **UPA-61**: *Macrophoma theicola* and **UPA-62**: *Macrophoma theicola.*

Plate.4 Microscopic view of pycnidia morphology (100×) in water mounting technique



Pycnidiospores (line arrow), spore stalk (dark arrow)

Plate .5 Microscopic view of branch canker pathogen spore morphology (10× and 100 ×) in water mounting technique

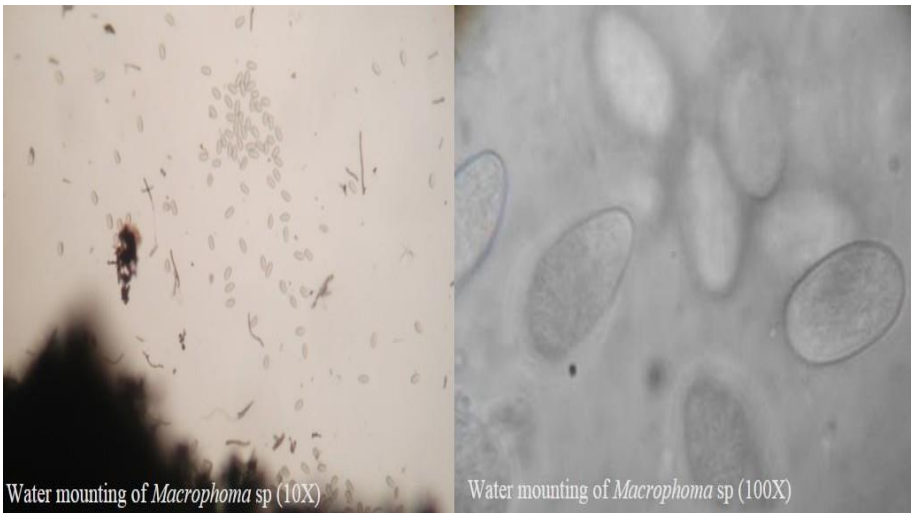


Plate.6 Lacto Phenol Cotton Blue Mounting of *Macrophoma* sp. spore structure (100×)

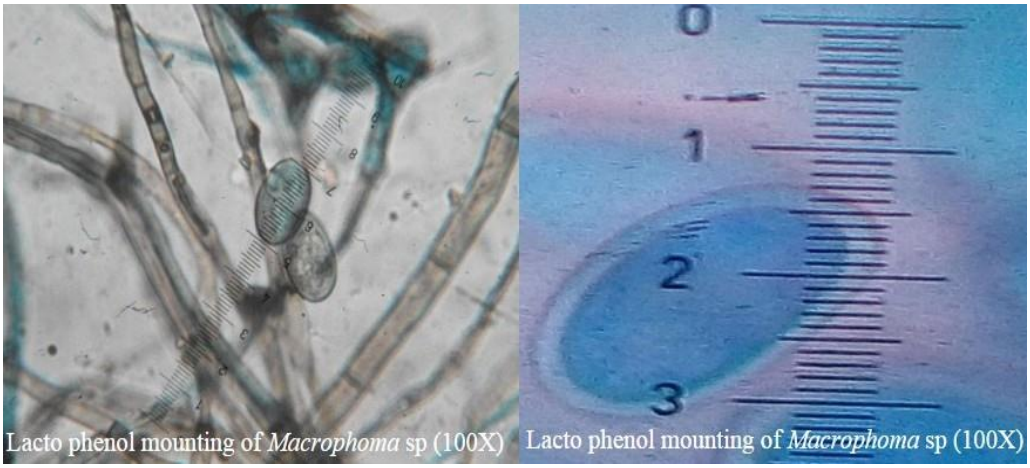


Table.2 Molecular identification of branch canker isolates

Strain code No	**Location**	**Identified as**	**NCBI Gen Bank Accession Number**
NBCHE-6	Coonoor	*Macrophoma* sp.	JQ234977
VPM	Vandiperiyar	*Macrophoma* sp.	KP004441
UPA-61	The Anamallais	*Macrophoma theicola*	KP179221
UPA-62	The Anamallais	*Macrophoma theicola*	KP179222
NBCC-2	The Nilgiris	*Macrophoma* sp.	JQ362417

1.3. Phylogenetic tree analysis of branch canker isolates

The isolated pathogen (NBCHE-6, VPM and UPA-61) was selected for phylogenetic tree construction through 18S rRNA sequence model. These nucleotide sequences were compared with sequences of other fungal genomes from the databases given in NCBI and a phylogenetic tree was constructed. The results indicated that strain of NBCHE-6, VPM and UPA-61 belonged to *Macrophoma* spp. and *Macrophoma theicola*. The respective isolates such as NBCHE-6 and UPA-61 showed 95% to 99% similarity with another fungal genomes of *Macrophoma* spp. (DQ100415.1 & DQ100414.1) and *Macrophoma theicola* KP179222.1. Another strain of VPM sequences exhibited less than 95% similarity with remaining species of the group of ascomycetes (Figure.1)

1.4. Culture characteristics feature of branch canker pathogen

The culture characteristics of the isolates were studied using both solid and liquid media. Radial growth of the pathogen, colony morphology and production of pycnidia were observed in solid media. Generally, the mycelium were whitish black, then it was changed to black color and the margin of the fungi were entire circular. The colony of all the isolates was hyaline with circular and regular entire margin. The NBCHE-6 was noticed completely with light blackish growth. Colony of VPM was initially white in color, which later turned to Whitish light black. The colony of UPA-61 was turned to completely blackish color and regular entire margin (Table.3). In case of different days of liquid culture, the mycelial dry weight was gradually increased till 7^{th} day after incubation and thereafter declined. The mycelial dry weight was recorded maximum in UPA-61 followed by VPM and NBCHE-6 (Figure. 2).

1.5. Pycnidia production of branch canker

The preliminary morphological colony characterization of the isolates was carried out in 7 day incubation and 15 day old cultures based on colony color, the ability to black tome pycnidia production, the distribution of tome shaped pycnidia within the colonies. The more number of pycnidia production of pathogen (UPA-61, VPM and NBCHE-6) was observed in MSM medium followed by SDA medium. The lowest pycnidia production of *Macrophoma* spp. isolates was recorded in soil medium followed by RBA medium (Table.4).

Figure.1 Phylogenetic tree constructed by NCBI - BLAST search methods

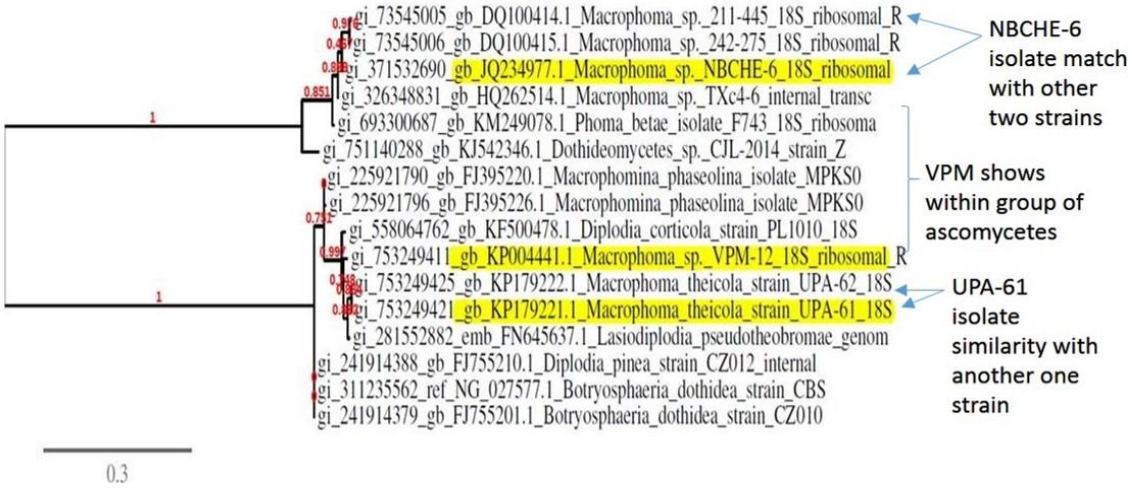


Table.3 Culture characteristic of branch canker pathogen isolates

Days	***Macrophoma theicola* isolates**		
	NBCHE-6	**VPM**	**UPA-61**
1	Entire and hyaline	Entire and hyaline	Entire and hyaline
2	Initially white	Initially white	Initially white
3	Irregular, flush growth	Irregular, flush growth	Irregular, flush growth
4	Pale white color	Pale white color	Pale white color
5	Blackish white and regular margin growth	Blackish white and regular margin growth	Blackish white and regular margin growth
6	Completely, light blackish regular margin growth	Whitish, light black regular margin growth	White, turned to blackish regular margin growth

Figure.2 Growth of *Macrophoma* spp. isolates in liquid medium

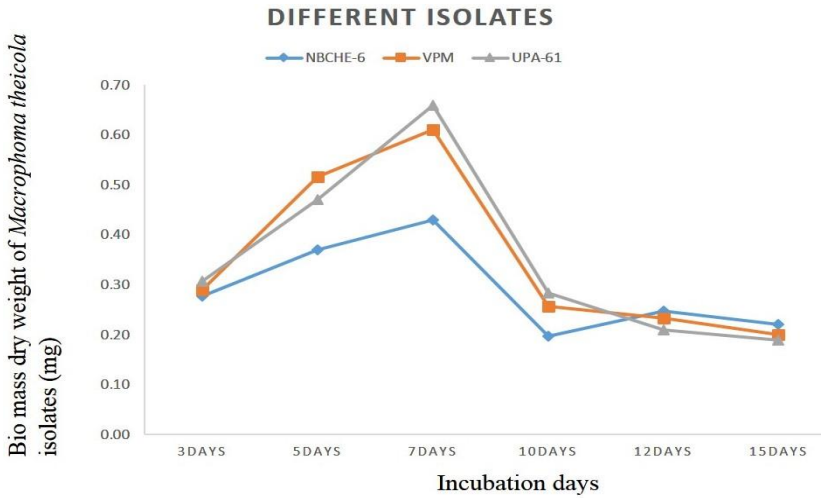


Table.4 Pycnidia production by *Macrophoma* spp. isolates on different solid medium

Different growth media	**Pycnidia production of different isolates on 15th day observation**		
	Macrophoma sp. (NBCHE-6)	*Macrophoma* sp. (VPM)	*Macrophoma theicola* (UPA-61)
PDA	29.67	33.33	38.00
CDA	22.33	24.00	24.33
YEMA	14.33	12.00	12.00
SDA	**45.33**	**46.33**	**48.67**
FIS	31.00	35.00	31.67
MSM	**106.00**	**141.00**	**158.33**
RBA	11.00	10.67	15.33
RICHARD	30.00	33.00	28.00
SOIL MEDIUM	3.33	5.00	5.33
CD at P=0.05	**10**	**13.2**	**11.7**

1.6. Growth characterization of branch canker pathogen in various nutrient media

Among the various growth media tested, the growth of *Macrophoma* spp. isolates (NBCHE-6, UPA-61 and VPM) was higher in Potato Dextrose Agar followed by Richard medium. Soil medium did not support the growth of pathogen followed by SDA (Table.5). In case of liquid media same trend of results was noticed, the higher growth of biomass dry weight was recorded in PDA followed by Richard medium (Table.5).

1.7. Pathogenicity of branch canker pathogen

The pathogenicity of branch canker was determined by inoculating the pathogen on the stem after removing the bark and development of symptoms were observed under *in vitro* glass house conditions. No symptoms were observed on the first three days after inoculation. The first initial symptom was noticed on the sixth day after inoculation, brownish margin started developing indicating the growth of pathogen. The black colour fungal mycelium with spores developed on the 18th day. A well-developed black color tome shaped of pycnidia production was observed on the 43rd day after inoculation. Finally the canker fully developed and entire branch was completely killed (Table.6). Characteristic of branch canker symptoms with canker size was noticed on 18th - 43rd day after inoculation. Pycnidia were noticed on the entire infected portion of the stem especially found in the inner epidermis layer of stem. Branch canker pathogen was re-isolated from artificially inoculated stem portions and the same pathogen was confirmed by Koch's postulate method.

IV. 2.1. Etiological characters of the pathogen

The etiology of the *Macrophoma theicola* isolates was studied *in vitro* with reference to abiotic and nutritional requirements of the pathogen.

2.2. Effect of abiotic factors

2.3. Effect of different temperature on branch canker pathogen

Results showed that, the radial growth and mycelial dry weight of the fungus were maximum at temperature 25 °C to 30 °C. When the temperature was less than 20°C, they reached declined growth and above 35 °C totally retorted the mycelial growth (Table.7 and 8).

Table.5 Colony growth of *Macrophoma* spp. in different growth media

Different media	Growth of different isolates on 7th day of incubation					
	Radial growth (cm)			Mycelial dry weight (mg)		
	NBCHE-6	VPM	UPA-61	NBCHE-6	VPM	UPA-61
PDA	**9.00**	**8.88**	**8.97**	0.93	1.01	1.03
CDA	7.98	6.25	8.30	0.55	0.66	0.71
YEMA	8.62	7.63	8.38	0.55	0.81	0.80
SDA	5.40	6.88	5.92	0.61	0.42	0.32
FIS	8.63	8.90	7.98	0.54	0.82	0.78
MSM	8.32	8.28	8.60	0.50	0.83	0.91
RBA	8.70	7.80	8.20	0.44	0.90	0.91
RICHARD	**8.92**	**8.10**	**8.88**	0.77	0.89	0.93
SOIL MEDIUM	3.67	3.93	4.15	0.05	0.24	0.25
CD at P=0.05	**0.4**	**0.5**	**0.6**	**0.1**	**0.1**	**0.1**

Table.6 Disease establishment pattern of the branch canker pathogens during Pathogenesis under glass house condition

Observation on	Pathogenicity test for Branch canker pathogen
3rd Day after incubation	No symptoms
6th Day after incubation	First initial symptoms noticed (brownish margin growth)
18th Day after incubation	Black color fungal mycelium and spores observed on stem portions
18th to 43rd Day after incubation	Complete callus formation and canker developed and entire branch was killed.

Table.7 Radial growth of *Macrophoma* spp. under different temperature

Different isolates	Growth of different isolates on 7th day observation Different Temperature (°C)						
	20 °C	**25 °C**	**30 °C**	**35 °C**	**40 °C**	**45 °C**	**50 °C**
Macrophoma sp. (NBCHE-6)	4.3±0.07	**8.7±0.04**	**8.3±0.10**	6.9±0.07	6.2±0.17	0.0±0.0	0.0±0.0
Macrophoma sp. (VPM)	4.8±0.04	**9.0±0.06**	**8.5±0.05**	7.0±0.10	6.5±0.13	0.0±0.0	0.0±0.0
Macrophoma theicola (UPA-61)	4.5±0.09	**8.9±0.03**	**8.2±0.12**	6.6±0.08	6.0±0.15	0.0±0.0	0.0±0.0

Values are mean ± S.D. in terms of growth in diameter (cm) of eight replicates

Table.8 Growth of *Macrophoma* spp. under different temperature in biomass dry weight (mg)

Different isolates	**Growth of different isolates on 7th day observation Temperature (°C)**						
	20 ºC	**25 ºC**	**30 ºC**	**35 ºC**	**40 ºC**	**45 ºC**	**50 ºC**
Macrophoma sp. (NBCHE-6)	2.47±0.18	**3.00±0.06**	**2.93±0.09**	2.07±0.04	1.80±0.06	0.0±0.0	0.0±0.0
Macrophoma sp. (VPM)	2.00±0.06	**3.13±0.09**	**2.90±0.06**	2.13±0.09	1.93±0.09	0.0±0.0	0.0±0.0
Macrophoma theicola (UPA-61)	2.07±0.09	**2.93±0.09**	**2.87±0.09**	2.27±0.09	2.00±0.06	0.0±0.0	0.0±0.0

Values are mean ± S.D. in terms of growth in dry weight (g) of eight replicates

2.4. Effect of different pH on branch canker pathogen

The results revealed that branch canker pathogen grow well at pH ranges between 5.0 and 5.5. The optimum pH 5.0 favored the growth of branch canker pathogen, whereas pH 7.0 does not support the branch canker pathogen growth. In solid media, all the isolates were recorded maximum growth at pH 5.0 while pH 5.5 supported the maximum growth of UPA-61 and VPM. The same tendency of results indicated that, in liquid media of all the isolates preferred pH 5.0 for better growth (Table.9 and 10)

2.5. Effect relative humidity on branch canker pathogen

Growth of the pathogen was kept in the relative humidity chamber at different level of RH such as 20, 30, 40, 50, 60, 70, 80 and 90 % for 7th days of incubation. After incubation period, the higher growth of isolates was recorded in 60 to 80 % RH. In case of 20-30 % RH did not support the fungus growth and also above 90 % RH level. Results revealed that the RH ranges between 60 to 80% preferred for the growth of the branch canker isolates (Figure.3).

2.6. Effect of different light condition on branch canker pathogen

The radial growth of the isolates was maximum in day light followed by dark light. The other light sources provided very less radial growth of the fungus. But this trend showed variation in liquid medium. Mycelial biomass was maximum on day light followed by dark light for UPA-61, VPM and NBCHE-6 in liquid medium, whereas UV light condition does not suitable for the growth of all the isolates (Table.11).

2.7. Effect of different nutrient sources

2.7.1. Effect of different carbon sources on branch canker

Among the different carbon sources studied, the maximum growth was observed in carbohydrate (polysaccharides) and fructose (monosaccharides) on 7th days of incubation. The higher mycelial dry weight was attained by the fungus in the above two groups of carbon sources (Table.12). The maximum pycnidia production of branch canker pathogen was recorded in sucrose followed by carbohydrate on 15th days of incubation (Table.13). This study will be helpful to find out their nutrient condition on branch canker pathogen in tea plant.

Table.9 Effect of pH on radial growth of *Macrophoma* spp.

Different isolates	Growth cm on 7th day observation Different pH						
	4.0	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**
Macrophoma sp. (NBCHE-6)	5.40±0.16	6.73±0.03	8.90±0.06	8.42±0.14	7.70±0.17	6.14±0.03	5.11±0.01
Macrophoma sp. (VPM)	4.93±0.09	6.47±0.18	8.90±0.06	8.67±0.15	7.63±0.12	6.33±0.19	5.03±0.07
Macrophoma theicola (UPA-61)	4.73±0.20	6.47±0.12	8.90±0.06	8.93±0.07	7.07±0.09	6.08±0.09	5.09±0.11

Values are mean ± S.D. in terms of growth in diameter (cm) of eight replicates

Table.10 Influence of pH on bio mass growth of *Macrophoma* spp.

Different isolates	Biomass dry weight of *Macrophoma* spp. (mg) on 7th day observation Different pH						
	4.0	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**
Macrophoma.sp. (NBCHE-6)	1.88±0.09	2.04±0.17	2.54±0.10	2.51±0.09	2.15±0.11	1.95±0.09	1.97±0.07
Macrophoma. sp. (VPM)	1.91±0.07	2.03±0.16	2.68±0.11	2.59±0.07	2.19±0.15	2.00±0.08	1.99±0.02
Macrophoma theicola (UPA-61)	1.90±0.10	2.00±0.09	2.50±0.13	2.48±0.10	2.13±0.09	1.98±0.12	1.94±0.05

Values are mean ± S.D. in terms of growth in dry weight (g) of eight replicates

Figure.3 Effect of humidity on different isolates of *Macrophoma* spp.

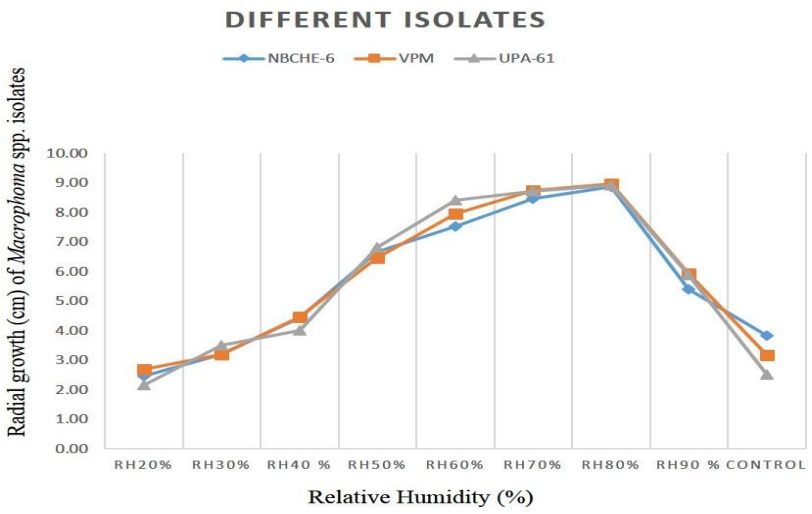


Table.11 Effect of illumination on growth of *Macrophoma* spp.

Different light condition	**Growth of different isolates on 7th day observation**					
	Radial growth (cm)			**Mycelial dry weight (mg)**		
	NBCHE-6	**VPM**	**UPA-61**	**NBCHE-6**	**VPM**	**UPA-61**
UV light	5.62	6.14	6.00	0.09	0.07	0.10
Fluorescent light	6.76	7.11	7.02	0.14	0.15	0.13
Dark condition	7.46	8.00	7.98	0.18	0.19	0.22
Day light (control)	8.50	8.78	8.76	0.63	0.68	0.68
CD at P=0.05	**0.4**	**0.3**	**0.3**	**0.1**	**0.1**	**0**

Values are Mean ± S.D. in terms of growth in dry weight and diameter of eight replicates

Table.12 Effect of various carbon sources on growth of *Macrophoma* spp.

Carbon compounds	Growth of *Macrophoma* spp. isolates on 7th day observation					
	Radial growth (cm)			Mycelial dry weight (mg)		
	NBCHE-6	VPM	UPA-61	NBCHE-6	VPM	UPA-61
Starch	8.50	8.44	8.45	0.08	0.08	0.08
Glucose	8.54	8.55	8.55	0.08	0.07	0.08
Carbohydrate	9.00	9.00	9.00	0.15	0.15	0.16
Sucrose	8.48	8.28	8.33	0.04	0.05	0.05
Fructose	8.69	8.63	8.60	0.10	0.09	0.09
Pectin	8.13	8.17	8.14	0.06	0.08	0.08
CD at P=0.05	**0.4**	**0.5**	**0.4**	**0**	**0**	**0**

Values are Mean ± S.D. in terms of growth in dry weight and diameter of eight replicates

Table.13 Pycnidia production influenced by various carbon sources

Carbon compounds	No of Pycnidia production on 15th day of observation		
	NBCHE-6	VPM	UPA-61
Starch	8.75	8.25	6.25
Glucose	21.00	21.00	18.50
Carbohydrate	30.50	30.25	31.75
Sucrose	52.75	51.25	55.75
Fructose	6.50	5.00	7.25
Pectin	16.75	16.00	15.25
CD at P=0.05	**5.8**	**4.3**	**3.5**

Values are Mean ± S.D. in terms of growth in dry weight and diameter of eight replicates

2.7.2. Effect of different nitrogen sources on branch canker

The growth of the pathogen was influenced by all the nitrogen compounds. In case of nitrogen compounds, the isolates UPA-61, VPM and NBCHE-6 maximum growth of the pathogen while using glycin, VPM recorded maximum biomass in the same (Table.14). The pycnidia production was highly observed in glycin followed by pottassium nitrate (Table.15).

IV. 3. Identification of toxic metabolites of branch canker pathogen through GC-MS analysis

Among the five isolates, the only two fungal isolates of toxins compounds of NBCHE-6 and NBCC-2 were identified through GC-MS analysis (Table.16 and 17). The culture filtrates of NBCHE-6 showed the nine compounds such as Butanoic acid, 2-oxo-, Undecane, 2-methyl-, Undecane, 2,6-methyl-, Octane,2-bromo-, Decane,2,4-dimethyl-, 3-Hexane, 4-ethyl-, Decane,2,5,9-trimethyl, 3-Buten-2-ol and Malonic acid, dihydroxy-,diisobutyl ester (Figure.4). In these toxins compounds may be responsible for induced the branch canker disease. Commonly, the mycotoxins are formed by plant fungal pathogens which are caused diseases in plant system.

The presence of seven compounds in the isolate of NBCC-2 *viz;* 1H-Indene, 1-methylene-, Propanedioic acid, phenyl-, 1-Methyldodecylamine, Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenylesters, 3-Trifluoroacetoxytridecane, Cyclooctasiloxane, hexadecamethyl- and 2-Aminononadecane (Figure.5). These compounds serve as toxin properties for disease development in host plant. In these two isolates were produced by many mycotoxin compounds, in these compounds are resemble like some other mycotoxin compounds of fungal plant pathogens. The remains isolates of UPA-61, UPA-62 and VPM were produced very trace level of metabolites compounds. But, in these case GC-MS graph did not indicated properly.

IV. 4. Biochemical Changes during Host-Pathogen Interaction

The changes in physiological and biochemical properties in young tea stem due to branch canker infection was studied with respective clones of UPASI-3, UPASI-17, UPASI-26, TRF-1, TRI-2025 and Yabukita. The biochemical constituents in the healthy plant and infected plant were subjected to analyses.

Table.14 Effect of Different Nitrogen Sources on growth of *Macrophoma* spp.

Nitrogen compounds	Radial growth (cm)			Mycelial dry weight (mg)		
	NBCHE-6	**VPM**	**UPA-61**	**NBCHE-6**	**VPM**	**UPA-61**
MSM (N_2Present)	6.03	6.07	6.43	0.02	0.02	0.02
MSM (N_2 Absent)	5.80	5.60	5.33	0.01	0.02	0.02
Sodium Nitrate	8.10	7.80	7.37	0.05	0.06	0.06
Glycin	8.77	8.93	8.97	0.08	0.09	0.08
Ammonium nitrate	8.10	7.80	7.77	0.05	0.04	0.05
Pottassium nitrate	8.12	8.60	8.60	0.05	0.06	0.07
Ammonium sulphate	7.80	7.63	7.43	0.05	0.05	0.05
Ammonium chloride	7.70	6.80	7.17	0.03	0.03	0.03
P value	**2.15**	**2.15**	**2.15**	**2.15**	**2.15**	**2.15**
CD@5 %	**0.58**	**0.46**	**0.72**	**0.1**	**0.02**	**0.01**
CD@1 %	**0.81**	**0.64**	**1.0**	**0.2**	**0.02**	**0.02**

Values are Mean ± S.D. in terms of growth in dry weight and diameter of eight replicates

Table.15 Pycnidia production on different Nitrogen sources

Nitrogen compounds	No of Pycnidia production on 15th day of observation		
	NBCHE-6	**VPM**	**UPA-61**
MSM (N2 Present)	9.00	6.33	13.67
MSM (N2 Absent)	6.33	5.67	7.67
Sodium Nitrate	4.00	3.00	6.00
Glysin	21.00	23.33	32.00
Ammonium nitrate	3.00	3.33	5.67
Pottassium nitrate	18.00	21.00	22.33
Ammonium sulphate	10.67	8.67	11.00
Ammonium chloride	3.67	3.00	5.33
P value	**2.15**	**2.15**	**2.15**
CD@5 %	**3.49**	**3.57**	**3.63**
CD@1 %	**4.85**	**4.96**	**5.04**

Values are Mean ± S.D. in terms of growth in dry weight and diameter of eight replicates

Table.16 Mycotoxin compounds identified in the culture filtrate of NBCHE-6 (*Macrophoma* sp.)

No	RT	Name of the compounds	Molecular formula	Molecular weight	Peak Area %
1	4.00	Butanoic acid, 2-oxo-	$C_4H_6O_3$	102	0.005
2	4.85	Undecane, 2-methyl-	$C_{12}H_{26}$	170	0.004
3	6.42	Undecane, 2,6-methyl-	$C_{13}H_{28}$	184	0.02
4	7.46	Octane,2-bromo-	$C_8H_{17}Br$	192	0.005
5	8.80	Decane,2,4-dimethyl-	$C_{12}H_{26}$	170	0.02
6	9.89	3-Hexane, 4-ethyl-	$C_8H_{16}O$	128	0.004
7	10.99	Decane,2,5,9-trimethyl	$C_{13}H_{28}$	184	0.009
8	13.22	3-Buten-2-ol	C_4H_{80}	72	0.004
9	33.49	Malonic acid, dihydroxy-,diisobutyl ester	$C_{11}H_{20}O_6$	248	0.03

Table.17 Toxins components identified in the sample of NBCC-2 (*Macrophoma* sp.)

No	RT	Name of the compound	Molecular formula	Molecular weight	Peak area %
1	3.533	1H-Indene, 1-methylene-	$C_{10}H_8$	128	7.79
2	3.844	Propanedioic acid, phenyl-	$C_9H_8O_4$	180	8.47
3	5.337	1-Methyldodecylamine	$C_{13}H_{29}N$	199	11.77
4	6.703	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	$C_{19}H_{30}O_3$	306	14.78
5	7.572	3-Trifluoroacetoxytridecane	$C_{15}H_{27}F_3O_2$	296	16.69
6	8.430	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	592	18.58
7	9.944	2-Amininonadecane	$C_{19}H_{41}N$	283	21.92

Figure.4 GC-MS Chromatogram of the sample NBCHE-6 (*Macrophoma* sp.)

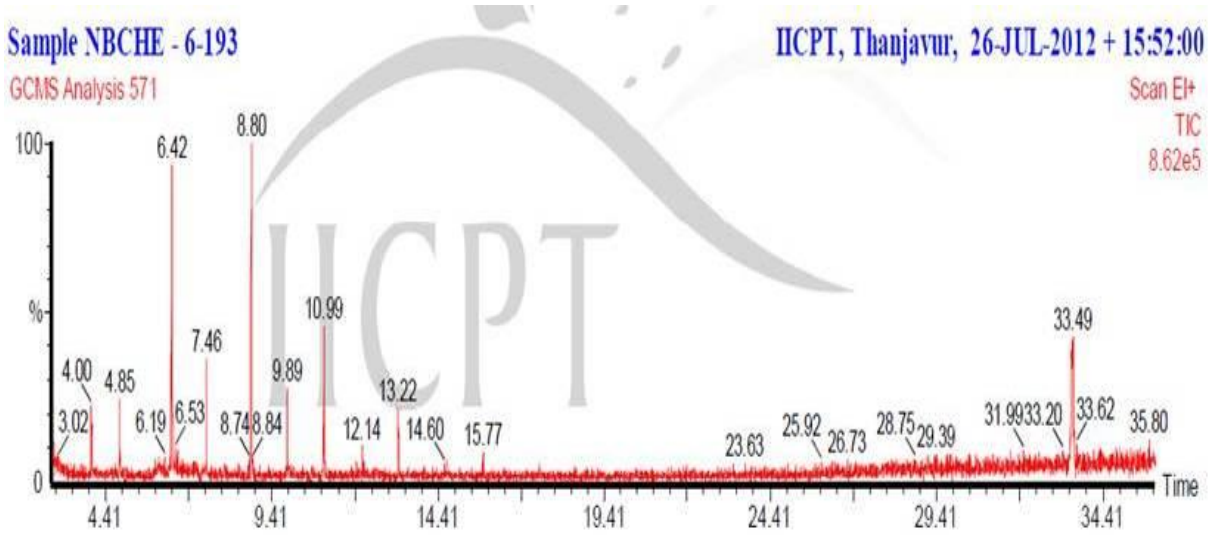
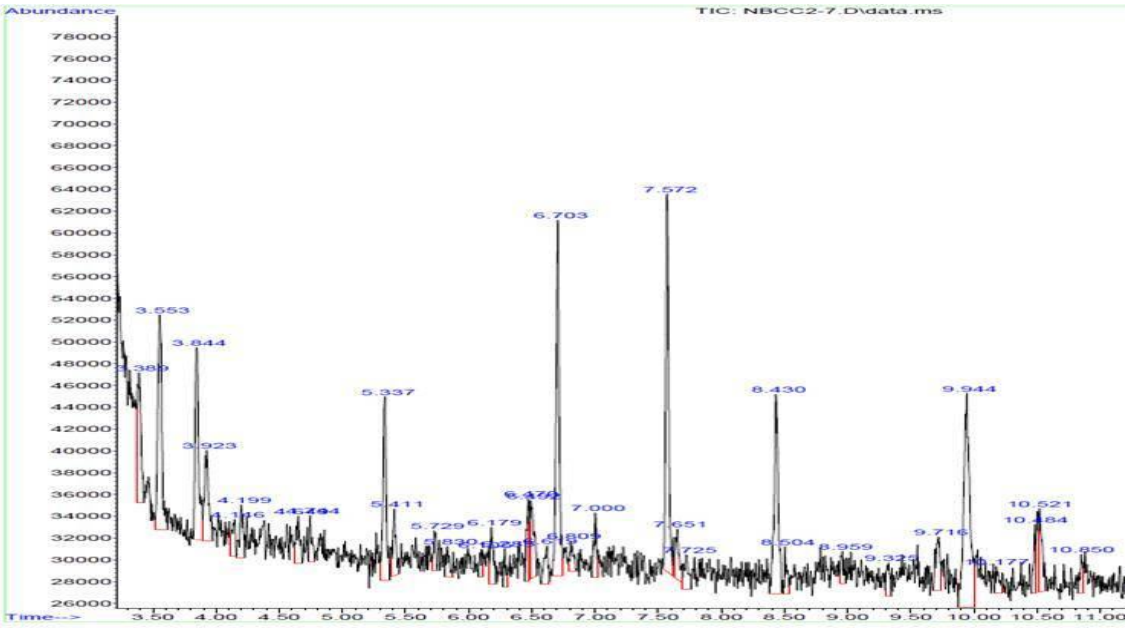


Figure.5 GC-MS Chromatogram of the culture filtrate of NBCC-2 (*Macrophoma* sp.)



IV. 4.1. Biochemical analysis

4.2.1. Effect of carbohydrate level on branch canker disease

The present study was focused to estimate the biochemical changes due to the infection of branch canker disease in the nursery tea plants. Host-pathogen interaction studies were carried out in different tea clones under glass house conditions. The pathogen was induced artificially in the stem portion of the nursery plants. After 43rd day from induction, the infected stem samples were collected and the analysis of carbohydrate was carried out. The total carbohydrate content in the infected plants show decreasing trend in all the tea clones when compared to the healthy plants (Figure.6). The carbohydrate level was decreased in all the clone due to branch canker pathogen infection, because it might utilized the carbohydrate content by branch pathogen.

4.2.2. Effect of reducing sugar on branch canker disease

The present investigation revealed that, the reducing sugar content was decreased in all the infected clones, except the clone of TRI-2025 (Figure.7). There was no major variation observed in the healthy and infected clone of TRI-2025. This study indicated that the clone of TRI-2025 showed better tolerance level against the fungus of *Macrophoma theicola*.

4.3. Nutrient analysis (Nitrogen, Phosphorous and Potassium)

4.3.1. Effect of NPK on branch canker disease

The biochemical changes of host-pathogen interaction studies were carried out the respective clone of above said the clones. The NPK level was carried out to respective infected clones and healthy clones. Similarly the nutrients (NPK) level of all the infected clones recorded lower values than the uninfected plants, whereas there is no notable difference in the nitrogen level of infected and healthy plants of the clone Yabukita (Table.18). In generally, the disease severity considerably increased due to pathogen infection. The present study examined that the fungus *Macrophoma theicola* might be utilized the nitrogen level from the infected plant. Results showed that the infected clone of Yabukita was accepted comparatively tolerant level of NPK content. This initial study will be helpful for further clonal screening against branch canker pathogen under nursery level condition.

Figure.6 Total carbohydrate level of infected and healthy tea cultivars due to branch canker disease

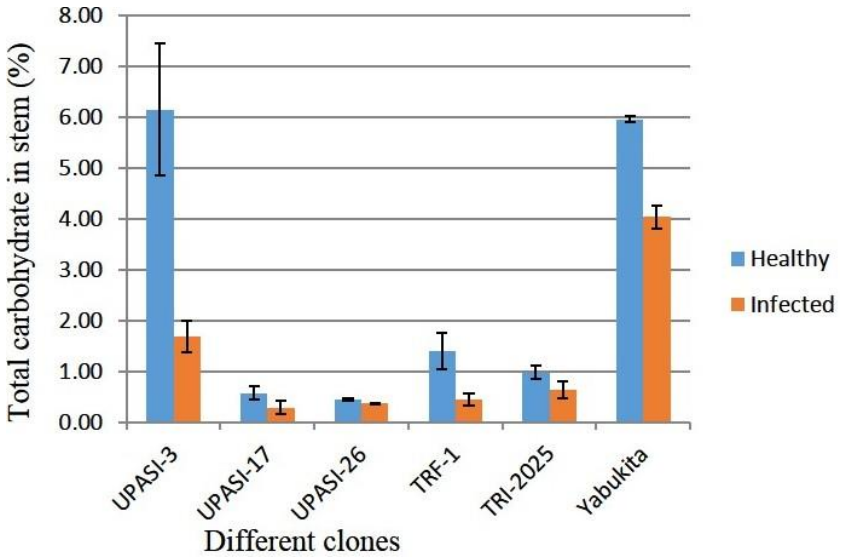


Figure.7 Reducing sugar level of infected and healthy tea cultivars due to branch canker disease

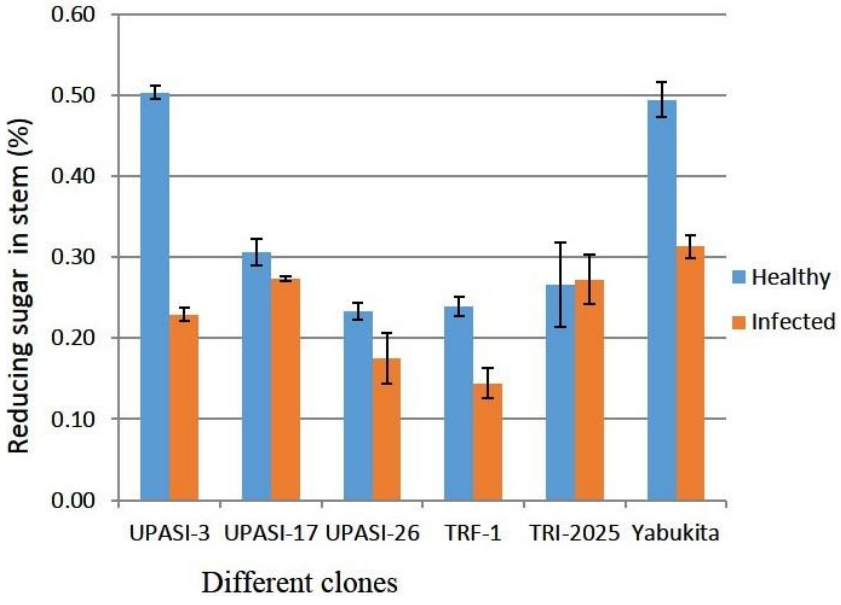


Table.18 NPK level on infected and healthy plants due to branch canker disease

Different Clones	Nitrogen (g/100g)		Phosphorous (g/100g)		Potassium (g/100g)	
	Healthy	**Infected**	**Healthy**	**Infected**	**Healthy**	**Infected**
UPASI-3	0.70±0.01	0.49±0.01	0.08±0.01	0.05±0.01	0.47±0.03	0.35±0.03
UPASI-17	0.59±0.02	0.51±0.02	0.08±0.01	0.07±0.01	0.41±0.01	0.40±0.01
UPASI-26	0.99±0.02	0.84±0.02	0.14±0.02	0.09±0.01	0.67±0.03	0.49±0.02
TRF-1	0.81±0.01	0.53±0.02	0.10±0.01	0.08±0.01	0.47±0.02	0.42±0.02
TRI 2025	0.84±0.01	0.73±0.02	0.08±0.01	0.06±0.01	0.51±0.03	0.49±0.02
Yabukita	0.73±0.01	0.73±0.02	0.14±0.02	0.10±0.01	0.40±0.01	0.30±0.02

Values are mean ± SD of three replications of three repeated analysis.

IV. 5. Histopathological Studies

5.1. Screening of tea cultivars for branch canker disease tolerance using histopathological studies

A glass house experiment was carried out for screening tea cultivars for their tolerance / susceptibility to branch canker disease. One year old nursery plants were selected for this study *viz*. TRF-1, UPASI-3, Yabukita, UPASI-6, TRF-2, CR-6017, UPASI-9, UPASI-17, UPASI-26, TRF-4 and TRI- 2025. This study revealed that UPASI-3 (21.00±1.53) and TRF-1 (19.67±1.20) were recorded higher number of pycnidiospores productions and bigger size of canker (Table.19). The lesion size was more in UPASI-3 (2.57±0.09) and TRF-1 (2.03±0.09) followed by Yabukita (Plate.7 a, b & c). These clones were categorized as highly susceptible (above 1.5 cm canker size). In CR-6017 (3.00±0.58), TRF-2 (1.67±0.67) and UPASI-6 (1.33±0.33) noticed less number of spore production in the epidermis layer of cell (Table.19 and Plate.8 a, b & c). The canker size was also recorded as very small in size. The tolerant clones of UPASI-9, UPAS-17, UPASI-26, TRF-4 and TRI-2025 were characterized by the absence of spore formation and below 1.0 cm of canker size (Table.19 and Plate. 9 a, b, c, d & e). These clones have been identified as tolerant varieties through histopathological studies and can be recommended for planting disease prone areas.

IV. 6. *In vitro* Bio efficacy of chemicals, botanical and plant extracts against branch canker

6.1. *In vitro* evaluation of chemical fungicides against branch canker pathogen

Among the fungicides tested at various dosages, the *in vitro* screening of systemic fungicides such as benomyl, carbendazim and companion showed 100 % growth inhibition against *Macrophoma* sp. at 10 ppm level followed by propiconazole and hexaconazole (Table.20). The contact fungicides of copper group *viz.*, copper oxychloride (50 % WP), copper hydroxide and copper oxychloride 435 (liquid) were tested against branch canker pathogen under *in vitro*. Among the copper group, copper oxychloride (50 % WP) was noticed highest growth inhibition against test pathogen followed by liquid copper oxy chloride 435 and copper hydroxide (Table.21).

Table. 19 Histopathological characterization of different tea cultivars against branch canker pathogen

Name of the clone	Canker size in cm (length)	No of spore production	Category	Histopathological observations
TRF-1	2.03±0.09	19.67±1.20	*Highly Susceptible	White circular patches, each surrounded by black ring was exposed by shaving. Minute black dome shaped pycnidium with brownish fungal mycelia were visible in the layer of epidermis and presence of enlarged canker size.
UPASI-3	2.57±0.09	21.00±1.53		
Yabukita	1.97±0.12	11.00±0.58		
UPASI-6	1.10±0.12	1.33±0.33	#Moderate Susceptible	Brownish mycelia with pycnidiospores were observed in the cortex layer.
TRF-2	1.37±0.15	1.67±0.67		
CR6017	1.23±0.09	3.00±0.58		
UPASI-9	0.50±0.02	0.00±0.00	$Less Susceptible	Few fungal mycelia were noticed in the layer of epidermis. Absence of pycnidiospores and less canker formations.
UPASI-17	0.58±0.02	0.00±0.00		
UPASI-26	0.58±0.02	0.00±0.00		
TRF-4	0.55±0.02	0.00±0.00		
TRI2025	0.50±0.01	0.00±0.00		

* - HS > above 1.5 cm, # - MS<1.5to>1 cm & $ - LS< 1.0 cm

Values are means ± SE of four replications and repeated twice.

Anatomical characterization of different tea cultivars under microscopic view (10X)

Plate.7 (a, b & c) Transverse section showing highly susceptible of different tea cultivars (T.S)

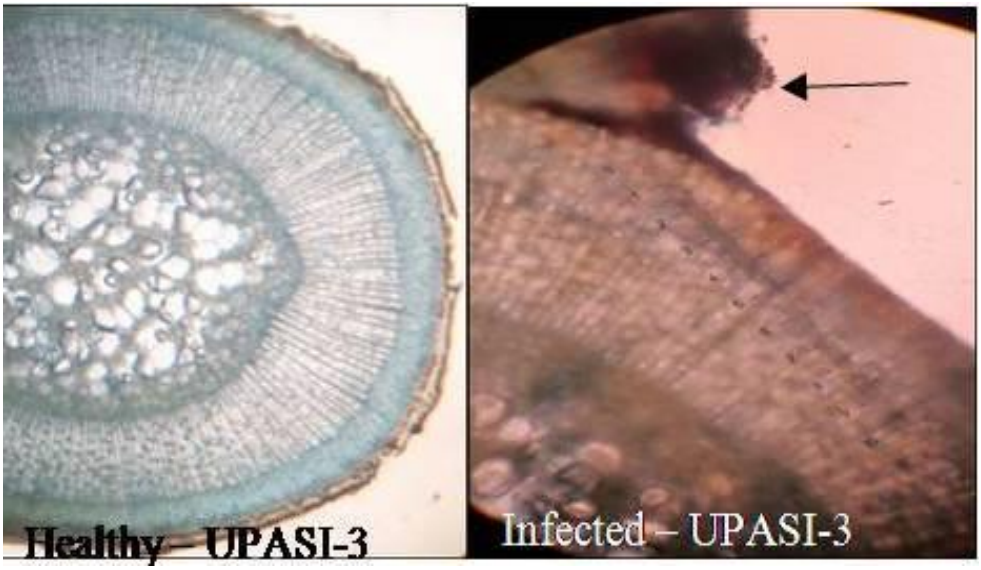


Plate.7(a) Brownish mycelia with pycnidiospore (Line arrow)

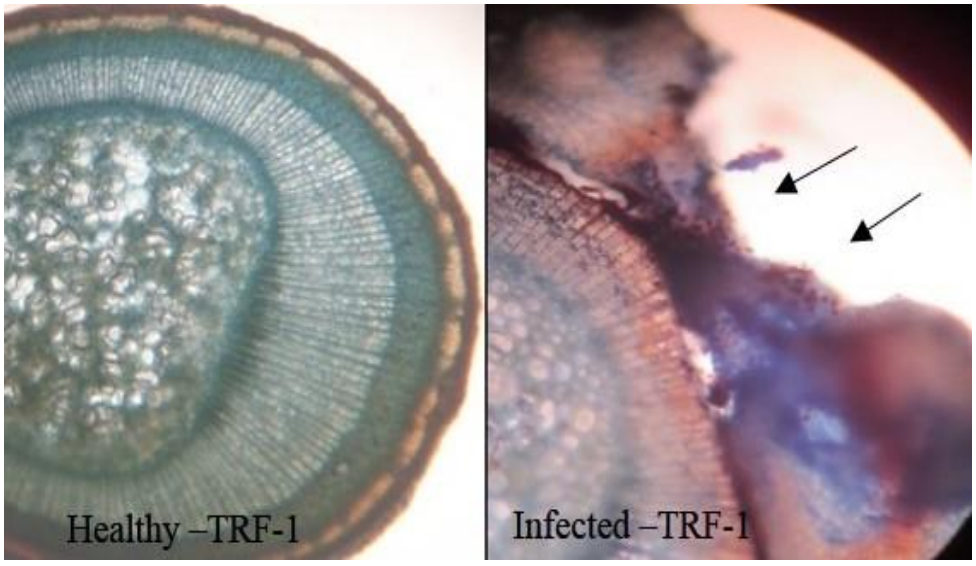


Plate.7 (b) More number of pycnidiospores present (Line arrows)

Plate.7 (c) Pycnidiospores noticed inner layer of cell (Line arrows)

Plate. 8 (a, b & c) Moderate susceptible of different tea clones (T.S)

Plate. 8(a) Mycelium present in inner epidermis (Line arrows)

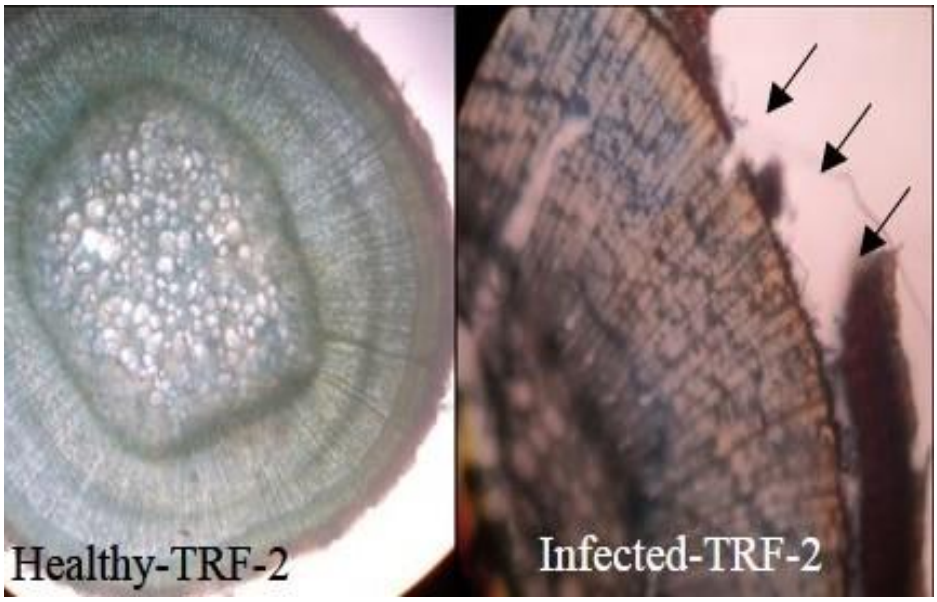


Plate. 8(b) Brownish mycelia present in inner layer (Line arrows)

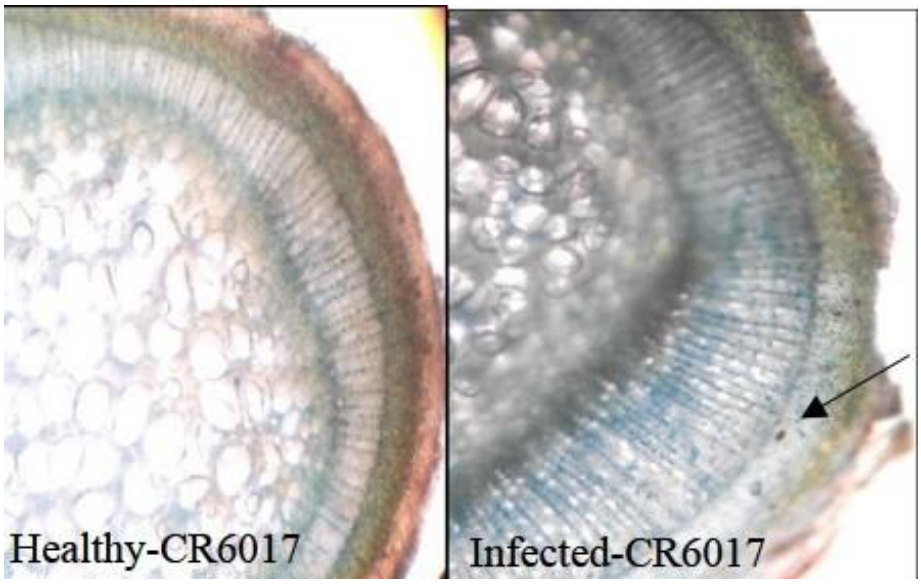


Plate.8(c) Pycnidiospore present in inner layer (Line arrows)

Plate. 9 (a, b, c, d & e) Less susceptible of tea clones (T.S)

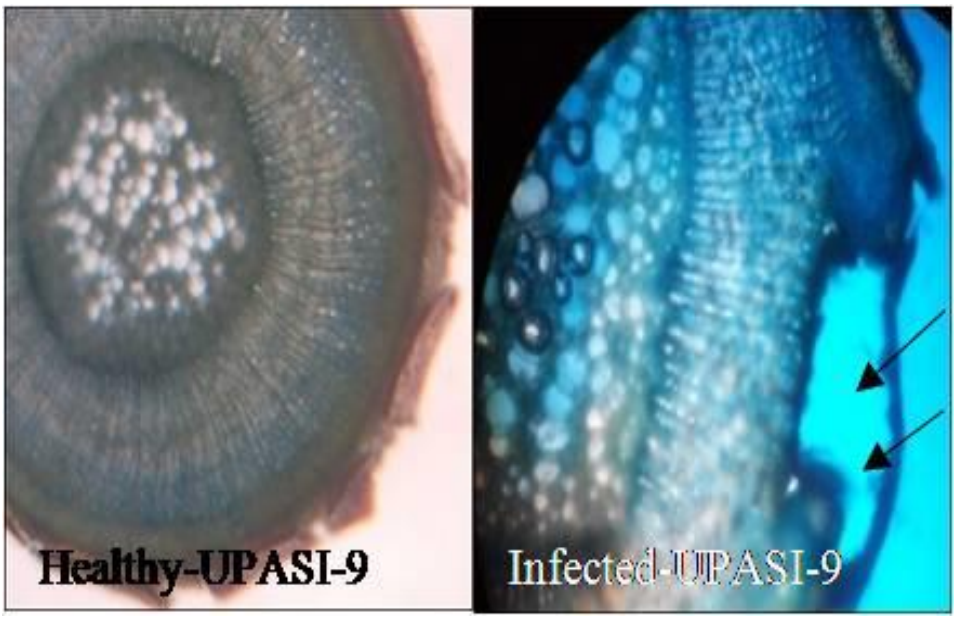


Plate. 9(a) Mycelium enter in to inner layer epidermis (Line arrows)

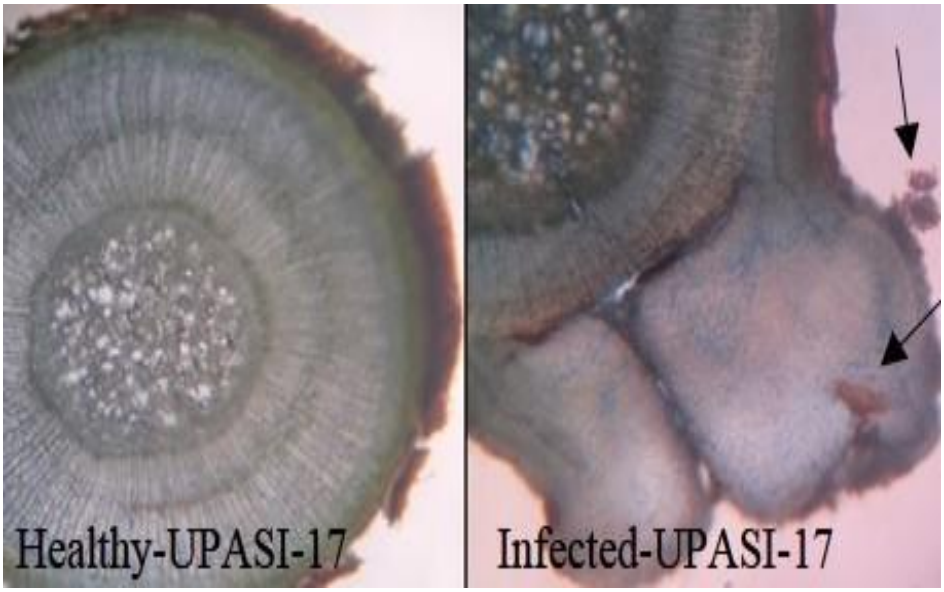


Plate. 9(b) Few fungal mycelia in outer epidermis layer (Line arrows)

Plate.9(c) Mycelia present in inner layer (Line arrows)

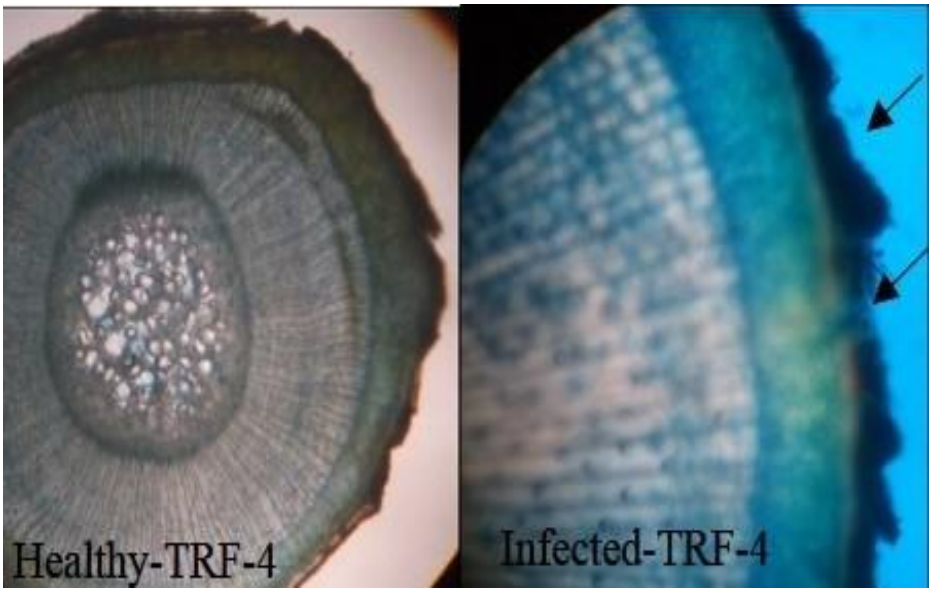


Plate. 9(d) Mycelia in outer epidermis layer (Line arrows)

Plate. 9(e) Brownish mycelium present in the inner layer (Line arrows)

Table.20 Bio efficacy evaluation of chemical fungicides against *Macrophoma* sp. under *in vitro*

Chemical fungicide	Percentage of growth inhibition at different concentrations level					
	10ppm	20ppm	30ppm	40ppm	50ppm	**CD at P= 0.05**
Benomyl (50 % WP)	100.00±00	-	-	-	-	**0.0**
Propiconazole (25 % EC)	75.00±1.15	78.22±0.44	81.44±0.38	85.44±0.48	89.22±0.57	**1.7**
Mancozeb (75 % WP)	31.44±1.00	45.33±1.98	49.86±1.06	55.33±0.74	62.66±0.99	**3.6**
Carbendazim (50 % WP)	100.00±00	-	-	-	-	**0.0**
Companion (carbendazim 12 % + Mancozeb 63 % WP)	100.00±00	-	-	-	-	**0.0**
Hexaconazole (5 % EC)	35.99±1.28	40.44±0.75	59.55±1.59	61.55±0.64	66.66±0.53	**3.0**

Values are Means ± SE of four replication of three repeated experiments.

Table.21 *In vitro* bioefficacy of Copper fungicides against branch canker pathogen

Copper group fungicides	**Concentrations**	**Growth inhibition (%)**	**C.D. at P=0.05**
Copper oxychloride 435 (liquid)	0.62%	74.42±0.39	
	1.00%	82.15±0.85	**1.7**
	1.24%	83.08±0.51	
	1.85%	87.33±0.47	
Copper hydroxide (77 % WP)	0.62%	60.91±0.73	
	1.00%	69.66±1.17	**4.7**
	1.24%	77.71±2.64	
	1.85%	82.71±0.49	
Copper oxychloride (50 % WP)	0.10%	3.55±0.28	
	0.30%	84.26±0.96	**2.0**
	0.45%	85.79±0.44	
	0.75%	88.75±0.42	

Values are Means ± SE of three replication of three repeated experiments.

6.2. Commercial botanical fungicides against branch canker pathogen under *in vitro* level

The commercial botanical fungicides were tested against *Macrophoma* sp. at different concentrations level. In this present study, botanical fungicides (Expel, Enroot and Attopsy) were found to be efficient results against the test pathogen at 0.1 % concentration level (Table.22).

6.3. *In vitro* screening of plant aqueous extracts against branch canker pathogen

The present study was carried out to evaluate different eco-friendly plant aqueous extract against branch canker disease under *in vitro* conditions. The antifungal activity of aqueous extract of *Acrous calamus* was found to fully inhibit growth of *Macrophoma* sp. at 10 percent concentration (Table.23 and Plate.10). The plant extracts of *Curcuma longa* and *Hibiscus rosasinensis* showed good results. The inhibitory potential of *Psidium guajava* and *Allamanda cathartica* indicated significant effect at 15 % concentrations followed by *Murraya koenigii, Azadirachta indica* and *Artemisia nilagirica* (Table.23 and Plate.11 & 12). Moderate inhibition was noticed by *Carica papaya* extracts followed by action of *Cinnamomum burmannii* and *Dryopteris linearis* extracts. The extracts of *Tithonia diversifolia, Conyza ambigua* and *Adhatoda vasica* level at 10% showed lowest control potential.

IV. 7. *In vitro* effect of fungal biocontrol agent against branch canker

7.1. *In vitro* effect of *Trichoderma* spp against branch canker pathogen

The fungal biocontrol agents were isolated from different tea growing areas of southern India such Vandiperiyar, Munnar, Wayanad and Coonoor. Among the total of 30 isolates, only 4 *Trichoderma* spp showed good antagonistic potential against branch canker pathogen (Table.24).

7.1(a). Dual plate method

In the present study the inhibitory effect of *Gliocladium virens* was higher against UPA-61 followed by *Trichoderma harzianum* against NBCHE-6 (Table.25 and Plate.13).

7.1(b). Culture filtrate method

Culture filtrate was tested, similar results were observed in *Gliocladium virens* and *Trichoderma harzianum* followed by *T.atroviride* (Table.26 and Plate.14).

Table.22 *In vitro* bio efficacy of botanical fungicides against branch canker pathogen

Botanical fungicide	**Concentrations**	**% inhibition of growth**	**C.D. at P=0.05**
Ecocare	0.10%	7.62±0.45	
	0.30%	23.91±2.05	
	0.50%	29.95±0.51	**3.6**
	0.75%	44.04±0.77	
	1%	50.06±1.03	
Fungfinish (5 % copper formulation)	0.10%	0.00±0.00	
	0.30%	1.55±0.44	
	0.50%	38.04±1.21	**2.1**
	0.75%	75.33±0.48	
	1%	85.55±0.38	
Tari (Organic Plus Tea special)	2%	0.00±0.00	
	4%	19.55±0.99	
	6%	54.53±5.17	**8.0**
	8%	69.77±2.83	
Nimbicidine (0.03 % Azadiractin EC)	2%	11.75±0.40	
	4%	33.26±1.10	**3.2**
	6%	45.68±0.91	
	8%	52.53±1.17	
Tricure (0.03 % Azadiractin EC)	2%	10.80±0.54	
	4%	43.37±1.23	**3.4**
	6%	74.95±2.14	
	8%	100.00±0.00	
Enroot	0.1%	100.0±0.00	**0.0**
Attopsy	0.1%	100.0±0.00	**0.0**
Expel (Combination of canolar extract Tea tree oil)	0.1%	100.0±0.00	**0.0**

Values are Means ± SE of three replication of three repeated experiments.

Table.23 *In vitro* screening of plant aqueous extracts against branch canker (*Macrophoma* sp.) pathogen

Plant extracts	Family	Growth Inhibition (%)		
		5 %	10 %	15 %
Curcuma longa	Zingiberaceae	59.25±2.09 ^{i}	79.44±0.32 ^{bc}	82.05±0.96 ^{b}
Dryopteris linearis	Dryopteridaceae	0.00±0.00 ^{q}	10.92±2.25 ^{p}	40.74±0.81 ^{l}
Cinnamomum burmannii	Lauraceae	23.33±1.47 ^{o}	39.81±2.73 ^{lm}	46.29±1.33 ^{k}
Carica papaya	Caricaceae	24.25±0.37 ^{o}	25.92±1.21 ^{o}	52.77±0.64 ^{j}
Tithonia diversifolia	Asteraceae	0.00±0.00 ^{q}	9.63±3.22 ^{p}	35.37±3.21 ^{n}
Allamanda cathartica	Apocynaceae	48.33±0.85 ^{jk}	68.52±1.82 ^{f}	77.59±1.13 ^{bcde}
Conyza ambigua	Asteraceae	0.00±0.00 ^{q}	3.14±0.49 ^{q}	33.88±4.44 ^{n}
Psidium guajava	Myrtaceae	74.25±1.82 ^{e}	74.63±0.98 ^{de}	79.04±2.02 ^{bcd}
Adhatoda vasica	Acanthaceae	0.00±0.00 ^{q}	3.51±0.49 ^{q}	25.03±0.58 ^{o}
Acorus calamus	Acoraceae	75.18±0.18 ^{cde}	100.00±00 ^{a}	100.00±0.00 ^{a}
Hibiscus rosasinensis	Malvaceae	65.26±0.62 ^{fg}	79.81±1.13 ^{b}	81.74±0.75 ^{b}
Artemisia nilagirica	Asteraceae	23.30±0.62 ^{o}	35.86±0.99 ^{mn}	61.66±1.93 ^{ghi}
Murraya koenigii	Rutaceae	48.51±0.50 ^{jk}	60.62±0.44 ^{hi}	73.70±1.22 ^{e}
Azadirachta indica (Neem kernel)	Meliaceae	48.70±0.74 ^{jk}	61.29±0.67 ^{ghi}	64.44±0.48 ^{fgh}

Values are Means ± SE of four replication of three repeated experiments. Means in the same column followed by the same letter are not significantly different at 0.05 level as determined by DMRT.

Plate.10 *In vitro* screening of plant extracts (*Curcuma longa* and *Acorus calamus*) against *Macrophoma* sp.

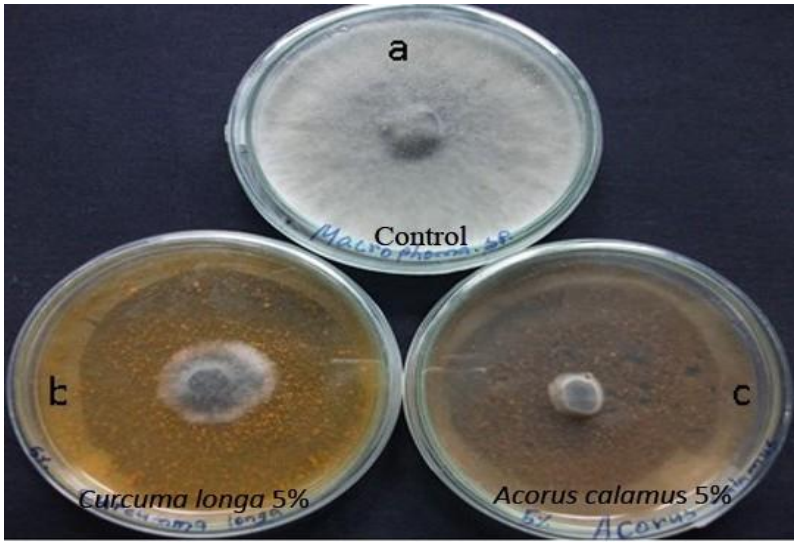


Plate.11 *In vitro* screening of plant extracts against branch canker pathogen

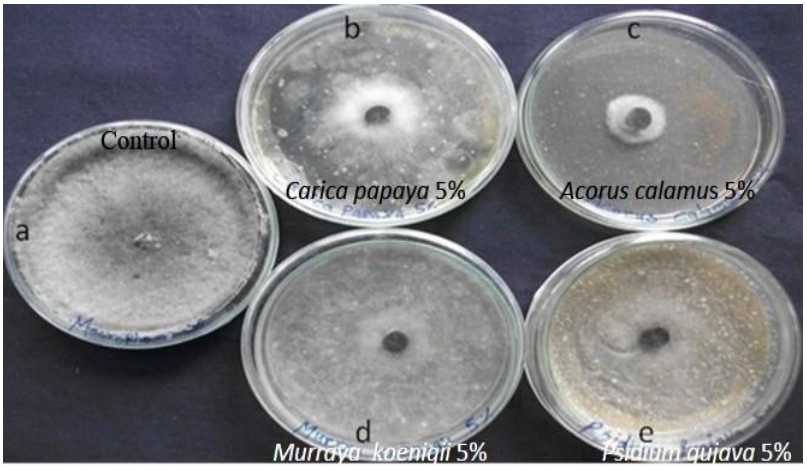


Plate.12 *In vitro* screenings of plant extract (*Azadirachta indica* Neem kernel) against *Macrophoma* sp.

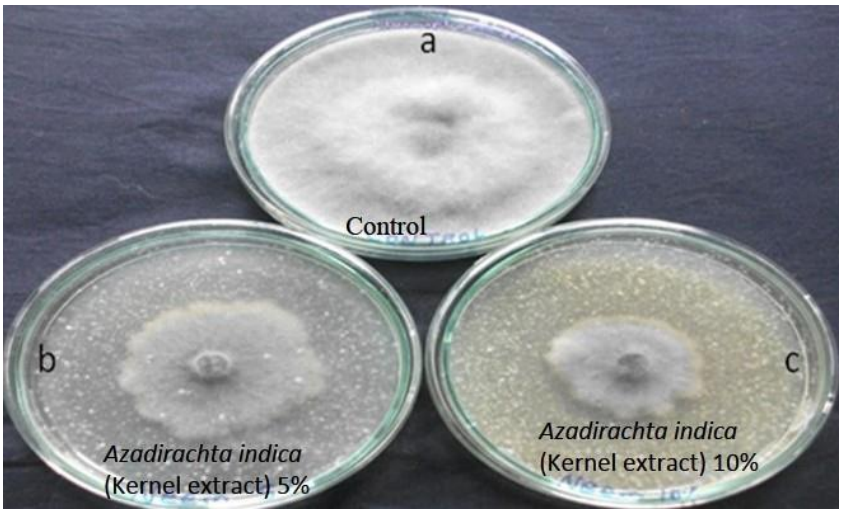


Table.24 *Trichoderma* spp isolated from different tea growing areas of southern India

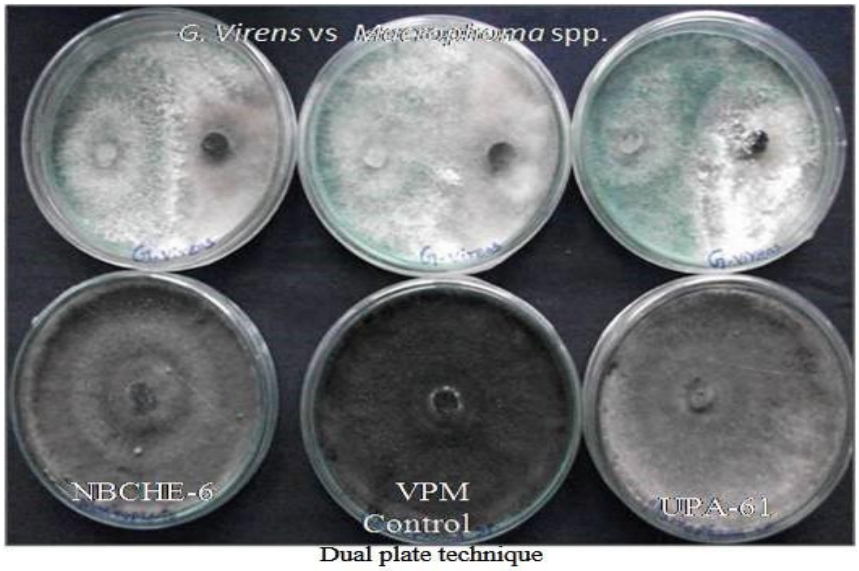
Tea growing districts	**Number of *Trichoderma* spp. isolates**	**Number of antagonistic against branch canker**
Vandiperiyar	13	1
Munnar	7	2
Coonoor	6	1
Wayanad	4	-

Table.25 *In vitro* screening of fungal biocontrol agents against branch canker isolates under dual plate technique

Fungal biocontrol agents	Growth inhibition (%)		
	***Macrophoma* sp. (NBCHE-6)**	***Macrophoma* sp. (VPM)**	***Macrophoma theicola* (UPA-61)**
T.viride	64.44±0.88[c]	63.77±1.15[d]	65.33±0.72[d]
T.harzianum	72.22±0.88[b]	70.91±0.74[b]	71.02±0.00[b]
T.atroviride	60.89±1.27[d]	66.88±1.07[c]	68.99±0.41[c]
G.virens	79.26±0.69[a]	80.33±0.54[a]	82.00±0.80[a]
C.D. at P=0.05	**2.3**	**2.1**	**2.0**

Values are Means ± SE of five replication of three repeated experiments. Means in the same column followed by the same letter are not significantly different at 0.05 % level as determined by DMRT.

Plate.13 *In vitro* efficacy of *Gliocladium virens* against *Macrophoma* spp. in dual plate technique



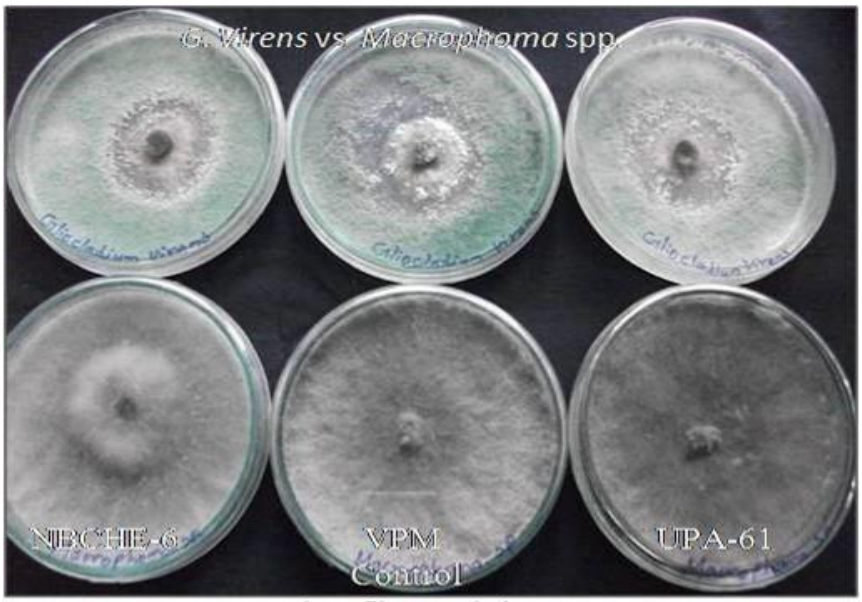
Dual plate technique

Table.26 *In vitro* screening of fungal culture filtrate at 2% against branch canker isolates

Culture filtrate of biocontrol (2%)	Growth inhibition (%)		
	***Macrophoma* sp. (NBCHE-6)**	***Macrophoma* sp. (VPM)**	***Macrophoma theicola* (UPA-61)**
T. viride	68.22±0.44[b]	63.88±1.09[c]	71.11±0.53[b]
T. harzianum	75.22±1.70[a]	69.77±0.75[b]	85.88±1.21[a]
T. atroviride	65.17±0.70[b]	69.80±0.54[b]	76.00±0.79[b]
G. virens	67.04±1.23[b]	81.33±0.85[a]	88.99±0.64[a]
C.D. at P=0.05	**3.1**	**2.5**	**5.7**

Values are means ± SE of five replication of three repeated experiments. Means in the same column followed by the same letter are not significantly different at 0.05 % level as determined by DMRT.

Plate.14 Culture filtrate studies on *Gliocladium virens* against branch canker pathogen isolates



Culture filtrate technique

7.1(c). Hyperparasitism study of fungal biocontrol agents

The hyphal interactions between *Gliocladium virens* and branch canker pathogen were observed to be coiled and shrunken. The pathogenic hyphal wall was penetrated by *G. virens* which produce large number of spores that interacts with hyphal wall (Plate.15). Thus the present study concludes that *G. virens* showed good antagonistic activities against the branch canker pathogen.

IV8. *In vitro* effect of entomopathogens against branch canker

8.1. *In vitro* antagonisms of entomopathogens against *Macrophoma* spp

An entomopathogens were procured from Microbial Type Culture Collection and Genebank (MTCC) Chandigarh, namely like *Beauveria bassiana, Paecilomyces fumosoroseus, Lecanicillium lecanii* and *Paecilomyces lilacinus.*

8.1(a). Dual plate method

In vitro studies revealed that *Beauveria bassiana* showed highest antagonistic effect against NBCHE-6 (64.22) followed by *Paecilomyces fumosoroseus* against UPA-61 (56.66) (Table.27 and Plate.16) *Paecilomyces lilacinus* significantly controlled VPM (54.66), while *lecanicillium lecanii* showed insignificantly control against VPM (47.33) (Table.27 and Plate.17).

8.1(b). Culture filtrate method

In culture filtrate studies revealed that *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* showed maximum control of VPM (68.44) and UPA-61 (65.59). *Beauveria bassiana* also showed significant control of two isolates VPM and UPA-61 (54.44). *Lecanicillium lecanii* showed least control of VPM (30.44) (Table.28 and Plate.18). This study concludes that entomopathogens can significantly control branch canker pathogen (*Macrophoma theicola*).

Plate.15 *Gliocladium virens* interact with pathogen hyphae cell wall 10 × view (Line arrow)

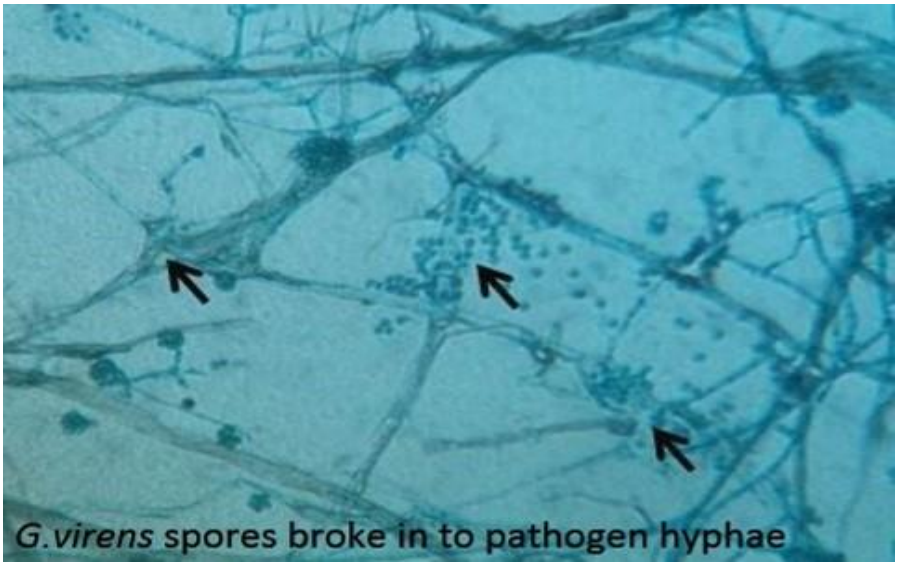


Table.27 *In vitro* screening of entomopathogens against branch canker isolates under dual plate technique

Entomopathogens culture (MTCC)	**Growth inhibition (%)**		
	***Macrophoma* sp. (NBCHE-6)**	***Macrophoma* sp. (VPM)**	***Macrophoma theicola* (UPA-61)**
Beauveria bassiana	64.22	61.55	62.66
Lecanicillium lecanii	45.33	47.33	44.22
Paecilomyces lilacinus	47.77	54.66	51.55
Paecilomyces fumosoroseus	54.44	56.66	56.66
C.D. at P=0.05	**3.1**	**2.8**	**2.8**

Values are means ± SE of four replication of three repeated experiments.

Plate.16 Growth inhibition of *Macrophoma* sp. by entomopathogens (*Beauveria bassiana* and *Paecilomyces lilacinus*)

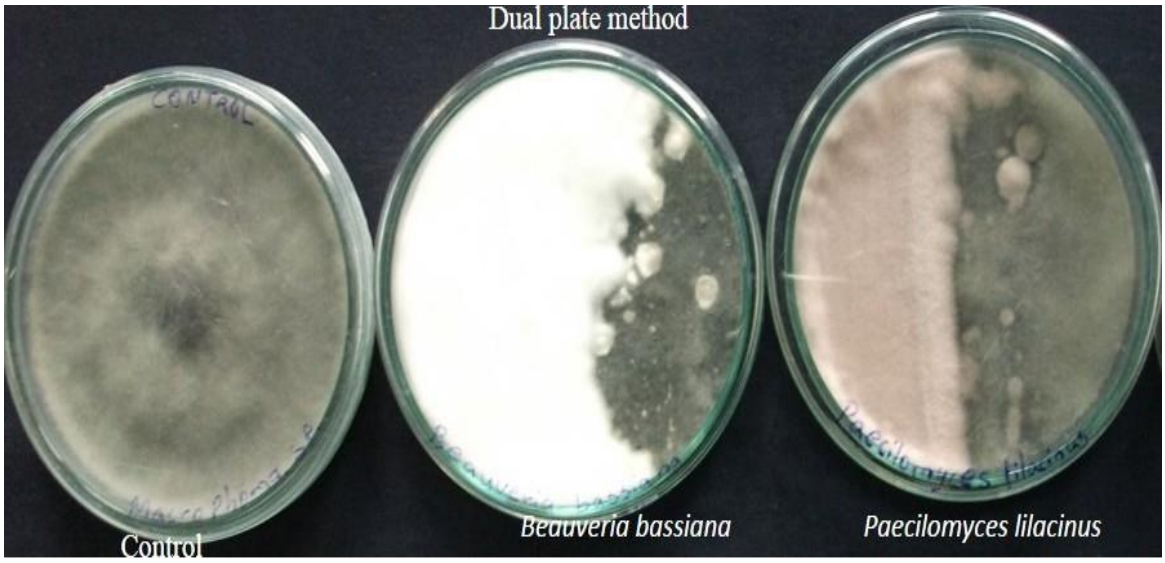


Plate.17 Growth inhibition of *Macrophoma* sp. by entomopathogens (*Paecilomyces fumosoroseus* and *Lecanicillium lecanii*)

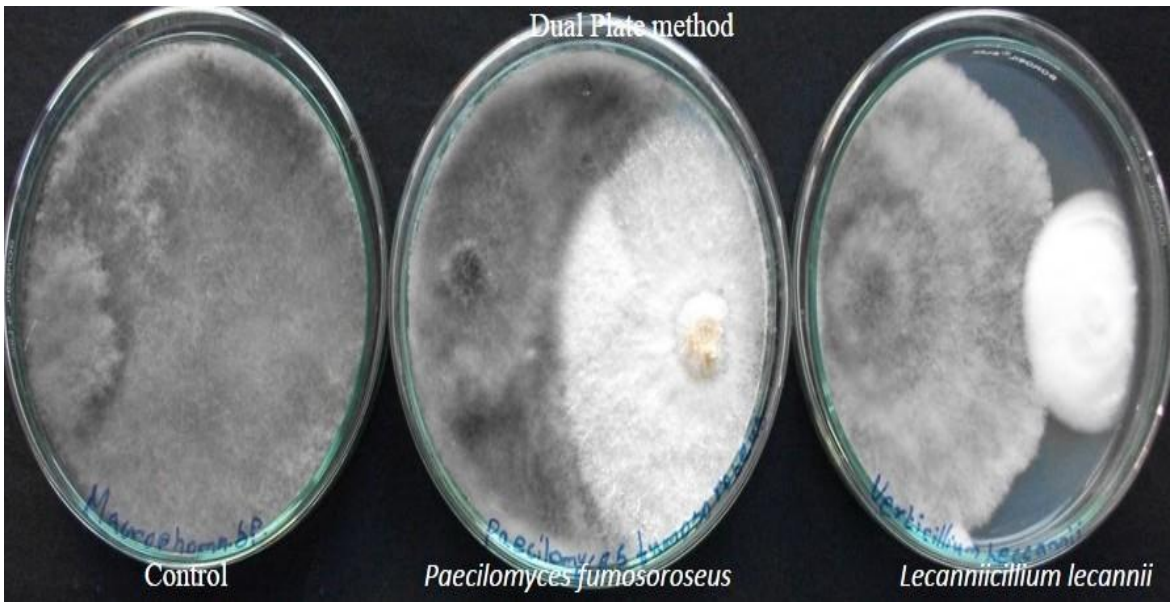
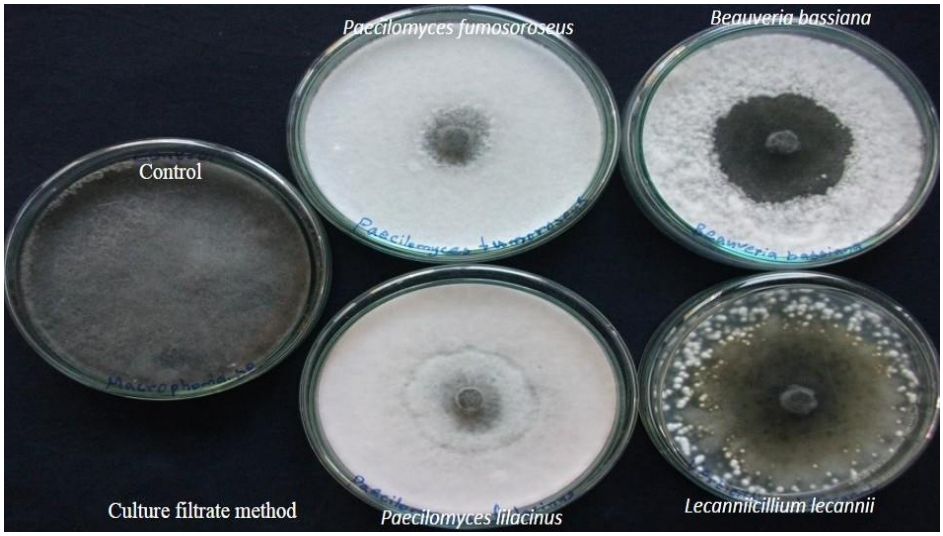


Table.28 *In vitro* screening of entomopathogens against branch canker isolates under culture filtrate method

Entomopathogens culture filtrates at 2% concentrations level (MTCC)	**Growth inhibition (%)**		
	***Macrophoma* sp. (NBCHE-6)**	***Macrophoma* sp. (VPM)**	***Macrophoma theicola* (UPA-61)**
Beauveria bassiana	53.33	54.44	54.44
Lecanicillium lecanii	18.55	30.44	26.55
Paecilomyces lilacinus	57.77	63.10	65.59
Paecilomyces fumosoroseus	60.04	68.44	66.77
C.D. at P=0.05	**7.1**	**4.9**	**4.4**

Values are means ± SE of four replication of three repeated experiments.

Plate.18 *In vitro* screening of entomopathogen against *Macrophoma* sp. (culture filtrate)



8.1(c). Hyperparasitism study of entomopathogens

The present investigation revealed that *Beauveria bassiana* (Plate.19) and *Paecilomyces lilacinus* coiled around and shrink branch canker pathogen hyphae. *Lecanicillium lecanii* breaks in to branch canker hyphae (Plate.20) and *Paecilomyces fumosoroseus* produces more spore to kill branch canker pathogen.

IV. 9. Isolation and identification of proven bacterial biocontrol agents

9.1. Isolation of bacterial biocontrol against branch canker disease

A total of 43 bacterial biocontrol was isolated from different tea growing region of southern India (Table.29). Ten bacterial strains were isolated from Coonoor, 5 isolates from Vandiperiyar, 14 strains from Koppa, 11 strains from Wayanad and 3 strains from the Anamallais. Among the 43 strains, only six strains were expressed antagonistic potential to branch canker disease.

9.2. Biochemical characterization of proven bacterial biocontrol agents

The bacterial strains isolated from the soils collected from three different tea growing regions were characterized morphological and biochemically as per procedure given in Bergey's Manual of systematic Bacteriology (Claus and Berkeley, 1986 and Stolp and Gadkari, 1981). The morphological and biochemical characterization of these bacterial strains revealed, presence of four *Bacillus* spp. and two *Pseudomonas* spp. The four *Bacillus* spp. were grown maintain on Luria Bertani Agar at 30 to 35 ˚C. The four different *Bacillus* spp. were identified as gram positive when analyzed as per the procedure given by Bergey's Manual of systematic Bacteriology. The two *Pseudomonas* spp. were grown on maintain on kings B medium at 30 to 35 ˚C and identified as gram negative. All the *Bacillus* spp. strains and two *Pseudomonas* spp. answered positive for catalase and oxidase. Whereas, the Methyl Red Test showed positive for the four *Bacillus* spp. and negative for *Pseudomonas* spp. (Table.30).

Plate.19 *Beauveria bassiana* interaction with branch canker pathogen hyphae (under 10× view)

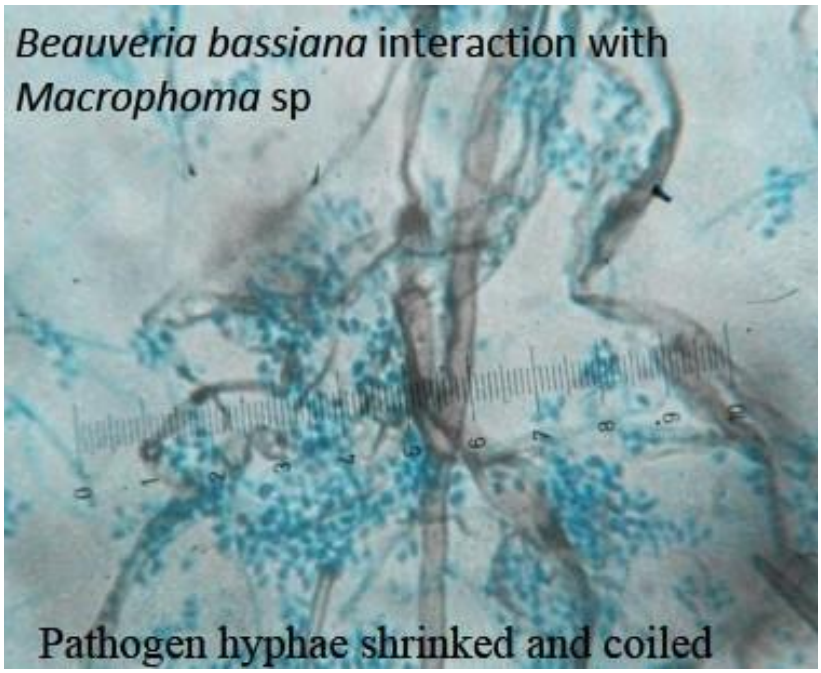


Plate.20 *Lecanicillium lecanii* interaction with branch canker pathogen hyphae (under 10× view)

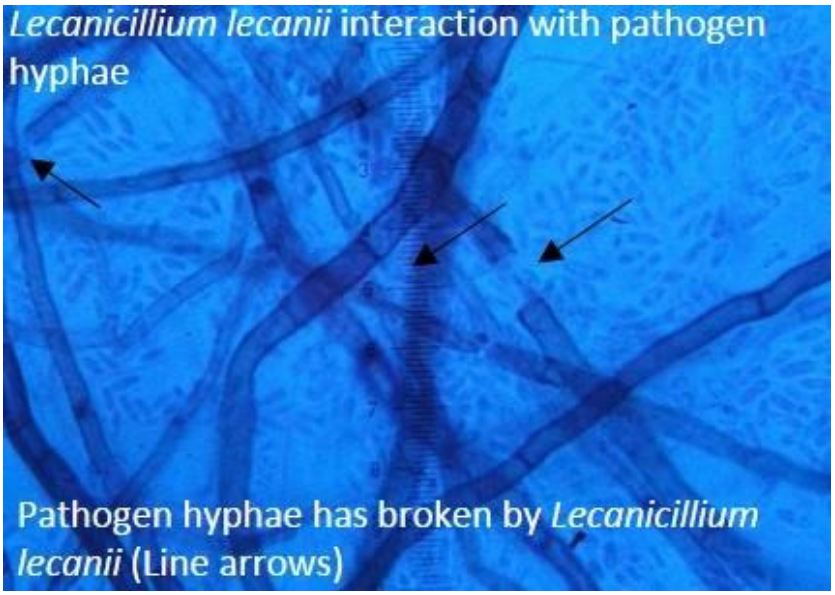


Table.29 Bacterial biocontrol isolated from different tea growing areas of southern India

Locations	**Total number of bacterial various isolates**	**Number of antagonistic bacteria**
Coonoor	10	1
Vandiperiyar	5	1
Koppa	14	2
Wayanad	11	1
The Anamallais	3	1

Table.30 The biochemical characterization of bacterial strains

Test	*Bacillus* spp. (4 strains)	*Pseudomonas* spp. (2strains)
Gram's stains	+	-
Catalase	+	+
Oxidase	+	+
Methyl Red Test	+	-

(+) Positive, (-) Negative

9.3. Molecular accession number of bacterial strains

These isolates identified based on the of genomic DNA sequencing, amplification by Polymerase Chain Reaction was performed with primer pairs targeted to the 16S rRNA gene of the bacterial strain. Molecular identifications of the four *Bacillus* spp. and the two *Pseudomonas* spp. were confirmed as, *Bacillus subtilis* (KM527836), *Bacillus licheniformis* (KM527838), *Bacillus amyloliquefaciens* (KM853034), *Bacillus* sp. (JN616373), *Pseudomonas* sp. (JQ319656) and *Pseudomonas fluorescens* (KM527837) through 16S rRNA sequence technique (Table.31).

9.4. Phylogenetic tree analysis of bacterial strains

An identification of bacterial nucleotide sequence was carried out in similarity search with the help of blast search method at NCBI data bases (Altschul *et al.,* 1990) and phylogenetic tree constructed following neighbor-joining method (Saitou and Nei, 1987). The bacterial nucleotide sequences data revealed 99% similarity with other bacterial nucleotide sequences of NCBI data bases (Figure.8).

IV. 10. *In vitro* effect of bacterial strains against branch canker

10.1. *In vitro* screening of bacterial biocontrol agent for their antagonism against *Macrophoma* spp

Among 43 bacterial strains isolates from the different agro climatic tea garden soil, four isolates proved higher efficacy and same were selected for further study. All these four isolates were abbreviated as WP104, TRB, CS-2 and AWRH-40B and they were three of *Bacillus* spp. and one each of *Pseudomonas* sp. (Plate.21 a, b, c, d & e).

10.1(a). Dual plate method

In this present study the identification of bacterial strains were evaluated for their antagonistic potential for the control of branch canker. Dual plate technique revealed that among the four bacterial strains *Bacillus amyloliquefaciens* showed maximum level of antagonism against all the three strains of *Macrophoma* spp. followed by *Bacillus subtilis* and *Bacillus licheniformis* (Table.32 and Plate.22).

Table.31 Isolation of bacterial strains from different tea growing areas of southern India

Strain Code No	Tea areas	Bacterial Strains Identified as	NCBI Gen Bank Accession Number
TRB	Koppa	*Pseudomonas fluorescens*	KM527837
AWRH-40B	Wayanad	*Bacillus licheniformis*	KM527838
WP-104	The Anamallais, UPASI-TRF, Farm	*Bacillus amyloliquefaciens*	KM853034
CS-2	Coonoor	*Bacillus subtilis*	KM527836
WR46-2	The Anamallais, UPASI-TRF, Farm	*Bacillus* sp.	JN616373
WR5-4	Vandiperiyar	*Pseudomonas* sp.	JQ319656

Figure.8 Phylogenetic tree constructed based on neighbor-joining method

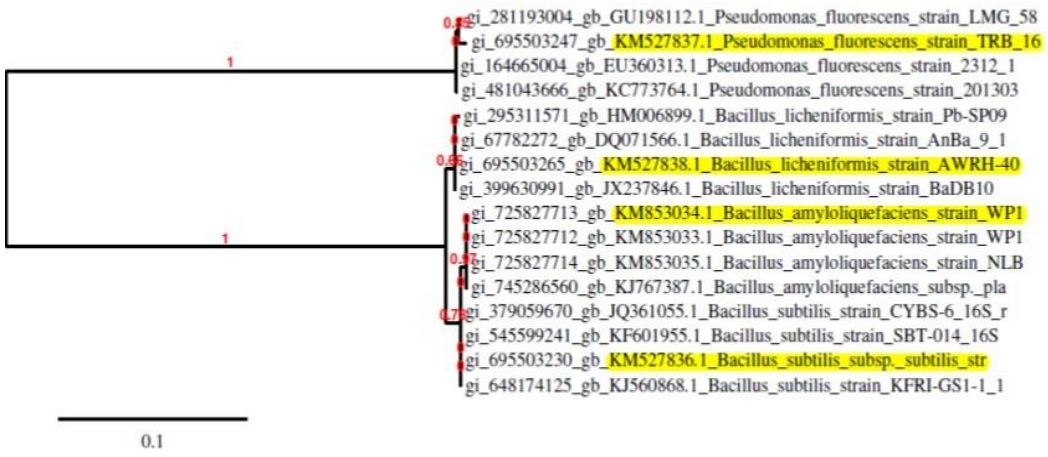
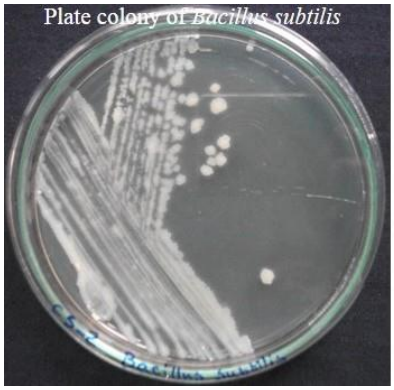
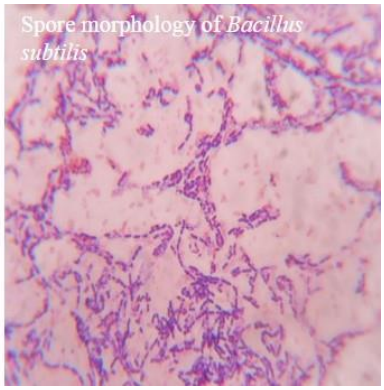
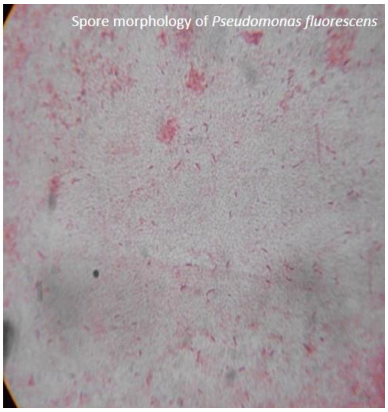


Plate.21 (a, b, c, d & e) Plate colony and spore morphology of bacterial biocontrol strains isolated from different tea growing agro climatic zones

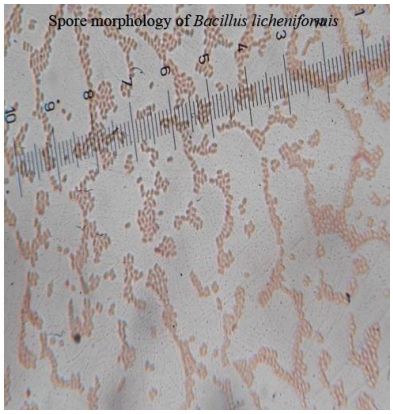




21.(a) CS-2 *Bacillus subtilis*



21.(b) TRB- *Pseudomonas fluorescens*



21.(c) *Bacillus licheniformis*

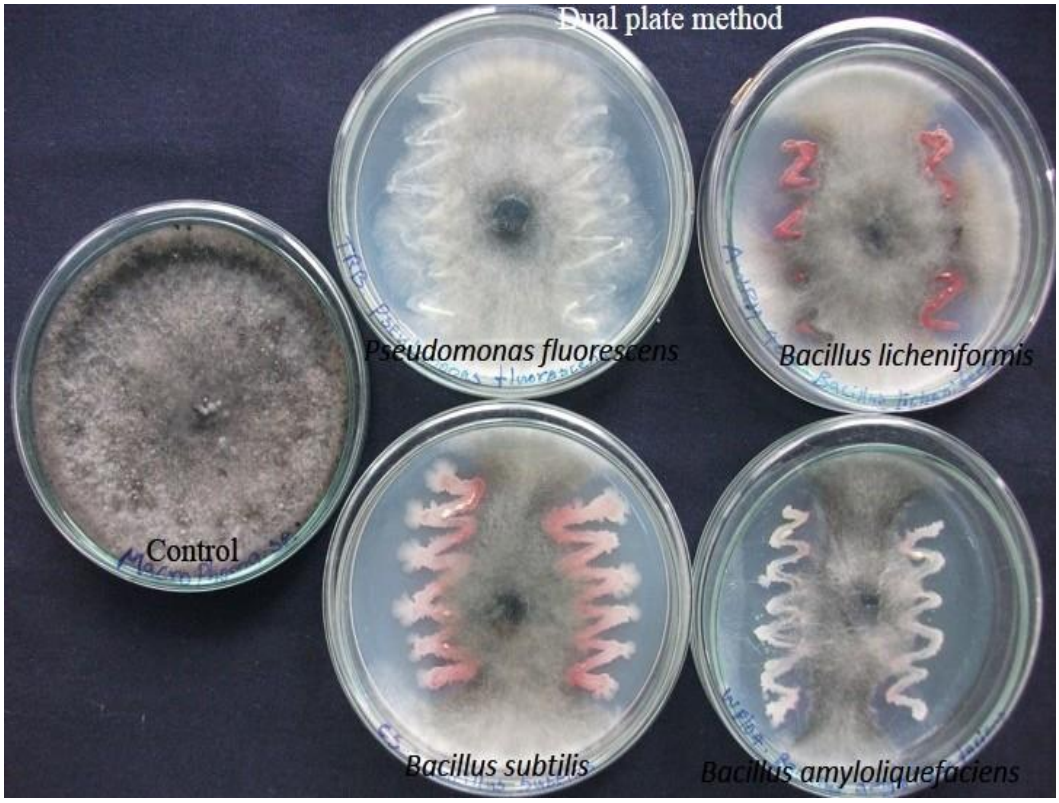
21.(d) *Bacillus amyloliquefaciens*

Table.32 *In vitro* screening of bacterial strains against branch canker isolates under dual plate technique

Bacterial isolates	Growth inhibition (%)		
	Macrophoma sp. (NBCHE-6)	*Macrophoma* sp. (VPM)	*Macrophoma theicola* (UPA-61)
Bacillus amyloliquefaciens (WP104)	78.88±0.44[a]	76.75±0.94[a]	75.53±0.88[a]
Bacillus subtilis (CS2)	70.11±0.53[b]	72.89±0.63[b]	71.44±0.67[b]
Pseudomonas fluorescens (TRB)	36.33±1.34[d]	33.44±0.93[d]	31.11±0.88[d]
Bacillus licheniformis (AWRH-40B)	64.22±0.72[c]	63.33±0.88[c]	62.22±0.88[c]
C.D. at P = 0.05	**2.4**	**2.5**	**2.5**

Values are Means ± SE of five replication of three repeated experiments. Means in the same column followed by the same letter are not significantly different at 0.05 % level as determined by DMRT.

Plate.22 *In vitro* effect of bacterial biocontrol strains against branch canker pathogen under dual plate method



10.1(b). Culture filtrate method

In culture filtrate studies also exposed that, *Bacillus amyloliquefaciens* was found to be highest antagonistic activity against the test pathogen followed by *Bacillus subtilis* and *Pseudomonas fluorescens* (Table.33).

IV. 11. Analysis of bacterial culture filtrate compounds by GC-MS

Among the four bacterial strains, the only one isolate of *Bacillus amyloliquefaciens* was taken for GC-MS study and their culture extract supernatant of bioactive compounds were identified (Table.34). In the potent strain was selected for based on their highest antagonistic potential properties. Culture filtrate of *Bacillus amyloliquefaciens* showed the presence of compounds namely as 1H-Indene, 1-methylene, Dichloroacetic acid, 4-hexadecy ester, 3-Hexadecene (Z)-, Benzeneacetic acid, Phenol, and caffeine. These compounds showed higher percentage peak area of GC-MS spectrum (Figure.9). These compounds are responsible for antagonistic related to antifungal activity against pathogen.

IV.12. Compatibility studies

The present study was carried out to evaluate the compatibility of commonly used agro chemicals at acceptable dosages with proven biocontrol agents such as *Bacillus subtilis, Pseudomonas fluorescens, Bacillus amyloliquefaciens, Bacillus licheniformis, Trichoderma viride, Trichoderma harzianum*, *Trichoderma atroviride* and *Gliocladium virens.* In these biological agents were isolated from tea soils of different tea growing areas of south India and their strains identified by respective methods. In commonly used agrochemical in tea plantation *viz.*, pesticides (Propargite and Deltamethrin), fungicides (Benomyl, Hexaconazole, Companian, Copper oxychloride and Propiconazole) and weedicides (Glyphosate and Ammonium salt of glyphosate) were tested for tolerance level with biocontrol agents under *in vitro* level. Among the agrochemicals, Propargite (pesticide), Glyphosate and Ammonium salt of glyphosate (weedicides) and Propiconazole (fungicide) were found as high compatibility with *Pseudomonas fluorescens* at 0.4 % concentration level (Table.35).

Table.33 *In vitro* screening of bacterial biocontrol against branch canker pathogen (culture filtrate method)

Bacterial culture filtrates at (4%)	**Growth inhibition (%)**								
	24hrs			**48hrs**			**72hrs**		
Code	**NBCHE-6**	**VPM**	**UPA-61**	**NBCHE-6**	**VPM**	**UPA-61**	**NBCHE-6**	**VPM**	**UPA-61**
WP104	80.22[a]	78.51[a]	79.89[a]	78.00[a]	79.37a	78.55[a]	75.99[a]	79.99[a]	78.64[a]
CS-2	70.00[b]	67.95[b]	68.57[b]	73.55[b]	74.82[b]	73.71[b]	61.33[b]	69.46[b]	63.86[c]
TRB	45.22[d]	49.73[c]	45.93[c]	69.00[c]	61.24[c]	66.97[c]	25.40[c]	31.66[d]	29.40[d]
AWRH-40B	17.18[c]	29.46[d]	25.19[d]	37.11[d]	40.86[d]	38.77[d]	62.89[b]	67.08[c]	65.88[b]
CD@P=0.05	**8.4**	**4.0**	**7.5**	**3.9**	**2.1**	**3.5**	**2.5**	**1.7**	**1.9**

Values are means of five replications of three repeated experiments. Means in the same column followed by the same letter are not significantly different at 0.05 % level as determined by DMRT.

Table.34 Compound identified in the bacterial culture filtrate (WP104 *Bacillus amyloliquefaciens*) by GC-MS

No	RT	Name of the compound	Molecular formula	Molecular weight	Peak area %
1	3.547	1H-Indene, 1-methylene-	$C_{10}H_8$	128	5.17
2	5.332	Dichloroacetic acid,4-hexadecy ester	$C_{14}H_{26}Cl_2O_2$	296	7.77
3	6.470	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	$C_{18}H_{52}O_7Si_7$	576	9.43
4	6.703	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	209	9.77
5	7.572	3-Hexadecene, (Z)-	$C_{16}H_{32}$	224	11.04
6	8.435	Benzeneacetic acid, α,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester	$C_{20}H_{40}O_5Si_4$	472	12.30
7	9.717	Benzenemethanol, α-(1-aminoethyl)	$C_9H_{13}NO$	151	14.16
8	9.950	4-Trifluoroacetoxytridecane	$C_{15}H_{27}F_3O_2$	296	14.50
9	10.876	Caffeine	$C_8H_{10}N_4O_2$	194	15.85

Figure.9 GC-MS spectrum of WP104 (*Bacillus amyloliquefaciens*)

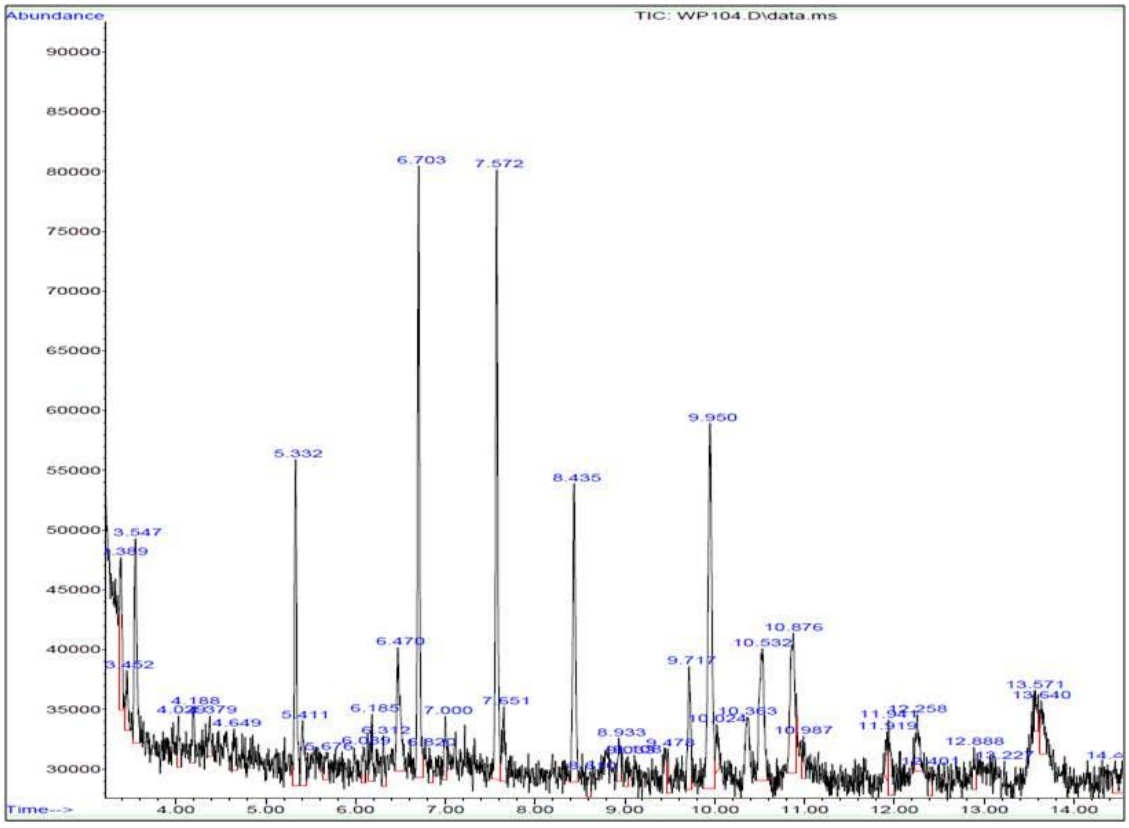


Table.35 *In vitro* compatibility of bacterial biocontrol agents with selected agrochemicals

Agrochemicals (0.4%)	**Colony Forming Unit/mL**			
	***Bacillus subtilis* ($\times 10^6$)**	***Pseudomonas fluorescens* ($\times 10^6$)**	***Bacillus amyloliquefaciens* ($\times 10^5$)**	***Bacillus licheniformis* ($\times 10^4$)**
Benomyl	7.0^{d}	7.0^{f}	3.0^{cd}	1.0^{f}
Copper oxychloride	11.3^{c}	12.7^{e}	3.3^{c}	2.0^{ef}
Companian	9.0^{cd}	5.3^{f}	8.0^{a}	0.0^{f}
Propiconazole	15.3^{b}	59.3^{b}	6.0^{ab}	3.0^{e}
Hexoconazole	22.3^{a}	19.3^{d}	6.0^{ab}	3.0^{e}
Propargite	7.3^{d}	79.7^{a}	1.0^{d}	7.0^{c}
Deltamethrin	3.0^{e}	38.7^{c}	4.3^{bc}	5.3^{d}
Ammonium salt of glyphosate	9.3^{cd}	76.0^{a}	1.7^{cd}	20.3^{a}
Glyphosate	3.0^{e}	79.7^{a}	2.7^{cd}	13.0^{b}
CD at 0.05	**2.4**	**5.5**	**2.2**	**1.7**

Values are means followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 0.05 % level.

Bacillus subtilis and *Bacilllus amyloliquefaciens* were lesser tolerant level with all the agrochemicals followed by *Bacillus licheniformis* (Table.35). Companian was most degree of toxicity to *Bacillus licheniformis* (Table.35).

The fungal bioagent of *Trichoderma* spp showed highest compatibility with copper oxychloride followed by hexaconazole and propiconazole at 10 ppm (Table.36 and 37). *In vitro* studies clearly examined that weedicide of ammonium salt of glyphosate (50 ppm) and pesticide of propargite (50 ppm) were found as tolerance with fungal biocontrol of *Trichoderma* spp (Table.38 and 39). The present results will be helpful for the possibility of combinations of chemicals and biological agents to integrated approaches for the control of pests, diseases and weeds.

IV. 13. Shelf-life studies

The shelf life of bacterial and fungal biocontrol agents were tested in coir pith and talc based formulations. Luria Bertani broth was used for bacterial formulations and potato dextrose broth was prepared for fungal biocontrol agents. The ratio of biocontrol agents were fixed at 1:2 in coir pith formulations and 1:4 in talc based formulations. In the present study, the initial value of CFU in bacterial biocontrol agent *Bacillus licheniformis* was131.14×10^6 cfu/g and in fungal biocontrol agent *Trichoderma harzianum* was 124.71×10^6 cfu/g showing higher population in coir pith formulation followed by talc based formulation of *Bacillus licheniformis* (75.9×10^6 cfu/g) and *Bacillus amyloliquefaciens* (66.2×10^6 cfu/g) after 30 days of storage (Table.41 and 40). Similar trend was recorded in talc based formulation of *Bacillus licheniformis* after 60 and 90 days of storage conditions followed by coir pith formulation of *Bacillus licheniformis* (94.90×10^6 cfu/g) after 60 days of storage (Table.40 and 41). After 90 days, the population was absent in talc based formulation, but in coir pith formulation, nearly 19.90×10^6 cfu/g to 61.44×10^6 cfu/g of population was recorded (Table.40 and 41). Thus, the minimum population was observed in coir pith formulation after 120 days of storage (Table.41). Finally results indicated that the CFU level was higher in coir pith formulation when compared than talc based formulation.

Table.36 Radial growth of fungal biocontrol agents with different fungicides

Fungal biocontrol agents	Fungicides (10 ppm)				
	Benomyl	**Companian**	**Propiconazole**	**Copper oxychloride**	**Hexoconazole**
T. harzianum	100.00±0.00	88.82±0.57	67.09±0.73	0.0±0.0	38.77±0.74
T. viride	100.00±0.00	100.00±0.00	100.00±0.00	0.0±0.0	89.06±0.58
T. atroviride	100.00±0.00	100.00±0.00	89.75±0.45	0.0±0.0	59.31±1.18
G. virens	100.00±0.00	100.00±0.00	68.24±1.03	0.0±0.0	26.64±0.34

Values are mean ± S.D. in terms of radial growth in diameter (cm) of eight replicates

Table.37 Fungicides compatible with fungal biocontrol agents in biomass dry weight (mg)

Fungal biocontrol agents	Fungicides (10 ppm)				
	Benomyl	**Companian**	**Propiconazole**	**Copper oxychloride**	**Hexoconazole**
T. harzianum	0.06±0.01	0.07±0.01	0.15±0.01	0.23±0.01	0.22±0.01
T. viride	0.07±0.01	0.06±0.01	0.08±0.01	0.20±0.01	0.10±0.01
T. atroviride	0.06±0.00	0.07±0.01	0.09±0.01	0.23±0.01	0.16±0.00
G. virens	0.05±0.00	0.07±0.01	0.17±0.01	0.24±0.01	0.22±0.01

Values are mean ± S.D. in terms of growth in dry weight (mg) of eight replicates

Table.38 *In vitro* tolerant study of fungal biocontrol agents with weedicides

Fungal biocontrol agents	Weedicides (50 ppm)			
	Inhibition of radial growth (cm)		Bio mass dry weight (mg)	
	Glyphosate	Ammonium salt of glyphosate	Glyphosate	Ammonium salt of glyphosate
T. harzianum	55.6±6.12	14.8±3.4	0.22±0.02	0.23±0.02
T. viride	66.5±0.85	35.8±1.9	0.15±0.02	0.20±0.02
T. atroviride	43.1±1.47	63.1±1.7	0.25±0.02	0.14±0.02
G. virens	26.3±2.72	18.3±6.3	0.28±0.02	0.26±0.02

Values are Mean ± S.D. in terms of growth in diameter and dry weight of eight replicates

Table.39 *In vitro* tolerant study of fungal biocontrol agents with pesticides

Fungal biocontrol agents	Pesticides (50 ppm)			
	Inhibition of radial growth (cm)		Bio mass dry weight (mg)	
	Propargite	Deltamethrin	Propargite	Deltamethrin
T. harzianum	35.7±1.63	47.0±1.30	0.47±0.03	0.49±0.02
T. viride	42.8±2.23	54.8±2.06	0.40±0.02	0.42±0.02
T. atroviride	69.3±0.79	68.0±1.39	0.21±0.02	0.22±0.02
G. virens	52.8±2.10	59.2±0.85	0.39±0.02	0.36±0.02

Values are Mean ± S.D. in terms of growth in diameter and dry weight of eight replicates

Table.40 Shelf-life of biocontrol agents establishment in talc powder formulation

Biocontrol agents	**Colony forming unit/gram of sample**		
	30 Days ($\times 10^6$)	**60 Days ($\times 10^6$)**	**90 Days ($\times 10^6$)**
Bacillus subtilis	31.5 ^{f}	19.3 ^{d}	8.87 ^{d}
Pseudomonas fluorescens	46.4 ^{d}	9.4 ^{e}	00.00 ^{f}
Bacillus amyloliquefaciens	66.2 ^{b}	31.6 ^{b}	16.69 ^{b}
Bacillus licheniformis	75.9 ^{a}	45.0 ^{a}	20.47 ^{a}
Gliocladium virens	49.3 c	19.7 ^{d}	6.76 ^{e}
Trichoderma viride	29.1 ^{g}	18.4 ^{d}	7.02 ^{e}
Trichoderma harzianum	60.0 ^{c}	28.7 ^{c}	12.33 ^{c}
Trichoderma atroviride	37.0 ^{e}	29.4 ^{c}	9.46 ^{d}
CD@5%	**2.28**	**2.32**	**2.08**

Values are means followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 0.05 % level.

Table.41 Shelf-life of biocontrol agents establishment in coir pith formulation

Biocontrol agents	**Colony forming unit/gram of sample**			
	30 Days ($\times 10^6$)	**60 Days ($\times 10^6$)**	**90 Days ($\times 10^6$)**	**120 Days ($\times 10^6$)**
Bacillus subtilis	110.3 ^{cd}	84.05 ^{b}	61.44 ^{a}	24.44 ^{a}
Pseudomonas fluorescens	83.62 ^{f}	74.99 ^{c}	29.67 ^{e}	12.01 ^{de}
Bacillus amyloliquefaciens	92.34 ^{e}	77.35 ^{c}	49.07 ^{b}	18.48 ^{b}
Bacillus licheniformis	131.14 ^{a}	94.90 ^{a}	36.40 ^{cd}	19.47 ^{b}
Gliocladium virens	107.46 ^{d}	63.27 ^{e}	19.90 ^{f}	9.25 ^{e}
Trichoderma viride	89.05 ^{ef}	45.75 ^{f}	31.99 ^{de}	16.62 ^{bc}
Trichoderma harzianum	124.71 ^{b}	66.38 ^{de}	22.61 ^{f}	11.78 ^{de}
Trichoderma atroviride	115.54 ^{c}	68.51 ^{d}	37.92 ^{c}	14.36 ^{cd}
CD@5%	**6.31**	**5.07**	**4.47**	**3.33**

Values are means followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 0.05 % level.

IV. 14. Bio-efficacy of fungicides and biocontrol agents against branch canker under glass house condition

14.1. Evaluation of chemical and biological control in glass house condition

The efficacy of fungicides and biocontrol were evaluated against branch canker disease under glass house experiment. Results noted that systemic fungicide of Benomyl (70 %) was found to give good results followed by Copper oxychloride (65 %) (Table.42). Both the biocontrol agent *viz., Bacillus* sp. (50 %) and *Trichoderma viride* (50 %) yielded significant activities (Table. 42). *Pseudomonas* sp. (45 %) showed moderate activity followed by *Trichoderma harzianum* (30 %) (Table. 42).

14.2. Integrated management of branch canker disease in glass house condition

An alternated of chemical, botanical and biological for implementation of integrated management of branch canker disease under glass house condition. The integrated manner of Propiconazole/*Bacillus amyloliquefaciens* and Propiconazole/*Bacillus subtilis* were recorded good results against branch canker disease followed by Hexaconazole/*Bacillus amyloliquefaciens* and Hexaconazole/*Bacillus subtilis* (Table. 43). The combination fungicide of companion and Propiconazole were most effectively (Table. 43). The organic fungicide of Tricure gave satisfactory result followed by *Gliocladium virens* (Table. 43).

Table.42 Potential of selected biocontrol agents and chemical fungicides on the control of *Macrophoma* sp. under greenhouse condition

Treatments	**Dosage in 10L**	**Branch canker Disease protection (%)**
Benomyl (Benofit 50%WP)	5 g	70[g]
Copper oxychloride (Fytolon 50% WP)	30 g	65[f]
Tebuconazole (Folicur 250 % EC)	5 g	60[e]
Tridemorph (Calixin 80% EC)	5 g	55[d]
Bacillus sp. (WR46-2)	30 g	50[c]
Pseudomonas sp. (WR5-4)	30 g	45[b]
T. harzianum	30 g	30[a]
T. viride	30 g	50[c]
CD at P= 0.05		**2.8**

Values are means followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 0.05 % level.

Table.43 Integrated management of branch canker pathogen under greenhouse condition

Treatments details	**Dosage in 10L**	**Pre-assessment in cm (L1)**	**Post – assessment in cm (L2)**	**Disease Protection (L1-L2)**
Propiconazole	7 mL	1.53[a]	0.87[a]	0.67
Hexaconazole	10 mL	1.37[a]	0.73[a]	0.63
Companion	20 g	1.80[ab]	1.10[a]	0.70
Propiconazole / *Bacillus amyloliquefaciens*	7 mL / 30 g	2.43[b]	1.03[a]	1.40
Propiconazole / *Bacillus subtilis*	7 mL / 30 g	1.93[ab]	0.97[a]	0.97
Hexaconazole / *Bacillus amyloliquefaciens*	10 mL / 30 g	2.00[ab]	1.10[a]	0.90
Hexaconazole / *Bacillus subtilis*	10 mL / 30 g	2.20[ab]	1.30[ab]	0.90
Gliocladium virens	30 g	1.73[ab]	1.17[a]	0.57
Tricure	30 mL	1.63[a]	1.03[a]	0.60
Control		1.43[a]	1.90[b]	-0.47
CD at P = 0.05		**0.7**	**0.6**	

Values are means followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 0.05 % level.

IV. 15. Disease management

15.1. Evaluation of chemical and biological control against branch canker disease under field conditions

Field experiment of an integrated management of branch canker disease was conducted at UPASI Tea Research Foundation, Tea Research Institute, Valparai located at 10° 23' North and 77° 0' East and about 1050 m above MSL. A field study was conducted to screen evaluation of chemical and biological control practices against branch canker disease caused by *Macrophoma* sp. There was 15 treatments with three replications. All the treatments laid out in randomized block design. The incidence of branch canker disease was measured at before and after treatment imposed.

In the present study, screening of four fungicides, two bacterial and fungal biocontrol agents were used at 0.05 %. Benomyl with *Bacillus* sp. (DPS, 5.07 cm) found to be superior to copper oxychloride alone (DPS, 1.03 cm) in controlling branch canker disease (Table.44). Tebuconazole + *Bacillus* sp. (DPS, 3.14 cm) and copper oxychloride + *Bacillus* sp. (DPS, 3.03 cm) excelled well (Table.44).

Botanical fungicide of Expel (5 %) (DPS, 2.07 cm) significantly reduces the canker size as compared with other treatments (Table.44). *Trichoderma viride* (DPS, 1.27 cm) and *Trichoderma harzianum* (DPS, 1.30 cm) usage in disease management showed the branch canker disease at very low level (Table.44). The treatment with Benomyl along with *Pseudomonas* sp. (DPS, 2.73 cm) and Tridemorph + *Bacillus* sp. (DPS, 2.70 cm) exhibits the reduction in the size of canker followed by copper oxychloride with *Pseudomonas* sp. (DPS, 2.39 cm) and Tridemorph with *Pseudomonas* sp. (DPS, 2.37 cm) (Table.44). Without usage of Biocontrol, Benomyl (DPS, 4.83 cm) higher values of branch canker disease were observed in the control. Thus, management of the branch canker disease with Benomyl along with bacterial biocontrol of *Bacillus* sp. found to be very effective (DPS, 5.07 cm) (Table. 44). In the case of untreated control, the canker size was increased than the treated plants.

Table.44 Field evaluation of certain chemicals and biological control against branch canker disease in tea

Treatments details	Branch Canker size (cm)				Disease Protection Score
	Pre-treatment		Post-treatment		Wound Healing (WH)
	Length (L_1)	Width (W_1)	Length (L_2)	Width (W_2)	WH= (L_1 - L_2) + (W_1- W_2) in cm
Benomyl	19.83	3.17	15.70	2.47	4.83
Benomyl + *Bacillus* sp (WR46-2)	23.87	3.27	19.70	2.37	5.07
Benomyl + *Pseudomonas* sp (WR5-4)	24.60	2.83	22.17	2.53	2.73
Tebuconazole + *Bacillus* sp.	18.30	2.80	16.03	1.93	3.14
Tebuconazole + *Pseudomonas* sp.	14.20	1.50	12.43	1.03	2.24
Trichoderma harzianum	3.30	2.53	2.50	2.03	1.30
Trichoderma viride	3.20	2.27	2.43	1.77	1.27
Bacillus sp. + *Pseudomonas* sp.	16.03	1.60	14.93	0.93	1.77
Copper oxychloride	6.50	3.23	5.90	2.80	1.03
Copper oxychloride + *Bacillus* sp.	23.43	4.43	21.60	3.23	3.03
Copper oxychloride + *Pseudomonas* sp.	14.50	2.03	12.67	1.47	2.39
Tridemorph + *Bacillus* sp.	27.47	2.10	25.33	1.53	2.70
Tridemorph + *Pseudomonas* sp.	9.60	1.93	7.80	1.20	2.37
Expel 5% (Botanical fungicides)	23.10	2.30	21.63	1.70	2.07
Control	35.10	6.17	38.63	9.50	- 6.86
CD @ 5 %	**9.1**	**1.5**	**5.5**	**1.4**	

Canker size (length and width in cm) was measured before (pre-treatment) and after (post-treatment) treatment and their values are given. **Bioformulation (1:1 Ratio):**

Talc (1 kg) + Biocontrol agent (1L liquid culture): 0.5 g + 99.5 g of biocontrol talc formulation to make it 100 g / bush. The chemical fungicides along with bioformulations (100 g) for wound pasting method. Adjuvant: Line seed oil use to make pasting and wound healing properties.

15.2. Evaluation of Integrated schedule against branch canker disease under filed level

The evaluated of various chemical, botanical and promising bacterial strains for implementation of integrated application on branch canker disease in field conditions. There was 11 treatments with three replications. Each replication consider as 25 bushes. They were followed same procedure for assessment of canker incident.

Field study was conducted in naturally infected pruned tea field at UPASI Tea Research Institute, Valparai, Tamil Nadu, India. Among these 11 treatments, the present investigation revealed that integrated application of Companion/*Bacillus amyloliquefaciens* showed superior control of branch canker disease followed by Companion under field condition (Table.45). Copper oxychloride/*Bacillus amyloliquefaciens* was moderately effective followed by Copper oxychloride (Table. 45). The resulting in the significantly canker size was recorded with treatment of *Bacillus amyloliquefaciens* followed by commercial organic fungicides of Tari (Organic Tea Special) and Tricure (0.03 % Azadirachtin). The lowest canker size was observed with *Gliocladium virens* followed by *Beauveria bassiana*. Branch canker disease incidence was increased in untreated control plants when compared to treated plants (Table. 45). Finally, this IDM package could be preferred for the control of branch canker disease under field condition.

Table.45 Field evaluation of integrated schedule against branch canker disease in tea

Treatment details	Branch Canker Size (cm)				Disease Protection
	Pre-Treatment		Post-Treatment		Wound Healing (WH)
	Length (L1)	Width (W1)	Length (L2)	Width (W2)	WH= $(L_1 - L_2) + (W_1 - W_2)$ in cm
Copper oxychloride (COC)	7.97	2.53	6.73	2.03	1.74
Companion [Carbendazim 12 % + Mancozeb 63 % WP]	13.60	4.73	11.19	3.03	4.11
Bacillus amyloliquefaciens	6.63	3.50	5.56	2.97	1.60
Companion/*Bacillus amyloliquefaciens*	14.43	4.10	10.95	1.82	5.76
COC/ *Bacillus amyloliquefaciens*	12.97	3.37	10.88	2.41	3.05
Trichoderma harzianum	13.10	3.00	12.61	2.67	0.82
Gliocladium virens	9.43	2.70	8.43	2.41	1.29
Beauveria bassiana	7.50	2.70	6.97	2.29	0.94
Tari (Organic Tea Special)	6.23	2.33	5.19	1.96	1.41
Tricure (0.03%)	7.87	2.90	7.08	2.33	1.36
Control	14.50	3.63	17.49	5.74	-5.10
CV %	**6.32**	**1.77**	**6.74**	**1.76**	
CD @ 5%	**12.31**	**1.07**	**11.84**	**0.89**	

Canker size (length and width in cm) was measured before (pre-treatment) and after (post-treatment) treatment and their values are given.

Chapter V
Discussion

Branch canker caused by *Macrophoma theicola* is the foremost disease of tea plants (*Camellia* spp.). In tea plantations, this disease causes crop loss and it is the major limiting factor for yield stagnation. The first part of the study describes the survey, isolation of the casual organism, molecular confirmations, culture characteristic features, etiology and life cycle of branch canker pathogen in tea. The second part analyzed the biochemical characters with host-pathogen interaction. The histopathology of the disease is introduced in the third section. The fourth part discussed about *in vitro* evaluation of synthetic fungicides, biocontrol agents, botanical fungicides and plant extracts tested against branch canker pathogen. The fifth section explained about various chemical, biological control and bio fungicides tested against branch canker disease under glass house condition. Finally, the out lines of implementation of integrated management of branch canker disease in field level and its control measures. The present findings are discussed in detail.

V. 1. Survey

Branch canker in tea was initially noticed in the southern parts of India around 1899, but in Srilanka it was recorded in 1904 and it becomes one of the most widespread and serious stem disease of the tea crop. But, *Poria hypobrunnea* has been reported as causal organism of branch canker disease in North Assam of India. In Taiwan, *Macrophoma theicola* has been stated as twig die-back of tea plants (Arulpragasam, 1992). In the present study, the branch canker disease specimens were collected from different tea growing districts of south India i.e., Anamallais, Nilgiris (in Tamil Nadu), Koppa (in Karnataka) and Vandiperiyar (in Kerala) (Table. 1). The observation is in agreement with Nepolean *et al*. (2014) the wood rot specimens were collected from various tea growing agro climatic zones of south India (Anamallais, Nilgiris, High range, and Koppa).

The current survey is generally focused on isolation and identification of branch canker pathogen in tea plants. After identification and confirmation, the study grips potential

in findings applications in etiology, host-pathogen interaction and for studying chemicals, biological and for its controlling measures.

V. 2. Identification

The first indication of the branch canker disease which usually appears in the rainy season and is confirmed by the visual identification of the branch canker pathogen which is likely to be small slightly sunken with dark patches and are internally blackened, soft, rotten, and separated from the wood. During moist weather, the sunscald injuries in the disease were observed under the microscope. The microscopic examination shows white circular patches each surrounded by a black ring. The overall sections of the fungus was identified as a minute black tome body as pycnidia. The pycnidia contain pycnidiospores and the spore is characterized by an oval shaped non septum which is arranged uniformly inside of the sac shape of pycnidia (Plate. 4). The morphology of pycnidiospore in under water mounting stains and lacto phenol cotton blue mounting showed the oval shape of pycnidiospore very clearly (Plate.5 & 6).

The spore morphology characters, culture and hyphae of branch canker pathogen were identified with the standard reference book by Burnett and Hunter (1972). The same fungal characters were found on tea twigs in Japan by Hara (1931). The result from present study also reveals the pathogen characters of branch canker pathogen reported by F. M. Thseng *et al.* (2004). Moreover, the sunken patches appeared as elongated lesions and minute black dome shaped pycnidia were also observed in most cankered areas.

V. 3. Molecular confirmation study

The DNA isolation, PCR amplification, sequencing and phylogenetic analysis are identical to those used by Crous and Palm (1999). PCR was performed with primer pairs targeted to the 18s rRNA gene. These fungal isolates were identified as *Macrophoma* spp. (Accession number JQ234977 for NBCHE-6, Accession number KP004441 for VPM, Accession number KP179221 for UPA-61, Accession number KP179222 for UPA-62 and Accession number JQ362417 for NBCC-2) through molecular tools and submitted to NCBI for public domain (Table. 2).

The different fungal isolates were confirmed as *Macrophoma* spp. through molecular techniques and the sequences were compared with other fungal genomes from NCBI and a phylogenetic tree was constructed using the neighbor-joining method (Altschul *et al*., 1990). The results indicated that the isolate of NBCHE-6 and VPM belonged to *Macrophoma* spp. and remain isolate of UPA-61 belonged to *Macrophoma theicola*. The isolate of NBCHE-6 and UPA-61 showed 95 % to 99 % similarity with another nucleotide sequences based on the phylogenetic tree analysis. Therefore, NBCHE-6 matches with another two nucleotide sequence number such as DQ100415 (*Macrophoma* sp.) and DQ100414 (*Macrophoma* sp.). UPA-61 matches with nucleotide sequence number KP179222 (*Macrophoma theicola*) whereas the VPM sequences exhibited less than 95 % similarity to the remaining species of ascomycetes group (Figure. 1).

V.4. Pathogenicity

The pathogenicity of *Macrophoma* sp. was tested *in vitro* and initial white circular patches symptom was noticed a sixth day of inoculation. Incubation of the inoculated stems after 18th to 43rd days resulted in the development of black fructifications formed by a black ring canker symptoms accompanied with the oozing of conidia from pycinidia from cankered tissues. The studies indicated that life cycle pattern of the pathogen was completed after 43 days of inoculation (Table. 6). The close results were recorded from pathogenicity test of Devanath (1985) *in vitro* studies indicated that the *Tunstallia aculeata* completes its life cycle i.e., ascospore formation in 45 days. Jee song chen (1987) reported that the incidence of twig die-back increased after 9-12 days of inoculation.

V.5. Growth characteristics of the pathogen

During the growth of branch canker pathogen in different solid and liquid medium, PDA and PDB medium exhibited the highest radial growth and mycelia dry weight of *Macrophoma* sp. respectively followed by Richard medium (Table. 5). Fundamentally reported that, the spores of some fungi is required nutrients for germination (Bilgrami and verma, 1978). This result is in negatively correlated with Jee song chen (1987) report of *Macrophoma theicola* grown on PDA failed, but when grown in wheat oat medium.

Among the different media tested, the branch canker pathogen produced pycnidia on solid medium from 15th day onwards. MSM supported the pycnidial production of the pathogen to the maximum followed by SDA and PDA (Table. 4). The growth of *Macrophoma* sp. was poor in soil extract medium. Tisserat (1988), reported that the pycnidia of *P. theae* contained numerous α and β conidia, which is distinctive to *Phomopsis* sp.

In vitro studies on the optimum temperature for the growth of branch canker pathogen indicated that 25 °C to 30 °C was optimum for the growth of pathogen (Table.7 & 8). Similar results were observed in relation to the seasonal release of ascospore in many pathosystems (Hershman and Perkins, 1995). Moreover, the wood rot pathogen also favored under the same temperature conditions (Venkata Ram 1973 and Onsando 1985). The pH of 5.0 to 5.5 was optimum for the mycelia growth of the isolates of *Macrophoma* spp. (Table. 9 &10). The same trend was observed with the tea pathogen *Hypoxylon serpens* (Venkata Ram 1973). RH ranges from 60-80 % were optimum for the growth of branch canker pathogen (Figure. 3). The study by Verma *et al.* (1996) describes that the relative humidity and rain fall shows a major influence on disease development. Moreover, similar results were observed with blister blight disease (60-90 %) and *Hypoxylonserpens* (60-90 %) on tea plants (Ajay, 2008 and Nepolean, 2014).

Among the different sources of light, day light was found to be the best for growth of *Macrophoma* sp followed by dark condition in PDA and PDB (Table.11). UV light was inhibitory to the vegetative growth of branch canker pathogen. Earlier findings were supported that the sporulation of *Alternaria solani* was stimulated by sunlight. Moreover, *Sclerotinia fructicola* sporulated well in dark conditions and not at all in light (Durbin 1959). The sporulation was found to be high in the UV treated *Cladosporium allii-cepae* (Hall and Kavanagh 1984).

The different carbon sources compounds were examined, the growth and utilization of branch canker pathogen was maximum in carbohydrate (Polysaccharides) followed by fructose such as monosaccharides and mycelial dry weight also noticed in same (Table.12). In the present study, pycnidia production was maximum noticed in sucrose followed by carbohydrate (Table.13). The observation made with Bilgrami and Verma (1992), Similarly, *Alternaria alternata* and *Clodosporium cladosporioides* (Pande and Varma 1992). *Glomerella cingulata* Singh and Shanker (1971) also preferred the carbon source.

Among the various nitrogen compounds studied, the maximum note on radial growth and mycelial dry weight of the branch canker pathogen in glysin compound followed by pottassium nitrate (Table.14) and also same result noticed in pycnidial production (Table.15). The medium amended with glysin, verified as a good nitrogen compound for *Macrophoma* sp. Tea root pathogen like *Fomes lamaoensis* showed good growth in complex organic nitrogen amended media (Barthakur and Samajpati 1986). Some fungi utilize many forms of complex nitrogenous bases like yeast, casein and glysin (Dan Santos 1963).

Among the 16 compounds detected from two indigenous isolates of branch canker pathogen through GC-MS spectrum method (Figure. 4 & 5). These isolates were produced several different mycotoxins like propanedioic acid, Phenyl, undecane, decane, methylene, butanic acid, hexane, octane and pentonic acid (Table. 16 and 17). This study coincidence with Schmelch *et al.* (2003) and Trucksee (2004) mycotoxins are produced by fungi that are causing disease in plants. Toxic compounds can be multifaceted by numerous properties: chemical characters (phenol, methylene, peptide, malonic acid, hydroxyl and indene). This result is in agreement with report of McCormick *et al.* (1998) normally toxin compounds are aflatoxin made by fungi such as *Aspergillus flavus, Fusarium* spp. and *Penicillium* sp.

V. 6. Biochemical study and host pathogen interaction

The present study was aimed to study the biochemical changes of canker infected tea plants under glass conditions to evaluate host-pathogen interaction of different tea clones. Host-pathogen interaction was demonstrated by biochemical analysis of carbohydrate and reducing sugar and nutrient analysis.

Total carbohydrate content in the infected plants showed decreasing trend in all the tea clones when compared to the healthy plants (Figure. 6). Reducing sugar level also decreased rapidly in all the infected clones, except the clone TRI-2025 in which no major variation was observed in the healthy and infected plants (Figure. 7). Similarly NPK level of all the infected clones recorded lower values than the uninfected plants, whereas there is no notable difference in the nitrogen level of infected and healthy plants of the clone Yabukita (Table. 18).

From the results it is inferred that, low level of carbohydrate and reducing sugars observed in the infected plants of all the tea clones was due to the infection of the fungus *Macrophoma theicola.*

Yadav, (1989) reported that, the primary metabolism of carbohydrate is altered because of the fungal pathogens resulting in the dissolution of the reserve. Similar result was obtained in the findings of Manik *et al.,* (2008) in the infected seeds of *Cassia angustifolia*. Similarly reduction in the NPK level of the infected plants than the healthy plants clearly demonstrated the host-pathogen interaction (Table. 18).

NPK level was considerably reduced in all the infected plants indicating that the fungus *Macrophoma theicola* might have utilized the NPK reserves from the infected plants. This finding is in accordance with the earlier report of Nepolean, (2014) in tea. Equal level of reducing sugars observed in the clone TRI-2025 and the same level of nitrogen content noticed in the clone Yabukita in both the infected and healthy plants shows their comparatively better tolerance level against the pathogen *Macrophoma theicola* than the other clones. Further field level studies are essential for the better understanding of the tolerance level of TRI-2025 and Yabukita to branch canker disease.

V. 7. Histopathological Studies

In present study, histopathological investigations have been attempted to find out the susceptible/tolerance level of various tea clones under glass house conditions. Histopathological studies showed that UPASI-3 followed by the cultivar TRF-1 supported maximum spore production and length of canker size compared to all other cultivars screened (Table.19). UPASI-3 and TRF-1, the brownish fungal mycelium with more number of pycnidiospores were recorded in the inner layer of epidermis cell (Plate.7a & b). But in other case of Yabukita showed hyphal with spores production presence in the outer layer of cortex region (Plate.7c). Present investigation revealed that UPASI-3, TRF-1 and Yebukita were highly susceptible cultivars under *in vitro* condition. This is in agreement with Hudson *et al.* (2002), the highly susceptible clone of UPASI-3 noted against collar canker disease. Several scientists reported that (Riedel *et al.* 2012; Parke *et al.* 2007 and Jung *et al.* 1996), woody tissue is damaged by the infection of *Phytophthora* spp. and presence of spores (chlamydospore or oospores) in infected tissue development. The same results are noticed by

Eziashi *et al*. (2007), the interaction between of host and pathogen showed that epidermis layer was macerated. Moreover, the findings of Oruade-Dimaro *et al.* (2010), the maceration of epidermis layer might have resulted due to production of fungal hydrolytic enzymes produced by *Glomerella cingulata* pathogen.

Present investigation showed that CR-6017 and TRF-2 were moderately susceptible clones followed by UPASI-6 (Table.19). Less number of pycnidiospores was observed in the inner layer of primary cortex layer of CR-6017 (Plate.8c). Brownish fungal hyphae penetrated to the inner epidermis layer of TRF-2 clone (Plate.8b). But in UPASI-6, only mycelia with spores were present in the outer layer of cortex region (Plate.8a). These lines are in agreement with Allen *et al*. (1983) the development of appressorium direct penetrates through the epidermis layer of sunflower. This study revealed that the mycelium of branch canker pathogen produced cell wall degradable enzymes and it may be degrading all layer of epidermis cells.

Cultivars, UPASI-9, UPASI-17, UPASI-26, TRF-4 and TRI-2025 were recorded as less susceptible clones (Table.19). Here, only few fungal mycelia were present in the epidermis and macerated the layer of outer epidermis cell due to pathogenic degradable enzymes. Pycnidiospore production was less in this case and canker size was small in size (Plate.9 a, b, c, d & e). So these clones are recorded in tolerance category (below 1.0 cm). In general, the histopathological studies showed that branch canker pathogen produced black tome shaped pycnidium and fungal mycelium penetrated through stem wound. Degradable enzyme of *Macrophoma theicola* might be involved in macerating host epidermis layer. This result is in concordance with Davison (2011), the infection of *Phytophthora* spp. released toxin compounds to disruption of host tissue and cause a variety of histopathological changes in host tissue. Furthermore, during infection of *Phytophthora* spp. the woody tissue are shrunken, cells shape are altered by infection of pathogen (Giesbrecht *et al.*, 2011 and Pogada and Werres, 2004). The present results revealed that UPASI-9, UPASI-17, UPASI-26, TRF-4 and TRI-2025 are tolerance clones to branch canker disease.

From the above study the clones are classified in to three category based on their canker size and spore productions *viz.*, highly susceptible (above 1.5 cm), moderately susceptible (below 1.5 cm to >1.0 cm) and less susceptible (below 1.0 cm). Infected clones of UPASI-3, TRF-1 and Yabukita were highly susceptible followed by moderately susceptible

clones, UPASI-6, TRF-2 and CR-6017. UPASI-9, UPASI-17, UPASI-26, TRF-4 and TRI-2025 clones have been identified as tolerant clones against branch canker disease. These clones will be helpful for replanting programme.

V. 8. *In vitro* effect of chemical fungicides

In vitro screening of systemic fungicides such as benomyl, carbendazim and companion showed 100 % growth inhibition against *Macrophoma* sp. at 10 ppm level followed by propiconazole and hexaconazole (Table.20). These results are in combine with the earlier report of suryawanshi *et al.* (2008) which demonstrated the efficacy of different fungicides against *Macrophomina phaseolina* blight of mungbean. Moreover, several workers reported that, (Gore *et al.,* 2008 and Gautham and Narain, 1996) similar inhibitory potential of different fungicides against *Macrophomia* sp.

The contact fungicides of copper group *viz.,* copper oxychloride (50 %WP), copper hydroxide and copper oxychloride 435 (liquid) were tested against branch canker pathogen under *in vitro*. Among the copper group, highest growth inhibition was noticed in copper oxychloride (50 % WP) followed by liquid copper oxy chloride 435 and copper hydroxide against branch canker pathogen (Table.21). Earlier researcher reported that, Sanjay *et al.* (2008) the contact fungicide of copper oxychloride gave the best disease control of grey blight pathogen in tea.

V. 9. *In vitro* effect of botanical fungicides

The commercial botanical fungicides were tested against *Macrophoma* sp. at different concentrations. In this present study, botanical fungicides (Expel, Enroot and Attopsy) were found to be efficient against the test pathogen at 0.1 % concentration (Table.22). These results are in agreement with Nepolean *et al.* (2014) biofungicide of expel (5000 ppm) showed maximum growth inhibitory effect of wood rot pathogen. However, the same report that, Anonymous (2015) the organic fungicide of Tricure noticed complete inhibition of branch canker pathogen. From this investigation, the efficacy of commercially available botanical fungicides against branch canker pathogen was established.

V. 10. *In vitro* effect of Plant extracts

In this present study, attempts were made to identify the plant aqueous extract to control branch canker disease under *in vitro* condition. Effect of different plant aqueous extracts on radial mycelia growth of pathogen on 7 days of incubation is presented in Table.23. *In vitro* screening of plant extracts against *Macrophoma* sp. showed that six plant extracts out of 14 tested were found to be effective. *Acorus calamus* at 10 % concentration showed (100 %) inhibition and superior to other plant extracts tested (Table. 23).

The maximum inhibitions were found at 15% concentration of plant aqueous extract of *Curcuma longa* (82.05±0.96) and *Hibiscus rosasinensis* (81.74±0.75) against branch canker pathogen. Plant extracts of *Psidium guajava* (79.04±2.02) and *Allamanda cathartica* (77.59±1.13) indicated significant control followed by *Murraya koenigii* (73.70±1.22), *Azadirachta indica* (64.44±0.48) and *Artemisia nilagirica* (61.66±1.93). Moderate inhibition was noticed with *Carica papaya* extract (52.77±0.64) followed by *Cinnamomum burmanii* (46.29±1.33) and *Dryopteris linearis* (40.74±0.81). Lowest inhibition was observed in case of *Tithonia diversifolia* (35.37±3.21) followed by *Conyza ambigua* (33.88±4.44) and *Adhatoda vasica* (25.03± 0.58).

The present study revealed that, the inhibitory potential of *Acorus calamus* extract was 100% effective against *Macrophoma* sp (Plate. 10). Similar results were reported by Jitendiya Devi and Chhetry (2013) the growth inhibition was observed with *Drechslera oryzae* may be due to the presence of antifungal compounds like ∞-asarone and β-asarone. *Curcuma longa* effectively suppressed the mycelial growth of the *Macrophoma* sp. pathogen (Plate. 10) followed by *Hibiscus rosasinensis*. These results were in line with Madhiazhagan *et al.* (2002) who found *Curcuma longa* very effective against bacterial blight of rice. Moreover, the antifungal activity of plant extract of *Curcuma longa* showed (100%) growth inhibition against tea pathogens such as *Pestalotipsis theae*, *Colletotrichum camelliae* and *Botryodiplodia theobromae* (Saha *et al* 2005). Wherever, Raja and Kurucheve (1998) reported that, the *Curcuma aromatica* showed (88.3 %) growth inhibition against *Macrophomina phaseolina*.

Previous finding supported that, the methanol extract of *Hibiscus rosasinensis* was recorded highest growth inhibition with concentration of 100 mg/mL against *Aspergillus flavus* (Rathi Sanjesh *et al* 2012). Generally, the medicinal plant extracts of *Azadirachta*

indica and Catharanthus roseus have been reported as antifungal activity against *Curvularia lunata* (Bhowmik and Varadhan 1981).

The present investigation revealed that, the inhibitory effect of plant extracts of *Psidium guajava, Allamanda cathartica* and *Murraya koenigii* showed good results. *Carica papaya* showed moderate results (Plate. 11). The results of the present study were in line with those reported by Neela *et al.* (2014) who reported superior inhibition of ethanol extract of *Psidium guajava* and acetone extract of *Carica papaya* against *Fusarium* sp.

Earlier reports says that, chloroform extract of *Allamanda cathartica* exhibited promising antifungal activity (Singha *et al* 2011) and *Murraya koenigii* showed strong toxicity and controlled damping-off disease of tomato (67 % and 71 %) infected with *Pythium aphanidermatum* and *Pythium debaryanum* (Pandey and Dubey 1994). Many workers proposed that, (Deo and Shastri 2003, Arima and Danno 2002) guava leaf extract is associated with flavinoids such as mosin glycosides and antimicrobial activity which play a role to resist to fungal attack, while other reported that, *Carica papaya* leaves exhibited a broad spectrum activity against pathogenic fungi *Fusarium* sp and *Colletotrichum* sp (Cha'Vez-Quintal *et al* 2011).

From the investigation, it was observed that plant extract of *Azadirachta indica* (Plate. 12) and *Artemisia nilagirica* effectively inhibited the mycelia growth of the test pathogen. Same results were noticed by Kamalakannan *et al* (2001) where they observed greater inhibitory effect of *Azadirachta indica* against *Pyricularia grisea* rice blast pathogen. Other scientists have also reported fungitoxic properties of phenolic compound and fungicidal spectrum of Azadirachtin compound in *Azadirachta indica* (Thapliyal and Nene 1961, Mukerjee and Kundu 1973, Subramanian 1993, Rahejha and Thakore 2002). Our results are in accordance with those put forth by Sati *et al.* (2013) who reported that plant extract of *Artemisia nilagirica* has antifungal activity and artimisinin constituents which act against phytopathogenic fungi *Rhizoctonia solani, Sclerotium rolfsii* and *Macrophomina phaseolina*. Our findings with *Cinnamomum burmannii* and *Dryopteris linearis* is in this, agreement with the studies of many scientists (Suresh *et al* 1992, Ooi *et al* 2006, Chami *et al* 2004, Bennis *et al* 2004, Pacheco *et al* 1993) who reported that *Cinnamomum burmannii* has a mixture of chemical constituents and possess cinnamaldehyde compound. These compounds have been proved to be active against many pathogenic bacteria, fungi and also

possess antibacterial and antifungal activity. Saha *et al.* (2005) used ethanol and aqueous extract of *Dryopteris filix-mas*. (L) Schoot and recorded 100 % inhibition of spore germination of fungal disease of tea.

The present result indicated that, very low inhibitory level of *Tithonia diversifolia, Conyza ambigua* and *Adathoda vesica*. The results are in agreement with those by Dissanayake (2014) and Dissanayake and Jayasinghe (2013) who recorded low activity of *Tithonia diversifolia* in inhibition of *Fusarim oxysporum*. The methanol extract of *Tithonia diversifolia* at all concentrations, showed less inhibition against *Colletotrichum musae, Rhizoctonia solani* and *Fusarium oxysporum*. The results indicated that plant extracts of *Tithonia diversifolia* very effectively controlled leaf spot diseases such as *Fusarium solani, Fusarium lateritium* and *Cochliobolus lunatus* (Ilondu *et al* 2014). *Tithonia diversifolia* has been identified as antifungal activity reported by Ragasa *et al* (2008). The antifungal activity of AgNPs synthesis from leaves of *Conyza ambigua* has also been reported against *Aspergillus flavus, Aspergillus niger* and *Sclerotium rolfsii* (Elumalai and Vinothkumar 2013). The phytotoxicity and fungitoxicity of *Conyza canadensis* is due to the presence various chemical constituents like, matricaria acid methyl ester, matricaria lactone and lachnophyllum lactone. These lactone compound inhibited growth of the pathogenic fungi *Colletotrichum acutatum, Colletotrichum gloeosporioides* and *Colletotrichum fragariae* (Sonia *et al* 2012).

In the present study very little inhibition was noticed in *Adhatoda vesica*. Previous information says that, *Adhatoda vesica* observed 57% reduction in percent of disease inhibition with maximum tannin (Rajeswari 1991). The plant extract of *Adhatoda vasica* was more efficient in dropping the disease incidence (25.37) (Madhiazhagan *et al* 2002). Henceforth, the professed of plants extract such as *Acorus calamus, Psidium guajava* and *Allamanda cathartica* could be used as biological control against branch canker as well as some other tea pathogens in way of ecological approaches for controlling plant disease.

From the above results, the selected plants extract of *Acorus calamus*, *Curcuma longa, Hibiscus rosasinensis*, *Psidium guajava* and *Allamanda cathartica* have been identified as highest antagonistic potential against branch canker pathogen. Further field study and screening of plants for antifungal and antimicrobial activities may elucidate various options, which may replace the use of chemical fungicides in future.

V. 11. *In vitro* effect of fungal biocontrol

11.1. Dual plate technique

The genus of *Trichoderma* sp. has been known since 1920s in controlling many fungal plant pathogens (Harman, 2006). The objective of this *in vitro* study was to identify the antagonistic potential of four fungal biocontrol agents against branch canker pathogen. The dual culture method revealed that *Gliocladium virens* (82 %) exhibited high potential to test pathogen of UPA-61 (Plate.13) followed by *T. harzianum* (72 %) against pathogen of NBCHE-6 (Table. 25). These results are in agreement with Ponmurgan and U.I. Baby (2003) the *in vitro* interaction of fungal antagonists was against *Phomopsis theae* the causal agent of collar canker disease in tea. Likewise Ramezani (2001) reported that *T. harzianum* significantly inhibited the growth of *Macrophomina phaseolina*. Baby and Chandramouli (1996) documented that *G. virens* exhibited high antibiosis to root pathogen of *Rosellina* sp in tea.

From the present study, *T. atroviride* (68%) and *T. viride* (65 %) showed similar results (Table. 25). A similar finding by Matroudi *et al.* (2009) on the antagonistic effect of *Trichoderma* spp. has been reported on *Sclerotinia sclerotiorum* the causal agent of canola stem rot. Various reports show that *T. atroviride* acts as a biocontrol agent against aerial and soil borne plant pathogens (Brunner *et al.* 2005). Moreover, *T. atroviride* was found to be effective against *Polymyxa betae* and this has been reported by Jaykubikova *et al.* (2006). *Trichoderma viride* documented greater inhibition growth on *Macrophomina phaseolina* (Ramezani, 2001).

11.2. Culture filtrate method

In cell free culture filtrate studies also illustrates the similar results of the dual plate technique. The present study indicates that *G. virens* was found to have a higher potential (88 %) against branch canker pathogen (UPA-61) (Plate.14) followed by *T. harzianum* (85 %) (Table.26). The culture filtrate of *G. virens* exhibited fairly high inhibitory effect on the tea root pathogen (Baby and Chandra mouli 1996). Similar results have been reported for *T. harzianum* against *Crinipellis perniciosa* (Marco *et al.* 2000) and *Rhizoctonia cerealis*

(Innocenti *et al*. 2003). Moreover, *T. harzianum* has been reported to sheath blight of rice pathogen (Tewari and singh 2005).

Culture filtrate of *T. atroviride* and *T. viride* were found to be similar results against test pathogen of (UPA-61) *Macrophoma theicola* (Table. 26). The use of *T. viride* for controlling sheath blight of rice has been reported by Das *et al*. (1996) and the use of *T. atroviride* against *Phomopsis theae* a casual organism of Collar canker in tea (Anita and Ponmurugan, 2011) has been documented.

11.3. Mycoparasitism study

The microscopic study of hyphal interaction between *G. virens* and branch canker pathogen cell wall shows that the hypal wall was lysed by the G. *virens* are some of the interesting results obtained from the study (Plate. 15). The similar results were noticed with *G. virens* lysis of *P. theae* (Ponmurugan and Baby 2003). However, mycoparasitism involves morphological changes, such as coiling and development of appressorium like structure that serves to penetrate the host (Mclntyre *et al*., 2004). *T. atroviride* hyphae comes into contact and attaches to the pathogenic fungi cell wall and *T. viride* produced more spore interaction with branch canker pathogen cell wall. Same findings in a study by Matroudi *et al.,* (2009) that showed coiled formation of *T. atroviride* and *T. viride* has been reported against *Sclerotinia scleotiorum*.

Many studies have reported that the antagonistic potential of *Trichoderma* spp. reduced the growth of plant pathogenic fungi and many other processes like competition, mycoparasitism, hyphal interaction and enzyme secretion (Cook and Baker 1983 and Deacon and Berry 1992). The hyphae of branch canker pathogen also showed shrinking and lysis by *T. harzianum* interactions. The properties of *Trichoderma harzianum* such as antibiotic production, nutrient competition and mycoparasitism could be useful (Ruiz-Herrera 1992 and Schirmabock *et al*. 1994). Antagonistic potential of *Trichoderma* spp. like coiling and cell wall degrading enzymatic activities against *M. phaseolina* (Gajera *et al*. 2012) has also been reported.

In conclusion, among the four fungal biocontrol agents, *G. virens* and *T. harzianum* showed the highest biocontrol activity against branch pathogen. These fungal biocontrol

agents can consequently be used for assessment against *Macrophoma theicola* in field condition.

V. 12. *In vitro* effect of entomopathogens

In this experiment results revealed that, the different tea growing area infected branch canker specimens were isolated and identified at molecular level. The entomopathogens have been tested for inhibition of branch canker pathogen. The inhibitory effects observed in this study were mainly for antagonistic and competition.

V.12.1.Dual plate method

The dual plate method showed maximum growth inhibition of *Beauveria bassiana* against branch canker isolates NBCHE-6 (64.22), UPA-61 (62.66) and VPM (61.55). The effect of *Paecilomyces fumosoroseus* against two isolates of VPM & UPA-61 (56.66) and *Paecilomyces lilacinus* against VPM (54.66) was significantly antagonistic potential followed by *Lecanicillium lecanii* observation against VPM (47.33), NBCHE-6 (45.33) and UPA-61 (44.22) (Table. 27 and Plate 16 & 17).

Mycoparasitism of both hyphal interaction vital role in mechanism of antagonistic potential capability. The hyphae of *Beauveria bassiana* and *Paecilomyces lilacinus* coiled around hyphae of *Macrophoma* sp. and shrunk it (Plate.19). However, same action *Beauveria bassiana* with pathogen of tomato root-rot *Pythium myrotylum* was reported by Klingeman *et al*. (2008). Kiss (2003) reported that, this *Beauveria bassiana* may control plant pathogens and can act through antibiosis and mycoparasitism. Pathogen hyphae were broken by *Lecanicillium lecanii* spores interaction (Plate.20). The result conform to the reports of Askary *et al*. (1997) who reported that, *Lecanicillium lecanii* acts as mycoparasitic attaching to powdery mildew mycelia and conidia, producing enzymes such as chitinase, which penetrates to the mildew spore and kills the pathogen. *Paecilomyces fumosoroseus* produced more hyphae and spores interact with pathogen hyphae. Several scientists reported the mycoparasitism interaction as main principle mechanism of biological control (Elad *et al*. 1983). This study results are in accordance with the reports of Kang *et al*. (1996), Verharr *et al*. (1997), Dik *et al*. (1998), Miller *et al*. (2004) and Kavkova and Curn (2005). This may also be the reason for its antagonistic effect on *Macrophoma* sp.

12.2. Culture filtrate method

Culture free extract of entomopathogen namely, *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* have showed maximum inhibition in growth of the pathogen at 2% concentrations. The maximum inhibition given by *Paecilomyces fumosoroseus* against VPM (68.44) followed by *Paecilomyces lilacinus* against UPA-61 (65.59). Significant inhibition observed by action of *Beauveria bassiana* against both isolates of VPM and UPA-61(54.44). *Lecanicillium lecanii* was seen to inhibit VPM sparsely (30.44) as compared to other antagonistic treatments (Table. 28 and Plate. 18).

The present studies reveals that, *Beauveria bassiana* and *Paecilomyce fumosoroseus* show higher control growth of *Macrophoma* sp. pathogen. Youssef and Hatem (2012) also reported the control of red palm weevil and *Rhizoctonia* –root-rot of date-palm with *Beauveria bassiana*. *Isaria fumosoroseus*, (formely *Paecilomyces fumosoroseus*) and *Lecanicillium* sp (formely *Verticillium lecanii*) are known entomopathogens and have good biopesticidal properties (Goettel *et al*. 2005). Wherever, these kinds of entomopathogens showed against to fungal plant pathogens (Benhamou and Brodeur 2000 and 2001). In our findings, we conclude that *Paecilomyces lilacinus* showed good antagonistic result. Similar findings were recorded by Perveen *et al*. (1998) with *Paecilomyces lilacinus* and *Pseudomonas aeruginosa* when used against Root rot (*Meloidogyne javanica*) and Root knot disease (*Macrophomina phaseolina*) in some vegetable crops. The applications of *Paecilomyces lilacinus* fungus protect plant roots from pathogens, increase plant growth and leaf yield (Manjula and Podile, 2001; Wraight *et al*., 2003 and Muthulakshmi *et al*., 2010).

Results of our experiment showed low inhibitory effect of *Lecanicillium lecanii*, though, these entomopathagenic fungus have activity against phytopathogenic fungi including powdery mildew (Verhaar *et al*., 1997 and 1998; Spencer and Atkey, 1981 and Askary *et al*., 1998). In the present investigation, all the entomopathogens studied, showed antagonistic potential and inhibitory effect against *Macrophoma* sp. pathogen. The evidence for role of competition and parasitism has been convinced and evidence established the importance of antibiosis.

From this study, it is evident that the entomopathogens reduced the growth of all isolates of branch canker pathogen causal organism by *Macrophoma theicola* significantly.

Mostly entomopathen can be used as pest and insect infection disease control. Hence, it may use in integrated approaches for managing plant disease and pest control.

V. 13. *In vitro* effect of bacterial strains

13.1. Effect of dual plate studies

In vitro screening of bacterial biocontrol strains against branch canker pathogen under dual plate technique showed that, the *Bacillus amyloliquefaciens* gave the maximum growth inhibition for all the three branch canker pathogens (Table. 32). The percentage of inhibition of *Bacillus amyloliquefaciens* (75 to 78 %) was the highest followed by *Bacillus subtilis* (70 to 72 %), *Bacillus licheniformis* (62 to 64 %) and *Pseudomonas fluorescens* (31 to 36 %) (Plate.22). The efficiency of *Bacillus amyloliquefaciens* as a potential biocontrol agent has been proved by several authors (Yuan *et al*., 2012 and Nam *et al*., 2009) against the pathogen of *Fusarium oxysporum f*. sp. *cubense.*

In the present study, *Bacillus subtilis* gave the growth inhibition of 70 to 72 % against *Macrophoma* spp. followed by *Bacillus licheniformis* (62 to 64 %). Similar results were reported that, *Bacillus* strains have been considered to be biologically active compounds and their involvement of microbial disease by Asaka and Shoda (1996) and Emmert and Handelsman (1999). The antagonistic potential of the *Bacillus licheniformis* strain has also been proved by Kamil *et al.* (2007) against damping disease. Mitoi *et al*. (2012) also reported the antagonistic potential of *Bacillus licheniformis* against the pathogen of *Alternaria alternata*. Though, several workers have reported that *Pseudomonas* sp gave superior antagonism against plant pathogens (Chakraborthy *et al.*, 1994). In the present study, *Pseudomonas fluorescens* gave the lowest growth inhibition of 31 to 36 % against all the three *Macrophoma* spp. (Table. 32).

13.2. Effect of culture filtrate studies

Free culture study revealed that, *Bacillus amyloliquefaciens* exhibited highest antagonistic potential against the pathogen *Macrophoma* spp. (Table. 33). This is in agreement with the result of Yuan *et al.* (2011) on the antagonistic potential of *Bacillus amyloliquefaciens* against the pathogen of *Fusarium oxysporum f*. sp. *cubens.* The bacterial strains *Bacillus subtilis* showed satisfactory level of antagonism against the test pathogen

(Table.33). Similar reports are published by Douville and Bolland (1992) and Romero *et al.* (2007) on the antagonistic potential of the same bacterial strain.

In the present investigation, *Pseudomonas fluorescens* and *Bacillus licheniformis* gave only minimum inhibitory effect against *Macrophoma* spp. (Table.33). The antagonistic potential of *Pseudomonas fluorescens* against certain root rot disease has been reported by Vanitha and Ramjegathesh (2014) and several scientists have reported that *Bacillus licheniformis* gave superior antagonistic potential against *Botrytis cinerea* and *Phytophthora capsici* (Moon *et al.*, 2002; Lee *et al.*, 2006 and Lim and Kim, 2010).

V.14. Effect of GC-MS studies

The potential bacterial strain of *Bacillus amyloliquefaciens* was subjected to GC-MS analysis to identify the compounds responsible for its antagonistic potential. Analytical research revealed that, nine compounds were detected which are known antifungal-antioxidant-antimicrobial and antibacterial activity (Table. 34). Among the nine compounds detected from the bacterial culture filtrate supernatant of *Bacillus amyloliquefaciens* compounds such as 1H-Indene, 1-methylene, Dichloroacetic acid, 4-hexadecy ester, 3-Hexadecene(Z)-, Benzeneacetic acid, Phenol, and caffeine were higher percentage peak area of GC-MS spectrum (Figure. 9). Elwenees and Derbalah (2011) reported that, the compound group of Hexadecanoic has got antifungal activity against powdery mildew pathogen. Goncalves *et al.* (2003) has also reported antifungal activity of benzoic acid. Similarly, Fernando *et al.* (2005) reported the antifungal activity of Benothiazole. The antagonistic potential of phenols and benzene compounds has been studied and prove to be useful against the pathogen of *Fusarium oxysporum* (Botta *et al.*, 2005; Yuan *et al.*, 2012). Therefore, it can be concluded that, the antifungal activity of *Bacillus amyloliquefaciens* against the pathogen *Macrophoma* spp. could be due to this compounds.

V.15. Compatibility studies

The present investigation revealed that the bacterial biocontrol of *Pseudomonas fluorescens* was more tolerant with propargite, glyphosate followed by ammonium salt of glyphosate (Table. 35). Companion was highly toxic towards the growth of *Bacillus licheniformis* (Table. 35). *Bacillus subtilis* showed tolerable growth in hexaconazole

followed by propiconazole (Table. 35). The CFU of *Pseudomonas fluorescens* and *Bacillus subtilis* were higher in all the agrochemicals at dilution (10^6) followed by *Bacillus amyloliquefaciens* (10^5) and *Bacillus licheniformis* (10^4) (Table. 35). There are very limited reports are available about the tolerant of agrochemicals with bacterial bioagents. However, the bacterial biocontol of *P. fluorescens* showed variable response which is tolerant with different concentrations of fungicides like Hexaconazole, Carbendazim and Thiram (Prakash and Puri, 2012). The other bacterial agent was observed to be lesser tolerant with all the agrochemicals. The bacterial growth was not affected by the selected agrochemicals at 0.4 % concentration except *Bacillus licheniformis* against on systemic fungicide of companion (Table. 35). The bacterial biocontrol of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis* and *P. fluorescens* were found to be most compatible with agrochemicals than fungal biocontrol agents. It might be due to the reason that, the bacterial agents utilized the agrochemicals as nutrient medium and therefore, the bacterial agents were found more tolerant with higher concentrations level of chemicals (Aislabie and Jones, 1995; Kishore and Jacob, 1987). Some other evidence supported that the *P. fluorescens* and *B. subtilis* were found as highly tolerant with azoxystrobin (Anand *et al.* 2007).

At 10 ppm concentration of copper oxychloride was supported to excellent growth of all the fungal biocontrol such as *Gliocladium virens*, *Trichoderma harzianum*, *Tichoderma atroviride* and *Tichoderma viride* (Table. 36). The growth of all fungal mycelia was noted at 10 ppm level of copper oxychloride without inhibition (Table. 36). Soumik *et al.* (2008) has also showed that the fungal biocontrol of *Trichoderma harzianum* more tolerant with copper oxychloride and copper hydroxide at 10 ppm level. Moreover, *Trichoderma harzianum* showed more compatible with copper oxychloride (Suseela and Thomas, 2010). Whereas not all the fungal biocontrol agent are compatible with benomyl and companion at 10 ppm level (Table. 36 & 37). Earlier report supported that the benzimidazole group of benomyl very toxic to the fungal bioagents of *T. harzianum* and *T. longibrachiatum* at dose of 1µM (Viji *et al.* 1997). Hexaconazole showed lesser toxic growth of all the fungal bioagents followed by propiconazole (Table 36&37).

The present study examined that the pesticide of propargite (50 ppm) showed to be lesser toxic towards the growth of all the fungal bioagents followed by deltamethrin (50 ppm) (Table. 39). Our findings are in concordant with the studies by used propargite showed moderate to good compatibility with *Trichoderma harzianum* (Soumik *et al.* 2008). Previous reports showed that the fungal bioagent of *Trichoderma* spp sensitivity with different pesticides (Koomen, 1993).

In vitro studies clearly indicated that the fungal biocontrol agents of *Trichoderma* spp showed to be more tolerant with weedicides of ammonium salt of glyphosate (50 ppm) followed by glyphosate (50 ppm) (Table. 38). Previous report recommended that all the herbicides were found as compatibility with fungal biocontrol of *Trichoderma harzianum* at all the dosages *viz.*, 2000, 1000, 500 and 250 ppm (Gangwar, 2013). The present study examined that, the combination of biological and chemical for use in an integrated approach to pest, disease and weeds management on tea plantation.

V.16. Shelf-life studies

16.1. Shelf life of biocontrol agents based on talc powder formulation

Shelf life of talc based formulation of biocontrol agents was studied at different days (30, 60 and 90) interval. The CFU of *Bacillus licheniformis* (75.9×10^6 cfu/g) was recorded in higher population followed by *Bacillus amyloliquefaciens* (66.2×10^6 cfu/g) after 30 days of storage of formulation (Table. 40). This result is in agreement with Amer and Utkhede (2000) who reported the shelf-life of talc based formulation of *Bacilllus subtilis* (1.0×10^6 cfu/g) found in higher number after 45 days of storage. Moreover, talc based formulation of biocontrol agents showed good result against wilt pathogen of *Fusarium* sp. (Lingan Rajendran *et al.*, 2014).

The present study revealed that the same trend population of *Bacillus licheniformis* (45.0×10^6 cfu/g) and *Bacillus amyloliquefaciens* (31.6×10^6 cfu/g) was observed after 60 and 90 days of storage. Even after 90 days, 6.79×10^6 cfu/g to 20.47×10^6 cfu/g of population was recorded in all the biocontrol agents (Table. 40). After 90 days of storage, no population was recorded in all the biocontrol agents. Several scientists reported that the average shelf-life of

talc based formulation of *Trichoderma harzianum* was recorded after 4-5 months of storage (Sankar and Jeyarajan, 1996a and Ramanujam *et al.,* 2010). However, addition of chitin might increase the shelf-life of talc based formulation *Trichoderma harzianum* (Sriram *et al.,* 2010). Many earlier reports suggested the use of different type of carrier materials for long storage of fungal biocontrol agent of *Trichoderma* spp. (Sarode *et al.,* 1998 and Saju *et al.,* 2002).

16.2. Shelf life of biocontrol agents based on coir pith formulation

Shelf life of coir pith formulations of biocontrol agents were carried out at different (30, 60, 90 and 120) interval. The initial CFU of *Bacillus licheniformis* (131.14×10^6 cfu/g) showed higher population followed by population of fungal biocontrol agent *Trichoderma harzianum* (124.71×10^6 cfu/g) after 30 days of storage. The same trend was observed in *Bacillus licheniformis* (94.90×10^6 cfu/g) after 60 days of storage (Table. 41). Therefore, *Bacillus subtilis* (84.05×10^{-6}) was found in higher number after 60 days of storage (Table. 41). Earlier workers found coir pith formulation as best carrier material used for good colonization of *Trichoderma viride* (Kumar and Marimuthu, 1997). Finally, the population of all biocontrol agents declined gradually after120 days of storage. Our results are in concordance with earlier findings of Sankar and Jeyarajan (1996b) who observed that the viability of *Trichoderma* sp formulation was reduced after 120 days of storage. From this study, it can be concluded that higher shelf-life has been found in coir pith based formulation of biocontrol agents than talc based formulation.

V.17. Evaluation of chemical and biological control in glass house condition

17.1. Evaluation of chemicals and biocontrol agents

Bacterial and fungal biocontrol agents were isolated from different tea growing areas of southern India. The selected biocontrol and chemicals were tested against branch canker disease causal organism by *Macrophoma* sp. under glass house condition. Benomyl (70 %) was noticed higher disease protection against branch canker pathogen followed by Copper oxychloride (65%) (Table.42). The results are in agreement with Nepolean *et al.*(2014) Benomyl at lowest concentration (0.01 %) totally inhibited the wood rot pathogen followed by copper oxyxhloride. Moreover, the systemic fungicide of benomyl (0.2 %) gave an effective activity against *Macrophomina* blight of mungbean (Suryawansi *et al.,* 2008). In the

present study, Tebuconazole (60 %) and Tridemorph (55 %) documented consequently results (Table. 42). These findings are in concordance with Mareeswaran *et al.* (2015) Tubeconazole at the all the concentrations inhibited the growth of *Macrophoma* sp. under *in vitro* conditions.

The biological agents such as *Bacillus* sp. (50 %) and *Trichoderma viride* (50 %) showed significant results against branch canker disease (Table. 42). Many workers have reported that *Trichoderma* sp. controls many fungal diseases (D'Ercole *et al.,* 1988 and Chowdappa *et al.,* 2013). Das *et al.*(1966) have reported that application of *T. viride* in *Rhizoctoina solani* injected soils reduced sheath blight pathogen of rice. In *Pseudomonas* sp. (45 %) yielded moderate inhibition of *Macrophoma* sp. followed by *T. harzianum* (30%) (Table.42). Moreover, *Pseudomonas fluorescens* showed control of pea wilt caused by *Fusarium* .sp (Narinder Singh *et al.,* 2007).

17.2. Integrated management of branch canker disease

Chemical, biological and integrated methods were tested to control branch canker disease. The size of canker reduced significantly after imposing the treatments under greenhouse conditions. The results were recorded no significance in the treatments. The present study has clearly indicated that integrated spraying of Propiconazole/*Bacillus amyloliquefaciens* was reduced the size of canker followed by Propiconazole/*Bacillus subtilis*. The same effects were observed in Hexaconazole/*B. amyloliquefaciens* and Hexaconazole/*B. subtilis* (Table. 43). The similar alternative schedule was reported by Narinder Singh *et al.* (2007) control of pea wilt. Several scientists have reported that Hexaconazole and Propiconazole were more effective against brown root rot of *Fomes lamoensis* (Morang *et al.,* 2012). However, Upmanyu (1999) has also reported that the efficacy of hexaconazole against purple blotch disease of Onion. Similarly *B. subtilis* has been reported to reduce disease of alfalfa seedlings (Douvile and Bolland, 1992). Furthermore, *B. amyloliquefaciens* acts as an effective biocontrol against *F. oxysporum* f. sp. *cubense* (Yuan *et al.,* 2012).

In the present investigation, the combination fungicide of companion and propiconazole were found to be more efficient against *Macrophoma* sp. followed by

hexaconazole (Table. 43). The results are in line with Kishore Khosla (2007) the different schedule of fungicides was screened against blight of garlic. The botanical fungicide of Tricure (0.03 % Azadiractin EC) gave satisfactory result followed by *Gliocladium virens* (Table.43). In the present findings are in concordance with Anonymous (2015) the organic fungicide of Tricure (8 %) showed complete inhibition of the growth of *Macrophoma* sp. under *in vitro* conditions. Moreover, *Gliocladium* has been reported as biological control of *Macrophomina phaseolina* in sesamum (Sankar and Jeyarajan, 1996). The investigation mainly highlights the integrated approaches of branch canker disease caused by *Macrophoma* sp.

V. 18. Integrated disease management

18.1. Evaluation of chemical and biological control in field conditions

Influence of different managing practices on branch canker disease assessed in the field conditions under integrated disease management (IDM). The size of branch cankers reduced considerably after the wound pasting treatment under field conditions (Table. 44). *Bacillus* sp. with systemic fungicide of Benomyl (0.05 %) and *Bacillus* sp. along with Tebuconazole (0.05 %) followed by contact fungicide of copper oxychloride (0.05 %) with *Bacillus* sp. gave good disease protection score followed by Tridemorph (0.05 %) with *Bacillus* sp. *Trichoderma viride* and *Trichoderma harzianum* exhibited very slow response on disease control. Without biocontrol of Benomyl (0.05 %), it was effective as compared with contact fungicide of copper oxychloride alone (0.05 %) (Table.44).

The reduction in canker size was maximum with treatments where integrated disease management of applied wound pasting. The control of canker size of (**T2**) length 23.87cm by width 3.27cm reduced to a length 19.70 cm by width 2.37 cm (DPS, 5.07 cm). Similarly, the control canker size of (**T4**) length 18.30 cm by width 2.80cm was reduced to length 16.03cm by width 1.93cm (DPS, 3.14 cm) followed by the integrated treatment of (**T10,** DPS**,** 3.03 cm) & (**T12,** DPS, 2.70 cm). However, there was a moderate results were noticed in (**T3**, DPS, 2.73 cm) Benomyl + *Pseudomonas* sp. and (**T11**, DPS, 2.39 cm) Copper oxychloride + *Pseudomonas* sp. followed by (**T13**, DPS, 2.37 cm). Expel 5 % (Botanical fungicide) showed significantly difference among these treatments. In case of untreated control, the canker size was larger than the treated plants (Table.44).

The present study revealed that the systemic fungicide without combinations of biocontrol agent, benomyl was more effective against branch canker disease pruned field conditions. Similar results were noticed by Hester (1973), Smyly (1973) and Paul (1980). The systemic fungicide was recorded higher values of control against branch canker disease in Sri Lanka. Moreover, efficacy of benomyl was examined *in vitro* and *in vivo* conditions and controlling in wood rot disease caused by *Hypoxylon serpens* (Onsando, 1986, Onsando, 1989 and Otieno, 19996). Previous report states that the systemic fungicide of Benomyl, Carbendazim and Companion were found to be very effective in control of branch canker disease under *in vitro* level (Mareeswaran and Nepolean, 2016). In an earlier study, it was reported that selective surgery and protection of wounds by applying copper oxychloride paste common practice followed to control the stem diseases in India (Premkumar and Baby, 2005).

In the present study the results recommends that the contact fungicide of copper oxychloride showed minimum disease protection (1.03 cm). Biocontrol agents, *Bacillus* sp. and *Pseudomonas* sp. along with copper oxychloride reduced the canker size. The study proved that the tested biocontrol agent along with tridemorph is effective in controlling branch canker disease. This line is in agreement with Mareeswaran *et al.* (2015) the bacterial agent *Bacillus* sp and *Pseudomonas* sp and fungicides tridemorph and tebuconazole showed highest antagonistic potential against branch canker disease under *in vitro* level.

Moreover, Dust formulations of carbendazim and tridemorph were found to be effective in controlling powdery milder (*Odium heveae*) and dry rot (*Ustilina desta*) in rubber (Jacob *et al.*, 1996 and Joy and Jacob, 2000). It has been suggested that *Trichoderma* sp. bioformulations proved to be effective in control of thorny stem blight disease (Chandramouli and Baby, 2002) and *Phomopsis* canker disease of tea (Ponmurugan and Baby, 2005). *Trichoderma* and *Gliocladium* sp. protected plum and peach from silver leaf caused by *Chondrostereum purpureum* when applied on the pruning cut (Barges, 1981). These studies suggest that spraying of the bacterial culture filtrate agents to reduced disease development in tea pathogens was reported, earlier by Chakraborthy *et al.* (1998). Similarly, a cell free culture of *Bacillus subtilis* has been reported to reduce disease incidence on

Alfalfa seedling by Douville and Boland (1992). Bacterial biocontrol of *Pseudomonas* sp. exhibited strong antifungal property against tea pathogens was reported by Chakraborthy *et al.* (1994).

It may be concluded that biocontrol agents can be incorporated with chemical fungicides in the integrated management of branch canker disease in tea fields. It has been suggested that branch canker disease can be controlled by cultural practices besides the eradications methods likely IDM which are ecofriendly system.

18.2. Evaluation of Integrated schedule against branch canker disease

The canker size was reduced significantly after explore of treatments under field condition. Generally, an integrated applications method showed more effectively than straight applications of fungicides. Among the various treatments, the present study revealed that highest reduction of canker size was measured with an integrated treatment of Companion/*B.amyloliquefaciens* followed by straight application of Companion (Table. 45). The moderate reduction of canker size was recorded with an integrated spraying of Copper oxychloride/*B.amyloliquefaciens* followed by separate application of Copper oxychloride (Table. 45). Furthermore, bacterial strain of *B.amyloliquefaciens* was considerably reduced canker size followed by the treatment of organic fungicides Tari (Organic Tea Special) and Tricure (0.03% Azadiractin). The lowest canker size was noted with fungal biocontrol agent of *Gliocladium virens*, *Beauveria bassiana* and *Trichoderma harzianum*. The untreated control plants clearly indicated that canker size was increased than the treated plants (Table. 45). Present examine showed that integrated application of Companion/*B.amyloliquefaciens* was found to be good result against branch canker disease followed by unaccompanied treatment of Companion (Table. 45). An earlier finding also supported that combination fungicide of Companion (mancozeb 63 % + carbendazim 12 %) showed good result against the root rot disease of chilli causal organism by *Sclerotium rolfsii* (Roy *et al.,* 2010). Moreover, the superior effect of Companion in controlling the botrytis gray mold disease of paprika plant causal agent by *Botrytis cinerea* (Yoon *et al.,* 2008).

The result of Copper oxychloride/*B.amyloliquefaciens* was noticed adequately against *Macrophoma theicola* (Table. 45). This line is in pledged with the contact fungicide of

Copper oxychloride has been reported as against the late blight and early blight in potato plant (Ferreira *et al.,* 2014). Mostly canker size was reduced in respective treatments due to foremost reason of *B.amyloliquefaciens*. Thus, the treatment of *B.amyloliquefaciens* was significantly reduced canker size when compared with other treatments of organic fungicides and fungal biocontrol agents (Table. 45). In our study concordance with Abdullah *et al.* (2008) bacteria strain of *B.amyloliquefaciens* has been act as powerful biocontrol against plant pathogens.

The results of organic fungicides obviously indicated that Tari (Organic Tea Special) and Tricure (0.03 % Azadiractin) were reduced minimal level of canker size (Table. 45). The similar observations were made with Mareeswaran *et al.* (2016) the same organic fungicides of Tari (Organic Tea Special) and Tricure (0.03 % Azadiractin) were noticed minimum growth inhibition against branch canker pathogen under *in vitro*. Finally, the fungal biocontrol of *Gliocladium virens* was noted lowest level of canker size (Table. 45). The result is in negatively correlated with the treatment of *Gliocladium virens* has been reported as better control of collar canker in tea plants (Ponmurugan and Baby, 2007).

From the above results, it might be concluded that branch canker disease has been successfully recovered from infected field by using integrated application of chemicals and biological control methods. As a final point, an integrated application of Companion/*Bacillus amyloliquefaciens* has been identified as superior control of branch canker disease under field condition.

Chapter-VI

Summary / Conclusion

Branch canker pathogen was isolated from different tea growing districts of southern India. These isolates were identified as *Macrophoma* spp (code as NBCHE-6, NBCC-2, VPM, UPA-61 and UPA-62) through molecular technique of 18S rRNA method.

The pathogen was tested on the two year old nursery plant and brownish colour was noticed on sixth day of after inoculation. The black colour fungal mycelium with spores was developed on the 18th day. A well-developed black colour tome shaped of pycnidia production was observed on the 43rd day after inoculation.

The growth of the pathogen was shown to be maximum in PDA followed by Richard medium. The temperature ranges between 25 ºC to 30 °C and the optimum pH ranges between 5.0 -5.5 favoured the growth of branch canker fungus

Carbohydrate and fructose were the favourable carbon sources for supporting the growth of pathogen. The maximum growth of the fungus was noticed in the nitrogen source of glycine compound. The toxic compounds of branch canker pathogen were studied through GC-MS analysis.

The carbohydrate level showed decreasing in all the infected clones when compared to the healthy clone. Similarly reducing sugar content was decreased in the infected clones except the clone of TRI-2025. The NPK level was recorded lower in all the infected clone. Moreover, there is no major variation level of N content in the infected and healthy plants of clone Yabukita. Therefore, the fungal pathogen was utilized the primary metabolism of carbohydrate and sugar from the infected plants.

This study revealed that tolerant clones of UPASI-9, UPAS-17, UPASI-26, TRF-4 and TRI-2025 were described by the absence of spore formation and canker size less on the respective clones. So these clones have been identified as tolerance varieties through histopathologically characterization. Therefore these plant materials are recommended for further disease prone areas.

The biological control was isolated from different tea growing region of southern India and their level of antagonistic activity against branch canker disease. The bacterial strains were identified by 16S rRNA level and fungal bioagents like as *Trichoderma* spp and *Gliocladium virens* were identified based on the colony morphology and spore characteristic feature.

Fungal biocontrol of *Gliocladium virens* and *Trichoderma harzianum* provided satisfactory control of branch canker pathogen. Bacterial biocontrol of *Bacillus amyloliquefaciens* and *Bacillus subtilis* proved their antagonistic potential against branch canker pathogen. Further, the entomopathogens of *Beauveria bassiana* showed highest antagonistic activity against *Macrophoma* spp.

Plant aqueous extract of *Acrous calamus, Curcuma longa* and *Hibiscus rosasinensis* were also effective.

Among the various fungicides, Benomyl, Carbendazim and Companion showed 100 % growth inhibition against *Macrophoma* sp at 10 ppm level followed by propiconazole and hexaconazole**.** The contact fungicide of copper oxychloride was noticed highest growth inhibition against branch canker pathogen. Biofungicides of Expel, Enroot and Attopsy were found to be efficient results against *Macrophoma* spp. at 0.1 % concentration level.

The efficacy of fungicides and biocontrol were evaluated against branch canker disease under glass house experiment. In the results revealed that systemic fungicide of Benomyl provided satisfactory control of branch canker disease followed by Copper oxychloride. Biological control of *Bacillus* sp. and *Trichoderma viride* yielded significant activities.

The integrated application of Propiconazole/*Bacillus amyloliquefaciens* was found to be good result followed by combination fungicide of Companion and Propiconazole were most effectively under glass house conditions.

In commonly used agrochemical in tea plantation *viz.*, pesticides, fungicides and weedicides were tested for tolerance level with biocontrol agents under *in vitro* level. Bacterial biocontrol agents were found to be most tolerant with above said agrochemical compared with the fungal biocontrol of compatibility studies.

The shelf life of bacterial and fungal biocontrol agents were tested in coir pith and talc based formulations. Results indicated that the CFU level was higher in coir pith formulation when compared than talc based formulation.

A field study was conducted to screen the integrated disease management (IDM) practices against branch canker disease. Thus, management of the branch canker disease in Benomyl along with bacterial biocontrol of *Bacillus* sp. was found to be very effective.

Our results proved that integrated application of Companion/*Bacillus amyloliquefaciens* controlled the branch canker disease well under field condition. Future it could be studied by conducting multi locational field trails. The outcome of this study resulted in evolving an integrated approach to control the branch canker by minimal use of chemical fungicides.

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*Originals not referred

APPENDIX – I

Chemicals, glassware's and media used

Chemicals and glassware

Analytical grade chemicals (Himedia and E.merck) and Borosil brand of glass ware were used for the studies.

Cleaning solution for glassware

Potassium dichromate - 80.0 g

Distilled water - 300.0 ml

Concentrated sulphuric acid - 400.0 ml

The glassware were cleaned with detergent solution and then immersed in cleaning solution for 12 h, washed with tap water then rinsed with distilled water and dried in a hot air oven before use.

Sterilization

Culture media and glassware except petri plates were sterilized in an autoclave at 15 p.s.i for 25 minutes. Petriplates were sterilized at 150°C in a hot air oven overnight. The thermolabile compounds were filter sterilized using membrane filtration unit.

Media

Water agar (WA)

Agar - 20.0 g

Distilled water - 1000.0 ml

Potato dextrose agar (PDA)

Peeled potato - 200.0 g

Dextrose - 20.0 g

Agar - 20.0 g

Distilled water - 1000.0 ml

Peeled potato were cut into small bits and boiled with 500 ml of water for 30 min. and the extract was filtered through strainer. To the extract dextrose was added. Agar was melted with another half of water and mixed with the extract and final volume was made upto1000 ml before sterilization.

Potato dextrose broth (PDB)

PDB was prepared as explained above without agar.

Potato dextrose yeast agar (PDYA)

Potato - 200.0 g

Dextrose - 20.0 g

Yeast- 10.0 g

Agar - 20.0 g

Distilled water - 1000.0 ml

Yeast extract malt agar (YEMA)

Malt extract - 25.0 g

Yeast- 10.0 g

Agar - 20.0 g

Distilled water - 1000.0 ml

Yeast extract agar (YEA)

Yeast extract - 10.0 g

Dextrose - 10.0 g

Agar - 15.0 g

Distilled water - 1000.0 ml

Richard's synthetic agar (RSA)

Potassium nitrate - 10.0 g

Potassium dihydrogen phosphate - 5.0 g

Magnesium sulphate - 2.50 g

Ferric chloride - 0.02 g

Sucrose - 50.0 g

Agar - 20.0 g

Distilled water - 1000.0 ml

Mineral salt agar medium (MSM)

Sucrose - 1.0 g

Ammonium nitrate - 1.0 g

Dipotassium hydrogen phosphate - 0.9 g

Potassium chloride - 0.2 g

Magnesium sulphate - 0.2 g

Manganese sulphate - 0.002 g

Potassium chloride - 0.5 g

Ferrous sulphate - 0.002 g

Zinc sulphate - 0.002 g

Agar - 20.0 g

Distilled water - 1000.0 ml

Mineral salt broth (MSB)

MSB was prepared as explained above without agar.

Czapek's Dox agar (CDA)

Sucrose - 30.0 g

Sodium nitrate - 2.0 g

Dipotassium phosphate - 1.0 g

Magnesium sulphate - 0.5 g

Potassium chloride - 0.5 g

Ferrous sulphate - 0.01 g

Agar - 20.0 g

Distilled water - 1000.0 ml

Rose Bengal agar (RBA)

Peptone - 5.0 g

D- Glucose - 10.0 g

Dipotassium hydrogen phosphate – 1.0 g

Magnesium sulphate – 0.500 g

Rose Bengal powder – 0.036 g

Agar - 20.0 g

Distilled water - 1000ml

Sabouraud's dextrose agar (SDA)

Peptone - 10.0 g
Dextrose - 40.0 g
Agar - 15.0 g
Distilled water - 1000ml

King's B medium (King *et al.,* 1954)

Proteose peptone – 20 g

Potassium sulphate – 10 g

Magnesium chloride – 1.64 g

Agar – 15 g

Distilled water - 1000ml

Fungal Indian Standard medium (FIS)

Yeast extract – 5 g

Dextrose – 20 g

Chloramphenical – 0.1 g

Agar – 20 g

Distilled water – 1000 ml

Soil extract media preparation

The soil samples were collected and boiled with 500 ml of water for 30 min. The soil extract was filtered through strainer.

Soil extract – 100 ml

Glucose – 2.5 g

Agar – 20 g

Distilled water – 1000 ml

***Trichoderma* Selective Medium (TSM)** (Elad and Chet, 1983)

Magnesium sulphate 0.20 g, dipotassium hydrogen phosphate 0.90 g, potassium chloride 0.15 g, ammonium nitrate 1 g, *Chloramphenicol 0.25 g, *Dexon 0.30 g, *Terractor 0.20 g (Plantachloro nitrobenzene), *Captan 0.02 g, *Dextrose 3g, Rose Bengal 0.15 g and Agar 20 g in 1 L of distilled water.

Nutrient Agar/ Nutrient Broth

Peptone 5.0 g, Beef extract 3.0 g, Agar* 20.0 g, Distilled water 1000 mL, pH 6.8- 7.0

* Agar was avoided in nutrient broth

Luria Bertaini medium (LB)

Tryptone 10.0 g, Yeast extract 5.0 g, Sodium chloride 10.0 g, Agar 18.0 g, Distilled Water 1000 ml, pH 7.2

Stain

Lacto phenol cotton blue

Cotton blue (1% aqueous solution) 2.00 g, Phenol crystals 20.00 g, Lactic acid 20.00, Glycerin 40.00 g

Appendix-II

Morphological and biochemical tests for bacterial isolates

The various morphological and biochemical tests were carried out according to the methods proposed by Stolp and Gadkari (1981).

1) Gram staining

Gram's crystal violet

Solution A

Crystal violet	-	2.0g
Ethanol	-	20.0mL

Solution B

Ammonium oxalate	-	0.8g
Distilled water	-	80.0mL

Gram's iodine

Iodine	-	1.0g
Potassium iodide	-	2.0g
Distilled water	-	50.0mL

Counter stain

Safranine	-	0.25g
Ethanol	-	10.0mL
Distilled water	-	90.0mL

The selected strains were uniformly spread on a clean glass slide and heat fixed by intermittent heating. A few drops of crystal violet was added and incubated for a min, followed by flooding with Gram's iodine solution. After a min, the slide was washed under running tap water for a few seconds and blot dried. It was decolorized with Gram's decolourizer (70 % ethanol) and immediately rinsed with tap water. The slide was then

flooded with safranine solution for a min and rinsed with tap water, blot dried and observed under a light microscope.

2) Oxidase test

A loopful of the selected strain was rubbed over the oxidase disc (Himedia, Mumbai, India) with the help of a clean glass rod. Then the colour change in the oxidase disc was observed.

3) Catalase test

A loopful of the selected cultures was transferred from agar plate to a sterile glass slide. Immediately, a drop of 3 % H_2O_2 was added onto the culture and observed for rapid evolution of air bubbles.

4) Methyl red and Voges-ProsKauer (MR -VP) Test

Test tubes containing MR-VP broth was prepared and sterilized by autoclaving. A loopful of 24 h old bacterial culture was inoculated into the tubes and incubated for 24 h at 37 °C. The broth was then divided into two parts. In one part, methyl red indicator was added and to other part Barrits reagent was added. Appearance of red colour indicates positive result for methyl red, whereas formation of pink colour indicates positive VP test. Uninoculated tubes served as control for both the tests.

MR-VP medium

Peptone	-	7.0g
Glucose	-	5.0g
Di potassium hydrogen phosphate	-	5.0g
Distilled water	-	1000mL
pH	-	6.9

Methyl red indicator

Methyl red	-	10.0g
Ethanol	-	300mL
Distilled water	-	1000mL

Voges -Proskauer indicator

Barrits A reagent

Alpha naphthol	-	5.0g
Ethanol	-	100mL

Barrits B reagent

Potassium hydroxide	-	40.0g
Distilled water	-	100mL

List of Publications

1. **J. Mareeswaran and** Premkumar Kumar Samuel Asir.R. (2017). Integrated management of branch canker disease (*Macrophoma* sp.) in tea under field level. *Journal of Plant Diseases and Protection*. 124(2), pp-115-119. DOI. 10.1007/s41348-017-0072-1. (**NAAS Score:6.48)**

2. **J. Mareeswaran**, P. Nepolean, R. Jayanthi, R. Premkumar Samuel Asir and B. Radhakrishnan. (2016) Bio efficacy of efficient entomopathogenic fungus against branch canker pathogen (*Macrophoma theicola*) in tea plantations of southern India. *Indian Journal of Agricultural Sciences*.86 (2): 242-6. **(NAAS Score:6.14)**

3. **J. Mareeswaran** and R. Premkumar Samuel Asir. (2016). Biochemical and nutritional changes due to branch canker disease (*Macrophoma theicola*) in tea. *Journal of Plant Disease Sciences*. Vol: 11 (1):29-31. (**NAAS Score:2.65)**

4. **J. Mareeswaran** and P. Nepolean. (2016). In vitro screening of chemical and organic fungicides against branch canker disease in tea. *Journal of Mycopathological Research*. 54(2):299-301. (**NAAS Score:3.17)**

5. **J. Mareeswaran** and Radhakrishnan.B. (2016). Shelf life of bacterial and fungal Biocontrol agents in different formulations. *Indian Phytopathology*. 69(4):422-423. (**NAAS Score: 4.59)**

6. **J. Mareeswaran**, P. Nepolean, R. Jayanthi, R. Premkumar Samuel Asir and B. Radhakrishnan. (2015) *In vitro* studies on branch canker pathogen (*Macrophoma* sp) infecting tea. *Journal of Plant Pathology and Microbiology*. 6:284. DOI: 10.4172/2157-7471.100284.

J Plant Dis Prot
DOI 10.1007/s41348-017-0072-1

ORIGINAL ARTICLE

Integrated management of branch canker disease (*Macrophoma* sp.) in tea under field level

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Received: 9 June 2016 / Accepted: 10 January 2017

Abstract A field study was conducted to screen the integrated disease management practices against branch canker disease caused by *Macrophoma* sp. In the present study, screening of four fungicides, two bacterial and fungal biocontrol agents were used at 0.05%. Benomyl with *Bacillus* sp. (DPS, 5.07 cm) found to be superior to copper oxychloride alone (DPS, 1.03 cm) in controlling branch canker disease. Tebuconazole + *Bacillus* sp. (DPS, 3.14 cm) and copper oxychloride + *Bacillus* sp. (DPS, 3.03 cm) excelled well. Botanical fungicide of Expel (5%) (DPS, 2.07 cm) significantly reduces the canker size as compared with other treatments. *Trichoderma viride* (DPS, 1.27 cm) and *Trichoderma harzianum* (DPS, 1.30 cm) usage in disease management showed the branch canker disease at very low level. The treatment with Benomyl along with *Pseudomonas* sp. (DPS, 2.73 cm) and tridemorph + *Bacillus* sp. (DPS, 2.70 cm) exhibits the reduction in the size of canker followed by copper oxychloride with *Pseudomonas* sp. (DPS, 2.39 cm) and tridemorph with *Pseudomonas* sp. (DPS, 2.37 cm). Without the usage of biocontrol, Benomyl (DPS, 4.83 cm), higher values of branch canker disease were observed in the control. Thus, management of the branch canker disease with Benomyl along with bacterial biocontrol of *Bacillus* sp. found to be very effective (DPS, 5.07 cm). In the case of untreated control, the canker size was increased than the treated plants.

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Keywords Biological control · Chemical fungicides · Field study · Nucleotide sequence · *Macrophoma* sp.

Abbreviations

T	Treatments
DPS	Disease Protection Score

Introduction

Tea is one of the most common and cheapest beverages in the world. Tea is manufactured from the crop shoots comprising at two to three leaves and a bud. The perennial shrub belongs to *Camellia* sp. in *Theaceae* family. It is commercially grown in more than 80 countries. India is the largest producer and consumer of black tea in the world [29].

Majority of tea pathogens are of fungal origin, and more than 300 species of fungi are reported to affect different parts of the tea plant [1, 11]. Stem diseases are reported to cause yield stagnation. Branch canker caused by *Macrophoma* sp. is most widespread and serious stem disease of tea plants nowadays. The practices of pruning tea bushes expose the woody stems to many parasitic fungi, and dry weather pruning exposes the frame to sun scorch injuries [27]. In Sri Lanka, *T. viride*, *Penicillium* sp. *Aspergillus niger* and *Bacillus* sp. showed antagonistic to stem rot caused by *Nimania diffusa* [4]. The action of *Trichoderma* and *Gliocladium* sp. against certain primary root diseases under in vitro condition has been studied [3]. Botanical fungicides, biological agents and chemical fungicides have been attempted against branch canker disease in tea plant under laboratory conditions [19, 20]. Even though time to time recommendations on these diseases are released with respect to cultural operations and chemical control

Published online: 18 January 2017

measures, no full-fledged integrated management of disease is available. Biological control of plant pathogens have been considered as potential control strategy in recent years. Minimizing the level of chemical and incorporating biological control agents under integrated management approach has been considered as a potential control strategy against branch canker disease. Evaluation of chemical, botanical and biological fungicides will be helpful to develop an appropriate control strategy for the management of branch canker disease in tea. In the present study, attempts made to control branch canker disease with fungicides and biocontrol agents under field conditions.

Materials and methods

Field experiment for an integrated management of branch canker disease was conducted at UPASI Tea Research Foundation, Tea Research Institute, Valparai located at 10° 23′ North and 77° 0′ East and about 1050 m above mean sea level. The bushes pruned at 60 cm height from ground level and treatments imposed. There were 15 treatments with three replications. All the treatments laid out in randomized block design. The branch canker disease protection calculated by using following formula, wound healing (WH) = $(L_1 - L_2) + (W_1 - W_2)$, where L_1—(pre-treatment) canker length in cm, L_2—(post-treatment) canker length in cm, W_1—(pre-treatment) canker size in width (cm), W_2—(post-treatment) canker size in width (cm). The canker size values are measured by before application of pre-treatment (length and width in cm) and after application of six months measured by post-treatment (length and width in cm).

The efficacy of three systemic fungicides, Benomyl 0.5 g (Benofit 50% WP), tebuconazole 0.5 g (Folicur 250 EC), tridemorph 0.5 g (Calixin 80% EC), contact fungicide of copper oxychloride 0.5 g (Fytolon 50% WP) and botanical extract of Expel (5% combination of canolar extract and tea tree oil procured from M/s Advance pesticides Pvt. Ltd, Nasik, Maharashtra) were selected and applied as wound paste. In this experiment, biocontrol agents' application (99.5 g) and chemical fungicides (0.5 g) were mixed with help of painting brush (total 100 g/bush) for making wound pasting after pruning. The bioformulation (1:1 ratio), talc (1.0 kg) + biocontrol agent (1 liter liquid culture) and chemical fungicides: 0.5 g + 99.5 g of biocontrol talc formulation to make it 100 g/bush. The chemical fungicides along with bioformulations (100 g) were applied. The adjuvant, linseed oil was used to make pasting and wound healing properties. For wound dressing biofungicides application was made into a paste with linseed oil and applied on the pruning cut with help of painting brush. The talc preparations were made into paste with distilled water (1:2 ratio) and applied on the pruned cuts and canker portion [26].

Isolation and identification of pathogen

The pathogen was isolated from naturally infected stem portion of tea bushes. The canker portions selected and placed on moistened filter paper in a Petri dish to induce oozing out of pycnidia. After 5 days incubation, the pycnidia oozed out from canker portions which were viewed under microscope to examine (Olympus microscope-Model CH20iBIMF) the structure and morphological characters of the fungus were confirmed as *Macrophoma* sp. Even though the section of pycnidium showed the spores under 45× lenses compared with standard reference images followed by the book of Diseases of Tea Bush [25] and Illustrated Genera of Imperfect Fungi [6]. The pure culture of the fungus again explored under microscopic level. The molecular identification of branch canker pathogen carried out using the molecular technique which includes DNA isolation, PCR amplification, sequencing and phylogenetic analysis is identical to those used by Crous and Palm [12]. PCR was performed with primer pairs targeted to the 18 s rRNA genes. The PCR-positive samples were identified by DNA sequencing of the internal transcribed spacer (ITS) region of the rRNA gene. The sequences were then analyzed using the option basic local alignment search tool (BLAST) software available in NCBI (accession No. JQ234977). In similarity search for the nucleotide sequence of (*Macrophom*a sp.) 18 s rRNA using a blast search methods at NCBI [2]. This fungal isolate was identified as *Macrophoma* sp. through molecular tools and submitted to NCBI for public domain.

Isolation and identification of biocontrols

The fungal biocontrol agents, *T. harzianum* and *T. viride* were isolated from the native tea soil (the Anamalis, Vandiperiyar and Munnar) and identified. The biocontrol agents were isolated by standard serial dilution techniques followed by biochemical tests as listed in Bergy's manual of determinative bacteriology [18] for *Pseudomonas* sp., whereas *Trichoderma* sp. was identified as reported by Barnett [7] and molecular identification of bacterial biocontrol agents using 16S rRNA methods. *Bacillus* sp. (accession No. JN616372) and *Pseudomonas* sp. (accession No. JQ319656) were explored. Seven-day-old culture of the fungal mat extracted and mixed with sterilized talc powder along with (0.5%) carboxy methyl cellulose (CMC) as adhesive agent. The bacterial suspension mixed next sterilized at 110 °C for 24 h with calcium carbonate to adjust the pH to neutral and CMC sterile conditions by Vidhyasekaran and Muthamilan [31].

Statistical analysis

The statistical analysis of obtained data was carried out using analysis of variance (ANOVA), and significant means segregated by critical difference (CD) at various levels of significance were presented [14].

Results

The effect of different managing practices on branch canker disease was assessed in the field conditions under integrated disease management (IDM). In this fungal pathogen sequence was compared with other fungal genomes from the databases given in NCBI and a phylogenetic tree constructed (Fig. 1). The results indicated that the strain of NBCHE-6 belongs to *Macrophoma* sp. and shared 99% similarity with *Macrophoma* sp. DQ100415.1, DQ100414.1 and *Macrophoma* sp. HQ262514.1 and less than 97% similarity to the remaining species of the group ascomycetes. The size of branch cankers reduced considerably after the wound pasting treatment under field conditions (Table 1). *Bacillus* sp. with systemic fungicide of Benomyl (0.05%) and *Bacillus* sp. along with tebuconazole (0.05%) followed by contact fungicide of copper oxychloride (0.05%) with *Bacillus* sp. gave good disease protection score followed by tridemorph (0.05%) with *Bacillus* sp. *Trichoderma viride* and *Trichoderma harzianum* exhibited very slow response on disease control. Without biocontrol of Benomyl (0.05%), it was effective as compared with contact fungicide of copper oxychloride alone (0.05%). The applied wound pasting treatment shows maximum reduction in canker size where integrated disease management was observed. The control of canker size of (T2) length 23.87 cm by width 3.27 cm reduced to a length 19.70 cm by width 2.37 cm (DPS, 5.07 cm). Similarly, the control canker size of (T4) length 18.30 cm by width 2.80 cm was reduced to length 16.03 cm by width 1.93 cm (DPS, 3.14 cm) followed by the integrated treatment of (T10, DPS, 3.03 cm) and (T12, DPS, 2.70 cm). However, moderate results were noticed in (T3, DPS, 2.73 cm) Benomyl + *Pseudomonas* sp. and (T11, DPS, 2.39 cm) copper oxychloride + *Pseudomonas* sp. followed by (T13, DPS, 2.37 cm). Expel 5% (botanical fungicide) showed significantly difference among these treatments. In case of untreated control, the canker size was larger than the treated plants.

Discussion

The present study revealed that the systemic fungicide without combinations of biocontrol agent Benomyl was more effective against branch canker disease-pruned field conditions. Similar results were noticed by Hester [15] Smyly [30] and Paul [24]. The systemic fungicide was recorded higher values of control against branch canker disease in Sri Lanka. Moreover, efficacy of Benomyl was examined in vitro and in vivo conditions and controlling in wood rot disease caused by *Hypoxylon serpens* [21–23]. Previous report states that the systemic fungicide of Benomyl, Carbendazim and Companion were found to be very effective in control of branch canker disease under in vitro level [19]. In an earlier study, it was reported that selective surgery and protection of wounds by applying copper oxychloride paste common practice followed to control the stem diseases in India [28]. In the present study the results recommends that the contact fungicide of copper oxychloride showed minimum disease protection (1.03 cm). Biocontrol agents *Bacillus* sp. and *Pseudomonas* sp. along with copper oxychloride reduced the canker size. The study proved that the tested biocontrol agent along with tridemorph is effective in controlling branch canker disease. This line is in agreement with Mareeswaran et al. [20] the bacterial agent *Bacillus* sp and *Pseudomonas* sp and fungicides tridemorph and tebuconazole showed highest antagonistic potential against branch canker disease under in vitro level.

Moreover, dust formulations of carbendazim and tridemorph were found to be effective in controlling powdery milder (*Odium heveae*) and dry rot (*Ustilina desta*) in rubber [16, 17]. It has been suggested that *Trichoderma* sp. bioformulations proved to be effective in control of thorny stem blight disease [10] and *Phomopsis* canker disease of tea [27]. *Trichoderma* and *Gliocladium* sp. protected plum and peach from silver leaf caused by *Chondrostereum*

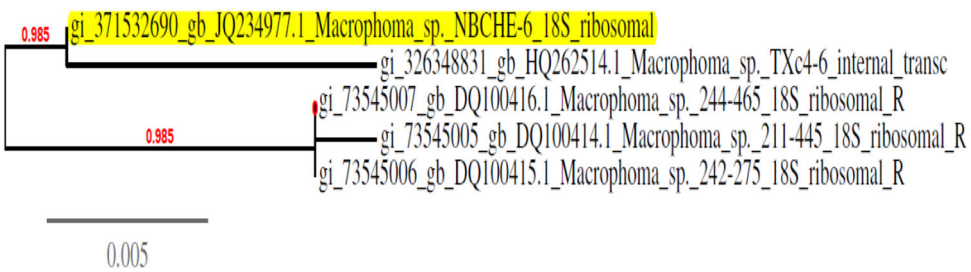

Fig. 1 Phylogenetic tree analysis of 18S rRNA gene method

Table 1 Integrated management of branch canker disease in tea

Treatments details	Branch canker size (cm)				Disease Protection Score
	Pre-treatment		Post-treatment		Wound healing (WH)
	Length (L_1)	Width (W_1)	Length (L_2)	Width (W_2)	WH = ($L_1 - L_2$) + ($W_1 - W_2$) in cm
Benomyl	19.83	3.17	15.70	2.47	4.83
Benomyl + *Bacillus* sp.	23.87	3.27	19.70	2.37	5.07
Benomyl + *Pseudomonas* sp.	24.60	2.83	22.17	2.53	2.73
Tebuconazole + *Bacillus* sp.	18.30	2.80	16.03	1.93	3.14
Tebuconazole + *Pseudomonas* sp.	14.20	1.50	12.43	1.03	2.24
Trichoderma harzianum	3.30	2.53	2.50	2.03	1.30
Trichoderma viride	3.20	2.27	2.43	1.77	1.27
Bacillus sp. + *Pseudomonas* sp.	16.03	1.60	14.93	0.93	1.77
Copper oxychloride	6.50	3.23	5.90	2.80	1.03
Copper oxychloride + *Bacillus* sp.	23.43	4.43	21.60	3.23	3.03
Copper oxychloride + *Pseudomonas* sp.	14.50	2.03	12.67	1.47	2.39
Tridemorph + *Bacillus* sp.	27.47	2.10	25.33	1.53	2.70
Tridemorph + *Pseudomonas* sp.	9.60	1.93	7.80	1.20	2.37
Expel 5% (botanical fungicides)	23.10	2.30	21.63	1.70	2.07
Control	35.10	6.17	38.63	9.50	−6.86
CD at 5%	9.1	1.5	5.5	1.4	

Canker size (length and width in cm) was measured before (pre-treatment) and after (post-treatment) treatment and their values are given

Bioformulation (1:1 ratio): Talc (1 kg) + Biocontrol agent (1 L liquid culture): 0.5 g + 99.5 g of biocontrol talc formulation to make it 100 g/bush. The chemical fungicides along with bioformulations (100 g) for wound pasting method. Adjuvant: Line seed oil use to make pasting and wound healing properties

T treatments, *DPS* Disease Protection Score

purpureum when applied on the pruning cut [5]. These studies suggest that spraying of the bacterial culture filtrate agents to reduced disease development in tea pathogens was reported, earlier by Chakraborthy et al. [9]. Similarly, a cell-free culture of *Bacillus subtilis* has been reported to reduce disease incidence on alfalfa seedling by Douville and Boland [13]. Bacterial biocontrol of *Pseudomonas* sp. exhibited strong antifungal property against tea pathogens was reported by Chakraborthy et al. [8].

Conclusion

It may be concluded that biocontrol agents can be incorporated with chemical fungicides in the integrated management of branch canker disease in tea fields. It has been suggested that branch canker disease can be controlled by cultural practices besides the eradications methods likely IDM which are ecofriendly system.

Acknowledgements The authors are thankful to Dr. B. Radhakrishnan, Director, UPASI Tea Research Foundation, Tea Research Institute, Valparai for providing the necessary facilities, constant encouragements and grateful to Tea Board of India, Kolkata, for the financial support. We wish to extend our heartfelt thanks to University of Calicut, Kerala, for affiliation of Doctoral studies to UPASI Tea Research Foundation, Tea Research Institute, Valparai.

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Indian Journal of Agricultural Sciences **86** (2): 242–6, February 2016/Article

Bioefficacy of efficient entomopathogenic fungus against branch canker pathogen (*Macrophoma theicola*) in tea plantations of southern India

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Received: 9 June 2015; Accepted: 28 September 2015

ABSTRACT

Three branch canker pathogens, viz. NBCHE-6, UPA-61 and VPM were isolated from different tea growing districts of south India and four entomopathogenic fungus, viz. *Beauveria bassiana*, *Paecilomyces lilacinus*, *Lecannicillium lecannii* and *Paecilomyces fumosoroseus* were procured from the microbial type culture collection and gene bank (MTCC), Chandigarh. *In vitro* studies revealed that *Beauveria bassiana* showed highest antagonistic effect against NBCHE-6 (64.22) followed by *Paecilomyces fumosoroseus* against UPA-61(56.66). *Paecilomyces lilacinus* significantly controlled VPM (54.66), while *Lecannicillium lecannii* showed insignificantly control against VPM (47.33). While *Beauveria bassiana* and *Paecilomyces lilacinus* coiled around and shrink branch canker pathogen, *Lecannicillium lecannii* breaks into branch canker hyphae and *Paecilomyces fumosoroseus* produces more spore to kill branch canker. In culture filtrate studies, *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* showed maximum control of VPM (68.44) and UPA-61 (65.59). *Beauveria bassiana* also showed significant control of two isolates VPM and UPA-61 (54.44). *Lecanniicillium lecannii* showed least control of VPM (30.44). This study concludes that entomopathogens can significantly control branch canker pathogen (*Macrophoma theicola*).

Key words: Antagonist, Entomopathogens, Hyperparasitism, *Macrophoma* sp, Non-volatile culture filtrate, Tea

Tea, being a perennial crop, provides a stable environment for a number of pests and diseases. Tea plantations suffer heavily from the infestation. Pests, pathogens and weeds are important factors limiting the productivity and quality of processed tea. Stem diseases are important as they stagnate crop and sometimes kill the bush. Pruning operation in tea increases the risk of stem diseases since it exposes the wood tissue to parasitic cuts and saprophytic fungi. Branch canker in tea was first noticed in southern India in 1899, but in Srilanka the diseases was recorded in 1904 (Petch 1923).The pathogen *Macrophoma* sp. is a wound pathogen.The fungal pathogen can easily enter through prune cuts or tissues damaged by sun-scorch. The pruning cuts also provide ideal surface for germination of spores (Otieno 1997). Stem diseases like wood rot (*Hypoxylon serpens*), collar canker (*Phomopsis theae*), branch canker (*Macrophoma theicola*) and thorny stem blight (*Tunstallia aculeata*) are predominant in southern India. Recent researchers reported that, entomopathogen could be used as plant fungal disease. Entomopathogenic fungi such as *Lecanicillium* sp. and *Beauveria bassiana* have shown to engage in plant pathogenic fungi interaction (Vega 2008 and Vega *et al.* 2008).These entomopathogens have been reported to very effectively control plant disease (Goettel *et al*. 2008 and Ownley *et al*. 2008). The entomopathogenic fungi are considered integrated control for chewing and sucking insect and pest (Gallego and Gallego 1988).The entomopathogens are (*Paecilomyces fumosoroseus*, *Lecanicillium*, *Beauveria bassiana* and *Metarhizium anisopliae*) commercially developed as biopesticides (Goettel *et al*. 2005). Moreover, these entomopathogens have been confirmed against plant fungal pathogen (Kavkova and Curn 2005). In our attempt, performance of entomopathogens like *Beauveria bassiana*, *Paecilomyces fumosoroseus*, *Lecanicillium lecanii* and *Paecilomyces lilacinus* have been evaluated against *Macrophoma* sp. The studies were conducted under *in vitro* level.

MATERIALS AND METHODS

The infected stem portions were collected from different tea growing district of south India, viz. Anamallais,

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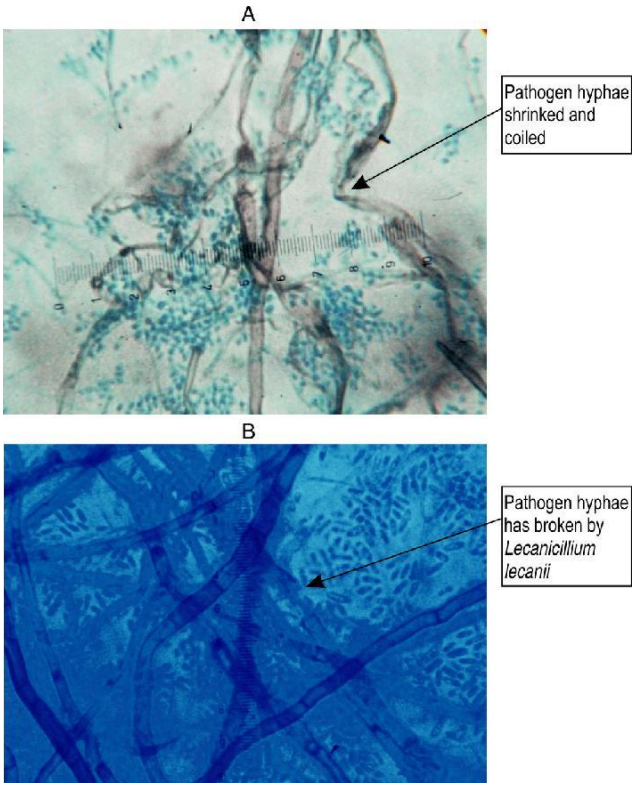

Fig 1 Hyphal interaction between entomopathogen and pathogen (under 10X view)

Coonoor, and Vandiperiyar. The plant part were then examined under microscope.The fungus was morphologically and cultural characteristically identified following "The diseases of tea bush" (Petch 1923).Isolation of genomic DNA amplification (PCR) was performed to18srRNA gene of the fungus sequencing analyses were identical used by Crous *et al.*(1999).Total of three strains were isolated namely VPM, UPA-61and NBCHE-6.These fungal pathogens were confirmed as *Macrophoma* sp and *Macrophoma theicola* through molecular technique identifications and submitted to NCBI (NBCHE-6-Accession No. JQ234977, VPM-Accession No. KP004441 and UPA-61-Accession No. KP17922).

The potential of entomopathogens were screened against *Macrophoma* sp. branch canker pathogen by dual culture method as described by Rajendiran *et al*. (2010).The entomopathogens were procured from Microbial Type Culture Collection and Genebank (MTCC), Chandigarh, namely like *Beauveria bassiana, Paecilomyces fumosoroseus, Lecanicillium lecanii* and *Paecilomyces lilacinus.* The pathogen and antagonist were inoculated in PDA plates on diametrically opposite points. Since the entomopathogens were slow growing in nature, the antagonists were inoculated only before the pathogen colony grew considerably therefore, after 2 days. Linear growth of the biocontrol agents colonizing either over or meet each other the pathogens growth was measured after 9 days of incubation. For the testing of antagonistic entomopathogens *Beauveria bassiana, Paecilomyces fumosoroseus, Lecanicillium lecanii* and *Paecilomyces lilacinus* 6 mm discs of antagonist and *Macrophoma* sp cut from the edge of 7 days old culture were placed 3 cm apart on potato dextrose agar (PDA) plate. The Petri plates were incubated at 27 ± 1ºC and periodical observations on the growth of the antagonist to colonize the pathogen were recorded. The experimental design used was completely randomized with four dishes for each isolate and control plate (without entomopathogen), a sterilized agar disc plate. Antagonistic activity was measured using Bell'scale method (Bell 1982).The percentage of inhibitions was calculated by the formula, PI=C-T/C×100.PI-percntage of inhibition,C-radial growth of the pathogen in control, T- radial growth of the pathogen in dual culture.

The entomopathogen and test pathogen at the opposite edges and were incubated for 7-9 days and interaction between the opposing cultures including hypal contact or coiling and lysis, which was observed under the microscope. Hyphal interaction gently from the zone of interaction in dual culture plates with the help of a needle and placed in a drop of lactophenol cotton blue on a microscopic slide (Elad *et al*. 1983).

The effect of culture filtrate of entomopathogen was studied following the method of Dennis and Webster (1971). The entomopathogens were inoculated in 100 ml sterilized Potato dextrose broth in 250 ml conical flasks and incubated at 27 ± 1ºC .The liquid culture medium was filtered through Whatman No.1. The filtrate was centrifuged at 10000 rpm for 15 min. The supernatant was filtered using millipore membrane filter paper (0.22µm).The entomopathogen filtrate was added to molten PDA to obtain final concentration of 2% (v/v). The medium was poured into petri plates (20 ml/ plate) and plates were inoculated with 6 mm disc of test pathogens. PDA plates inoculated with *Macrophoma* sp.but amended with sterile distilled water served as control. The plates were incubated at 27 ± 1ºC for 6 days.The percentage of inhibitions was calculated by the above formula.

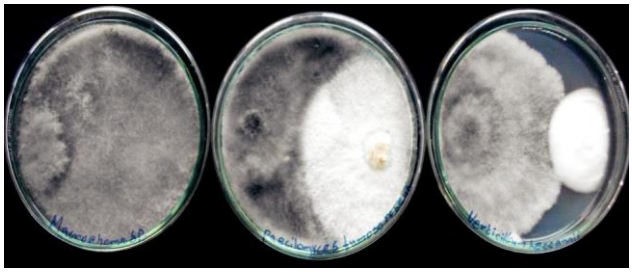

Fig 2 Growth inhibition of *Macrophoma* sp. by entomopathogen (*Paecilomyces fumosoroseus* and *Lecanicillium lecanii*)

Table 1 Isolation of branch canker pathogen from tea growing districts of southern India

Strain code No	Location	Identified as	NCBI Gen Bank Accession Number
NBCHE-6	Coonoor	*Macrophoma* sp	JQ234977
VPM	Vandiperiyar	*Macrophoma*	KP004441
UPA-61	Anamallais	*Macrophoma theicola*	KP179221

Table 2 *In vitro* screening of entomopathogen against branch canker pathogen (Dual plate technique)

Entomopathogen culture (MTCC)	Growth inhibition (%)		
	Macrophoma sp (NBCHE-6)	*Macrophoma* sp (VPM)	*Macrophoma theicola* (UPA-61)
Beauveria bassiana	64.22	61.55	62.66
Lecanicillium lecanii	45.33	47.33	44.22
Paecilomyces lilacinus	47.77	54.66	51.55
Paecilomyces fumosoroseus	54.44	56.66	56.66
CD (P=0.05)	3.1	2.8	2.8

Values are means ± SE of four replication of three repeated experiments.

Table 3 *In vitro* screening of entomopathogen against branch canker pathogen (Non-volatile culture filtrate)

Entomopathogen culture filtrates at 2% concentrations (MTCC)	Growth inhibition (%)		
	Macrophoma sp (NBCHE-6)	*Macrophoma* sp (VPM)	*Macrophoma theicola* (UPA-61)
Beauveria bassiana	53.33	54.44	54.44
Lecanicillium lecanii	18.55	30.44	26.55
Paecilomyces lilacinus	57.77	63.1	65.59
Paecilomyces fumosoroseus	60.04	68.44	66.77
CD (P=0.05)	7.1	4.9	4.4

RESULTS AND DISCUSSION

In this experiment results revealed that, the different tea growing area infected branch canker specimens were isolated and identified at molecular level. The details of branch canker isolate NBCHE-6,VPM and UPA-61 with source of location is given (Table 1). The entomopathogens have been tested for inhibition of branch canker pathogen.The inhibitory effects observed in this study were mainly for antagonistic and competition.

The dual plate method showed maximum growth inhibition of *Beauveria bassiana* against branch canker isolates NBCHE-6 (64.22), UPA-61 (62.66) and VPM (61.55). The effect of *Paecilomyces fumosoroseus* against two isolates of VPM and UPA-61 (56.66) and *Paecilomyces lilacinus* against VPM (54.66) was significantly antagonistic potential followed by *Lecannicillium lecannii* observation against VPM (47.33),NBCHE-6 (45.33) and UPA-61 (44.22) (Table 2 and Fig 2).

Mycoparasitism of both hyphal interaction vital role in mechanism of antagonistic potential capability. The hyphae of *Beauveria bassiana* and *Paecilomyces lilacinus* coiled around hyphae of *Macrophoma* sp. and shrunk it (Fig 1A). However, same action *Beauveria bassiana* with pathogen of tomato root-rot *Pythium myrotylum*was reported by Klingeman *et al*. (2008). Kiss (2003) reported that, this *Beauveria bassiana* may control plant pathogens and can act through antibiosis and mycoparasitism. Pathogen hyphae was broken by *Lecannicillium lecannii* spores interaction (Fig 1B). The result conform to the reports of Askary *et al*. (1997) who reported that, *Lecannicillium lecannii* acts as mycoparasite attaching to powdery mildew mycelia and conidia, producing enzymes such as chitinase, which penetrates to the mildew spore and kills the pathogen. *Paecilomyces fumosoroseus* produced more hyphae and spores interact with pathogen hyphae. Several scientists reported the mycoparasitism interaction as main principle mechanism of biological control (Elad *et al*. 1983). This study results are in accordance with the reports of Kang *et al*. (1996), Verharr *et al*. (1997), Dik *et al*. (1998), Miller *et al*. 2004 and Kavkova and Curn (2005). This may also be the reason for its antagonistic effect on *Macrophoma* sp.

Culture free extract of entomopathogen namely, *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* have showed maximum inhibition in growth of the pathogen at 2% concentrations. The maximum inhibition given by *Paecilomyces fumosoroseus* against VPM (68.44) followed by *Paecilomyces lilacinus* against UPA-61 (65.59). Significant inhibition observed by action of *Beauveria bassiana* against both isolates of VPM and UPA-61(54.44). *Lecannicillium lecannii* was seen to inhibit VPM sparsely (30.44) as compared to other antagonistic treatments (Table 3).

The present studies reveal that, *Beauveria bassiana* and *Paecilomyce fumosoroseus* show higher control growth of *Macrophoma* sp. pathogen. Youssef and Hatem (2012) also reported the control of red palm weevil and *Rhizoctonia* – root-rot of date-palm with *Beauveria bassiana*. *Isaria fumosoroseus* (formely *Paecilomyces fumosoroseus*) and *Lecannicillium* sp (formely *Verticillium lecannii*) are known entomopathogens and have good biopesticidal properties (Goettel *et al*.2005). Wherever, these kind of entomopathogens against fungal plant pathogens (Benhamou and Brodeur 2000, 2001). In our findings, we conclude that *Paecilomyces lilacinus* showed good antagonistic result. Similar findings were recorded by Perveen *et al*. (1998) with *Paecilomyces lilacinus* and *Pseudomonas aeruginosa* when used against root rot (*Meloidogyne javanica*) and root knot disease (*Macrophomina phaseolina*) in some vegetable crops. The applications of *Paecilomyces lilacinus* fungus protect plant roots from pathogens, increase plant growth and leaf yield (Manjula and Podile 2001. Wraight *et al*. 2003 and Muthulakshmi *et al*. 2010).

Results of our experiment showed low inhibitory effect of *Lecannilium lecanni*, though, these entomopathagenic fungus have activity against phytopathogenic fungi including powdery mildew (Verhaar *et al*. 1997, 1998), Spencer and Atkey 1981, Askary *et al*. 1998). In the present investigation, all the entomopathogens studied, showed antagonistic potential and inhibitory effect against *Macrophoma* sp. pathogen. The evidence for role of competition and parasitism has been convinced and evidence established the importance of antibiosis.

From this study, it is evident that the entomopathogens reduced the growth of all isolates of branch canker pathogen causal organism by *Macrophoma theicola* significantly. Mostly entomopathogen can be used as pest and insect infection disease control. Hence, it may use in integrated approaches for managing plant disease and pest control.

ACKNOWLEDGEMENTS

The authors are thankful to UPASI Tea Research Foundation,Tea Research Institute,Valparai, for facilities and encouragement. We wish to extend our heartfelt thanks to Calicut University, Kerala, for affiliation of Doctoral studies to UPASI Tea Research Foundation,Tea Research Institute,Valparai.

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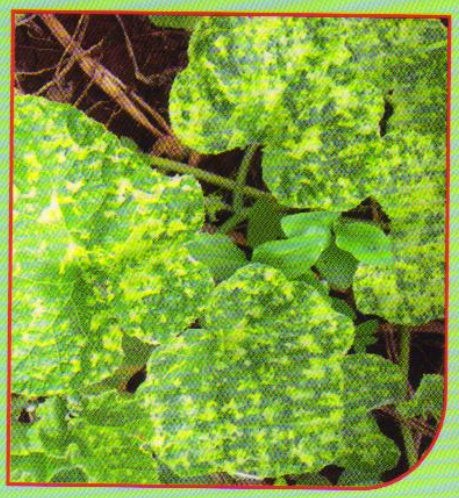
Volume 11 (1) January, 2016

ISSN 0973-7456
Online ISSN 0976-2388

Journal of Plant Disease Sciences



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BIOCHEMICAL AND NUTRITIONAL CHANGES DUE TO BRANCH CANKER DISEASE (*MACROPHOMA THEICOLA*) IN TEA

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ABSTRACT

Branch canker disease is caused by *Macrophoma theicola* belonging to the family *Botryo sphaeriaceae*. In tea plantations, disease causes considerable crop loss resulting in yield stagnation. The present study was focused to estimate the biochemical changes due to infection of branch canker disease in the nursery tea plants. Host-pathogen interaction studies were carried out in different tea clones under glass house conditions. The pathogen was induced artificially in the stem portion of the nursery plants. After 43rd days from induction, the infected stem samples were collected and the analysis of carbohydrate, reducing sugar and nutrients level (N, P and K) were carried out. The total carbohydrate content in the infected plants showed decreasing trend in all the tea clones when compared to the healthy plants. Similarly reducing sugar level also decreased rapidly in all the infected clones, except the clone TRI-2025 in which no major variation was observed in the healthy and infected plants. Similarly the nutrients (NPK) level of all the infected clones recorded lower values than the uninfected plants, whereas there is no notable difference in the nitrogen level of infected and healthy plants of the clone Yabukita.

Keywords: Branch canker, Nucleotide sequence, Nutrient analysis, Carbohydrate and Reducing sugar

Tea is the most common and inexpensive beverages consumed by over two thirds of the world population. Tea is obtained from the young shoots of the tea plant comprising two to three leaves and bud. Majority of the tea diseases are fungal origin pathogens and more than 300 species of fungal pathogens are reported in tea plant (Agnihothrudu, 1967; Chen and Chen, 1989). Branch canker disease is caused by *Macrophoma theicola,* is serious widespread stem disease in south India. The main objective was aimed to study the biochemical changes of canker infected tea plants under glass conditions to evaluate host-pathogen interaction of different tea clones. Host-pathogen interaction was demonstrated by biochemical analysis of carbohydrate, reducing sugar and nutrient analysis.

MATERIAL AND METHODS

The study was carried out in UPASI Tea Research Foundation located in Valparai, Coimbatore District, Tamilnadu, India. One year old nursery plants of the tea clones UPASI-3, UPASI-17, UPASI-26, TRF-1, TRI-2025 and Yabukita were used in the study. The infected canker portion was isolated from tea fields and identified through 18S rRNA technique. The identified spores were compared with the spore morphology of *Macrophoma theicola* reported by Petch, (1924). The fungus was confirmed as *Macrophoma theicola* and their nucleotide sequence submitted to NCBI Gene bank (Accession no - KP179222 for *M. theicola* code as UPA- 62).

The healthy plants of stem portion was gently cracked with a sterile scalpel to remove epidermis and freshly prepared spores suspension (1×10^5 spores ml^{-1}) of *Macrophoma theicola* was inoculated on the cracked portion. Then the treated portion was covered with wet cotton and parafilm and kept

Received on: 04-01-2016 Accepted on: 11-02-2016

under 80% relative humidity for proliferation of fungal mycelium. After completion of the life cycle of the fungus (43rd days after incubation), the infected stem portions were collected from glass house for analysis of biochemical parameters. Healthy plants served as control (without inoculation). Total carbohydrates and reducing sugars were estimated by pathogens using the procedures described by Mc Cready *et al.,* (1950) and Hedge and Hofreiter (1962). For nutrient analysis, the respective stem samples were separated into infected and healthy stem portion. The samples were washed with tap water followed by double distilled water and kept in enamel plates overnight and dried in an oven at 70°C. The dried samples were ground using a mortar and pestle and stored carefully in polythene bags for further analysis. The oven dried stem (canker infected and Healthy plant) samples were ground to fine powder and used for estimation of nitrogen, potassium and phosphorus contents. Estimation of nitrogen was made as per the procedure given by Page *et al.,*(1982). Estimation of potassium was done as per the method out lined by Hanway and Heidal (1952). The method of estimation of phosphorus was adopted from Bray and Kurtz (1945).

RESULTS AND DISCUSSION

Total carbohydrate content in the infected plants showed decreasing trend in all the tea clones when compared to the healthy plants (Figure 1). Reducing sugar level also decreased rapidly in all the infected clones, except the clone TRI-2025 in which no major variation was observed in the healthy and infected plants (Figure 2). Similarly NPK level of all the infected clones recorded lower values than the uninfected plants, whereas there is no notable difference in the nitrogen level of infected and healthy plants of the clone Yabukita (Table 1).

From the results it is inferred that, low level of carbohydrate and reducing sugars observed in the infected plants of all the tea clones was due to the infection of the fungus *Macrophoma theicola*. Yadav, (1989) reported that, the primary metabolism of carbohydrate is altered because of the fungal pathogens resulting in the dissolution of the reserve. Similar result was obtained in the findings of Manik *et al.,* (2008) in the infected seeds of *Cassia angustifolia*. Similarly reduction in the NPK level of the infected plants than the healthy plants clearly demonstrated the host-pathogen interaction (Table 1). NPK level was considerably reduced in all the infected plants indicating that the fungus *Macrophoma theicola* might have utilized the NPK reserves from the infected plants. This finding is in accordance with the earlier report of Nepolean, (2014) in tea. Equal level of reducing sugars observed in the clone TRI-2025 and the same level of nitrogen content noticed in the clone Yabukita in both the infected and healthy plants shows their comparatively better tolerance level against the pathogen *Macrophoma theicola* than the other clones. Further field level studies are essential for the better

Figure 1. Total carbohydrate level of infected and healthy plants due to branch canker disease

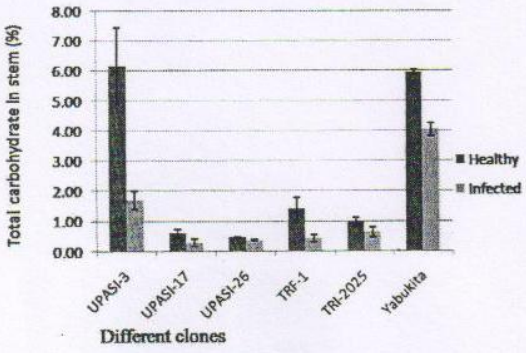

Figure 2. Reducing sugar level of infected and healthy plants due to branch canker disease

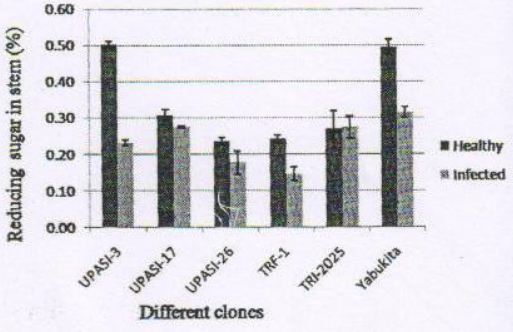

Table 1. NPK level infected and healthy plants due to branch canker disease

Different Clones	Nitrogen (g/100g)		Phosphorous (g/100g)		Potassium (g/100g)	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
UPASI-3	0.70±0.01	0.49±0.01	0.08±0.01	0.05±0.01	0.47±0.03	0.35±0.03
UPASI-17	0.59±0.02	0.51±0.02	0.08±0.01	0.07±0.01	0.41±0.01	0.40±0.01
UPASI-26	0.99±0.02	0.84±0.02	0.14±0.02	0.09±0.01	0.67±0.03	0.49±0.02
TRF-1	0.81±0.01	0.53±0.02	0.10±0.01	0.08±0.01	0.47±0.02	0.42±0.02
TRI 2025	0.84±0.01	0.73±0.02	0.08±0.01	0.06±0.01	0.51±0.03	0.49±0.02
Yabukita	0.73±0.01	0.73±0.02	0.14±0.02	0.10±0.01	0.40±0.01	0.30±0.02

Values are mean ± SD of three replications of three repeated analysis.

understanding of the tolerance level of TRI-2025 and Yabukita to branch canker disease.

Acknowledgements

The authors are thankful to the Director, UPASI Tea Research Institute, Valparai, for all the facilities and encouragement provided during the study. We wish to extend our heartfelt thanks to Calicut University, Kerala, for affiliation of Doctoral studies to UPASI Tea Research Institute, Valparai.

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In vitro screening of chemical and organic fungicides against Branch Canker disease in tea

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J. Mycopathol, Res, 54(2) : 299 -301, 2016;
ISSN 0971-3719

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J. Mycopathol. Res. ***54(2)*** *: 299-301, 2016;* (ISSN 0971-3719)

SHORT COMMUNICATION

In vitro screening of chemical and organic fungicides against Branch Canker disease in tea

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Received : 20.01.2016 RMS Accepted : 16.03.2016 Published : 25.07.2016

Branch canker disease is caused by *Macrophoma* sp . The infected stem portions of tea plant were collected and identified by using 18S rRNA method. *In vitro* experiment was carried out to evaluated various fungicides against branch canker pathogen. Out of the ten chemical fungicides tested, Benomyl, Carbendazim and Companian were found to be efficient against test pathogen at 10 ppm level. Copper oxychloride was noticed maximum growth inhibition of *Macrophoma* sp . The commercial botanical fungicides of Expel, Enroot and Attopsy were recorded highest inhibition of Branch canker pathogen at 0.1 % concentrations.

Key words: Antagonistic potential, fungicides, *Macrophoma* sp , nucleotide sequence.

Tea is the most popular beverage consumed in many parts of the world. Tea is produced from the young shoots of the commercially cultivated tea plant [*Camellia sinensis* (L.) O. Kuntze]. Tea in south India is cultivated in the hilly tracts of the Western Ghats at an altitude ranging from 500 to 2200 m above mean sea level. The Branch canker disease is caused by *Macrophoma* sp. and serious stem disease in southern India. The present study aimed to evaluate the effect of different chemical and organic fungicides against Branch canker pathogen under *in vitro* condition. The branch canker pathogen was isolated from tea growing area and identified through 18S rRNA methods. PCR amplification, DNA sequencing of the ITS region of the rRNA gene and finally sequences were submitted to NCBI (Accession No. KP004441 for Branch canker pathogen - VPM). The different concentration of fungicides and botanical fungicides were evaluated *in vitro* against branch canker pathogen applying Food Poisoned Technique using PDA as conventional medium. All the PDA plate containing fungicides were inoculated with 5 mm disc test pathogen from seventh days old culture. The plate containing PDA without fungicides were maintained as control and all treatments were replicated thrice. After seven days incubation, the treatment plates were measured along with fully grown control plate and per cent of inhibition (PI) was calculated by Bell'scale method. *In vitro* screening of systemic fungicides such as benomyl, carbendazim and companion showed 100 % growth inhibition against *Macrophoma* sp. at 10 ppm level followed by propiconazole and hexaconazole (Table 1). The same results are in agreement with Suryawanshi (2008) *et al,* who *evaluated* efficacy of different fungicides evaluated against *Macrophomina phaseolina* blight of

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Table 1 : Evaluation of chemical fungicides against *Macrophoma* sp. under *in vitro* level

Chemical fungicides	Percentage of growth inhibition at different concentrations of chemical fungicides (ppm)					
	10	20	30	40	50	CD at P= 0.05
Benomyl (50 % WP)	100.00±00	-	-	-	-	0.0
Propiconazole (25 % EC)	75.00±1.15	78.22±0.44	81.44±0.38	85.44±0.48	89.22±0.57	1.7
Mancozeb (75 % WP)	31.44±1.00	45.33±1.98	49.86±1.06	55.33±0.74	62.66±0.99	3.6
Carbendazim (50 % WP)	100.00±00	-	-	-	-	0.0
Companian (carbendazim 12 % + mancozeb 63 % WP)	100.00±00	-	-	-	-	0.0
Hexaconazole (5 % EC)	35.99±1.28	40.44±0.75	59.55±1.59	61.55±0.64	66.66±0.53	3.0

Values are Means ± SE of four replication of three repeated experiments

Table 2 : *In vitro* screening of Copper fungicides against branch canker pathogen

Copper group fungicides	Concentrations	Growth inhibition (%)	C.D. at P=0.05
Copper oxychloride 435 (liquid)	0.62%	74.42±0.39	
	1%	82.15±0.85	1.7
	1.24%	83.08±0.51	
	1.85%	87.33±0.47	
Copper hydroxide (77 % WP)	0.62%	60.91±0.73	
	1%	69.66±1.17	4.7
	1.24%	77.71±2.64	
	1.85%	82.71±0.49	
Copper oxychloride (50 % WP)	0.10%	3.55±0.28	
	0.30%	84.26±0.96	2.0
	0.45%	85.79±0.44	
	0.75%	88.75±0.42	

Values are Means ± SE of three replication of three repeated experiments

Table 3 : Bio efficacy of botanical fungicides against branch canker pathogen under lab condition

Botanical fungicides	Concentrations	% inhibition of growth	C.D. at P=0.05
Ecocare	0.10%	7.62±0.45	
	0.30%	23.91±2.05	
	0.50%	29.95±0.51	3.6
	0.75%	44.04±0.77	
	1%	50.06±1.03	
Fungfinish (5 % copper formulation)	0.10%	0.00±0.00	
	0.30%	1.55±0.44	
	0.50%	38.04±1.21	2.1
	0.75%	75.33±0.48	
	1%	85.55±0.38	
Tari (Organic Plus Tea special)	2%	0.00±0.00	
	4%	19.55±0.99	
	6%	54.53±5.17	8.0
	8%	69.77±2.83	
Nimbicidine (0.03 % Azadiractin EC)	2%	11.75±0.40	
	4%	33.26±1.10	3.2
	6%	45.68±0.91	
	8%	52.53±1.17	
Tricure (0.03 % Azadiractin EC)	2%	10.80±0.54	
	4%	43.37±1.23	3.4
	6%	74.95±2.14	
	8%	100.00±0.00	
Enroot	0.1%	100.0±0.00	0.0
Attopsy	0.1%	100.0±0.00	0.0
Expel (Combination of canolar extract Tea tree oil)	0.1%	100.0±0.00	0.0

Values are Means ± SE of three replication of three repeated experiments.

mungbean. Moreover, several workers (Gore *et al,* 2008) reported similar inhibitory potential of different fungicides against *Macrophomina* sp. The contact fungicides of copper group *viz.,* copper oxychloride (50 %WP),copper hydroxide and copper oxychloride 435 (liquid) were tested against branch canker pathogen under *in vitro*. Among the copper group, copper oxychloride (50 % WP) was noticed highest growth inhibition against test pathogen followed by liquid copper oxychloride 435 and copper hydroxide (Table.2). Earlier researchers like, Sanjay *et al,* (2008) reported that the contact fungicide of copper oxychloride gave the best disease control of grey blight pathogen in tea. The commercial botanical fungicides were tested against *Macrophoma* sp. at different concentrations level. In this present study, botanical fungicides (Expel, Enroot and Attopsy) were found to be efficient against the test pathogen at 0.1 % concentration level (Table 3). These results are in agreement with Nepolean *et al,* (2014).

ACKNOWLEDGEMENT

The authors are thankful to UPASI – TRF, TRI, for providing the necessary facilities to carry out the experiment work.

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Indian Phytopath. 69 (4) : 422-423 (2016)

SHORT COMMUNICATION

Shelf life of bacterial and fungal biocontrol agents in different formulations

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Key words: Coir pith formulation, colony forming unit, shelf life, talc formulation

The biocontrol agents have been identified as potential agents for the management of plant diseases. Commercially, successful use of biocontrol agents like *Trichoderma* spp. has been done for the control of soil borne plant pathogens (2). Biological control has been used for the control of plant diseases to replace the usage of harmful chemicals in agriculture field. The mass production of biocontrol agents is an active process and reasonable inexpensive. Talc based formulation of *Pseudomonas* sp. and *Trichoderma* sp. have been reported as potential biocontrol agents for *Fusarium* sp. wilt pathogen (6). Krishnamurthy and Gnanamanikam (4) reported that the talc based formulation of *P. fluorescens* controlled the rice blast pathogen caused by *Pyricularia grisea*. The combination product of PY15, TDK1 and *Pseudomonas* sp. was found to be satisfactory for control of sheath rot disease in rice (9). The present investigation is aimed to study the effect of carrier material on population level and shelf-life of different biocontrol agents.

Mass production of bacterial biocontrol agents

Bacterial biocontrol agents were isolated from different districts of tea growing regions and then used for the shelf-life studies. The bacterial strains were confirmed by using 16S rRNA molecular method and their nucleotide sequences were submitted to NCBI gene bank. The molecular accession number were given by NCBI data base for respective isolates such as *Bacillus subtilis* (Accession No: KM527836), *Pseudomonas fluorescens* (Accession No: KM527837), *Bacillus amyloliquefaciens* (Accession No: KM853034) and *Bacillus licheniformis* (Accession No: KM527838) were grown on the respective medium of Nutrient Broth and Luria Bertani (NB and LB). The talc and coir pith formulations of bio control agents were prepared based on the technique of Jeyarajan *et al.* (3). A loopful of inoculam of bacterial strains was transferred into conical flask of 250 ml and 500 ml LB medium to be grown for 3 days. Then 250 ml of culture was mixed with sterilized talc powder (1 kg) and 500 ml of culture was mixed with sterilized carrier material of coir pith (1 kg) [Talc based formulation in the 1:4 ratio and Coir pith formulation in 1:2 ratio]. These isolates are being maintained in the repository of UPASI Tea Research Institute, Valparai.

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Mass production of fungal biocontrol agents

The fungal biocontrol agents were procured from Microbial Type Culture Collection and Genebank (MTCC), Chandigarh. These fungal biocontrol agents were maintained on PDA media for further studies. Fungal biocontrol agents such as *Gliocladium virens*, *Trichoderma viride*, *Trichoderma harzianum* and *Trichoderma atroviride* were grown on potato dextrose broth medium. The medium is inoculated with 1 disk of 5 mm of 6 days old culture of fungal biocontrol agents and incubated at room temperature (25-27°C) for 7 days. The formulation of fungal biocontrol was prepared by following the procedure as described earlier. The formulations were packed in polythene bags and stored at 25-28°C for different interval. The formulations were serially diluted at respective intervals (30, 60, 90 and 120 days) to obtain 10^6 dilution by adopting pour plate method. The colony forming (CFU) unit of biocontrol formulation was calculated.

Shelf life of biocontrol agents based on talc powder formulation

Shelf life of talc based formulation of biocontrol agents was studied at different days (30, 60 and 90) interval. The CFU of *Bacillus licheniformis* (75.9×10^6 cfu/g) was recorded in higher population followed by *Bacillus amyloliquefaciens* (66.2×10^6 cfu/g) after 30 days of storage of formulation (Table 1). This result is in agreement with Amer and Utkhede (1) who reported the shelf-life of talc based formulation of *Bacilllus subtilis* (1.0×10^6 cfu/g) found in higher number after 45 days of storage. Moreover, talc based formulation of biocontrol agents showed good result against wilt pathogen of *Fusarium* sp. (6).

The present study revealed that the same trend in population of *B. licheniformis* (45.0×10^6 cfu/g) and *B. amyloliquefaciens* (31.6×10^6 cfu/g) was observed after 60 and 90 days of storage. Even after 90 days, 6.79×10^6 cfu/g to 20.47×10^6 cfu/g of population was recorded in all the biocontrol agents (Table 1). After 90 days of storage, no population was recorded in all the biocontrol agents. Several scientists reported that the average self-life of talc based formulation of *T. harzianum* was recorded

Table 1. Viability of biocontrol agents in talc powder formulation

Biocontrol agents	Colony forming unit/gram of sample		
	30 Days ($x10^6$)	60 Days ($x10^6$)	90 Days ($x10^6$)
Bacillus subtilis	31.5 f	19.3 d	8.87 d
Pseudomonas fluorescens	46.4 d	9.4 e	00.00 f
B. amyloliquefaciens	66.2 b	31.6 b	16.69 b
B. licheniformis	75.9 a	45.0 a	20.47 a
Gliocladium virens	49.3 c	19.7 d	6.76 e
Trichoderma viride	29.1 g	18.4 d	7.02 e
T. harzianum	60.0 c	28.7 c	12.33 c
T. atroviride	37.0 e	29.4 c	9.46 d
CD@5%	2.28	2.32	2.08

Values are means followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 0.05% level

after 4-5 months of storage (7). However, addition of chitin might increase the self-life of talc based formulation *T. harzianum* (11). Many earlier reports suggested the use of different type of carrier materials for long storage of fungal biocontrol agent of *Trichoderma* spp. (8,10)

Shelf life of biocontrol agents based on coir pith formulation

Shelf life of coir pith formulations of biocontrol agents were carried out at different (30, 60, 90 and 120) interval. The initial CFU of *B. licheniformis* (131.14×10^6 cfu/g) showed higher population followed by population of fungal biocontrol agent *T. harzianum* (124.71×10^6 cfu/g) after 30 days of storage. The same trend was observed in *B. licheniformis* (94.90×10^6 cfu/g) after 60 days of storage (Table 2). Therefore, *B. subtilis* (84.05×10^{-6}) was found in higher number after 60 days of storage (Table 2). Earlier workers found coir pith formulation as best carrier material used for good colonization of *T. viride* (5). Finally, the population of all biocontrol agents declined gradually after120 days of storage. From this study, it can be concluded that higher self-life has been found in coir pith based formulation of biocontrol agents than talc based formulation.

ACKNOWLEDGEMENT

The authors are grateful to UPASI Tea Research Institute, Valparai for facilities and encouragement provided during the study.

Table 2. Viability of biocontrol agents in coir pith formulation

Biocontrol agents	Colony forming unit/gram of sample			
	30 Days ($x10^6$)	60 Days ($x10^6$)	90 Days ($x10^6$)	120 Days ($x10^6$)
Bacillus subtilis	110.3 cd	84.05 b	61.44 a	24.44 a
P. fluorescens	83.62 f	74.99 c	29.67 e	12.01 de
B. amyloliquefaciens	92.34 e	77.35 c	49.07 b	18.48 b
B. licheniformis	131.14 a	94.90 a	36.40 cd	19.47 b
Gliocladium virens	107.46 d	63.27 e	19.90 f	9.25 e
Trichoderma viride	89.05 ef	45.75 f	31.99 de	16.62 bc
T. harzianum	124.71 b	66.38 de	22.61 f	11.78 de
T. atroviride	115.54 c	68.51 d	37.92 c	14.36 cd
CD@5%	6.31	5.07	4.47	3.33

Values are means followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 0.05% level

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Received for publication: May 10, 2016
Accepted for publication: September 20, 2016

Plant Pathology & Microbiology

Mareeswaran et al., J Plant Pathol Microb 2015, 6:7
http://dx.doi.org/10.4172/2157-7471.1000284

Research Article Open Access

In vitro Studies on Branch Canker Pathogen (*Macrophoma* sp.) Infecting Tea

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Abstract

Branch canker is the main stem disease of *Camellia* sp. caused by *Macrophoma* sp. In this study, branch canker pathogen was isolated, bought to pure culture and maintained in potato dextrose agar medium (PDA). A total number of 150 bacterial and 40 fungal strains were isolated from different agro climatic zone of south India, which are region specific and native strains (resembling *Pseudomonas* spp. *Bacillus* spp. and *Trichoderma* spp.). Among the total number of bacterial and fungal isolates, 6 bacterial and 3 *Trichoderma* spp. Showed antagonistic effect against the branch canker pathogen. The study clearly indicates that *Bacillus* spp. *Pseudomonas* spp. followed by *Trichoderma* spp. showed higher antagonistic potential against the test pathogen. The study also includes that, the selected botanical fungicides, neem kernel extract, garlic extract, *Aloe vera*, Tulsi and Expel (Botanical fungicides) at different concentration were carried out against *Macrophoma* sp. Results showed that, commercially available botanical fungicide (Expel) is effective to control the growth of branch canker pathogen compare then other chemical and botanical fungicides. The commonly used fungicides in tea plantation such as Hexaconazole (Contof 5E), Tebuconazole (Folicur) and Tridemorph (Calixin) were evaluated against *Macrophoma* sp. under *in vitro* conditions. The results indicated that Tebuconazole all the three concentrations at 1.78 ppm was found to be the most effective in suppressing the growth of branch canker pathogen. The results concluded that biocontrol agents (*Bacillus* spp. *Pseudomonas* spp and *Trichoderma* spp.), botanical fungicide (Expel) and chemical fungicide (Tebuconazole) are very effective to control the branch canker pathogen under *in vitro* conditions.

Keywords: Biocontrol agents; Botanical and chemical fungicides; *Camellia* sp.; *Macrophoma* sp.

Introduction

Tea, an evergreen plant is one of the most popular, non-alcoholic beverages consumed by nearly half the world population. Tea is produced from the young shoots of the commercially cultivated tea plant (*Camellia* sp.). India is the one of the largest producer and consumer of tea in the world with an area of 5.75 lacks/ha under tea cultivation. Tea is attacked by number of pests and diseases which are the major limiting factors in crop productivity. The first comprehensive account on the pests and diseases of tea was presented by Watt [1]. Majority of tea pathogens are of fungal origin and more than 300 species of fungi are reported to affect different parts of the tea plant [2-4]. Mann and Hutchinson [5] recorded various diseases and that was substantiated by Petch [6]. Sarmah [7] described all parasitic and non-parasitic/ physiological diseases. Among the stem diseases of tea, branch canker caused by *Macrophoma theicola* is a predominant stem inhabiting fungal disease which has been reported from Ceylon. Branch canker, *Macrophoma theicola* occurs in drought susceptible areas where soil is poor. In Kangra valley, Himachal Pradesh this disease was observed after rainy season, whereas the occurrence of the disease was very rare in Darjeeling [7]. *M. theicola* has been observed to cause twig die-back of mature tea in Taiwan [8]. In general, tea bush affected by sun-scorch is prone to this disease. The diseased patches on the branches appear as slightly sunken lesions surrounded by a ring of callus growth [7]. The affected branches are killed slowly by the invading fungus until the disease spreads to the collar when upper portion of the plant dies. In mild infestations, the canker is callused over completely within a few months, but the fungus may renew its growth forming concentric cankers under adverse conditions. Fructifications are produced on the dead bark during wet weather conditions. To control the disease; the affected branches should be cut out to clean healthy wood. Plants should be protected from sun-scorch and pruning should be avoided during dry weather. The crop loss due to this disease depends upon pathogen and the geographical area [9]. In Taiwan, around 40% of the tea bushes were killed by twig dieback and in south-east Asian countries, root rot disease was responsible for major crop loss [4,10]. Low yield due to incidence of collar and branch canker caused by *Phomposis theae* and *Macrophoma theicola* was reported from central Africa [11]. It has been difficult to control branch canker as it grows with the saprophytic fungi on the plant stem. Being a related anamorph genera of *Botryosphaeria* along with *Botryodiplodia, Diplodia, Fusicoccum, Lasiodiplodia, Macrophomposis* and *Sphaeropsis,* it was difficult to separate it from others as its morphological features were poorly defined [12]. The present study involves the isolation, morphological identification and the effect of different chemical and botanical fungicides, bio-control agents on *Macrophoma* sp.

Materials and Methods

Sample collection

Survey was conducted in major tea growing areas of south India (The Anamallais, Central Tranvancore, High Range, Wayanad, Coonoor and Koppa) to collect soil samples in order to isolate biocontrol agents and branch canker fungal pathogens.

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Received April 29, 2015; **Accepted** July 09, 2015; **Published** July 16, 2015

Citation: Mareeswaran J, Nepolean P, Jayanthi R, Premkumar Samuel Asir R, Radhakrishnan B (2015) *In vitro* Studies on Branch Canker Pathogen (*Macrophoma* sp.) Infecting Tea. J Plant Pathol Microb 6: 284. doi:10.4172/2157-7471.1000284

Isolation of branch canker pathogen

The infected stem portions were collected. The samples were washed in distilled water and were cut into small piece. Surface sterilized with 0.1% mercuric chloride for few seconds followed by sterile distilled watering, 2-3 times. After surface sterilization the infected portions were blotted on sterile filter paper and then inoculated on water agar plates amended with streptomycin (50 mg/lit). Plates were incubated for 3 to 5 days. The grown mycelial tips from water agar plates were aseptically transferred to potato dextrose agar medium (PDA). Pure cultures were obtained from the primary plates by colonies initiated from single spores or from hyphal tips. Single-spore cultures were made by preparing a suspension of spores in distilled sterile water and spreading it over water agar plates. Single germinated spores were removed on a small amount of agar with a transfer needle to a PDA medium. Distinct hyphal tips were cut from the well grown water agar plate and then sub-cultured repeatedly on PDA to obtain pure culture of the fungus.

Isolation of bio-control agents from soil

Soil samples at 0"- 9" depth were collected from three tea growing districts, High range Munnar, Central Travancore, Koppa and The Annamallais for isolation of biocontrol agents (*Trichoderma* spp. *Bacillus* spp. and *Pseudomonas* spp.). The Biocontrol agents were isolated by standard serial dilution plating techniques, sub cultured, brought to purity and stored in slants at 4°C. The cultures were identified using standard bacteriological techniques.

Screening for antagonism

The isolated bacterial and fungal strains (*Trichoderma* spp. *Bacillus* spp. and *Pseudomonas* spp.) were screened for their antagonistic potential against the pathogen, following dual culture technique [13]. The mycelial plug of four day old, actively growing *Macrophoma sp*. was ground and spread uniformly on PDA medium plate with the help of a sterilized spatula. These plates were then spot inoculated within 24 h culture of isolated bacterial strains. Plates were incubated at 30 ± 2°C for 3-5 days. The antagonism was graded by measuring the zone of inhibition produced around the bacterial strains. The grading was given as (-) no antagonism, (+) those showing inhibition zone of <0.5 cm, (++) with 0.5 cm to 1 cm and (+++) those with >1.0 cm. In the case of *Trichoderma*, *in vitro* screening was done by placing a mycelial plug of 4 days old culture of both pathogens and the antagonist. Time for the first contact between the antagonist and the pathogen and the advancement of the antagonist on the pathogen colony was noted and the efficient strains were short listed. A control plate was maintained for comparison. Radial growth of the pathogenic fungi was measured after comparison with control. Percent inhibition was calculated by the formula given by Bell et al. [14]

Compatibility of pathogen against chemical fungicides

Three systemic fungicides, hexaconazole (Contof 5E), tebuconazole (Folicur) and tridemorph (Calixin) which are commercially being used in the tea fields for the control of various fungal pathogens were selected for the compatibility study of the branch canker pathogen. Three dosages of the systemic chemical fungicide were tested. RD- recommended dosage (3.57 ppm), LR- lower recommended dosage (1.78 ppm) and HR- higher recommended dosage (5.35 ppm). These dosages were mixed with PDA and poured in sterile petri plates and allowed to solidify. Small blocks of the pure culture of the pathogen were cut using sterile cork borer (7 mm) and inoculated onto the solid medium. A control plate, devoid of the chemical fungicide was made as reference. The growth of the pathogen was observed for 10 days (3rd, 5th, 7thand 10th days) and recorded.

Compatibility of pathogen towards botanical fungicides

Neem kernel extract: Dry neem seeds (approximately 100 g) were ground using a mortar and pestle. The powder was tied in a sterile muslin cloth and soaked in 250 ml sterile distilled water and left to stand overnight at room temperature. The extract was filtered using Whatmann filter paper No 1. The filtrate was added to the PDA medium at different concentrations (5%, 7.5% and 10%) to find out the effective dosage at which the pathogen cannot survive. The plates along with the extract, at various doses were inoculated by placing small block of the pure culture (7 mm). A control plate devoid of fungicide was maintained as the reference. The growth is observed for 10 days (at 3rd, 5th, 7thand 10th days) measured and recorded.

Garlic: 30g of garlic was made to paste using mortar and pestle and mixed with 30 ml of sterile distilled water. The extract was filtered using muslin cloth and the extract was added to the PDA medium at different concentrations (1%, 2.5% and 5%) to find the percent inhibition at various dosage of garlic extract. The plates were inoculated the growth was measured and recorded as mentioned as earlier for neem kernel extract.

Tulsi: Tulsi leaves were cleaned with sterile distilled water and ground with 5 ml of 95% ethanol using mortar and pestle. The ground paste was centrifuged at 5000 rpm for 5 min. The collected extract was used to prepare the disc**.**

Disc preparation: Discs were prepared with Whatmann No. 1 filter paper. The extract was added to 95% ethanol at various concentrations (5%, 7.5% and 10%) and the prepared discs were immersed in it. The control discs were prepared by soaking the discs to 95% ethanol. The discs were kept in hot air oven at 45°C and left overnight to dry. The PDA plates were swabbed with the pure culture over the entire surface of the plate. This procedure was repeated twice and the plate was rotated 60º each time to ensure an even distribution of the culture. The appropriate discs were placed (with plant extracts) evenly (no closer than 24 mm from centre to centre) on the surface of the agar plate either by using sterile forceps or the dispensing apparatus. After 7 days of incubation, each plate was examined and measured for the diameters of the zones of complete inhibition. The zones were measured to the nearest mm using a ruler.

Aloe vera: Gel portion of the leaf was separated using a sterile blade and ground using a mortar and pestle. The ground gel was filtered and the extract was collected. The collected extract was mixed up to 95% ethanol at various concentrations (5%, 7.5%, 10% and 100%). The control discs were prepared using in 95% ethanol and dried in hot air oven at 45°C overnight. The disc preparation and the procedure were same as that of tulsi.

Expel: The commercially available botanical fungicide Expel which is being widely used in the tea field was evaluated at low dose- 1.5 ppm, recommended dose- 3 ppm and high dose- 4.5 ppm. The procedure was same as that of chemical fungicide method.

Results and Discussion

Survey was conducted in major tea growing areas of south India to collect the soil samples and disease specimens to isolate bio-control agents and branch canker fungal pathogen. A total of four branch canker pathogen and biocontrol were isolated from different tea growing district like the Anamallais (MT APF1), Central Tranvancore

(MT HE 02), Coonoor (MT C2 03) and Koppa (MT KH 04) also specific same areas (The Anamallais - 2 *Bacillus* spp. and 2 - *Trichoderma* spp. Central Travancore - 1 *Bacillus* sp., Koppa – 2 *Pseudomonas* spp. and The Nilgiris - 1 *Pseudomonas* sp and 1- *Trichoderma* sp.) were showed bacterial and fungal biocontrol agents. The branch canker pathogen was morphologically, spore characteristically identified used as standard reference book image Petch [6] and confirmed as *Macrophoma* sp (Figure 1). A total number of 150 bacterial and 40 fungal isolate (resembling *Pseudomonas* spp. *Bacillus* spp. and *Trichoderma* spp.), were isolated and screened six bacterial and three *Trichoderma* spp. showed higher antagonistic effect against branch canker pathogen (Table 1). The antagonism was graded by recording the zone of inhibition produced around the bacterial strains. From this study it was concluded that *Bacillus* spp. *Pseudomonas* spp. followed by *Trichoderma* spp. were more inhibitory effect against branch canker pathogen (Figures 2 and 3). Three fungicides, hexaconazole (Contof 5E), tebuconazole (Folicur) and tridemorph (Calixin) were evaluated against *Macrophoma* sp. under *in vitro* condition. The results indicated that, Tebuconazole all the three concentrations at 1.78 ppm was found to be the most effective in suppressing the growth of pathogen followed by hexaconazole and tridemorph. Hexaconazole at 1.78 ppm and tridemorph at 3.57 ppm were found to be optimum for the control of pathogen growth (Table 2). The study revealed that Tebuconazole completely inhibited the growth of branch canker pathogen compared

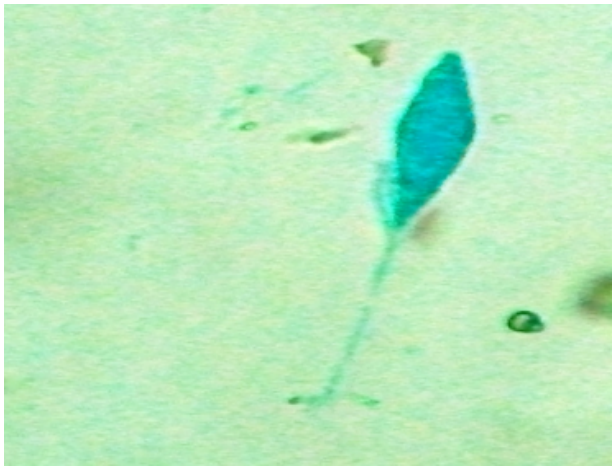

Figure 1: Microscopic view of *Macrophoma* sp. A single pycno spore is magnified through 40x which was isolated from Branch canker infected stem obtained from the Nilgiris.

Tea growing districts	Number of bacterial isolates	Number of *Trichoderma* spp. isolates	No. of antagonist against *Macrophoma* sp.	
			Bacterial strains (*Bacillus* spp. and *Pseudomonas* spp.)	*Trichoderma* spp.
1. The Anamallais	25	15	2 (2 cm)*	2
2. The Nilgiris	50	5	1 (>1 cm)	1
3. Central Travancore	50	15	1 (>1 cm)	-
4. Koppa	25	5	2 (1-2 cm)*	-
Total	150	40	6	3

Table 1: List of biocontrol bacterial and fungal strains isolated from various tea growing areas *Values in parentheses indicate inhibition zone produced by bacterial antagonist.

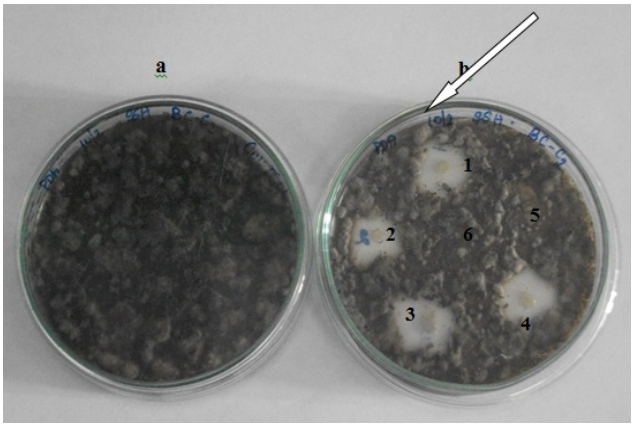

Figure 2: Control plate (a) *Macrophoma* sp. spreaded PDA plate is free from bacterial antagonist and (b) *Bacillus* spp. and *Pseudomonas* spp. inhibited the growth of *Macrophoma* sp. (Arrow indicates the zone of inhibition between fungal pathogen & bacterial antagonist).

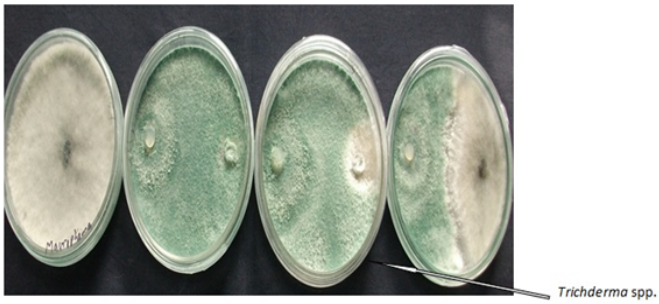

Figure 3: *Trichderma* spp. against *Macrophoma* sp. pathogen.

Fungicides		Fungicide concentration (ppm)	Mean radial growth (cm)	% inhibition of growth (%)
1.Hexaconazole	RD	3.57	0.00	100
	LR	1.78	7.58	75.8
	HR	5.35	0.00	100
2.Tebuconazole	RD	3.57	0.00	100
	LR	1.78	0.00	100
	HR	5.35	0.00	100
3.Tridemorph	RD	3.57	0.00	100
	LR	1.78	0.62	6.24
	HR	5.35	0.00	100
Control plate	UT	-	9.00	0

Table 2: *In vitro* efficacy of different fungicides on *Macrophoma* sp. *On 10th day. Values in the parentheses indicate percent inhibition of the pathogen. RD: Recommended Dosage; LR: Lower Recommended Dosage; HR: Higher Recommended Dosage and UT: Untreated control.

to that other two fungicides. There was absolutely no growth in the fungicide amended plates even at a lower concentration (Figure 4). Among the botanical fungicides tested, expel showed the highest percentage of inhibition against *Macrophoma* sp under *in vitro* condition (Table 3). While Tulsi, Neem kernel, *Aloe vera* and garlic extract had no growth effect of *Macrophoma* sp. (Table 4). In this study, commercially available botanical fungicide (Expel) is effective to control the branch canker disease without any residual effect and maintaining the soil structure, bush health when compared to the

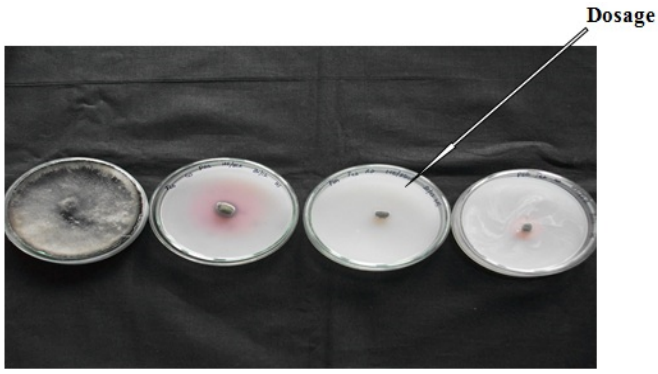

Figure 4: Tebuconozole inhibitied the growth of *Macrophoma* sp. with three different concentration. UT-Untreated control, LD: Lower Recommended Dosage, RD: Recommended Dosage, HR: Higher Recommended Dosage.

Isolates	3rd day				5th day				10th day			
Dosage	U	LD	RD	HD	U	LD	RD	HD	U	LD	RD	HD
MT APF1	8.50	-	-	-	9.00	-	-	-	9.00	74.4	-	-
MT HE 02	9.00	85.0	86.6	87.7	9.00	64.4	67.7	85.0	9.00	60.3	62.3	76.1
MT C2 03	3.50	48.2	54.9	-	4.55	47.4	54.0	69.2	8.10	40.0	52.8	65.8
MT KH 04	9.00	86.6	88.8	89.9	9.00	83.3	87.7	87.7	9.00	75.4	82.4	84.4

Table 3: Effect of botanical fungicide (Expel) on *in vitro* growth of Macrophoma spp. Means of 5 replicates and four different isolates. *(-) no growth *the values indicate percentage inhibition *the values of untreated are indicated in centimeters. U: Untreated, LD: Low Dose, RD: Recommended Dose and HD: High Dose.

Fungicides		Fungicide Concentration (ppm)	Mean radial growth	% inhibition of growth (%)
1. *Aloe vera*		5%	-	-
		10%	-	-
		100%	-	-
2. Tulsi		5%	-	-
		10%	-	-
		100%	-	-
Control plate	UT	-	+	+

Table 4: *In vitro* efficacy of different botanical fungicides on Macrophoma spp. (Means of 5 replicates and 4 different isolates). Garlic and Neem Kernel were noticed same results (-) samples showed no growth. *On 10th day. Values in the parentheses indicate percent inhibition of the pathogen. (+) samples showed growth (-) samples showed no growth.

other chemical fungicides. Same result recorded with Nepolean et al. [15] expel botanical fungicide and bacterial biocontrol agents were showed good results against wood rot pathogen. Long term application of PGPR resulted in reduced disease incidence in field grown tea plants. When fungicide or biocontrol agents were incorporated their efficiency in controlling the disease was also improved. Continuous application of PGPR helped the plants to build up natural resistance to the disease. Silva et al. [16] reported reduction of fungicide application number of rounds (50%) in tomato plants treated with *Bacillus cereus*, which provided protection against multiple diseases. In recent studies on antagonistic potential of biocontrol agents against tea pathogens, *Hypoxylon* sp. and *Pestalotiopsis* sp. were tested under *in vitro* level and the results indicated that *Pseudomonas* sp. and *Trichoderma* sp. exhibited superior antagonistic potential against the grey blight and wood rot pathogens [17]. The study clearly indicated that each 3 strains of *Bacillus* and *Pseudomonas* that showed higher antagonism against branch canker pathogen. *Trichoderma* spp. isolated from such a region showed effective antagonism against *Macrophoma* sp. and *Bacillus* spp. provided excellent control of the branch canker disease. Similar results were reported by Nandakumar et al. [18], Vivekananthan et al. [19], Vidhyasekaran and Muthamilan [20] and Ramamoorthy et al. [21], for the control of various fungal pathogens. When groundnut plants were sprayed with *P. fluorescencs*, increased activity of PAL was observed and correlated with the lesser disease incidence [22]. In the present study, *Bacillus* spp. and *Pseudomonas* spp. followed by *Trichoderma* spp. showed more inhibitory effect against *Macrophoma* sp. under *in vitro* condition. Standard fungicides and biological control agents provided satisfactory control of the disease under the field conditions without any residual effects on tea. In this result accordance with Premkumar and Baby [23] have published the latest recommendations on the control of blister blight and grey blight in tea and also Karthika and Muraleedharan [24] supported that, fungicides residues were lost during the shoot expansion time and the 10th day, the level of residues on tea shoots are definitely lower than the limits of residue effect. Hence upon the climatic factor, i.e., due to such as mainly growth dilution, rainfall elution, thermal degradation and photodegradation. Both the fungal and bacterial biocontrol agents provided superior control for the integrated management of grey blight disease. Jo and willson [25] found that the exogenous application sof carbon and nitrogen sources increased the population of biocontrol agent, *P. syringae* in the phyllosphere and increased the biocontrol efficacy. The present study revived that the potential of the selected chemical fungicides (hexaconazole, tebuconazole and tridemorph). To sum up, the present investigation proved beyond doubt that various botanical fungicides like (Expel) neem kernel extract, garlic extract, aloe vera, tulsi and expel were experimented. It was found that expel showed the highest percentage of inhibition against *Macrophoma* sp. while Tulsi, Neem kernel, Garlic extract, and *Aloe vera* had no growth effect of test pathogen.

Conclusion

The study indicated that biocontrol agents (*Bacillus* spp. *Pseudomonas* spp. and *Trichoderma* spp.), botanical fungicide (Expel) and chemical fungicide (Tebuconazole) are very effective to control the branch canker pathogen under *in vitro* conditions. There was absolutely no growth in the fungicide amended plates even at a lower concentration. From this study, it was critically evaluated that *Bacillus* spp. and *Pseudomonas* spp. followed by *Trichoderma* spp. botanical fungicide (Expel) and chemical fungicide (tebuconazole) strengthens the integrated disease management of branch canker disease in tea.

Acknowledgements

The authors are thankful to UPASI Tea Research Institute, Valparai, Coimbatore dist. for their constant encouragement. We wish to extend our heartfelt thanks to Tea Board of India, Kolkata, for the financial support.

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List of submitted sequences

NCBI GENBANK SUBMISSION LIST

Branch canker pathogen strains

1. **J. Mareeswaran**, P. Nepolean, R. Jayanthi, R. Premkumar Samuel Asir, A. Balamurugan and T. Princy (2015).Branch canker tea pathogen (*Macrophoma* sp.) isolated from infected tea stem portions in India. Code No: VPM (Molecular Accession No: KP004441).

2. **J. Mareeswaran**, P. Nepolean, R. Jayanthi, and R. Premkumar Samuel Asir, (2015).Branch canker tea pathogen (*Macrophoma theicola*) isolated from infected tea stem portions in India. Code No: UPA-61 (Molecular Accession No: KP179221).

3. **J. Mareeswaran**, R. Jayanthi, P. Nepolean, R. Premkumar Samuel Asir and T. Princy (2015).Branch canker tea pathogen (*Macrophoma theicola*) isolated from infected tea stem portions in India. Code No: UPA-62 (Molecular Accession No: KP179222).

Bacterial Biocontrol strains

1. **J. Mareeswaran**, R. Jayanthi, P. Nepolean R. Premkumar Samuel Asir, T. Kuberan and A. Balamurugan (2014). Bioefficacy of *Bacillus subtilis* subsp. subtilis against branch canker tea pathogen (*Macrophoma* sp.) isolated from infected tea soils in India. Code No: CS-2(Molecular Accession No: KM527836).

2. **J. Mareeswaran**, P. Nepolean R. Jayanthi, and R. Premkumar Samuel Asir (2014). Bioefficacy of *Pseudomonas fluorescens* against branch canker tea pathogen (*Macrophoma* sp.) isolated from infected tea soils in India. Code No: TRB (Molecular Accession No: KM527837).

3. **J. Mareeswaran**, P. Nepolean R. Jayanthi, A. Balamurugan R. Premkumar Samuel Asir and T.Princy (2014). Bioefficacy of *Bacillus licheniformis* against branch canker tea pathogen (*Macrophoma* sp.) isolated from infected tea soils in India. Code No: AWRH-40B (Molecular Accession No: KM527838).

4. A. Balamurugan, M. Jayaprakashvel, S. Soumik, P. Nepolean, R. Jayanthi, **J. Mareeswaran**, T.Princy and R. Premkumar Samuel Asir (2014). Bioefficacy of *Bacillus amyloliquefaciens* (*Bacillus velezensis*) against grey blight and branch canker diseases isolated from infected tea soils in India. Code No: WP104 (Molecular Accession No: KM853034).

Nucleotide

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Macrophoma sp. VPM-12 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KP004441.1

FASTA Graphics

Go to:

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  TITLE     Direct Submission
  JOURNAL   Submitted (17-OCT-2014) Plant Pathology, Upasi Tea Research
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      901 cgatagcgca caagtagagt gatcgaaaga tgaaaagcac tttggaaaag agagttaaaa
      961 agtacgtgaa attgttgaaa gggaagcgct tgcaaccaga cttgtccgca gttgctcagc
     1021 cggtctcctg accggcgtac tcttctgcgg ccaggccagc atcagttcgg gcggtcggat
     1081 aaagacctcg ggaatgtagc tcctctcggg gagtgttata gcccggggtg gaatgcggcc
     1141 agcctggact gaggatctcg cttcggcaag gatgctggcg taatggttgt aagcggcccg
     1201 tcttgaaaca cggaccaagg agtctaacat ctatgcgagt gttcgggtgt caaacccgta
     1261 cgcgtaatga aagtgaacgg aggtgggaac ccgcaagggt gcaccatcgg ccgatcctga
     1321 tgtcttcgga tggatttgag caagagcata gctgttggga cccgaaagat ggtgaactat
     1381 gcctgaatag ggtgaagcca gaggaaactc tggtggaggc tcgcagcggt tctgacgtgc
     1441 aaatcgatcg tcaaatttgg gtataggggc gaaagactaa tcgaaccatc tagtagctgg
     1501 ttcctgccga agtttccctc aggatagcag taacgtattc agttttatga ggtaaagcga
     1561 atgattagag gccttggggc tgaaacagcc ttaacctatt ctcaaacttt aaatatgtaa
     1621 gaagtccttg ttacttaat
```

Nucleotide

Display Settings: GenBank

Macrophoma theicola strain UPA-61 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KP179221.1

FASTA Graphics

Go to:

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LOCUS       KP179221               1652 bp    DNA     linear   PLN 10-FEB-2015
DEFINITION  Macrophoma theicola strain UPA-61 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,
            and internal transcribed spacer 2, complete sequence; and 28S
            ribosomal RNA gene, partial sequence.
ACCESSION   KP179221
VERSION     KP179221.1  GI:753249421
KEYWORDS    .
SOURCE      Macrophoma theicola
  ORGANISM  Macrophoma theicola
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetes incertae sedis; Botryosphaeriales;
            Botryosphaeriaceae; Macrophoma.
REFERENCE   1  (bases 1 to 1652)
  AUTHORS   Mareeswaran,J., Nepolean,P., Jayanthi,R. and Premkumar Samuel
            Asir,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-NOV-2014) Plant Pathology, Upasi Tea Research
            Institute, Nirar Dam, Valparai, Tamil Nadu 642127, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /organism="Macrophoma theicola"
                     /mol_type="genomic DNA"
                     /strain="UPA-61"
                     /db_xref="taxon:1609051"
     misc_RNA        <1..>1652
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
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       61 tagaggaagt aaaagtcgta acaaggtttc cgtaggtgaa cctgcggaag gatcattacc
      121 gagttttcga gctccggctc gactctccca ccctttgtga acgtacctct gttgctttgg
      181 cggctccggc cgccaaagga ccttcaaact ccagtcagta aacgcagacg tctgataaac
      241 aagttaataa actaaaactt tcaacaacgg atctcttggt tctggcatcg atgaagaacg
      301 cagcgaaatg cgataagtaa tgtgaattgc agaattcagt gaatcatcga atctttgaac
      361 gcacattgcg ccccttggta ttccgggggg catgcctgtt cgagcgtcat tacaaccctc
      421 aagctctgct tggaattggg caccgtcctc actgcggacg cgcctcaaag acctcggcgg
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      541 cgccggacga accttctgaa cttttctcaa ggttgacctc ggatcaggta gggatacccg
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      661 ggcgagtgaa gcggcaacag ctcaaatttg aaagctggcc cttttagggt ccgcattgta
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      781 gagggtgaga atcccgtatg cggtaggttg ccttagccat gtgaatctcc ttcgacgagt
      841 cgagttgttt gggaatgcag ctctaaatgg gaggtaaatt tcttctaaag ctaaataccg
      901 gccagagacc gatagcgcac aagtagagtg atcgaaagat gaaaagcact ttggaaagag
      961 agttaaaaag tacgtgaaat tgttgaaagg gaagcgcttg caaccagact tgtccgcagt
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     1441 tgacgtgcaa atcgatcgtc aaatttgggt ataggggcga aagactaatc gaaccatcta
     1501 gtagctggtt cctgccgaag tttccctcag gatagcagta acgtattcag ttttatgagg
     1561 taaagcgaat gattagaggc cttggggctg aaacagcctt aacctattct caaactttaa
     1621 atatgtaaga agtccttgtt acttagttga ac
//
```

Nucleotide

Display Settings: GenBank

Macrophoma theicola strain UPA-62 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GenBank: KP179222.1

FASTA Graphics

Go to:

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LOCUS       KP179222               1656 bp    DNA     linear   PLN 10-FEB-2015
DEFINITION  Macrophoma theicola strain UPA-62 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,
            and internal transcribed spacer 2, complete sequence; and 28S
            ribosomal RNA gene, partial sequence.
ACCESSION   KP179222
VERSION     KP179222.1  GI:753249425
KEYWORDS    .
SOURCE      Macrophoma theicola
  ORGANISM  Macrophoma theicola
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetes incertae sedis; Botryosphaeriales;
            Botryosphaeriaceae; Macrophoma.
REFERENCE   1  (bases 1 to 1656)
  AUTHORS   Mareeswaran,J., Jayanthi,R., Nepolean,P., Premkumar Samuel Asir,R.
            and Princy,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-NOV-2014) Plant Pathology, Upasi Tea Research
            Institute, Nirar Dam, Valparai, Tamil Nadu 642127, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="UPA-62"
                     /db_xref="taxon:1609051"
     misc_RNA        <1..>1656
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
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       61 atttagagga agtaaaagtc gtaacaaggt ttccgtaggt gaacctgcgg aaggatcatt
      121 accgagtttt cgagctccgg ctcgactctc ccacccttg tgaacgtacc tctgttgctt
      181 tggcggctcc ggccgccaaa ggaccttcaa actccagtca gtaaacgcag acgtctgata
      241 aacaagttaa taaactaaaa ctttcaacaa cggatctctt ggttctggca tcgatgaaga
      301 acgcagcgaa atgcgataag taatgtgaat tgcagaattc agtgaatcat cgaatctttg
      361 aacgcacatt gcgccccttg gtattccggg gggcatgcct gttcgagcgt cattacaacc
      421 ctcaagctct gcttggaatt gggcaccgtc ctcactgcgg acgcgcctca aagacctcgg
      481 cggtggctgt tcagccctca agcgtagtag aatacacctc gctttggagc ggttggcgtc
      541 gcccgccgga cgaaccttct gaacttttct caaggttgac ctcggatcag gtagggatac
      601 ccgctgaact taagcatatc aataagcgga ggaaaagaaa ccaacaggga ttgccttagt
      661 aacggcgagt gaagcggcaa cagctcaaat ttgaaagctg gccctttag ggtccgcatt
      721 gtaatttgta gaggatgatt cggcgagggc tcctgcctaa gtcccctgga acggggcgtc
      781 atagagggtg agaatcccgt atgcggtagg ttgccttagc catgtgaatc tccttcgacg
      841 agtcgagttg tttgggaatg cagctctaaa tgggaggtaa atttcttcta aaagctaaat
      901 accggccaga gaccgatagc gcacaagtag agtgatcgaa agatgaaaag cactttggaa
      961 agagagttaa aaagtacgtg aaattgttga aagggaagcg cttgcaacca gacttgtccg
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     1501 tctagtagct ggttcctgcc gaagtttccc tcaggatagc agtaacgtat tcagttttat
     1561 gaggtaaagc gaatgattag aggccttggg gctgaaacag ccttaaccta ttctcaaact
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Nucleotide

Display Settings: GenBank

Bacillus subtilis subsp. subtilis strain CS-2 16S ribosomal RNA gene, partial sequence

GenBank: KM527836.1

FASTA Graphics

Go to:

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LOCUS       KM527836               1357 bp    DNA     linear   BCT 14-OCT-2014
DEFINITION  Bacillus subtilis subsp. subtilis strain CS-2 16S ribosomal RNA
            gene, partial sequence.
ACCESSION   KM527836
VERSION     KM527836.1  GI:695503230
KEYWORDS    .
SOURCE      Bacillus subtilis subsp. subtilis
  ORGANISM  Bacillus subtilis subsp. subtilis
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1357)
  AUTHORS   Mareeswaran,J., Jayanthi,R., Nepolean,P., Premkumar Samuel Asir,R.,
            Kuberan,T. and Balamurugan,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-SEP-2014) Plant Pathology, Upasi Tea Research
            Institute, Nirar Dam, Valparai, Tamilnadu 642127, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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     rRNA            <1..>1357
                     /product="16S ribosomal RNA"
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      121 actgggataa ctccgggaaa ccggggctaa taccggatgg ttgtttgaac cgcatggttc
      181 aaacataaaa ggtggcttcg gctaccactt acagatggac ccgcggcgca ttagctagtt
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      301 cactgggact gagacacggc ccagactcct acgggaggca gcagtaggga atcttccgca
      361 atggacgaaa gtctgacgga gcaacgccgc gtgagtgatg aaggttttcg gatcgtaaag
      421 ctctgttgtt agggaagaac aagtaccgtt cgaatagggc ggtaccttga cggtacctaa
      481 ccagaaagcc acggctaact acgtgccagc agccgcggta atacgtaggt ggcaagcgtt
      541 gtccggaatt attgggcgta aagggctcgc aggcggtttc ttaagtctga tgtgaaagcc
      601 cccggctcaa ccggggaggg tcattggaaa ctggggaact tgagtgcaga agaggagagt
      661 ggaattccac gtgtagcggt gaaatgcgta gagatgtgga ggaacaccag tggcgaaggc
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      781 taccctggta gtccacgccg taaacgatga gtgctaagtg ttaggggttt tccgcccctt
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      901 caaaggaatt gacgggggcc cgcacaagcg gtggagcatg tggtttaatt cgaagcaacg
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     1141 ggtgactgcc ggtgacaaac cggaggaagg tggggatgac gtcaaatcat catgcccctt
     1201 atgacctggg ctacacacgt gctacaatgg acagaacaaa gggcagcgaa accgcgaggt
     1261 taagccaatc ccacaaatct gttctcagtt cggatcgcag tctgcaactc gactgcgtga
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```

Nucleotide

Display Settings: GenBank

Pseudomonas fluorescens strain TRB 16S ribosomal RNA gene, partial sequence

GenBank: KM527837.1

FASTA Graphics

Go to:

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LOCUS       KM527837               1469 bp    DNA     linear   BCT 14-OCT-2014
DEFINITION  Pseudomonas fluorescens strain TRB 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KM527837
VERSION     KM527837.1  GI:695503247
KEYWORDS    .
SOURCE      Pseudomonas fluorescens
  ORGANISM  Pseudomonas fluorescens
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 1469)
  AUTHORS   Mareeswaran,J., Nepolean,P., Jayanthi,R. and Premkumar Samuel
            Asir,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-SEP-2014) Plant Pathology, Upasi Tea Research
            Institute, Nirar Dam, Valparai, Tamilnadu 642127, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /strain="TRB"
                     /db_xref="taxon:294"
     rRNA            <1..>1469
                     /product="16S ribosomal RNA"
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      661 ggtgaaatgc gtagatatag gaaggaacac cagtggcgaa ggcgaccacc tggactgata
      721 ctgacactga ggtgcgaaag cgtggggagc aaacaggatt agataccctg gtagtccacg
      781 ccgtaaacga tgtcaactag ccgttggaag ccttgagctt ttagtggcgc agctaacgca
      841 ttaagttgac cgcctgggga gtacggccgc aaggttaaaa ctcaaatgaa ttgacggggg
      901 cccgcacaag cggtggagca tgtggtttaa ttcgaagcaa cgcgaagaac cttaccaggc
      961 cttgacatcc aatgaacttt ccagagatgg attggtgcct tcgggaacat tgagacaggt
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     1201 acgtgctaca atggtcggta caaagggttg ccaagccgcg aggtggagct aatcccataa
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//
```

Nucleotide

Display Settings: GenBank

Bacillus licheniformis strain AWRH-40B 16S ribosomal RNA gene, partial sequence

GenBank: KM527838.1

FASTA Graphics

Go to:

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LOCUS       KM527838               1380 bp    DNA     linear   BCT 14-OCT-2014
DEFINITION  Bacillus licheniformis strain AWRH-40B 16S ribosomal RNA gene,
            partial sequence.
ACCESSION   KM527838
VERSION     KM527838.1  GI:695503265
KEYWORDS    .
SOURCE      Bacillus licheniformis
  ORGANISM  Bacillus licheniformis
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1380)
  AUTHORS   Mareeswaran,J., Nepolean,P., Jayanthi,R., Balamurugan,A., Premkumar
            Samuel Asir,R. and Princy,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-SEP-2014) Plant Pathology, Upasi Tea Research
            Institute, Nirar Dam, Valparai, Tamilnadu 642127, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:1402"
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                     /product="16S ribosomal RNA"
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//
```

Nucleotide

Display Settings: GenBank

Bacillus amyloliquefaciens strain WP1O4 16S ribosomal RNA gene, partial sequence

GenBank: KM853034.1

FASTA Graphics

Go to:

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LOCUS       KM853034               1405 bp    DNA     linear   BCT 30-NOV-2014
DEFINITION  Bacillus amyloliquefaciens strain WP1O4 16S ribosomal RNA gene,
            partial sequence.
ACCESSION   KM853034
VERSION     KM853034.1  GI:725827713
KEYWORDS    .
SOURCE      Bacillus amyloliquefaciens (Bacillus velezensis)
  ORGANISM  Bacillus amyloliquefaciens
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1405)
  AUTHORS   Balamurugan,A., Jayaprakashvel,M., Soumik,S., Nepolean,P.,
            Jayanthi,R., Mareeswaran,J., Princy,T. and Premkumar Samuel Asir,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-SEP-2014) Plant Pathology, Upasi Tea Research
            Institute, Nirar Dam, Valparai, Tamil Nadu 642127, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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