ECOFRIENDLY MANAGEMENT OF ANTHRACNOSE OF BETEL VINE (*Piper betle* L.)

by

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(2016-11-021)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Ecofriendly management of anthracnose of betel vine (*Piper betle* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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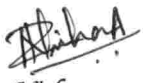
" I will give thanks to you LORD, with all my heart; I will tell of all your wonderful deeds"



Nisha

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
°C	Degree Celsius
CD	Critical difference
cfu	Colony forming units
et al .	And other co-workers
h	Hours
sec	Seconds
min	Minutes
i.e.	that is
ha	Hectares
L	Litre
ml	Millilitre
µl	Microlitre
cm	Centimeter
mm	Millimeter
µm	Micrometer
kg	Kilogram
g	Gram
mg	Milligram
µg	Microgram
viz.	Namely
rpm	Revolutions per minute
sp. or spp.	Species (Singular and plural)
S.Em±	Standard error of mean
DAI	Days after inoculation
rDNA	Ribosomal deoxy ribo nucleic acid

DNA	Deoxyribonucleic acid
ITS	Internally transcribed spacers
ppm	Parts per million
PDI	Percentage disease index
DI	Disease incidence
PDA	Potato dextrose agar
nos.	Numbers
Sl. No.	Serial number
NA	Nutrient agar
SDW	Sterile distilled water
bp	Base pairs
PCR	Polymerase chain reaction
WP	Wettable powder
EC	Emulsifiable concentrate

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Introduction

1. INTRODUCTION

Betel vine (*Piper betle* L.) is an important cash crop cultivated for its green heart shaped leaves and widely used as masticatory. Betel leaves are rich in protein, vitamins (A, B, C) and minerals. It is an important source of essential oils and alkaloids (Guha, 2006). When chewed along with arecanut, it acts as cheap source of dietary calcium (Shahzad and Zareen, 1999). It is also used in various religious ceremonies in India as token of respect. It is shade loving crop grown in moist tropical regions of the world especially in India, Bangladesh, Srilanka, Malayasia, Thailand and other Southeast Asia.

India is the leading producer of betel leaves (Arulmozhiyan *et al.,* 2005). In India, the crop is cultivated in an area of 75,000 ha with an annual production worthing Rs. 1000 million (Dasgupta *et al*, 2011; Vijayakumar and Arumugam, 2012) Das *et al.* (2017) reported that the cultivation of betel leaves creates livelihoods to about 20.8 lakh people of the country. Betel vine cultivation is highly labour intensive and particularly suited for small holdings to justify the term house hold bank. The leaves of betel are designated as green ATM for rural women (Das *et al.,* 2017).

The lucrative crop is commercially cultivated under shady and humid conditions in conservatories called *Boroj* which provide amble climatic conditions for the incidence of various diseases (Chattopadhyay and Maiti, 1990). The crop is prone to many fungal and bacterial diseases which limit its cultivation. Anthracnose or leaf spot disease caused by *Colletotrichum* sp. causes severe damage in the growing tracts. The incidence of the disease was first reported by Hector in 1925 from West Bengal. The infection caused 20 - 80 per cent leaf (Singh and Shankar, 1971) and 10 - 35 per cent stem damage (Dasgupta and Sen, 1999). Several species of *Colletotrichum* had been reported to incite anthracnose in betel vine, but *C. gloeosporioides* had wide spread occurrence.

C. gloeosporoides is an asexual facultative parasite belonging to the Division: Ascomycota, Order: Phyllachorales and family Phyllachoraceae

(Gautam, 2014). The fungus could survive in crop debris and soil during unfavourable climatic conditions (Sankar, 2002; Poornima, 2007).

The widely recommended management practice of the disease is foliar spray of 0.5% Bordeaux mixture. Hazardous fungicides were widely used by the farmers for the management of the disease apart from the traditional practice. The crop is mainly used for masticatory purpose, so indiscriminate use of the toxic fungicides lead to health hazards and environmental pollution. Hence, there is an urge for developing an integrated management strategy against the disease.

The present study entitled as "Ecofriendly management of anthracnose of betel vine (*Piper betle* L.)" was undertaken with an objective to develop an integrated management strategy involving organic preparations, biocontrol agents and new generation fungicides for anthracnose of betel vine. The study emphasised on the following aspects :

1. Symptomatology of the disease
2. Isolation of the pathogen and pathogenic studies
3. Cultural, morphological and other variability studies of the pathogen
4. *In vitro* studies on pathogen suppression using indigenous organic preparations
5. Isolation and evaluation of phyllosphere and rhizosphere fungal and bacterial antagonists
6. *In vitro* studies of pathogen suppression using fungicides
7. *In vivo* studies of disease suppression

Review of Literature

2. REVIEW OF LITERATURE

Betel vine is an important cash crop of India, widely known as "Green Gold". It is grown under cool shady climatic conditions, which promotes the incidence of many fungal diseases.

2.1. SYMPTOMATOLOGY, ISOLATION AND PROVING PATHOGENICITY OF *COLLETOTRICHUM* SP. CAUSING ANTHRACNOSE OF BETEL VINE

2.1.1. Importance, Disease Severity and Predisposing Factors

Anthracnose caused by *Colletotrichum* sp. limits the commercial cultivation of betel vine. *Colletotrichum* spp. were listed as one of the top ten fungal pathogens of scientific and economic importance (Dean *et al,* 2012).

Hector (1925) reported the incidence of anthracnose for the first time in India from Midnapur in West Bengal. The disease caused wide spread damage to the crop and the extent of damage varied with the plant parts affected. Singh and Shankar (1971) reported 20 - 80 per cent leaf damage while the crop loss due to leaf spot of betel vine ranged about 10 - 35 per cent (Maiti and Sen, 1982). The yield loss due to anthracnose was estimated to be around 10 - 60 per cent (Singh and Joshi, 1971; Maiti and Sen, 1982). Stem infection leading to gridling of vine resulted in 10 - 60 per cent damage (Dasgupta and Sen, 1999).

Different pathogens have been reported to cause leaf spot and stem anthracnose on betel vine in India. The four fungal pathogens *viz., Colletotrichum piperis* (Dastur, 1935), perithecial stage of *Glomerella cinganulata* (Stonem.) Spauld and Schrank (Dastur, 1931), *C. dasturi* Roy (Roy, 1948) and *C. capsici* Butler and Bibsy (Singh and Shankar, 1971) were reported to be associated with the disease. *C. dematium* was also reported to cause anthracnose in betel vine from Bangladesh (Ahmed *et al.,* 2014).

A survey conducted during 1996-1999 in betel vine farms of Thatta and Hub regions of Balochistan reported 15 and 30 per cent disease incidence of *C. capsici* whereas *C. gloeosporioides* incidence was 10 per cent in Karachi

region (Shahzad, 2000). Ahmed *et al.* (2014) observed 10.87 per cent and 80.50 per cent disease incidence of betel vine anthracnose during December and July from the Shalon village in Moulvibazar district.

The occurrence of disease was highly correlated with weather parameters *viz.*, temperature, relative humidity and rainfall. Huq (2011) reported that the incidence of leaf spot of betel vine was severe during March - May. The incidence of leaf spot was high at an average temperature of 26.7° C, 88.3 per cent relative humidity, 19.4 mm of rainfall in Barisal region of Bangladesh.

Dasgupta and Datta (2012) correlated meteorological parameters like temperature, rainfall and relative humidity with disease severity of betel vine anthracnose. The disease severity was positively correlated to maximum relative humidity and negatively to high temperature.

Minimum temperature, number of rainy days and rainfall had positive correlation while maximum temperature had negative correlation on the incidence of anthracnose of black pepper in Karnataka (Biju *et al.*, 2013). The disease incidence was mainly noticed during June - September with peak incidence in August and September. The minimum temperature of 17.6°- 18.7°C with monthly rainfall of 306.6 - 584.7 mm in 22 - 31 rainy days were reported to be highly favourable for peak incidence of disease.

2.1.2. Symptomatology

Hector (1925) observed that anthracnose caused foliar yellowing and wilting of vines. Dastur (1935) described the symptom initiated as circular spots with brown to black centre having yellow halo. Apart from leaves, the stem infection occurred in vines as black lesions. On severe infection withering, drying and death of affected vines were noticed.

The symptoms on leaves were characterised by the presence of one or more irregular spots with black centre having a diffused chlorotic yellow halo. The spots coalesced to form large lesions covering entire leaf lamina (Maiti and

Sen, 1979; Chandra and Sagar, 2004). The leaf blades drooped off when lesions were noticed in leaf margin due to shrinkage of the tissues. Premature defoliation was also noticed (Chattopadhyay and Maiti, 1990).

The stem infection could occur both in petiole and vines (Mehrotra, 1984). The symptom initially appeared as circular black specks which enlarged to form narrow streaks under moist conditions covering the entire stem. As the lesion coalesced, nodal infection led to wilting of stem. Development of vertical cracks with gummy exudations and splitting of internodes were also noticed. Under severe infection death and drying of affected vines were noticed (Chattopadhyay and Maiti, 1990).

Shahzad (2000) also reported similar symptoms which appeared on the stem initially as small black spots enlarged to black streaks leading to constriction and splitting of the stem. The old plantations were highly prone to the attack of anthracnose pathogen than newly planted ones.

2.1.3. Isolation of Pathogen

Roy (2001) isolated *Colletotrichum* sp. from the infected betel leaf and stem portions by tissue isolation technique. The infected portion along with healthy parts were surface sterilized in 0.1 per cent mercuric chloride followed by 3 - 4 washings with sterile water. The washed specimens were placed in the slides and incubated in humid chamber for four days for the formation of acervuli. The acervuli were picked and suspended in sterile water to obtain spore suspension. The suspension was serially diluted and single spore isolation was done for the purification of the culture. Tissue isolation method was used for the isolation of anthracnose pathogen from betel vine by Haralpatil (2006).

Isolation of *C. gloeosporioides* from mango leaves by surface sterilizing the infected bits with 0.1 per cent mercuric chloride followed by washing in sterile water was attempted by Sundaravadana *et al.*, 2007 and Koshale *et al.*, 2015.

Ann and Mercer (2017) isolated *C. gloeosporioides* from the infected leaf tissue and berries of black pepper by treating in 5 per cent sodium hypochlorite for five min followed by washing in sterile distilled water. The surface sterilized bits were placed in PDA medium under aseptic conditions and incubated at 30°C for 48 h. Kendre *et al.* (2017) isolated *C. gloeosporioides* from leaf blight affected thippali by leaf tissue method on PDA medium.

2.1.4. Proving Pathogenicity

Roy (2001) conducted the pathogenicity test of anthracnose pathogen by making wounds with carborandum powder on the leaf surface on to which spore suspension (5×10^5 conidia ml^{-1}) was sprayed.

Sankar (2002) proved pathogenicity of *C. gloeosporioides* on black pepper by pin prick method. Seven day old culture of the pathogen along with mycelium and conidia were inoculated on both the surface of the pin pricked portion of the leaves. A thin layer of moist cotton was placed over the injured portions in order to provide humidity for symptom development.

Chandrakant (2005) performed the pathogenicity test of *C. gloeosporioides* on black pepper seedlings by makings abberations using sterilized sand paper. On the aberrated surface, mycelial bits along with spores were placed and kept in humid chamber for the development of symptoms.

Haralpatil (2006) conducted the pathogenic studies of *C. gloeosporioides* in betel vine by leaf inoculation technique. The detached leaves were initially sterilized in 70 per cent alcohol followed by 3 - 4 washings in sterilized distilled water. The leaves injured using carborandum powder were inoculated with the spore suspension along with bits of mycelium and kept for symptom development.

Poornima (2007) proved the pathogenicity of *C. gloeosporioides* on thippali by inoculating the adaxial surface of the leaves with seven day old sporulated culture of the pathogen by pin prick method and incubated in a humid chamber.

2.2. MORPHOLOGICAL AND PATHOGENIC VARIABILITY OF *COLLETOTRICHUM* ISOLATES AND IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF VIRULENT ISOLATE

2.2.1. Culture and Morphological Characteristics of Isolates

C. gloeosporioides is an asexual facultative parasite belonging to the Division : Ascomycota, Order : Phyllachorales and representative of the family Phyllachoraceae (Gautam, 2014).

Sutton (1992) observed the production of variable white to dark grey aerial colonies with tufty appearance and on reverse side, it appeared as evenly white to dark grey in *C. gloeosporioides*. The pathogen produced pale salmon coloured conidial masses with straight cylindrical conidia measuring 12 - 17 µm x 3.5 - 6 µm.

The conidia of *C. gloeosporioides* were oblong or cylindrical with rounded edges (Prasad and Singh, 1960) and conidial size varied from 9 - 24 µm x 3 - 6 µm in length and breadth respectively. These conidia were formed on phialadic condiophore which was one celled and hyaline in nature (Mordue, 1971).

Naik and Hiremath (1986) reported that the *C. gloeosporioides* of betel vine produced smooth greyish black colonies with hyaline and oblong conidia with round ends and oil globules at centre. The size of the conidia varied about 8.6 - 19.9 µm x 3.5 - 6.5 µm.

Kanapathipillai (1996) studied the culture characters of 18 isolates of *C. gloeosporioides* from different hosts. The colony appearance of *C. gloeosporioides* on both sides were grey to orange coloured with regular margin. The mycelium was hyaline and growth rate of the isolates varied from 1.0 - 1.5 cm day^{-1}. *C gloeosporioides* from black pepper had conidial size of 14.31 µm x 4.33 µm and sub globose shaped appressoria having size of 6.55 µm x 5.28 µm.

The mycelial characteristics of *C. gloeosporioides* isolates of black pepper varied from cottony to appressed growth having colony colour either white, light grey to dark greyish in centre. The colour of the margin varied from dark grey to black. The average conidial size varied about 13.10 - 16.85 µm x 3.28 - 5.43 µm (Sankar, 2002).

The colony colour of *C. gloeosporioides* isolates of black pepper were light to dark grey. The mycelial width of the pathogen ranged about 1.25 - 4.00 µm while the spore size ranged about 13.45 - 16.45 µm x 3.86 - 5.36 µm (Mammooty, 2003).

Vivekananth (2006) stated that *C. gloeosporioides* produced white to greyish white, cottony growth in PDA. The conidia were hyaline, single celled with an oil globule at centre with diverse shapes of ovoid to cylindrical. The spore size varied from 16 µm x 4 µm in length and breadth respectively.

Poornima (2007) compared the culture and morphological characters of *C. gloeosporioides* from thippali, black pepper and betel vine. The thippali and betel vine isolates had evenly felt growth with white coloured colony while black pepper isolate had fluffy uneven white growth with grey margins. The colour of spore mass varied from light to dark salmon among thippali, black pepper and betel vine isolates. The sporulating nature also varied from shy, moderate and high among the *C. gloeosporioides* isolates of thippali, black pepper and betel vine respectively. The morphological studies revealed that the conidia of isolates were cylindrical, hyaline with oil globule at centre. The average size varied from 12.67 µm x 4.7 µm, 14.63 µm x 4.7 µm and 15.52 µm x 4.7 µm for thippali, black pepper and betel vine isolates respectively.

Ahmadi (2011) described that the colony nature of *C. gloeosporioides* of anthracnose in pomegranate as fluffy cottony white with regular margins which became greyish on sporulation. The study also revealed that spore morphology as hyaline, oblong conidia measuring 12.5 - 18.5 µm x 3.0 - 7.5 µm in size.

Studies on culture and morphological features of *C. gloeosporoides* from chilli revealed that their colonies were cottony to appressed having greyish black to white colour, while on rear side it appeared as creamish white. The pathogen produced 63.77 -74.16 µm x 40.89 - 53.85 µm sized acervulii having conidiophore measuring 30.05 - 56.60 µm x 3.40-6.64 µm. The conidial size varied between 29.26 µm - 32.10 µm x 2.82 - 6.07 µm and appressorial size from 11.06 - 12.95 µm x 10.08 - 12.10 µm (Parashar, 2013).

Balaso (2014) observed that *C. gloeosporioides* isolate obtained from pomegranate produced light grey to brown coloured colony in PDA. The morphological studies of the pathogen revealed that mycelium was hyaline and septate with 1.31 - 4.1 µm width. The conidia were hyaline, oblong to cylindrical in shape with rounded ends measuring 11.46 µm x 4.72 µm.

Gautam (2014) reported that *C. gloeosporioides* produced cottony circular colonies on culture media. The mycelium was hyaline, septate and branched. The conidia were hyaline, cylindrical with obtuse ends or dumbbell shaped with an average size of 10 - 15 µm x 5 - 7 µm.

Sreeja (2014) described the colony nature of cowpea isolate of *C. gloeosporioides* and it varied from dense to sparse. The colony colour varied from off white to grey colour on upper side while on reverse side it appeared as dark grey to black coloured colonies. The pathogen could complete the full growth in petridish within 6 - 10 days with an average growth rate of 0.80 - 1.38 cm day $^{-1}$. The mycelium was hyaline, septae and branched. The conidia were cylindrical with rounded apices having size of 8.6 - 11.3 µm x 3.5 - 4.3 µm.

Jagtap *et al.* (2015) observed that *C. gloeosporioides* produced light pinkish pigmented raised fluffy growth colony with intermixed black and white colour in PDA. The conidia were oblong or dumbbell shaped with one or two oil globules measuring an average size of 15.74 µm x 5.43 µm.

Aswani *et al.* (2016) studied the morphological characters of *Colletotrichum* sp. causing anthracnose of snakegourd and observed the hyphae as

hyaline, septate and had a width ranging from 2.22 - 3.86 µm. The conidia was hyaline, cylindrical with obtuse to round ends having 11 - 15 µm x 4 - 5 µm size.

2.1.2. Pathogenic Variability Studies of the Isolates

Sankar (2002) performed the pathogenic variability studies with different isolates of *C. gloeosporioides* from black pepper for their virulence based on the lesion diameter at 10 days after inoculation (DAI) and rated as highly, semi and mildly virulent.

The comparative virulence of *C. gloeosporioides* isolates from cowpea were assessed based on the time taken for symptom development by Sreeja (2014). The study reported that minimum time for symptom expression was positively correlated to high virulence.

2.1.3. Molecular Characterization of the Virulent Isolate

Molecular techniques of fungal identification are the one of the most reliable method for species level identification. The molecular techniques provided alternate methods for taxonomic and species demarcation studies (MacLean *et al.*, 1993). Cai *et al.* (2009) stated that the preliminary identification of *Colletotrichum* spp. could be done by phylogenetic analysis of ITS (Internally Transcribed Spacer) region.

The amplification of conserved regions of ITS using polymerase chain reaction was done for the molecular level identification of *C. gloeosporioides* (Raj *et al,* 2013). Abang *et al.* (2002) confirmed the identity of *C. gloeosporioides* by analysing the sequence of ITS region of the ribosomal DNA. Photita *et al.* (2005) molecularly characterized the *Colletotrichum* sp. affecting herbaceous plants by the amplification of the ITS regions 1 and 2 using primers ITS 4 and ITS 5. The characterization of *Colletotrichum* sp. associated with coffee berry disease was done by using amplification of rDNA ITS region (Prihastuti *et al.,* 2009). Zakaria *et al.* (2015) performed the molecular characterization of *Colletotrichum* sp. of mango by the amplification of ITS region of ribosomal DNA using ITS 4 and 5

and β- tubulin using Bt 2a and Bt 2b. The characterization of the *Colletotrichum* sp. using the amplified product of ITS region were also reported by Lu *et al.* (2004) and Xie *et al.* (2010) in strawberry.

2.3. SCREENING OF INDIGENOUS ORGANIC PREPARATIONS FOR *IN VITRO* PATHOGEN SUPPRESSION

Rajput (2011) evaluated the effectiveness of organic preparations *viz.*, panchagavya, jeevamruth, cow's urine and vermiwash against *Colletotrichum capsici* under *in vitro* conditions. The 10 per cent organic preparations of panchagavya, jeevamruth, cow's urine and vermiwash recorded 5.6 per cent, 5.6 per cent, 11.13 per cent and 11.10 per cent mycelial inhibition respectively. Similarly at 20 per cent the organic preparations recorded 5.6 per cent, 11.06 per cent, 12.03 per cent and 11.06 per cent mycelial inhibition respectively.

The effectiveness of organic preparations like panchgavya, vermiwash and cow's urine against soil borne pathogens was investigated by Chadha *et al.* (2012). The study revealed that panchgavya, vermiwash and cow's urine at 2 per cent inhibited 85.73 per cent, 91.00 per cent and 81.52 per cent of mycelial growth of *C. capsici* under *in vitro* conditions respectively.

Kambar *et al.* (2013) reported the efficacy of cow's urine in inhibiting the growth of *C. capsici* of chilli under *in vitro* conditions. The mycelial growth of the pathogen showed an inhibition of 75.4 per cent and 79.7 per cent at 10 per cent and 15 per cent concentrations respectively. The study also revealed that cow's urine at 5 per cent could inhibit 56.5 per cent mycelial growth.

Effect of vermiwash on incitant of mango anthracnose was studied by Kumari *et al.* (2013). They observed that vermiwash at 1 per cent was effective in inhibiting the spore germination of the pathogen by 64.24 per cent.

Ashlesha and Paul (2014) evaluated the anti-fungal effect of cow's urine, vermiwash, butter milk, biosol and panchagavya against damping off and fruit rot

pathogens of bell pepper under *in vitro*. Complete growth inhibition of *C. capsici* was noticed at 10 per cent fermented cow's urine in amended media.

Chacko (2015) reported complete growth inhibition of fruit rot pathogen of chilli by panchagavya at 2.5 per cent, 5 per cent and 10 per cent concentrations. Jeevamruth at 5 and 10 per cent concentrations inhibited 75.45 per cent and 96.39 per cent of mycelial growth of the pathogen respectively under *in vitro*.

Jandaik *et al.* (2015) reported the inhibitory effect of 15 per cent concentrated cow's urine against soil borne pathogens in chilli *viz., Fusarium oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii*. The treatments recorded 82.62 per cent, 76.92 per cent and 76.28 per cent mycelial inhibition of the pathogens. *F. oxysporum, R. solani* and *S. rolfsii* respectively. Patel *et al.* (2015) reported that 2 per cent cow's urine inhibited 26.08 - 48.76 per cent mycelial growth of different isolates of *C. falcatum* under *in vitro* conditions.

Parvathy and Girija (2016) evaluated the efficacy of organic preparations *viz,* compost tea, vermiwash, fish amino acid and jeevamruth on the growth of *C. gloeosporoides* of black pepper under *in vitro*. Complete growth inhibition of pathogen was recorded at 2.5 per cent, 5 per cent and 10 per cent concentrations of jeevamruth. Vermiwash at 10 per cent was also effective in inhibiting the mycelial growth of the pathogen (79.68 per cent).

Jandaik and Sharma (2016) assessed the efficacy of panchagavya against soil borne pathogens. Panchagavya at 15 per cent concentration recorded 82.62 per cent, 76.28 per cent and 76.92 per cent of mycelial inhibition of *F. oxysporum, S. rolfsii* and *R. solani* respectively.

Karthika *et al.* (2017) reported that panchagavya at 5 per cent completely inhibited the mycelial growth of *R. solani* causing sheath blight of rice. They observed that the sclerotia failed to germinate after dipping for 24 h in panchagavya.

2.4. SCREENING OF BIOCONTROL AGENTS FOR *IN VITRO* PATHOGEN SUPPRESSION

Tucci *et al.* (2011) reported that biological control was the safest method to reduce plant disease severity without collateral damages to the environment as well as to human health induced by indiscriminate use of fungicides.

Souza *et al.* (2001) reported the antagonistic potential of *Trichoderma harzianum* isolated from the soils of betel vine tracts against *C. capsici, Phytophthora parasitica, S. rolfsii* and *R. solani* under *in vitro* conditions.

Sankar (2002) evaluated the effectiveness of phyllosphere and rhizosphere fungal antagonist against *C. gloeosporioides* of black pepper. The two phyllosphere isolates of *Aspergillus niger* recorded 53.06 - 54.08 per cent growth inhibition of the pathogen. Two isolates of *T. harzianum* were also effective in inhibiting the pathogen (49.97 per cent and 43.61 per cent).

The efficacy of fungal and bacterial biocontrol agents against *C. gloeosporioides* of vanilla was evaluated by Ashoka (2005). The fungal bioagents were reported to be highly effective than bacterial bioagents. *T. harzianum* recorded 64.65 per cent of mycelial inhibition of the pathogen under *in vitro* conditions. Similarly *T. koningii, T. virens, T. viride, Pseudomonas fluorescens* and *Bacillus subtilis* also showed inhibition of 46.72 per cent, 54.50 per cent, 55.38 per cent, 50.41 per cent and 42.48 per cent on the mycelial growth respectively.

Haralpatil (2006) reported antagonistic ability of *T. viride* against *C. gloeosporioides* of betel vine. *T. viride* recorded 89.66 per cent of mycelial growth inhibition under *in vitro* condition. The study also revealed that bio control agents *viz., T. koningii, Gliocladium virens, P. fluorescens* and *T. harzianum* were also effective in suppressing the pathogen with an inhibition percentage of 80.77, 77.44, 77.44 and 75.22 respectively.

Poornima (2007) evaluated fungal antagonists against anthracnose of thippali and observed that *T. viride* and *A. terreus* were effective in suppressing the growth of pathogen under *in vitro* conditions. The fungal antagonists recorded 84.3 per cent and 80 per cent mycelial inhibition of *C. gloeosporioides* respectively.

Deka *et al.* (2008) evaluated the efficacy of fungal antagonists *viz., T. harzianum, A. terreus* and bacterial antagonist *viz., B. subtilis* and *P. fluorescens* against leaf spot disease complex of betel vine. The study demonstrated *B. subtilis* to be highly effective in controlling the disease complex under *in vitro* conditions. The biocontrol agent recorded an inhibition percentage of 79.5, 68.1 and 76.4 on growth of *C. capsici, Xanthomonas axonopodis* pv. *betlicola* and their complex respectively.

Prapagdee *et al.* (2008) reported the antagonistic ability of soil actinomycete, *Streptomyces hygroscopicus* against *C. gloeosporoides* and *S. rolfsii* under *in vitro* conditions.

Shovan *et al.* (2008) assessed the antagonistic potential of 20 different isolates of *Trichoderma* spp. against *C. dematium* of soyabean. The different isolates showed mycelial inhibition ranging from 50.93 - 89.44 per cent against *C. dematium.*

The *Bacillus* sp. isolated from the rhizosphere of healthy black pepper showed antagonistic ability against its major pathogens. *B. subtilis* was reported to be most effective antagonistic bacterial isolate under *in vitro* inhibiting *C. gloeosporioides, C. capsici, F. solani and Streptobasidium* sp. This antagonistic bacteria recorded 40.4 - 54.2 per cent mycelial growth inhibition which was attributed to the lysis of the cell wall by cellulase and protease enzymes. Hyphal malformations *viz.,* hyphal thickening, vacuolar formation and swellings were also induced by the biocontrol agent (Ann, 2012).

Patil *et al* (2009a) reported *T. viride* and *T. harzianum* as effective fungal antagonist inhibiting the mycelial growth of *C. gloeosporiodes* from betel vine

under *in vitro* conditions with 66.29 per cent and 58.06 per cent respectively. Patil *et al.* (2009b) recorded 70 42 per cent and 66.90 per cent growth inhibition of *C. gloeosporioides* causing leaf blight in thippali by *T. viride* and *T. harzianum* respectively under *in vitro* conditions. Zivkovic *et al.* (2010) reported *S. natalensis* and *S. noursei* were able to inhibit the mycelial growth of *C. gloeosporioides* under *in vitro* conditions.

Tasiwal *et al.* (2009) evaluated the efficacy of bioagents against the anthracnose pathogen of papaya. The study revealed that *T. virens* was superior to other biocontrol agents inhibiting the pathogen (60.87 per cent). The other bioagents *viz., T. harzianum, T. viride, T. koningii, P. fluorescens and B. subtilis* recorded 51.89 per cent, 50.11 per cent, 53.32 per cent, 42.87 per cent and 50.97 per cent mycelial inhibition respectively.

Jayalakshmi (2010) reported that, among the fungal bioagents screened against *C. gloeosporioides*, *T. viride* showed a maximum inhibition of 86.82 per cent on mycelial growth. The other bioagents *viz., T. harzianum, P. fluorescens* and *B. subtilis* recorded 72.47 per cent, 67 per cent and 53.88 per cent mycelial inhibition respectively.

Rajput (2011) evaluated the efficacy of bioagents against anthracnose of chilli. The fungal bioagent *T. harzianum* was effective in managing the pathogen with an inhibition percentage of 71.80 under *in vitro* conditions. The other bioagents *T. virens, T. viride, P. fluorescens* and *B. subtilis* which were screened for their antagonistic potential recorded per cent mycelial inhibition of 65.22, 69.58, 46.77 and 41.02 respectively.

Azad *et al.* (2013) assessed the efficacy of *Trichoderma* spp. against *C. gloeosporioides* of banana under *in vitro* conditions. Of the different *Trichoderma* spp. evaluated, *T. harzianum* was superior to *T. viride* and *T. virens* in inhibiting the pathogen.

Rahman *et al.* (2013) isolated a potential antagonistic strain of *T. harzianum* (IMI-392433) from the rhizosphere of chilli against *C. capsici*. The

metabolites of the antagonist inhibited the spore germination and germ tube elongation of *C. capsici.*

A. niger was a potent antagonist against *C. gloeosporioides* of betel vine under *in vitro* conditions (Ahmed *et al.,* 2014). Srividya (2014) reported *B. subtilis* as an effective bacterial biocontrol agent against *C. gloeosporioides* of chilli.

Thakur and Harsh (2014) screened fungal antagonists against *C. gloeosporioides* of thippali. They identified *T. harzianum* ISO-1, *T. harzianum* ISO-2 and *T. piluliferum* as effective with 90 per cent mycelial inhibition. The antagonistic ability attributed was mycoparasitism of hyphae.

A promising bacterial biocontrol agent *B. subtilis* CBF was isolated from black pepper which inihibited mycelial growth (50.1 per cent) and conidial germination (83.8 per cent) of *C. gloeosporioides* (Ann *et al.,* 2015).

Chacko (2015) evaluated the efficacy of antagonistic microbes from the pomoplane and phyllosphere of chilli against *C. capsici.* The fungal antagonist *T. viride* recorded a mycelial inhibition of 68.40 per cent while, *Pencillium citrinum* and *Aspergillus* sp. recorded 54.73 per cent and 43.04 per cent inhibition. *P. fluorescens* and *Bacillus* sp. exhibited 73.33 and 88.88 per cent mycelial inhibition on pathogen.

Hosen *et al.* (2016) attributed the antagonistic ability of *A. niger* and *T. viride* against *C. gloeosporioides* of jute to volatile and non-volatile metabolites. The volatile metabolites *A. niger* and *T. viride* recorded 25 and 46 percentage of mycelial growth inhibition.

Anusree and Bhai (2017) evaluated the efficacy of actinobacteria *viz, Streptomyces, Actinomycetes* and *Micromonospora* against major fungal pathogens affecting black pepper. *Streptomyces* sp. isolated from rhizosphere was most effective against *C. gloeosporioides* with 90 per cent mycelial growth inhibition.

2.5. SCREENING OF FUNGICIDES FOR *IN VITRO* PATHOGEN SUPPRESSION

Efficacy of systemic fungicides *viz.*, carbendazim, propiconazole and carbendazim + mancozeb at 0.1 per cent concentration were tested against *C. gloeosporioides* of vanilla. The study revealed that carbendazim, propiconazole and carbendazim + mancozeb were highly effective in managing the pathogen *in vitro* (93.70 per cent, 100 per cent and 100 per cent inhibition respectively) (Ashoka, 2005).

Chandrakrant (2005) reported the effectiveness of triazole fungicides in managing *C. gloeosporioides* of black pepper. Propiconazole, difenconazole and hexaconazole at 0.05per cent inhibited the mycelial growth.

Haralpatil (2006) reported that propiconazole and hexaconazole at 0.05per cent were highly effective in controlling the growth of *C. gloeosporioides* of betel vine under *in vitro* condition (100 per cent).

Strobilurin fungicide azoxystrobin at 1.0, 2.0, and 4.0 ppm concentrations completely inhibited mycelial growth of *C. gloeosporioides* of mango under *in vitro* conditions (Sundaravadana *et al.*, 2007).

Shovan *et al.* (2008) evaluated the efficacy of propiconazole, carboxin, Iprodione, mancozeb and copper oxy chloride at 100, 200 and 400 ppm against *C. dematium* of soyabean and concluded that propiconazole even at 100 ppm concentrations completely inhibited the mycelial growth of the pathogen.

Patil *et al.* (2009a) evaluated the efficacy of propiconazole, carbendazim + mancozeb and mancozeb in inhibiting mycelial growth of *C. gloeosporioides* in betel vine at 0.1 per cent and 0.2 per cent. It was found that propiconazole and carbendazim + mancozeb recorded 100 per cent inhibition on growth while mancozeb recorded only 36.58 per cent.

The systemic, contact and combination fungicides tested against *C. gloeosporioides* of thippali by Patil *et al.* (2009b) revealed that combination

fungicide of carbendazim + mancozeb at 0.2 per cent recorded highest mycelial inhibition (96.25 per cent).

Tasiwal *et al.* (2009) reported the effectiveness of propiconazole (25% EC), hexaconazole (5% EC) and mancozeb (75% WP) against anthracnose pathogen of papaya. These fungicides at 0.15 per cent recorded 100 per cent, 94.33 per cent and 88.61 per cent mycelial inhibition respectively under *in vitro* conditions.

Jayalakshmi (2010) evaluated systemic, non-systemic and combination fungicides against anthracnose caused by *C. gloeosporioides* of pomegranate. The study revealed that mancozeb and carbendazim + mancozeb at 0.3 per cent inhibited 22.99 per cent and 81.88 per cent mycelial growth of the pathogen respectively. Azoxystrobin and propiconazole at 0.15 per cent recorded 62.77 per cent and 87.10 per cent of mycelial inhibition under *in vitro* conditions. Ahmadi (2011) observed complete growth inhibition of *C. gloeosporioides* at 100 ppm of mancozeb and while systemic fungicide propiconazole was effective even at 25 ppm.

Basalingappa (2011) reported that the systemic fungicides propiconazole and tebuconazole at 400 ppm effectively inhibited *C. gloeosporioides* of mango with 83.11 per cent and 80.33 per cent mycelial inhibition.

Pandit (2012) evaluated the efficacy of propiconazole, tebuconazole, mancozeb and copper oxychloride against *C. gloeosporioides* of Sarpagandha. The propiconazole and copper oxychloride (0.2 per cent) recorded 100 per cent growth inhibition under *in vitro* conditions. Mancozeb (0.2 per cent) was least effective with 27.77 per cent of mycelial inhibition.

Devamma *et al.* (2012) reported that mancozeb at 500 ppm inhibited the mycelial growth of *C. gloeosporioides* of mango by cent percent. The study also revealed that new generation fungicides *viz.*, propiconazole and hexaconazole at 25 ppm and contact fungicide copper oxychloride at 500 ppm recorded 82.91 per cent, 87.53 per cent and 81.97 per cent of growth suppression respectively.

Jayalakshmi *et al.* (2013) evaluated the effectiveness of copper hydroxide, mancozeb, azoxystrobin, hexaconazole, propiconazole and carbendazim + mancozeb against *C. gloeosporioides* of pomegranate under *in vitro* conditions. Carbendazim + mancozeb at 0.3 per cent and propiconazole at 0.15 per cent were the best among the fungicides tested. These fungicides recorded 81.88 per cent and 87.10 per cent mycelial inhibition.

Complete growth inhibition of *C. gloeosporioides* of betel vine by propiconazole, tebuconazole at 50 ppm and 300 ppm respectively under *in vitro* condition (Ahmed *et al.*, 2014).

Balaso (2014) reported that tebuconazole and azoxystrobin at 0.1 per cent recorded good mycelial inhibition of *C. gloeosporioides* of pomegranate. The respective fungicides at 0.05 per cent inhibited 84.32 per cent and 73.69 per cent of the mycelial growth under *in vitro* conditions.

Kolase *et al.* (2014) reported that carbendazim + mancozeb (0.1 per cent) inhibited 56.32 per cent mycelial growth of anthracnose pathogen of mango, while mancozeb at 0.25 per cent recorded 89.38 per cent of mycelial growth.

Sreeja (2014) reported complete mycelial inhibition of *C. gloeosporioides* of cowpea by propiconazole, tebuconazole and mancozeb at 0.25 per cent. The study also revealed copper oxychloride at 0.2 per cent and captan + hexaconazole at 0.1 per cent recorded 30.09 per cent and 87.06 per cent of mycelial inhibition respectively under *in vitro* conditions. The least mycelial inhibition of 25.37 per cent was reported for azoxystrobin at 0.15 per cent.

Parvathy and Girija (2016) evaluated the efficacy of different fungicides *viz.* propiconazole , fluzilazole, azoxystrobin, tebuconazole, copper hydroxide, captan + hexaconazole and carbendazim + mancozeb against *C. gloeosporioides* of black pepper. The study indicated that tebuconazole and combination fungicide of carbendazim + mancozeb (0.1 per cent) completely inhibited mycelial growth of the pathogen *in vitro*. Propiconazole, fluzilazole, combination fungicide of captan + hexaconazole at (0.1 per cent) and azoxystrobin (0.15 per

cent) inhibited 93, 88.24, 86.62 and 86 per cent of growth of pathogen under *in vitro* respectively.

The efficacy of systemic and contact fungicides against *C. gloeosporioides* of mango was studied by Kumari *et al.* (2017). The study revealed that propiconazole at 500 ppm could completely suppress the pathogen. The fungicides recorded 75.1 per cent and 82.3 per cent growth inhibition at 100 and 250 ppm respectively. Among the non-systemic fungicides screened, mancozeb at 100 ppm completely inhibited the growth of the pathogen.

2.6. POT CULTURE TRIAL TO DEVELOP INTEGRATED DISEASE MANAGEMENT PACKAGE FOR ANTHRACNOSE OF BETEL VINE

2.6.1. Organic Preparations

Rajput (2011) reported that the practice of seedling dip in panchagavya (3 per cent) followed by spraying of sulphur (0.2 per cent) along with 3 per cent of panchagavya at 15 days interval reduced the disease incidence of fruit rot of chilli. This application reduced the disease severity to 46.2 per cent and 54.9 per cent during first and second picking respectively. Disease suppression of 57.6 per cent had been reported on fruit rot of chilli by the foliar application of panchagavya at 2.5 per cent (Chacko, 2015). The foliar application of panchagavya reduced amaranth leaf blight by 47.29 per cent over control (Anuja, 2010)

2.6.2. Biocontrol Agents

Deka *et al.* (2008) reported the practice of sanitation coupled with the application of *B. subtilis* as an effective management strategy for the leaf spot complex caused by *C. capsici, Xanthomonas axonopodis* pv. *betlicola* in betel vine gardens.

Ann *et al.* (2015) reported that different strains of *Bacillus* were effective in managing the anthracnose of black pepper under field conditions. Five foliar applications of *Bacillus* strain CBF at 10^9cfu ml^{-1} recorded highest biocontrol

efficiency of 80.45 per cent with a disease incidence of 6.01 per cent and index 2.25 per cent respectively.

Chacko (2015) reported that foliar spray of *Bacillus* sp. at 10^8 cfu ml^{-1} reduced the disease incidence of fruit rot of chilli by 59.59 per cent at three weeks after spraying.

The disease index on anthracnose of grapes was reduced by 25.50 per cent with the foliar application of *Bacillus* strain (TL-171) at 1 x 10^8cfu ml^{-1} under field conditions (Sawant *et al.*, 2016)

Narasimhan and Shivakumar (2014) reported that seed treatment, root dip, soil and foliar application of *B. subtilis* successfully reduced the disease incidence of anthracnose of chilli and also increased the biometric characters and yield.

2.6.3. Fungicides

Two foliar sprays of Bordeaux mixture at 1 per cent during pre-monsoon period (May and September) were reported to be effective in reducing the disease incidence and severity of anthracnose in black pepper (Anandraj and Sharma, 1995).

Two foliar applications of benzimidazole carbamate or thiophanate methyl at 0.5 per cent was found highly effective in controlling anthracnose of betel vine in Karachi (Shahzad, 2000).

Gopinath *et al.* (2006) reported that propiconazole (0.1 per cent) was effective in controlling anthracnose of chilli under greenhouse condition and the spraying reduced the disease incidence by 70 per cent.

Sundaravadana *et al.* (2007) evaluated the field efficacy of azoxystrobin at 0.25, 0.05, 1.0, 2.0 and 4.0 ppm concentrations for anthracnose disease in mango. At 1.0, 2.0 and 4.0 ppm, the disease severity was reduced by 65.39 per cent, 68.29 per cent and 69.92 per cent respectively.

Devasahayam *et al.* (2008) reported that prophylatic spraying of Bordeaux mixture at 1 per cent or carbendazim + mancozeb at 0.1 per cent reduced the anthracnose disease in black pepper.

Kurian *et al.* (2008) reported that foliar spray of 0.1 per cent carbendazim + mancozeb was effective in controlling the pollu disease in black pepper and could reduce the leaf and spike infection by 77.5 per cent and 82.7 per cent respectively.

Patil *et al.* (2009a) reported that spraying of 0.2 per cent of mancozeb + carbendazim at monthly intervals recorded 33.38 per cent of disease suppression of anthracnose in thippali over control under field conditions.

Jayalakshmi (2010) reported that the disease severity of *C. gloeosporioides* of pomegranate was reduced by the seven applications of propiconazole (0.1 per cent) or carbendazim + mancozeb (0.3 per cent) and controlled the disease.

Basalingappa (2011) reported that the *C. gloeosporioides* infection on mango could be reduced by pre harvest spray of either propiconazole or tebuconazole (0.2per cent) with a disease suppression of 95 per cent.

Kumbhar and More (2013) tested the efficacy of triazole fungicides in reducing the infection of *C. capsici* in chilli under field conditions. Spraying of propiconazole and tebuconazole reduced fruit rot incidence and severity. Foliar spray of 0.1 per cent tebuconazole showed 57.60 and 65.48 per cent disease reduction over leaf spot and fruit rot in pomegranate (Balaso, 2014).

Spraying of fungicides *viz.*, propiconazole (0.1 per cent), mancozeb (0.25 per cent) and captan + hexaconazole (500g ha^{-1}) for management of anthracnose in cowpea recorded a percentage disease index of 13.90, 40.70 and 33.0 respectively (Sreeja, 2014).

Foliar spraying of 0.1 per cent propiconazole thrice from November to January at monthly interval reduced the anthracnose disease in mango (Bhagwat

et al., 2016). Kumari *et al.* (2017) reported that two foliar sprays of 0.05 per cent of propiconazole at 15 days interval reduced disease intensity of mango anthracnose.

Kendre *et al.* (2017) reported that application spraying of carbendazim + mancozeb at 1kg ha^{-1} reduced the disease severity of leaf blight in thippali from 4.66 to 2.16 and disease incidence from 15.44 per cent to 9.75 per cent.

Kumar *et al.* (2018) tested the efficacy of chemicals in the management of the leaf spot disease of betel vine under field conditions. They reported that two foliar spray of carbendazim + mancozeb (0.2 per cent) was effective in managing *C. gloeosporioides* in betel vine with 73 per cent reduction in disease severity.

Materials and Methods

3. MATERIALS AND METHODS

3.1. SYMPTOMATOLOGY, ISOLATION AND PROVING PATHOGENICITY OF *COLLETOTRICHUM* SP. CAUSING ANTHRACNOSE OF BETEL VINE

3.1.1. Collection of Diseased Samples

A survey was conducted during December 2016 - April 2017 in Southern districts of Kerala *viz.*, Thiruvananthapuram, Kollam and Alappuzha. Three locations from Thiruvanathapuram (Kalliyoor, Vellayani and Kattakada), one location each from Kollam (Kareepra) and Alappuzha (Cherthala) were surveyed for the disease incidence and severity. The weather parameters recorded during the survey were maximum temperature, minimum temperature and relative humidity. Ten plants were selected randomly from each field and five leaves from each plant were graded for determining the percentage disease index (PDI).

Percentage disease index was calculated using the formula of Mayee and Datar (1986),

$$PDI = \frac{\text{Sum of the grades of each leaf}}{\text{Number of leaves assessed}} \times \frac{100}{\text{Maximum grade used}}$$

Disease incidence was calculated using the formula of Singh (2002).

$$\text{Disease incidence} = \frac{\text{Total number of infected plants}}{\text{Total number of plants observed}} \times 100$$

3.1.2. Symptomatology

Symptoms of anthracnose of betel vine in the fields were studied on leaves and stem.

3.1.3. Isolation of Pathogen

Infected betel leaves were collected from various locations of South Kerala *viz*., Thiruvananthapuram (Kalliyoor, Kattakada, Vellayani), Kollam (Kareepra) and Alappuzha (Cherthala) regions. For the isolation of the pathogen, the diseased leaves were washed under running water and cut into small bits containing diseased portion along with healthy portion. The bits were surface sterilized using 0.1% mercuric chloride for 1 min followed by three washings in sterile distilled water. The excess moisture was removed by placing the surface sterilised bits over a sterile filter paper. The surface sterilised bits were transferred on to petri dishes containing potato dextrose agar (PDA) medium under aseptic conditions. The petri dishes were incubated at room temperature (27 ± 3^{0}C) for 24 – 48 h. The fungal growth observed on the petri dishes was transferred to PDA slant (Aneja, 2003).

3.1.3.1. Purification of Isolates

The fungal isolates of anthracnose pathogen obtained were purified by single spore isolation technique (Dhingra and Sinclair, 1985). The spore suspension of *Colletotrichum* sp. was prepared by placing mycelial bits along with spore mass in test tubes containing sterile water. The spore suspension was serially diluted and plated on 1 per cent sterilized plain agar in petri dishes. Single spores located under microscope were marked with a fine tip marker and then transferred into PDA slants for growth and stored for further studies.

3.1.4. Pathogenicity Studies

The pathogenicity studies were carried out on excised leaves of *Pannikarpooram* variety. The leaves were initially washed under running tap water followed by surface sterilization with 70 per cent ethanol. Artificial inoculation of the pathogen was done by gentle aberrations on the leaves by pin pricks and placed 5 mm mycelial discs from actively growing seven day old culture using cork borer. The inoculated leaves were incubated in moist chamber

at room temperature for symptom development. The control treatment was also maintained without inoculation of the pathogen. The pathogen was reisolated from the infected tissue and was compared to the original isolated culture to prove the pathogenicity.

3.2. MORPHOLOGICAL AND PATHOGENIC VARIABILITY OF *COLLETOTRICHUM* ISOLATES, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF VIRULENT ISOLATE

3.2.1. Culture Characteristics of Isolates

The culture characteristics of the isolates were studied by growing on PDA medium. The sterilised PDA medium was poured in petri dishes and allowed to solidify under aseptic conditions. Mycelial disc of 5 mm from five day old culture was placed at the centre of the petri dish. The petri dishes were incubated at room temperature (27 ± 3^0C). Five replications were maintained for each treatment. Observations were made on the radial growth, rate of growth of each isolates, growth pattern, colony colour and number of days taken to cover petri dishes.

3.2.2. Morphological Characterization of Isolates

The morphological characters of the various isolates *viz.*, the mycelial characters, sporulation, size and shape of conidia and appresoria were studied by preparing slides stained with cotton blue and observed under 400X and 1000X magnification of Leica DM 750.

3.2.3. Pathogenic Variability of the Isolates

The pathogenic variability of *Colletotrichum* isolates were studied by virulence rating. Healthy leaves of betel vine (var. *Pannikarpooram)* collected from farmer's field were used for the study. The leaves were initially washed under running tap water and surface sterilized by cotton swabbing the leaves with 70 per cent ethanol. The collected isolates of *Colletotrichum* sp. were grown in PDA medium. The mycelial bit of 5 mm from five day old culture was used for

inoculating the leaves. Prior to the artificial inoculation of the pathogen, the leaves were wounded gently by pin pricks on both sides and inoculum was placed at the site of injury. The inoculated leaves were kept in moist chamber for providing humidity. Observations were made at daily interval on the lesion size, time for symptom appearance and rate of lesion development.

3.2.4. Molecular Characterization of the Virulent Isolate

The molecular characterization of virulent isolate was done by using universal primers of ITS by the method of DNA barcoding.

3.2.4.1. Isolation of DNA using NucleoSpin® Plant II Kit (Macherey-Nagel)

Hundred mg of the mycelium of *Colletotrichum* sp. was homogenised using liquid nitrogen. The powdered tissue was then transferred to micro centrifuge tubes on to which 400 µl of buffer PL1 was added and vortexed for 1 min. After vortexing, 10 µl of RNase A solution was added and it was inverted to mix well. Then the homogenate was incubated for 10 min at 65°C. After incubation, the lysate was transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 min. The flow through liquid was collected and the filter was discarded. On to the collected liquid / filtrate, 450 µl of buffer PC was added and mixed thoroughly. Then the solution was transferred to a Nucleospin Plant II column, centrifuged for 1 min and the flow through liquid was discarded, on to which 400 µl buffer PW1 was added to the column, centrifuged at 11000 x g for 1 min and flow though liquid was discarded. Then 700 µl PW2 was added, centrifuged at 11000 x g and flow through liquid was again discarded. Finaliy 200 µl of PW2 was added and centrifuged at 11000 x g for 2 min in order to dry the silica membrane. Then the column was transferred to a new 1.7 ml tube and 50 µl of buffer PE was added and incubated at 65°C for 5 min. The column was then centrifuged at 11000 x g for 1 min to elute the DNA. The eluted DNA was stored at 4°C.

3.2.4.2. DNA Quality Check by Agarose Gel Electrophoresis

The isolated DNA was checked for quality using agarose gel electrophoresis. For checking the quality, 1 µl of 6X gel-loading buffer containing 0.25% bromophenol blue, 30% sucrose in TE buffer with pH-8.0 was added to 5 µl of DNA. Then the samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg ml^{-1} ethidium bromide. Electrophoresis was performed using 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front had migrated to the bottom of the gel. The gels were, then visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.2.4.3. PCR Analysis

Twenty µl reaction volume containing 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg ml^{-1} BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers was used for PCR amplification reactions.

3.2.4.3.1. Primers used for PCR Analysis

Target	Primer Name	Direction	Sequence (5' → 3')
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTGCGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

3.2.4.3.2. PCR Amplification Profile

ITS

98 °C	-	30 sec	
98 °C	-	5 sec	40 cycles
58 °C	-	10 sec	
72 °C	-	15 sec	

72 °C - 60 sec

4 °C - ∞

3.2.4.3.3. Agarose Gel Electrophoresis of PCR Products

The amplified PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg ml^{-1} ethidium bromide. One µl of 6X loading dye was mixed with 5 µl of PCR amplified products and it was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 h, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.2.4.4. ExoSAP-IT Treatment

ExoSAP-IT treatment was done to remove unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer. Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 min.

3.3.4.5. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated) - 10-20 ng

Primer - 3.2 pM (either Forward or Reverse)

Sequencing Mix - 0.28 µl

5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10 µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 min followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 min for all the primers.

3.3.4.6. Post Sequencing PCR Clean Up

Post sequencing PCR clean up was done using Master mix I and II. The master mix I consisted of 10 µl milli Q and 2 µl 125 mM EDTA per reaction, while master mix II had 2 µl of 3 M sodium acetate (pH 4.6) and 50 µl of ethanol per reaction. For each reaction, 12 µl of master mix I and 50 µl of master mix II were added. The contents were thoroughly mixed by inverting. The mixture was incubated at room temperature for 30 min and then centrifuged at 14000 rpm for 30 min. After centrifugation, the supernatant was decanted and 100 µl of 70% ethanol was added to the residue. This was centrifuged at 14000 rpm for 20 min. The supernatant was again decanted and repeatedly washed with 70% ethanol. The pellets were air died. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Bio Systems).

3.2.4.6. Analysis of Sequence

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems).

3.3. SCREENING OF INDIGENOUS ORGANIC PREPARATIONS FOR *IN VITRO* PATHOGEN SUPPRESSION

3.3.1. Preparation of Panchagavya

Panchagavya was prepared by following the procedure described as in Organic POP, KAU (2009). The five dairy products were used for its preparation includes cowdung, cow urine, ghee, curd and milk. Cow dung (7 kg) and cow ghee (1 kg) were mixed and kept in clean plastic bucket for three days with thorough mixing in morning and evening hours. After three days, cow's urine

(10 L) and water (10 L) were added. The mixture was kept for 15 days with regular mixing in morning and evening hours. After 15 days, 3 L of cow's milk, cow's curd (2 L), tender coconut water (3 L), jaggery (3 kg) and well ripened poovan banana (12 nos.) were added in to this. The mix was kept under shade and mouth of the bucket was covered with mosquito proof net. The solution was used for *in vitro* studies 30 days after preparation.

3.3.2. Preparation of Jeevamruth

Jeevamruth was prepared by the method described by Chadha *et al.* (2012). It was prepared by mixing 1 kg cow dung, 1 L cow's urine, 200 g jaggery, 200 g pulse flour, a handful of fertile soil and 1 L of water in drum with the help of wooden stick. The mixture was swirled regularly twice daily in clock-wise and anticlock wise direction for 5 - 7 days for proper fermentation.

3.3.3. Preparation of Vermiwash

Vermiwash was prepared by the method described in organic POP, KAU (2009). The vermiwash unit consisted of a cement tank with tap on the side for the collection of vermiwash. The bottom of the tank was lined with small brick pieces or gravel to layer of 5 cm thickness. This layer was lined above with coconut fibre of 3 - 4 cm thickness. Four kg of biowaste was added along with 2 kg of earthworms to the system. After two weeks the biomass would turn to compost having brownish black in colour. About 2 L of water was sprinkled over the freshly formed compost containing earthworms. The leachate (vermiwash) was collected after 24 hours through side tap.

3.3.4. Preparation of Fermented Cow's urine

The fermented cow's urine was prepared following the procedure mentioned by Ashlesha and Paul (2014). The fresh cow's urine was collected from Department of Animal Husbandry. The cow's urine was mixed with water in 1: 2 ratio and allowed to ferment for 10 days.

The population of microflora in the organic preparations were estimated by the serial dilution technique described by Johnson and Curl, (1972).

3.3.5. *In vitro* Evaluation of Organic Preparations Against *C. gloeosporioides*

The organic preparations like panchagavya, jeevamruth, vermiwash and fermented cow's urine were evaluated for their suppression of the pathogen *C. gloeosporioides* by poisoned food technique described by Nene and Thapliyal (1993). All the organic preparations were tested at 5 per cent and 10 per cent concentration. For the study, 45 ml and 40 ml of sterile water was taken in two 250 ml conical flask. Similarly in another two 250 ml conical flask, 50 ml each of double strength PDA were taken and sterilized by autoclaving. The organic preparations were initially filtered through Whatman No. 1 filter paper. The filtrate was then centrifuged at 3000 rpm for 30 min to remove the residues. The filtrates were sterilised by passing through bacterial filter before amending the media. The measured quantity *i.e.* 5 ml and 10 ml of the organic preparations were pippeted out and added to 45 ml and 40 ml of the sterile water respectively under aseptic condition and mixed thoroughly. This mixture of different concentrations was then added to 50 ml of molten double strength PDA contained in two different conical flasks to get desired concentrations as 5 and 10 per cent respectively. The amended medium (20 ml) was then poured in to the sterile petri plates and was allowed to solidify. The 5 mm mycelial disc of five day old culture of *C. gloeosporioides* was inoculated at the centre of the amended medium under aseptic conditions. The plates were incubated at room temperature (27 ± 3^{0}C). Four replications were maintained for each treatment.

In another method, the organic preparations of desired concentrations were poured directly into the desired quantity of sterile water after passing through Whatman No. 1 filter paper, followed by centrifugation and filter sterilization. Then it was mixed with double strength PDA and autoclaved. The autoclaved amended medium (20 ml) was poured into sterile petri dishes and a 5 mm mycelial disc of five day old culture of pathogen was inoculated at the centre.

Unamended PDA medium inoculated with the pathogen served as the control. Radial growth of the pathogen was recorded when pathogen in control attained full growth. Four replications for each treatment were maintained.

Percentage inhibition of growth over control was calculated using the formula (Vincent, 1927)

$$I = C\text{-}T/C \times 100$$

Where,

I = Percentage inhibition

C = Growth of *C. gloeosporioides* in unamended medium (Control)

T= Growth of *C. gloeosporioides* in amended medium

3.4. SCREENING OF BIOCONTROL AGENTS FOR *IN VITRO* PATHOGEN SUPPRESSION

3.4.1. Isolation of Micro Flora from Rhizosphere and Phyllosphere of Healthy Betel Vine Leaves

Saprophytic micro flora present on the healthy leaves of betel and rhizosphere collected from diseased field were used for the study. The serial dilution technique described by Johnson and Curl (1972) was used for the isolation of biocontrol micro flora from the healthy betel leaves and soil. Ten gram of healthy betel leaves were weighed out, added to 90 ml of sterile water and was shaken in rotary shaker for 20 min. From this aliquot serial dilutions were prepared upto 10^{-7}. 10^{-3} and 10^{-4} dilutions were used for isolation and enumeration of fungi whereas 10^{-6} and 10^{-7} dilutions for bacteria. In the case of rhizosphere microflora, soil particles adhering to the roots of betel vine plants were collected from the field.

One ml aliquots from 10^{-3} and 10^{-4} dilutions were plated on to Martin's Rose Bengal Agar medium and swirled gently clock wise and anticlock wise for

uniform dispersal of the microorganisms. Similarly one ml from 10^{-6} and10^{-7} dilutions were transferred into Nutrient Agar (NA) medium for isolation of bacteria. The plates were incubated at room temperature (27 ± 3^{0}C) for 24-72 h. The plates were examined for the growth of fungal and bacterial colonies. Observations were recorded on microbial population and expressed as colony forming units (cfu) per gram of sample. The colonies of the fungi and bacteria were transferred to PDA slants and NA slants respectively. The fungal colonies were purified by hyphal tip method. Purification of bacteria was done by simple streak and single colonies were transferred to NA slants. These cultures were maintained under refrigerated conditions for further studies.

3.4.2. *In vitro* Evaluation of the Antagonistic Fungi and Bacteria for Suppression of the Pathogen by Dual Culture Technique

3.4.2.1. Antagonistic Fungi

The fungal isolates obtained through serial dilution technique were evaluated for suppression of *C. gloeosporioides* by dual culture method described by Skidmore and Dickinson (1976). The mycelial disc of 5 mm diameter from three day old culture of the saprophytic fungus was placed 2.5 cm away from the periphery of sterilized petriplates containing PDA. Similarly 5 mm mycelial disc of five day old pathogen was also placed 2.5 cm away from the edge of the petridish opposite to the antagonist. Four replications were maintained for each treatment. The plates containing the pathogen alone served as control. The observations were recorded on the radial growth of the pathogen in control and treated plates.

The percentage inhibition of growth over control was calculated using the formula (Vincent, 1927)

$$I = C1 - C2 / C1 \times 100$$

Where,

I = Percentage inhibition

C1 = Growth of the pathogen in control (cm)

C2 = Growth of the pathogen in treatment (cm)

3.4.2.2. Antagonistic Bacteria

The bacterial antagonists obtained through serial dilution were evaluated for antagonism against the pathogen by dual culture technique described by Utkhede and Rahe (1983). NA medium was melted and was poured into sterile petridishes. After solidification, 5 mm disc of five day old culture of the pathogen was placed 2.5 cm away from the periphery and the respective bacterial isolate was streaked 2.5 cm away parallel to the inoculated pathogen. The percentage of inhibition was calculated using the formula given in 3.4.2.

3.4.3. Identification and Cultural Characterization of Selected Fungal and Bacterial Antagonist

3.4.3.1. Identification of Antagonistic Fungi

The cultural characteristics of the selected antagonistic fungi were studied by growing on PDA medium. The sterilised PDA medium was poured on petri dishes and allowed to solidify under aseptic conditions. Mycelial disc of 5 mm from five day old culture was placed at the centre of the petri dish. The petri dishes were incubated at room temperature (27 ± 3^{0}C). Five replications were maintained for each treatment. Observation was made on the colony colour.

3.4.3.2. Identification of Antagonistic Bacteria

The bacterial isolates obtained through serial dilution were streaked on NA medium in sterile petri plates. The bacterial isolates obtained were subjected to gram staining technique to identify the bacterium. Gram staining was done based on the technique devised by Gram (1884). Bacterial smear prepared on a glass slide was heat fixed followed by air drying. After air drying, smear was flooded with crystal violet for one min. The excess stain was drained off and washed in running water. The smear was then flooded with Gram's iodine and was kept for

one min and the excess stain was washed off in running water. The bacterial cells were washed with 95 per cent ethanol to decolourise the stain and then counter stained using safranin. The excess stain was washed off after one min and smear was air dried. A drop of cedar oil was placed into slide and was observed under oil immersion objective (100X).

3.5. SCREENING OF FUNGICIDES FOR *IN VITRO* PATHOGEN SUPPRESSION

The *in vitro* suppression of the *C. gloeosporioides* using fungicides was done by poisoned food technique described by Nene and Thapliyal (1993). Seven commercially available fungicides were used for evaluation against the pathogen. The fungicides used in the study are given in Table 1.

Fifty ml of sterilised water and 50 ml of double strength PDA was used for the *in vitro* evaluation of the fungicide. The desired concentration of the fungicides was weighed out, added into the sterilized water, shaken thoroughly under aseptic conditions and then added into 50 ml of molten double strength PDA to get the desired concentrations. This amended medium was poured into sterile petri dishes under aseptic conditions and allowed to solidify. The same procedure was repeated for all the test fungicides under this study. A 5 mm mycelial disc from five day old culture of *C. gloeosporioides* was inoculated at the centre of the amended medium under aseptic conditions. The plates were incubated at room temperature (27 ± 3^0C).

Un amended PDA medium inoculated with the pathogen at centre served as the control. Observations were recorded when control plate attained full growth.

The percentage inhibition of growth over control was calculated using the formula (Vincent, 1927)

$$I = C1 - C2 / C1 \times 100$$

Table 1. Dosages of fungicides screened against *C. gloeosporioides* under *in vitro* conditions

Treatments	Fungicides	Nature of fungicide	Trade name	Lower dose (%)	Recommended (%)	Higher dose (%)
T1-T3	Propiconazole 25.0 % EC	Systemic	Tilt	0.05	0.1	0.2
T4 -T6	Tebuconazole 25.9 % EC	Systemic	Folicur	0.05	0.1	0.2
T7 - T9	Azoxystrobin 23 % SC	Systemic	Amistar	0.05	0.1	0.2
T10 - T12	Mancozeb 75% WP	Contact	Indofil M-45	0.1	0.2	0.4
T13 - T15	Copper hydroxide 53.8 % WP	Contact	Kocide	0.1	0.2	0.4
T16 - T18	Captan (70%) + Hexaconazole (50%) WP	Combination	Taquat	0.05	0.1	0.2
T19 - T21	Carbendazim (12%) + Mancozeb (63%) WP	Combination	Saaf	0.05	0.1	0.2

Where,

I = Percentage inhibition

C1= Growth of pathogen in control (cm)

C2 = Growth of pathogen in treatment (cm)

The best systemic fungicide, contact fungicide and combination fungicide was used for further pot culture trials.

3.6. POT CULTURE TRIAL TO DEVELOP INTEGRATED DISEASE MANAGEMENT PACKAGE FOR ANTHRACNOSE OF BETEL VINE

A pot culture experiment was conducted in Complete Randomised Design at Department of Plant Pathology with eight treatments and four replications to evaluate the efficacy of the selected treatments from the above experiments (Table 2) against anthracnose of betel vine under natural conditions. The experiment was laid in pots filled with solarized potting mixture consisting of sand, soil and cowdung in 1: 2:1 ratio enriched with *Trichoderma*. The variety *Pannikarpooram* was used for the study. Each pot were planted with vine cuttings having 3 nodes in such a way that one node beneath the soil with the second node on the surface of the soil and third above the soil. Mulching was done to facilitate easy germination. The treatments were given as foliar application on the leaves after the challenge inoculation of the pathogen. The best treatments from 3.3, 3.4, 3.5 and were used for further studies

3.6.1. Soil Solarization

Soil solarisation was carried out during summer months (March-April). The potting mixture consisting of sand, soil and cowdung in 1: 2: 1 ratio was mixed, moistened, levelled and made into beds prior to soil solariziation. The beds were then covered with 120 gauge thick transparent polythene sheets. The edges were sealed with moist soil to keep in position, to maintain the temperature and moisture inside the sheet. Adequate care was taken to keep the sheet in

contact with the surface of the soil in order to prevent air pockets inside. After 45 days, the sheet was removed and potting mixture was used.

3.6.2. Preparation of Enriched *Trichoderma* Neem Cake Organic Manure Mixture

The enriched *Trichoderma* was prepared by mixing powdered dry neem cake and cowdung in 1 : 9 ratio to get a coarse texture and was moistened by sprinkling water. The commercial preparation of *Trichoderma viride* was added at the rate of 1kg per 100 kg of neem cake - cowdung mixture. After thorough mixing, it was heaped, covered with moistened gunny bags and kept in shade for 10 days for multiplication. Again it was mixed well and kept for five more days for further multiplication.

3.6.3. Preparation of Pathogen Inoculum

For the preparation of pathogen inoculum Potato Dextrose broth (PDB) was prepared and autoclaved. The broth was then inoculated with 5 mm mycelial discs of five day old cultures of *C. gloeosporioides* under aseptic conditions. The broth was incubated at room temperature ($27 \pm 3^{O}C$).

3.6.4. Application of Pathogen Inoculum

For the application of the pathogen on the betel vine, the mycelial mats were harvested from fourteen day old culture grown in PDB. The harvested mats were homogenised in a warring blender for one min after suspending in sterile distilled water (SDW). The homogenate was then strained through double layered muslin cloth and again diluted with SDW in order to get 10^6 conidia ml^{-1}. Then it was sprayed on to the leaves using an atomiser (Golda, 2010).

3.6.5. Application of Treatments

Foliar application of the selected treatments at recommended dose was done at 10 days after inoculation.

Table 2. Treatments selected for pot culture experiment for the management of anthracnose of betel vine

	Treatments
T1	Foliar application of the effective organic preparation
T2	Foliar application of the effective biocontrol agent
T3	Foliar application of the effective systemic fungicide
T4	Foliar application of the effective contact fungicide
T5	Foliar application of the effective combination fungicide
T6	0.5% Bordeaux mixture (Chemical control Check)
T7	Inoculated control
T8	Un inoculated control

3.6.6. Disease intensity

The disease intensity was recorded at 10 days after inoculum application and also at ten days intervals after treatment application. Scoring of the disease was done according to the 0-9 score chart (Sankar, 2002) where,

0- No infection

1- Lesions covering upto 10 per cent leaf area

3- Lesions covering 11- 25 per cent leaf area

5- Lesions covering 26- 50 per cent leaf area

7- Lesions covering 51-75 per cent leaf area

9- Lesions covering > 75 per cent leaf area

Percentage disease index was calculated using the formula of Mayee and Datar (1986),

$$PDI = \frac{\text{Sum of the grades of each leaf}}{\text{Number of leaves assessed}} \times \frac{100}{\text{Maximum grade used}}$$

3.6.7. Disease Incidence

The disease incidence was recorded 10 days after inoculum application and also at ten days intervals after treatment application.

Percentage disease incidence was calculated using the formula of Singh (2002),

$$\text{Disease incidence} = \frac{\text{Total number of infected leaves}}{\text{Total number leaves observed}} \times 100$$

Observations on parts affected and biometric characters were recorded.

3.7. STATISTICAL ANALYSIS

The data obtained from *in vitro* and *in vivo* studies were subjected to analysis of variance (ANOVA) after appropriate transformation where ever needed. When the effects were found to be significant, critical difference value were calculated for each observation using 't' values at 5 per cent level of significance. Significant treatments were compared with critical difference value. All the treatments were analysed in WASP 2.0 software

Results

4. RESULTS

The present study on " Ecofriendly management of anthracnose of betel vine (*Piper betle* L.)" was conducted during the period 2016 - 2018 in the Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The objective of the study is to evolve an integrated management strategy for anthracnose of betel vine involving organic preparations, biocontrol agents and new generation fungicides. The results obtained from the *in vitro* and *in vivo* studies were summarized below.

4.1. SYMPTOMATOLOGY, ISOLATION AND PROVING PATHOGENICITY OF *COLLETOTRICHUM* SP. CAUSING ANTHRACNOSE OF BETEL VINE

4.1.1. Collection of Diseased Samples

A survey was conducted during the period from December 2016 - April 2017 in Southern districts of Kerala *viz.,* Thiruvananthapuram, Kollam and Alappuzha. Three locations from Thiruvanathapuram (Kalliyoor, Vellayani and Kattakada), one location from Kollam (Kareepra) and Alappuzha (Cherthala) were surveyed for the disease. The weather parameters observed during survey were maximum and minimum temperature and relative humidity as they were the most crucial factors in disease development. The maximum temperature ranged from 31 to 34°C and minimum temperature from 20.7 to 27°C (Table 3). The relative humidity during the survey period ranged from 82 - 94.4 per cent. In surveyed locations, the disease incidence ranged from 4 – 80 per cent. The maximum disease incidence and severity was noticed in betel vine cultivation at Cherthala with 80 per cent and 20 per cent respectively. Kalliyoor and Vellayani area had a disease incidence of 20 per cent and disease index of 16.42 and 17.86 respectively. The least disease incidence was noticed in Kareepra (4 per cent). The PDI (20.00) and DI (80.00) recorded was higher in Cherthala region of Alappuzha (Table 4). The prominent symptom observed during the survey was necrotic spots with a yellow halo and stem lesions (Plate 1).

Table 3. Temperature and relative humidity recorded during survey period at various locations

Locations	**Temperature (^{0}C)**		**Relative humidity (%)**
	Maximum	**Minimum**	
Kalliyoor	31.0	26.0	82.0
Vellayani	31.9	20.7	94.4
Kattakada	32.7	20.9	91.7
Kareepra	34.0	26.0	84.0
Cherthala	33.0	27.0	84.0

Table 4. Disease incidence and severity of betel vine anthracnose in survey locations

Sl. No.	Locations	Disease incidence (%)	Per cent disease index*
1.	Kalliyoor	20	17.86
2.	Vellayani	20	16.42
3.	Kattakada	70	13.57
4.	Kareepra	4	5.70
5.	Cherthala	80	20.00

*Mean of 50 leaves per location

Location I- Kalliyoor

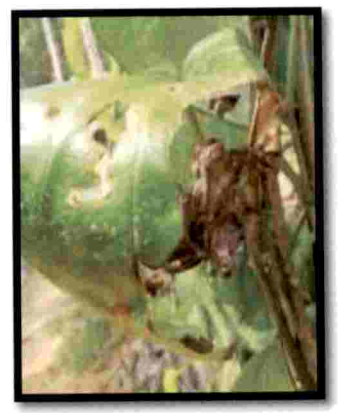
Location II-Vellayani

Location III- Alappuzha

Necrotic spots with yellow halo

Location IV- Kattakada

Necrotic spots with yellow halo along the margin



Location V- Kollam

Leaf blight

Plate 1. Symptoms of anthracnose of betel vine in survey locations

4.1.2. Symptomatology of the Disease

The symptoms observed on the leaves initially as small circular necrotic spots with a yellow halo, which started from different parts of the leaf lamina even from the tips. Under high humid conditions, these spots coalesced together causing extensive leaf blight. The affected leaves became pale yellow and resulted in defoliation. Under conducive climatic conditions, the pathogen produced elongated black lesions on the petiole and stem portion. As the disease progressed, these elongated lesions enlarged and coalesced resulting in the girdling, withering and drying up of entire vines.

4.1.2.1. Stages in Symptom Development

The disease symptom during Stage 1 produced small necrotic spots with a yellow halo, followed by enlargement of spots (Stage 2). In Stage 3 enlarged necrotic spots coalesced together forming larger blighted area. In this stage petioles and stem lesions were also formed followed by drying and death of the entire vine (Table 5) (Plate (2 A-D)).

4.1.3. Isolation of the Pathogen

The anthracnose pathogen *Colletotrichum* sp. was isolated from the infected leaves showing typical leaf spot symptoms. The infected leaf samples were collected from Southern districts of Kerala *viz.,* Thiruvananthapuram (Kalliyoor, Vellayani and Kattakada), Kollam (Kareepra) and Alappuzha (Cherthala) regions. The pathogen was isolated from the infected leaves by standard procedure described in 3.1.

Five isolates of *Colletotrichum* sp. were obtained and labelled as C1, C2, C3, C4 and C5. The pure cultures of the five isolates were made using single spore isolation technique (Plate 3).

Table 5. Stages in symptom development of anthracnose in betel vine

Stage 1	Necrotic spots surrounded by an yellow halo
Stage 2	Enlargement of spots
Stage 3	Spots coalesce together causing leaf blighting and development of stem lesions
Stage 4	Drying of the vines

A. Stage 1- Necrotic spots on the leaf lamina

B. Stage 2- Enlargement of spots

C. Stage 3- Leaf blight and stem lesions

D. Stage 4- Drying of vines

Plate 2 (A-D). Stages in symptom development of anthracnose of betel vine

C1- Isolate from Kalliyoor, C2- Isolate from Vellayani, C3 - Isolate from Cherthala, C4- Isolate from Kattakada, C5- Isolate from Kareepra

Plate 3. Pure cultures of five isolates of *Colletotrichum* causing anthracnose of betel vine on 7th day

4.1.4. Pathogenicity Studies

The pathogenicity studies of the collected isolates of *Colletotrichum* spp. were conducted by artificial inoculation on to healthy leaves by pin prick method. The pathogen produced symptoms on leaves as necrotic spots with yellow halo within 2 - 4 days of inoculation The isolate C2 produced a mean lesion size of 3.27 mm at 5 DAI and was more virulent compared to other isolates (Table 6) (Plate 4). The other isolates C3, C5, C1 and C4 produced 1.97 mm, 1.70 mm, 1.45 mm and 1.20 mm sized lesion at 5 DAI respectively and were on par. The pathogen was re-isolated from the artificially inoculated leaves. The re-isolated cultures were identical to the original culture and hence the pathogenicity was proved.

4.2. MORPHOLOGICAL AND PATHOGENIC VARIABILITY OF *COLLETOTRICHUM* ISOLATES AND IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF VIRULENT ISOLATE

4.2.1. Culture Characteristics

The culture characters of the five different isolates were studied by growing them on PDA medium as mentioned under 3.2.1.

4.2.1.1. Colony Characters

The isolates had either fluffy or sparse nature of mycelial growth with regular margin. The isolate C2 had a sparse growth when compared to others. The colony colour of the C1 isolate varied from whitish to dark grey on the front view and on rear view appeared as dark grey. The isolate C2 appeared as dark greyish on upper side while on rear view as orangish with concentric zonation. The colony growth of the isolate C3 and C4 appeared as whitish on front and rear view. While C5 appeared as off white on rear view and orange to pinkish on rear view (Table 7) (Plate 5).

Among the isolates C2 was comparatively a fast grower with an average growth of 1.28 cm day^{-1} and took 7 days to complete full growth (9cm) in petri

Table 6. Lesion size and days taken for symptom expression by different *Colletotrichum* isolates in betel vine

Isolates	Locations	Mean lesion size at 5th DAI (mm^2) *	Days taken for symptom expression
C1	Kalliyoor	1.45 [b]	4
C2	Vellayani	3.27 [a]	2
C3	Cherthala	1.97[b]	3
C4	Kattakada	1.20[b]	4
C5	Kareepra	1.70[b]	4
CD (0.05)		1.014	
SEm±		0.673	

*Mean of four replications
DAI – Days after inoculation
Values followed by similar superscripts are not significantly different at 5% level

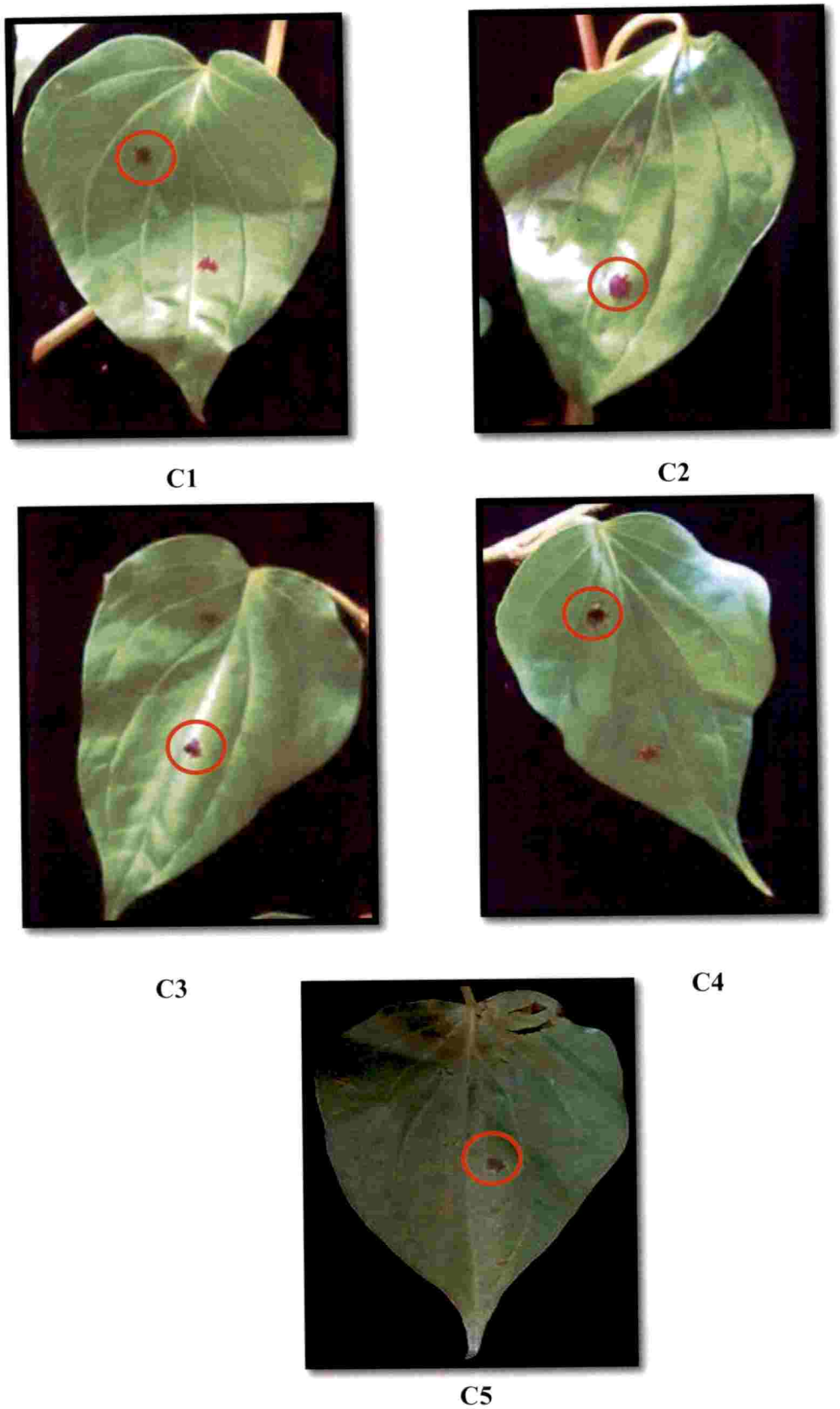


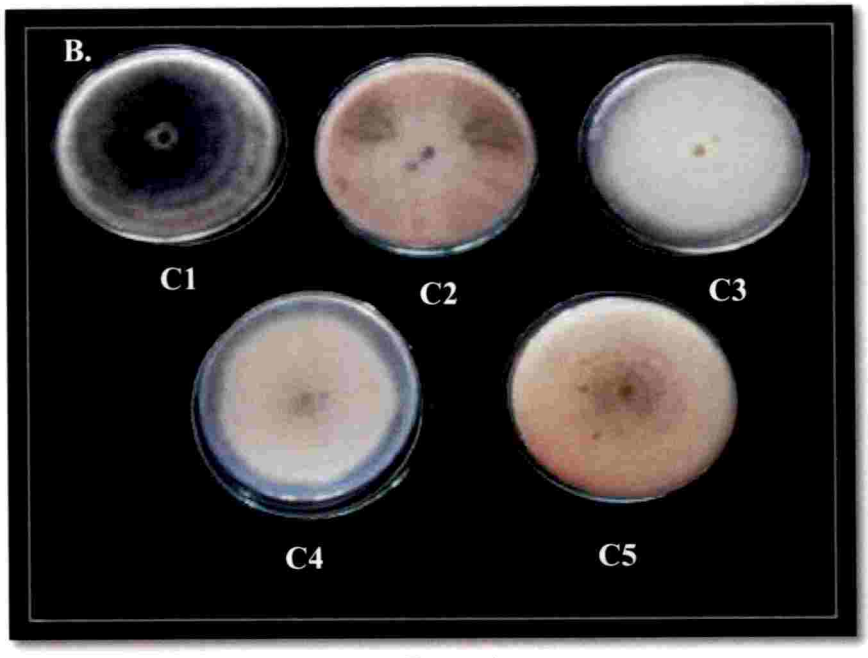
Plate 4 (C1-C5). Lesion development by *Colletotrichum* isolates causing anthracnose of betel vine on excised leaves (5DAI)

Table 7. Culture characters of *Colletotrichum* isolates causing anthracnose in betel vine

Isolates	Growth in petri dish (cm) after 7 days *	Rate of growth (cm/day) *	Characteristics of the mycelium				DTCP
			Growth pattern	Appearance			
				Front view	Rear view	Margin	
C1	8.1	1.15	Fluffy	White to dark grey	Dark grey	Regular	8
C2	9.0	1.28	Sparse	Dark grey	Orangish	Regular	7
C3	8.5	1.21	Fluffy	White	White	Regular	7
C4	7.3	1.04	Fluffy	White	White	Regular	9
C5	8.6	1.22	Fluffy	Off white	Orange to pinkish	Regular	8
CD (0.05)	0.269	0.037					0.224
SEm±	0.178	0.031					0.148

*Mean of four replications
DTCP: Days taken to cover 9 cm petri dish

Front view



Rear view

Plate 5 (A-B). Mycelial growth of *Colletotrichum* isolates causing anthracnose of betel vine on PDA medium (7th day)

dish. The isolate C4 was a slow grower which took 9 days for complete growth in petridish and had the lowest growth rate of 1.04 cm day^{-1}. The isolate C3, C1 and C5 took 7, 8 and 8 days respectively for complete growth in petri dish.

4.2.2. Morphological Characters

The morphological characters of the different isolates were studied using the technique mentioned in 3.2.2.

The mycelium of the isolates were hyaline, branched and septate. The mycelial width varied from 2.65 µm to 3.45 µm. The highest mycelial width of 3.45 µm was recorded for the isolate C4 and the lowest width of 2.65 µm for the isolate C1. The mycelial width of 3.32 µm was recorded for the isolate C3 followed by C5 and C2 with 3.02 µm and 2.98 µm respectively. The septal distance was also varied among the isolates from 9.80 to 25.56 µm. The maximum septal distance was recorded for C1 (25.56 µm). This was followed by C2, C3 and C4 with 19.15 µm, 13.86 µm and 9.80 µm respectively. The minimum septal distance was recorded for the isolate C5 (8.50 µm) (Table 8) (Plate 6).

Conidia were borne on elongated acervular conidiomata. The conidial shape varied from cylindrical, oblong to dumbbell with an oil globule at centre. The size also varied from 9.6 µm to 12.2 µm in length, 3.8 µm to 4.7 µm in breadth among the isolates (Table 9) (Plate 7). The C1 isolate had a conidial size of 11.4 µm x 4.7µm. The isolate C5 had 9.6 µm x 3.8 µm cylindrical shaped conidia. The isolate C3 had oblong shaped conidia with the size of 11.2 µm in length and 3.7 µm in width. The isolate C4 and C2 had oblong and dumbbell shaped conidia with a dimension of 12.2 µm x 4.3 µm and 11.3 µm x 3.9 µm respectively.

The appressorial size also varied among isolates from 8.73 to 10.08 µm and 5.02 to 6.28 µm length and breadth respectively. The highest appressorial length was recorded for the isolate C2 and breadth for the isolate C3. The isolate

Table 8. Mycelial characters of *Colletotrichum* isolates causing anthracnose in betel vine

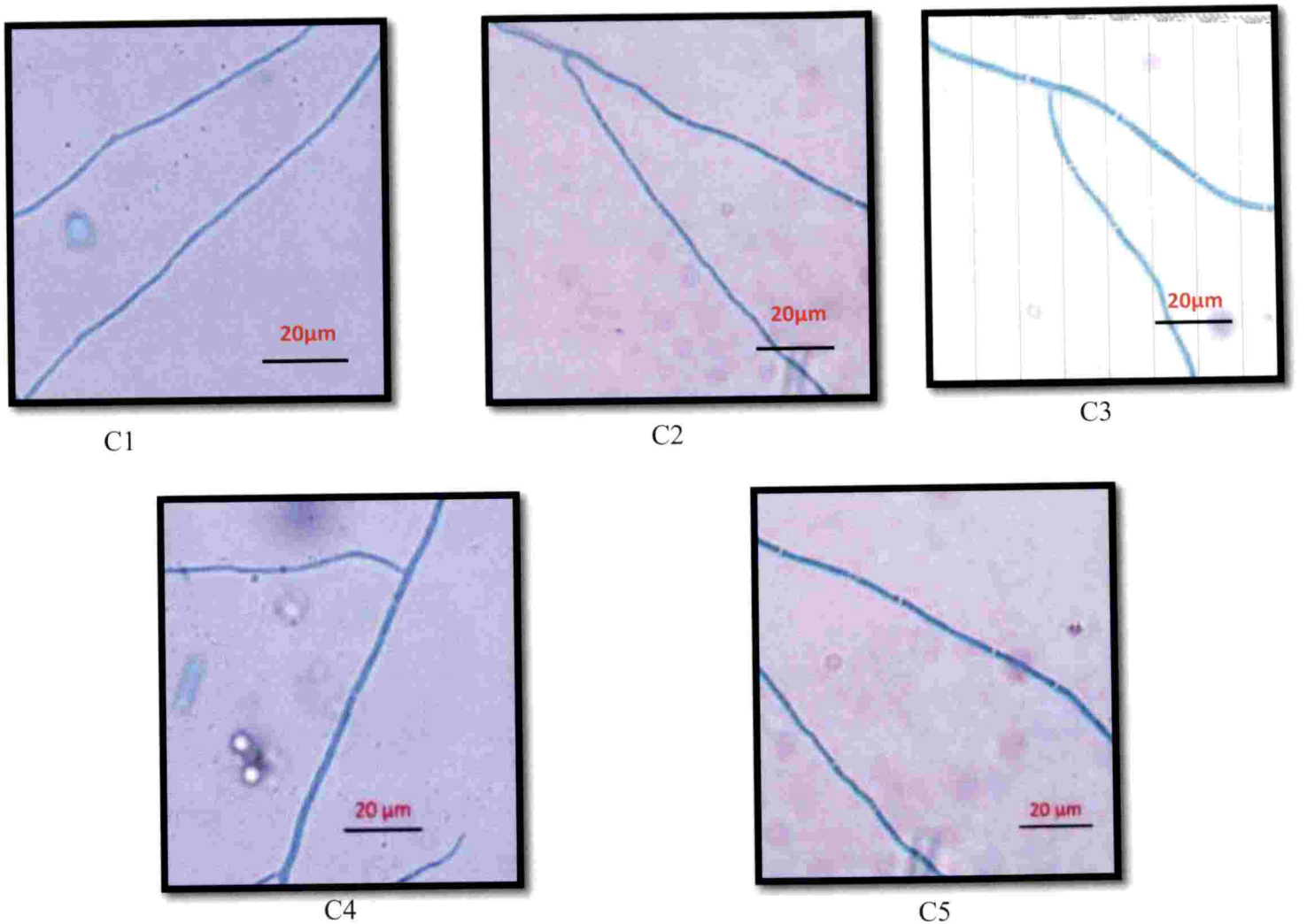
Isolates	Mycelial width (µm*)	Septal distance (µm*)	Nature of mycelium	Colour
C1	2.65	25.56	Septate	Hyaline
C2	2.98	19.15		
C3	3.32	13.86		
C4	3.45	9.80		
C5	3.02	8.50		

Table 9. Conidial and appressorial characters of *Colletotrichum* isolates causing anthracnose in betel vine

Isolates	Conidia		Appressoria
	Size (length x breadth) (µm)*	Shape	Size (length x breadth) (µm)*
C1	11.4 x 4.7	Cylindrical	8.73 x 5.02
C2	11.3 x 3.9	Dumbbell	10.08 x 5.23
C3	11.2 x 3.7	Oblong	9.57 x 6.28
C4	12.2 x 4.3	Oblong	9.28 x 5.71
C5	9.6 x 3.8	Cylindrical	8.37 x 5.11

*Mean of ten replications

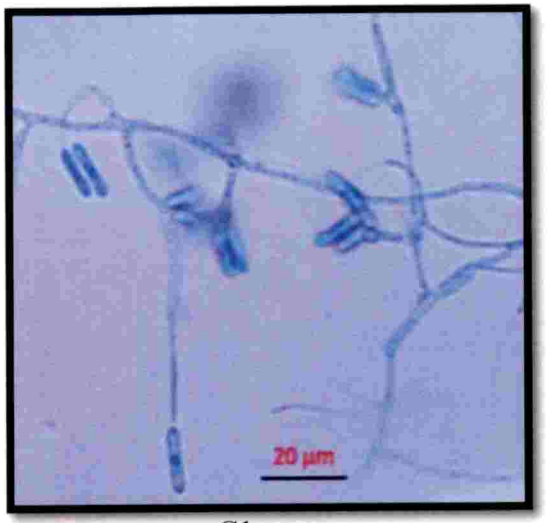
Observations were made from seven day old culture



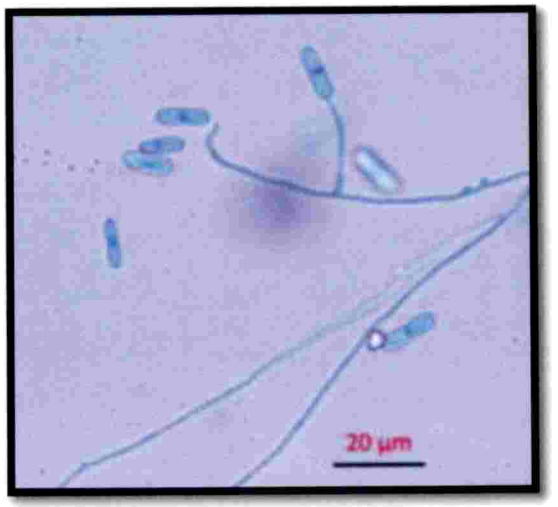
C1 C2 C3

C4 C5

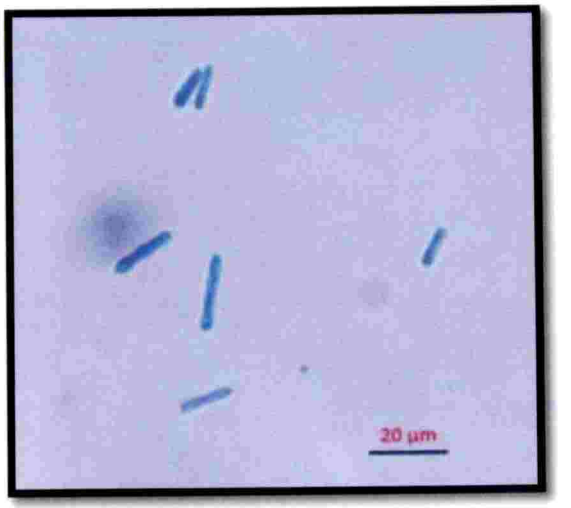
Plate 6 (C1-C5). Mycelia of *Colletotrichum* isolates causing anthracnose of betel vine (400X)



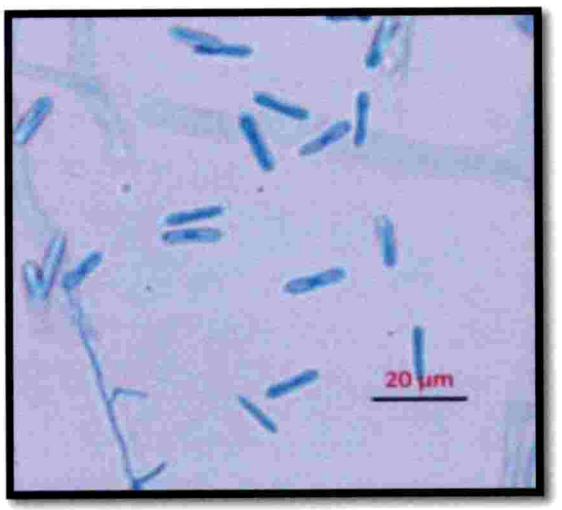
C1



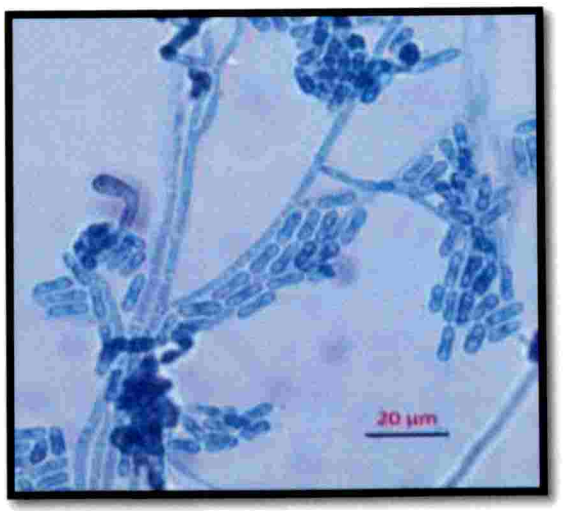
C2



C3



C4



C5

Plate 7 (C1-C5). Conidia of *Colletotrichum* isolates causing anthracnose of betel vine (400X)

C1 had an appressorial size of 8.73 µm x 5.02 µm, while the isolate C2 had 10.08 µm x 5.23 µm sized appressoria. Similarly, the isolate C3 had an appressorial size of 9.57 µm x 6.28 µm while for the isolates C4 and C5 the size ranged about 9.27 µm x 5.71 µm and 8.37 µm x 5.11 µm respectively. From the morphological studies the pathogen was identified as *C. gloeosporioides* (Plate 8).

4.2.3. Virulence Rating of the Isolates

The five different isolates *viz.*, C1, C2, C3, C4 and C5 were subjected to pathogenic variability study by artificial inoculation of the pathogen on the excised leaves (Table 10) (Plate 9).

The virulence rating among the different isolates was based on the lesion size, rate of lesion development and time taken for symptom expression. The isolate C2 showed initiation of symptom within two DAI and produced 14.75 mm sized lesion on 5th day of inoculation. Similarly the isolates C5, C3, C4, C1 produced 5.07 mm, 3.52 mm, 3.00 mm and 2.50 mm sized lesions at 5 DAI. The rate of lesion development varied from 0.5 mm day^{-1} to 2.95 mm day^{-1}. Similarly, the days for symptom expression varied from 2 to 5 days among the isolates. The highest rate of lesion development and minimum days for symptom initiation was recorded for the isolate C2, while the isolate C1 was the least virulent with respect to the lesion development. Based on the lesion size produced, rate of lesion development and days for symptom expression, C2 was found to be most virulent among the isolates of *Colletotrichum* sp. and used for further studies.

4.2.4. Molecular Characterization of Virulent Isolate

The molecular characterization was done through the method of DNA barcoding using universal primers. The ITS - rDNA region of the virulent isolate of *C. gloeosporioides* was sequenced and amplified for the identification and molecular characterization. The amplification resulted in an amplified product of approximately 500 bp long using ITS - 1F (forward) and ITS - 4R (reverse) primers. The sequence of the virulent isolate was obtained as

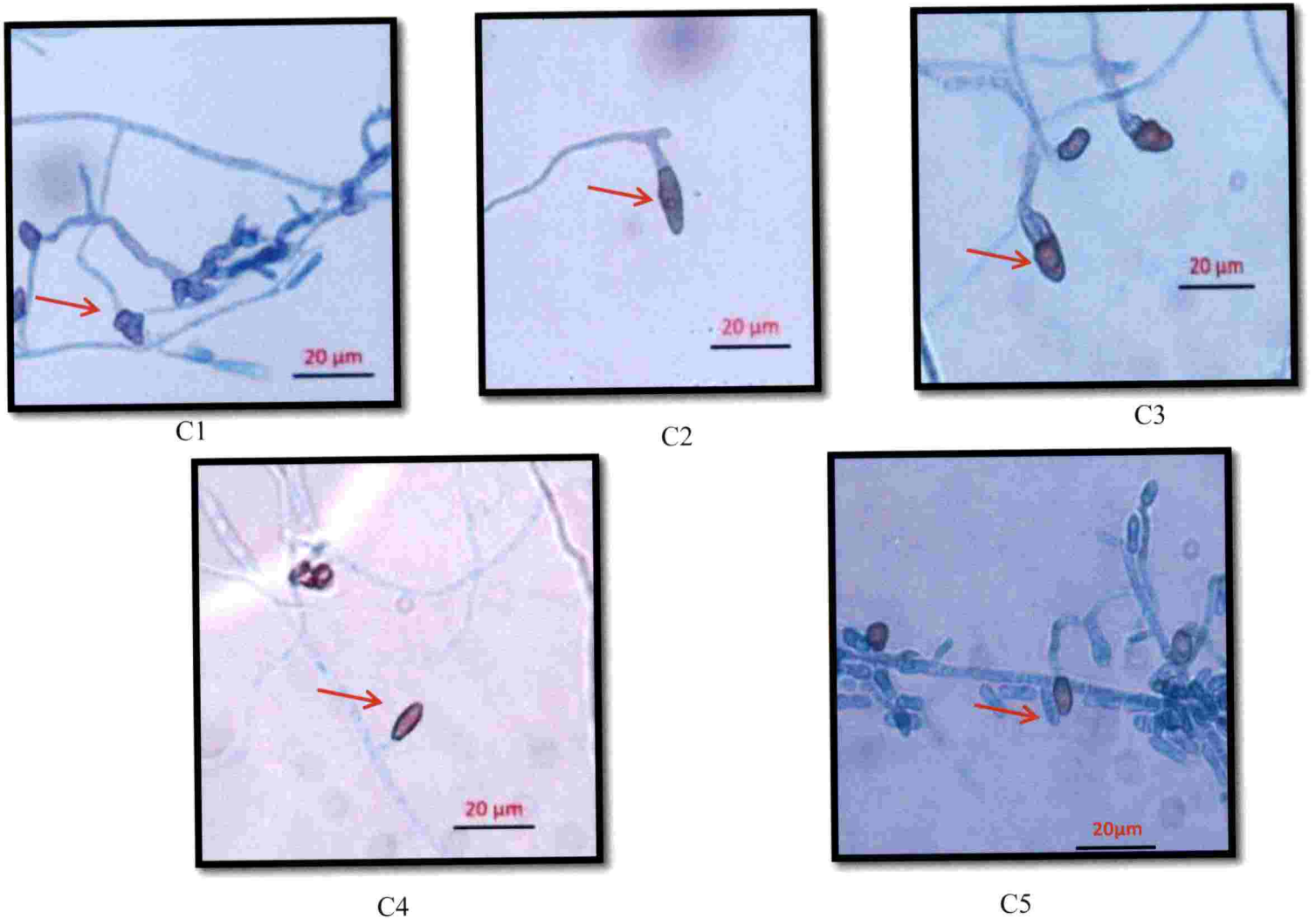


Plate 8 (C1-C5). Appressoria of *Colletotrichum* isolates causing anthracnose of betel vine (400X)

Table 10. Lesion size, rate of lesion development and days for symptom expression for assessing the virulence of *Colletotrichum* isolates causing anthracnose in betel vine

Isolates	**Mean lesion size at 5 DAI (mm^2) ***	**Time taken for symptom development (days)**	**Rate of lesion development ($mm\ day^{-1}$)**
C1	2.50^b	5	0.5
C2	14.75^a	2	2.95
C3	3.52^b	4	0.70
C4	3.00^b	4	0.60
C5	5.07^b	4	1.01
CD (0.05)	6.95		
SEm±	4.615		

*Mean of four replications
Values followed by similar superscripts are not significantly different at 5% level
DAI- Days after inoculation

C1

C2

C3

C4

C5

Plate 9 (C1-C5). Lesion enlargement of *Colletotrichum* isolates causing anthracnose of betel vine on excised leaves (5 DAI)

>*Colletotrichum gloeosporioides* Vellayani isolate
GAATCCTACGTGATCCGAGGTCACCTTTGGAAATTGGGGGGTTTTACG
GCAAGAGTCCCTCCGGATCCCAGTGCGAGACGTAAAGTTACTACGCAA
AGGAGGCTCCGGGAGGGTCCGCCACTACCTTTGAGGGCCTACATCGGC
TGTAGGGCCCCAACACCAAGCAGAGCTTGAGGGTTGAAATGACGCTC
GAACAGGCATGCCCGCCAGAATGCTGGCGGGCGCAATGTGCGTTCAA
AGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTT
CGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTAAAAG
TTTTGATTATTTGCTTGTACCACTCAGAAGAAACGTCGTTAAATCAGAG
TTTGGTTATCCTCCGGCGGGCGCCGACCCGCCCGGGGGGGCGGGAGGC
CGGGAGGGTCACGGAGACCCTACCCGCCGAAGCAACAGTTATAGGTA
TGTTCACAAAGGGTTGTAGAGCGTAAACTCAGTAATGATCCCTCCGCA
GGTTCACCCTACGGAGGTTACAGAGTTTACGCTCTACACCTATGTGAA
ATACTTCTCATTTCGCTTCGCGGGAGGGTCTCCTGACCTCTGCCCCGCC
CCCCGGGGGGGGTCGGCTCCGCCCGAGAAACCAACTCTTAAATTAAGA
ACATTTGCTGCTGAGATGGGTACAGGCCGATTAAACAAAACTC

and was blasted in the Gene Bank of NCBI data base using BLAST program to identify the similar sequences. The blast results showed that the virulent isolate had 99 per cent ITS nucleotide identity with other *C. gloeosporioides.* The isolated virulent culture of *Colletotrichum* sp. had 99 per cent nucleotide identity with isolates of *C. gloeosporioides* infecting *Mangifera indica*, Nagpur mandarin and *Lantana camara* (Table 11). The virulent pathogen was thus confirmed as *C. gloeosporioides* (Figure 1).

4.3. SCREENING OF INDIGENOUS ORGANIC PREPARATIONS FOR *IN VITRO* PATHOGEN SUPPRESSION

The microbial population of the organic preparations were evaluated at 1st, 5th and 7th day after preparation both in filtered and non-filtered extract (Table 12). The bacterial population showed an increasing trend in filtered and non-filtered extracts of organic preparations *viz.*, panchagavya, vermiwash and fermented

Table 11. Nucleotide identity of virulent isolate (C2) causing anthracnose in betel vine with reported isolates of *C. gloeosporioides*

Accession no.	Description	Nucleotide identity (%)	Crop
MH221089.1	*C. gloeosporioides* isolate NCG	99	Nagpur Mandarin
KU671363.1	*C. gloeosporioides* strain Ps-36	99	*Premna serratifolia*
KF192821.1	*C. gloeosporioides* strain EGJMP6	99	*Lantana camara*
KC790938.1	*C. gloeosporioides* strain MTCC10339	99	*Mangifera indica*
KM044006.1	*C. gloeosporioides* strain GZBY01	99	*Castanopsis fissa*
KP686471.1	*Colletotrichum* sp. BAB-4679	99	-
KX022506.1	*C. gloeosporioides* strain SY	99	*Dracaena sanderiana*
KX022503.1	*C. gloeosporioides* strain LB	99	*Dracaena deremenis*
GU810510.1	*C. gloeosporioides* isolate CBE(Ro)	99	
GU819238.1	*C. gloeosporioides* strain TEN2	99	-

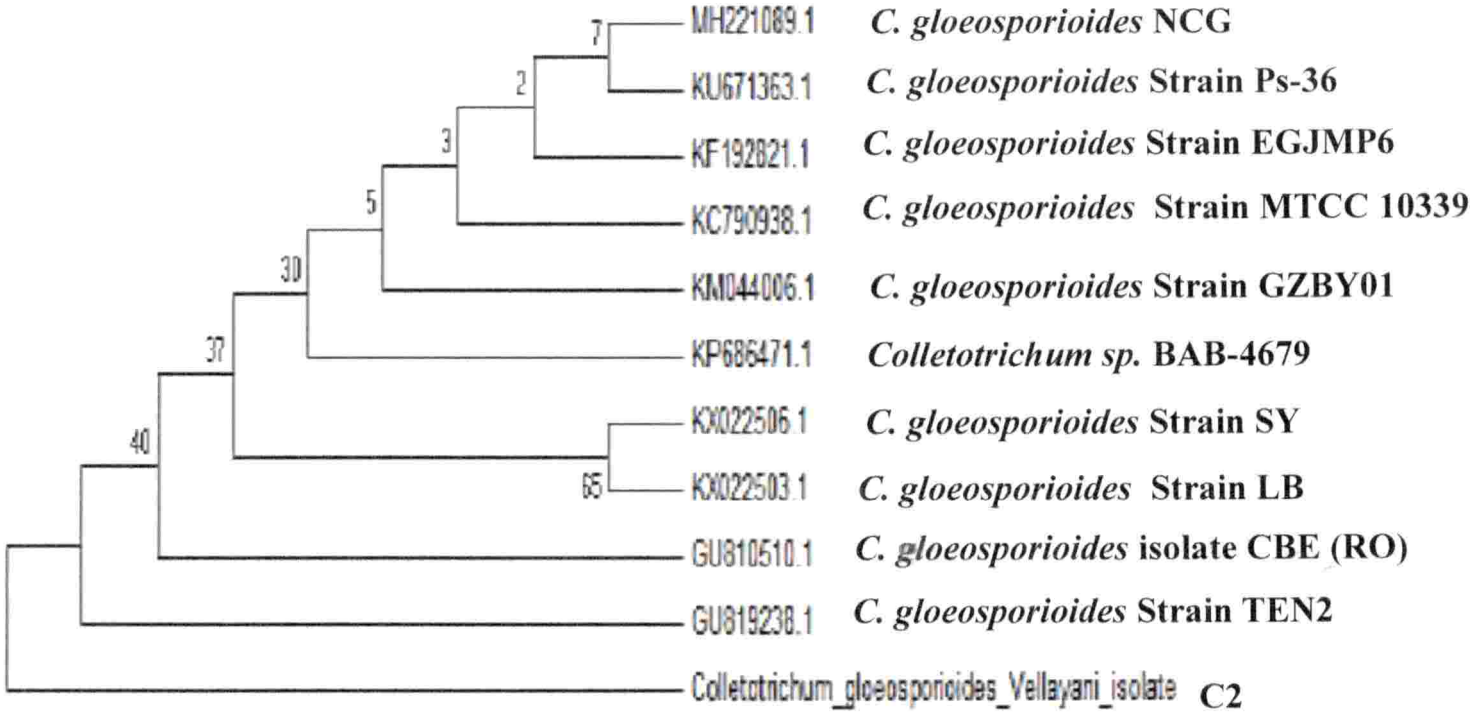


Figure 1. Dendogram showing the relationship of C2 isolate of *C.gloeosporioides* causing anthracnose in betel vine with reported isolates of *C. gloeosporioides*

Table 12. Fungal and bacterial population in the filtered and non-filtered organic preparations at different period of its preparations

Organic amendments	1st day (cfu ml -1)				5th day (cfu ml-1)				7th day (cfu ml -1)			
	Filtered		Non filtered		Filtered		Non filtered		Filtered		Non filtered	
	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria
Panchagavya	-	2	1	160	-	4	-	189	-	-	-	212
Jeevamruth	-	33	-	163	1	-	1	60	1	-	1	30
Vermiwash	3	20	12	32	-	27	8	41	-	35	2	49
Fermented cow's urine	-	1	-	12	-	5	-	20	-	15	-	35

Fungal population at 10^{-4} dilution

Bacterial population at 10^{-6} dilution

cow's urine, while the population was decreasing in jeevamruth as the number of days increased. The bacterial colonies were higher in non-filtered extract than in filtered ones. The fungal colonies were prominent in vermiwash than other indigenous organic preparations.

In vitro evaluation of the indigenous organic preparations *viz.*, panchagavya, jeevamruth, vermiwash and fermented cow's urine was done against *C. gloeosporioides* using poisoned food technique in PDA.

The assay was conducted in autoclaved and non-autoclaved organic preparations amended PDA medium. The result of the experiment with non-autoclaved extract showed that fermented cow's urine at 5 per cent recorded highest growth inhibition of 100 per cent. This treatment was followed by panchagavya and jeevamruth which inhibited 36.39 per cent, 26.94 per cent mycelial growth of the pathogen respectively. The least inhibition percentage was recorded for vermiwash (12.78 per cent) (Table 13) (Plate 10).

The organic preparations at 10 per cent concentration, fermented cow's urine and panchagavya recorded complete growth inhibition of the pathogen. The treatment was followed by jeevamruth which recorded 30.83 per cent mycelial inhibition. The least inhibition of the pathogen was recorded by vermiwash (10 per cent) with an inhibition percentage of 13.89. The growth of *C. gloeosporioides* in the amended medium were either fluffy or sparse with uniform pattern.

At 5 per cent concentration of autoclaved organic preparations, panchagavya had the highest mycelial inhibition of the pathogen (24.89 per cent) which was statistically superior than the other organic treatments (Table 14) (Plate 11). This was followed by jeevamruth and vermiwash which recorded 8.56 per cent and 6.44 per cent mycelial inhibition respectively. The least inhibition was noted for fermented cow's urine (5.67 per cent).

Among the various the indigenous organic preparations at 10 per cent, panchagavya recorded complete mycelial inhibition (100 per cent) of the

Table 13. Effect of non-autoclaved organic preparations on mycelial growth and inhibition of *C. gloeosporioides* causing anthracnose in betel vine

Treatments	Organic preparations	Dosage (%)	Nature of mycelium	Growth pattern	Percentage inhibition of mycelial (%)*
T1	Panchagavya	5	Fluffy	Uniform	36.39 (37.00)[b]
T2		10	No growth	No growth	100.00 (89.05)[a]
T3	Jeevamruth	5	Fluffy	Irregular	26.94 (31.26)[c]
T4		10	Fluffy	Irregular	30.83 (33.72)[bc]
T5	Vermiwash	5	Sparse	Uniform growth with concentric zonations	12.78 (20.11)[d]
T6		10	Sparse	Uniform growth with concentric zonations	13.89 (21.19)[d]
T7	Fermented cow's urine	5	No growth	No growth	100.00 (89.05)[a]
T8		10	No growth	No growth	100.00 (89.05)[a]
T9	Control		Fluffy	Uniform growth with concentric zonation	0.00 (0.96)[e]
CD (0.05)					4.983
SEm±					3.434

*Mean of four replications

Values in parenthesis are arc sin transformed values

Values followed by similar superscripts are not significantly different at 5% level

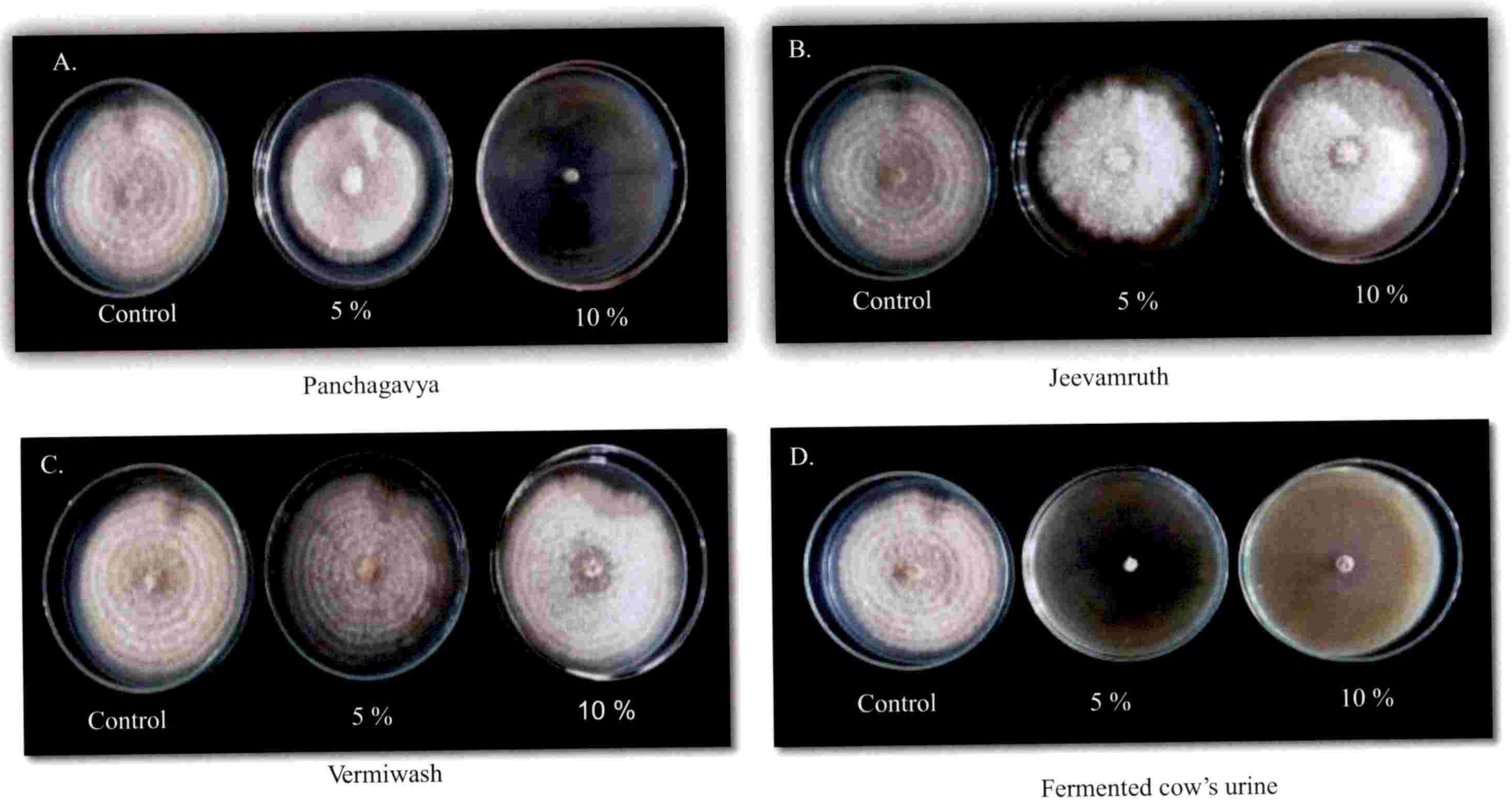
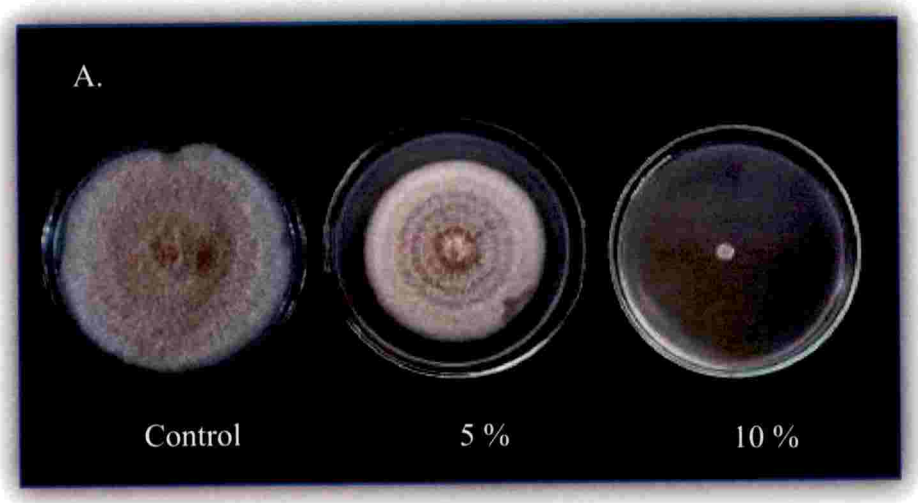


Plate 10 (A-D). Effect of non-autoclaved organic preparations on mycelial growth of *C. gloeosporioides* causing anthracnose of betel vine

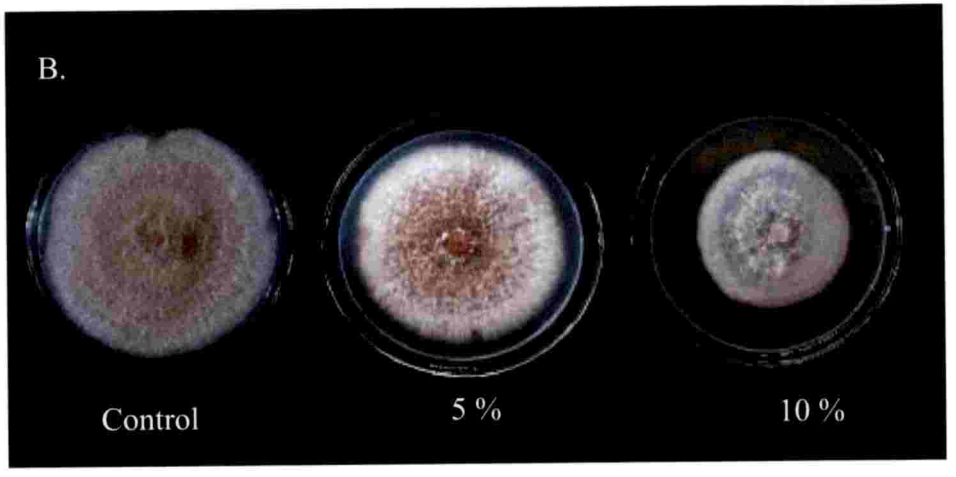
Table 14. Effect of autoclaved organic preparations on mycelial growth and inhibition of *C. gloeosporioides* causing anthracnose in betel vine

Treatments	Organic preparations	Dosage (%)	Nature of mycelium	Growth pattern	Percentage of mycelial inhibition (*)
T1	Panchagavya	5	Fluffy	Uniform	24.89 (29.72)^{c}
T2		10	No growth	No growth	100.00 (89.05)^{a}
T3	Jeevamruth	5	Fluffy	Uniform	8.56 (16.79)^{d}
T4		10	Sparse	Uniform	30.33 (33.41)^{b}
T5	Vermiwash	5	Fluffy	Uniform	6.44 (14.65)^{de}
T6		10	Sparse	Uniform	7.33 (15.55)^{de}
T7	Fermented cow`s urine	5	Sparse	Uniform	5.67 (13.69)^{e}
T8		10	Sparse	Uniform	8.68 (17.08)^{d}
T9	Control		Fluffy	Uniform growth with zonations	0.00 (0.96)^{f}
CD (0.05)					3.084
SEm±					2.404

*Mean of four replications
Values in parenthesis are arc sin transformed values
Values followed by similar superscripts are not significantly different at 5% level

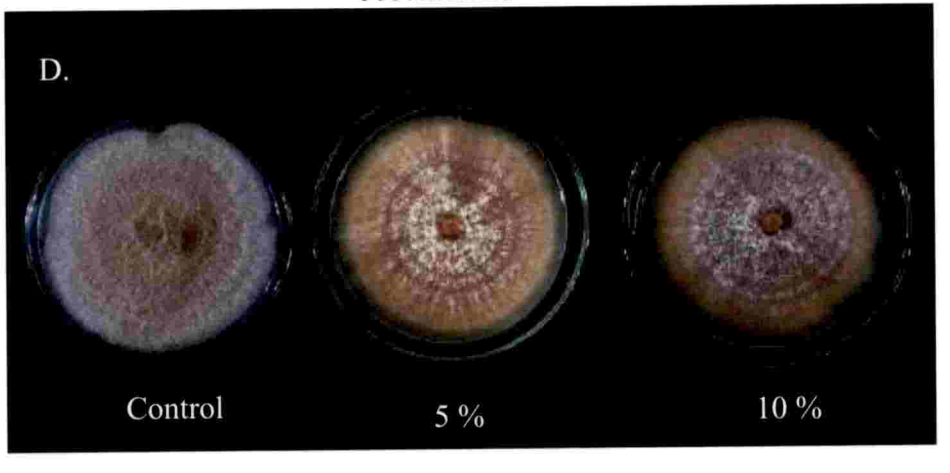


Panchagavya



Jeevamruth

Vermi wash



Fermented cow's urine

Plate 11 (A-D). Effect of autoclaved organic preparations on mycelial growth of *C. gloeosporioides* causing anthracnose in betel vine

pathogen. The treatment was statistically superior to the other treatments. This was followed by jeevamruth and fermented cow's urine with 30.33 per cent and 8.68 per cent mycelial inhibition of the pathogen respectively. The least inhibition of the mycelial growth of the pathogen was observed for vermiwash (7.33 per cent). Similar to non-autoclaved organic preparation, nature of mycelial growth was either fluffy or sparse with uniform growth.

4.4. SCREENING OF BIOCONTROL AGENTS FOR *IN VITRO* PATHOGEN SUPPRESSION

4.4.1. Isolation of Micro Flora from Rhizosphere and Phyllosphere of Healthy Betel Vine Leaves

The antagonistic microflora were isolated from the rhizosphere and phyllosphere of healthy betel vine from four different locations *viz.*, Kalliyoor, Vellayani, Cherukoodu and Kattakada. The details of the fungal and bacterial isolates were given in the Table 15. The fungal colonies were higher in rhizosphere than phyllosphere.

4.4.2. *In vitro* Evaluation of the Antagonistic Fungi and Bacteria for Suppression of the Pathogen by Dual Culture Technique

4.4.2.1. Antagonistic Fungi

Two fungal genera *viz.*, *Trichoderma* sp. and *Aspergillus* sp. were isolated from the rhizosphere of the healthy betel vine. Two *Trichoderma* sp. and four *Aspergillus* sp. were evaluated for their antagonistic potential against *C. gloeosporioides* under *in vitro* conditions. The effective *Trichoderma* isolates were obtained from Kalliyoor and Cherukoodu and *Aspergillus* from Vellayani and Kattakada. The antagonistic fungi were designated as *Trichoderma* 1, *Trichoderma* 2, *Aspergillus* sp.1, *Aspergillus* sp. 2, *Aspergillus* sp. 3 and *Aspergillus* sp. 4. All the fungal isolates screened were superior in inhibiting the mycelial growth of the pathogen (Table 16) (Plate 12).

Table 15. Population of fungi and bacteria from rhizosphere and phyllosphere of healthy betel vine

Location	**Fungal colonies (10^{-4} cfu ml^{-1})**		**Bacterial colonies (10^{-6} cfu ml^{-1})**	
	Rhizosphere	**Phyllosphere**	**Rhizosphere**	**Phyllosphere**
Kalliyoor	19	3	4	8
Vellayani	8	5	12	14
Cherukoodu	15	1	12	2
Kattakada	10	3	18	12

Table 16. Mycelial inhibition of *C. gloeosporioides* causing anthracnose in betel vine by rhizosphere fungi

Isolates	Fungal antagonist	Locations of collection	Percentage of mycelial inhibition (*)
F1	*Trichoderma* 1	Kalliyoor	78.89 (62.64)[a]
F2	*Trichoderma* 2	Cherukoodu	74.72 (59.86)[b]
F3	*Aspergillus* sp.1	Vellayani	56.11 (48.51)[d]
F4	*Aspergillus* sp.2		54.72 (47.71)[d]
F5	*Aspergillus* sp.3	Kattakada	60.56 (51.09)[c]
F6	*Aspergillus* sp.4		56.94 (48.99)[d]
	Control		0.00 (0.96)[e]
	CD (0.05)		1.700
	SEm±		1.168

*Mean of four replications
Values followed by similar superscripts are not significantly different at 5% level
Values in parenthesis are arc sin transformed

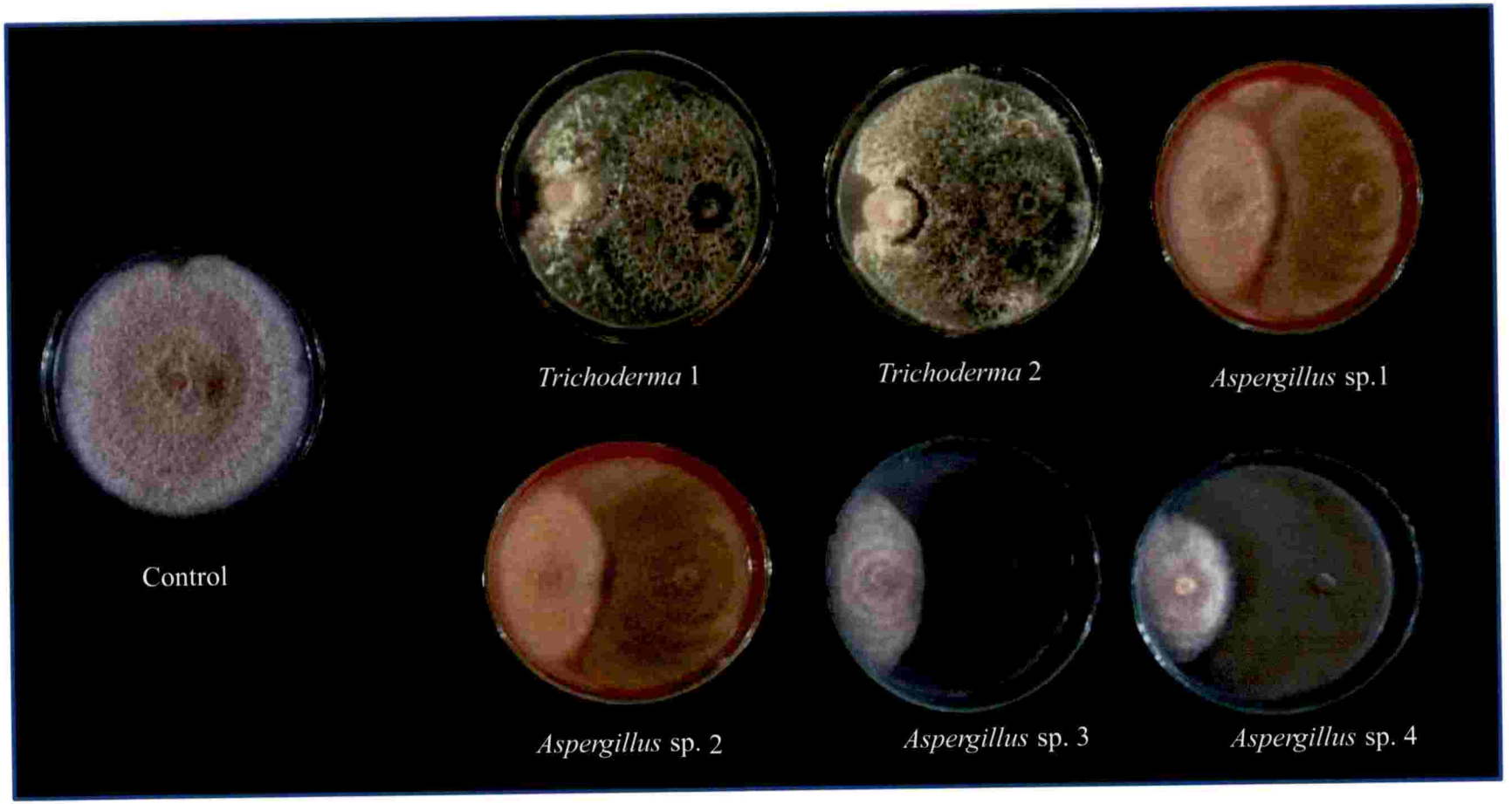


Plate 12. *In vitro* inhibition of *C. gloeosporioides* causing anthracnose of betel vine by selected fungal antagonists from rhizosphere

The *Trichoderma* 1 recorded highest mycelial inhibition of the pathogen (78.89 per cent) which was statistically superior to other treatments. This was followed by *Trichoderma* 2 which had an inhibition percentage of 74.72 under *in vitro* conditions. An inhibition percentage of 60.56 was recorded for the *Aspergillus* sp. 3. The *Aspergillus* sp. 1, *Aspergillus* sp. 2 and *Aspergillus* sp. 4 had an inhibition percentage of 56.11, 54.72 and 56.94 respectively and were statistically on par.

4.4.2.2. Antagonistic Bacteria

Thirteen antagonistic bacterial isolates were obtained from rhizosphere and phyllosphere of betel vine from Kalliyoor, Kattakada, Vellayani and Cherukoodu. These bacterial isolates were evaluated for their antagonistic potential against *C. gloeosporioides* under *in vitro* conditions (Table 17) (Plate 13). The isolates were designated as B1, B2, B3, B4, B5, B6, B7, B9, B10, B11, B13 and B14. The bacterial isolate B5 was found superior among the different isolates screened with an inhibition percentage of 45. This isolate was on par with isolate B11 which recorded 43.61 per cent mycelial inhibition. This treatment was followed by B14 (40.27 per cent). The bacterial isolate B11 was statistically on par with B14. The bacterial isolate B14 was followed by B4 with 37.22 per cent mycelial inhibition. This was followed by bacterial isolates B10, B2, B9, B6, B13, B3 and B1 with 31.39 per cent, 30.78 per cent, 29.72 per cent, 28.02 per cent, 24.72 per cent and 21.38 per cent of mycelial inhibition of the pathogen respectively. The bacterial isolate B2, B9 and B6 was statistically on par in inhibiting the mycelial growth. The least inhibition of 19.72 per cent was observed for the isolate B7. B5 the phyllosphere isolate showing highest mycelial inhibition of the pathogen under *in vitro* condition was taken for the further study

Table 17. Mycelial inhibition of *C. gloeosporioides* causing anthracnose in betel vine by antagonistic bacteria from the rhizosphere and phyllosphere

Bacterial isolates	**Percentage of mycelial inhibition(*)**
B 1	21.38 (27.47)^{gh}
B 2	31.38 (34.07)^{de}
B 3	24.71 (29.82)^{fg}
B 4	37.22 (37.59)^{bc}
B 5	45.00 (42.12)^{a}
B 6	29.72 (33.03)^{de}
B 7	19.71 (26.20)^{h}
B 9	30.27 (33.35)^{de}
B 10	32.77 (34.92)^{cd}
B 11	43.61 (41.33)^{a}
B 13	28.02 (31.95)^{ef}
B 14	40.27 (39.38)^{ab}
Control	0.00 (0.96)^{i}
C.D.(0.05)	2.786
SEm±	1.947

*Mean of four replications
Values followed by similar superscripts are not significantly different at 5% level
Values in parenthesis are arc sin transformed

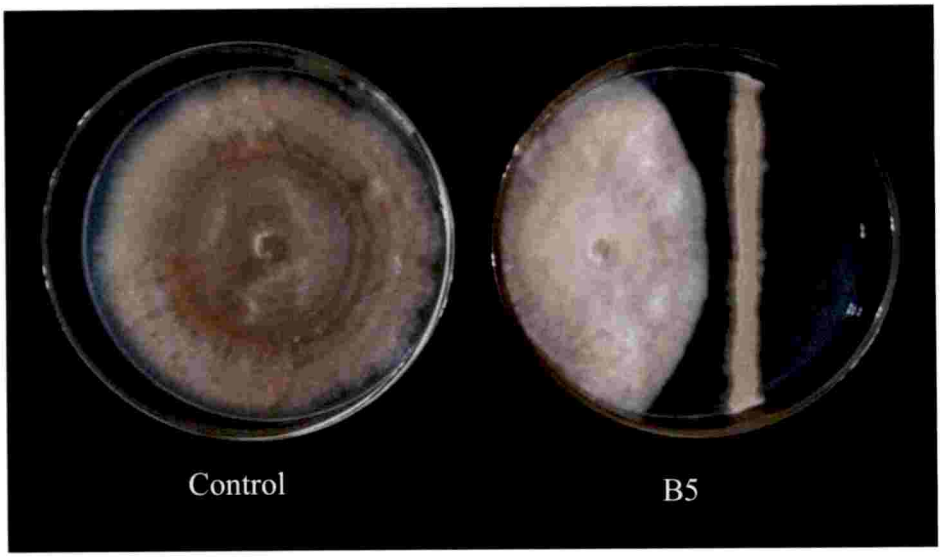


Plate 13. Inhibition of *C. gloeosporioides* causing anthracnose of betel vine by bacterial antagonist (B 5)

4.4.3. Identification and Cultural Characterization of Selected Fungal and Bacterial Antagonist

4.4.3.1. Identification of Fungal Antagonist

The six selected fungal antagonists were morphologically studied (Plate 14). The colony colour of fungal antagonist *Trichoderma* 1 was greenish white initially then turning to dark green in colour. Similarly *Trichoderma* 2 produced colony which was white initially gradually turning to green in colour. Both *Aspergillus* sp. 1 and *Aspergillus* sp. 2. produced light greenish coloured colonies. *Aspergillus* sp. 3 and *Aspergillus* sp. 4 produced white coloured colony turning black and dull greenish coloured colony respectively.

4.4.3.2. Identification of Antagonistic Bacteria

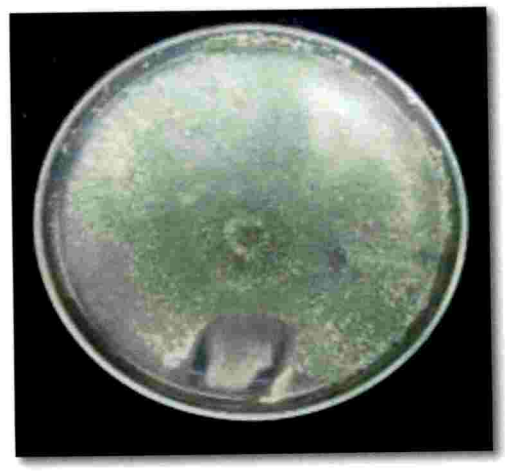
The antagonistic bacteria B5 was studied by gram staining method. The 24 h old culture was used for gram staining procedure. The gram stained culture showed a Gram +ve reaction and was rod shaped. The bacteria was identified as *Bacillus* sp.

4.5. *IN VITRO* EVALUATION OF FUNGICIDES AGAINST *C. GLOEOSPORIOIDES*

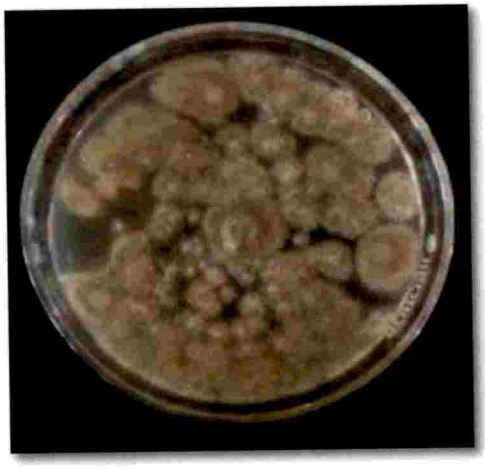
The *in vitro* inhibition of *C. gloeosporioides* by the fungicides was done by poisoned food technique. Seven commercially available fungicides were used for the study (Table 18) (Plate 15, 16 & 17).

In vitro study revealed that at recommended dosage, fungicides *viz.*, propiconazole (0.1 per cent), tebuconazole (0.1 per cent), captan + hexaconazole (0.1 per cent) and carbendazim + mancozeb (0.1 per cent) recorded 100 per cent growth inhibition of the pathogen under *in vitro* conditions. Copper hydroxide (0.2 per cent) recorded 69.56 per cent mycelial inhibition of the pathogen. This treatment was followed by azoxystrobin (0.1 per cent) with 55.33 per cent inhibition of the mycelial growth of the pathogen. The lowest inhibition of the mycelia was recorded for mancozeb (0.2 per cent) *i.e.* 48.22 per cent.

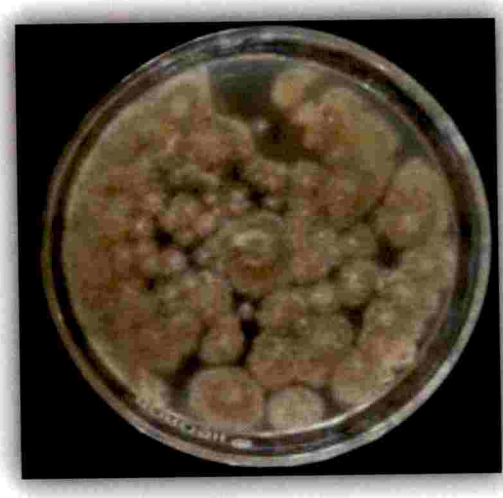
Trichoderma 1



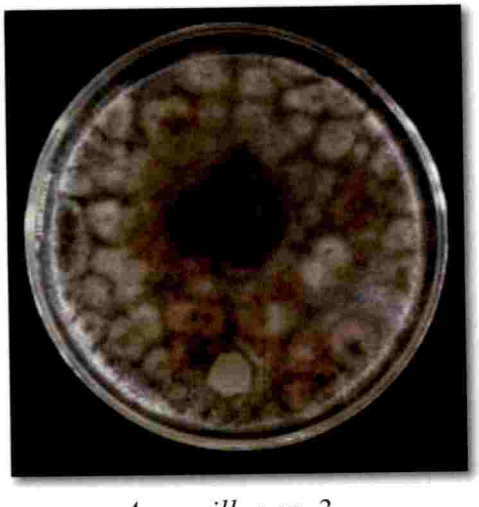
Trichoderma 2



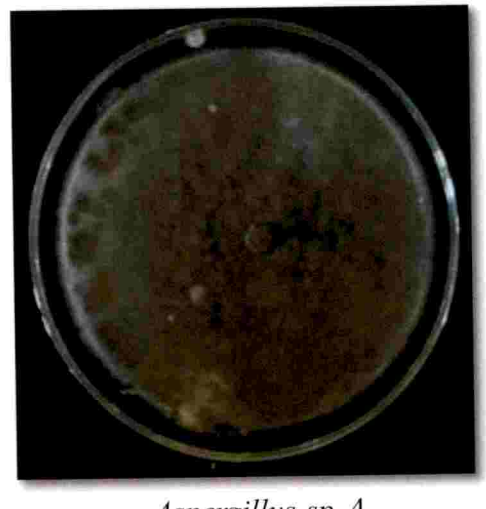
Aspergillus sp. 1



Aspergillus sp. 2



Aspergillus sp. 3



Aspergillus sp. 4

Plate 14. Antagonistic fungi isolated from rhizosphere of betel vine

Table 18. Evaluation of fungicides for *in vitro* inhibition of C. *gloeosporioides* causing anthracnose in betel vine

Treatments	Fungicide	Percentage of mycelial inhibition *		
		Lower dose	Recommended dose	Higher dose
T1- T3	Propiconazole 25.0% EC	100 (89.05)^{a}	100 (89.05)^{a}	100 (89.05)^{a}
T4 – T6	Tebuconazole 25.9% EC	100 (89.05)^{a}	100 (89.05)^{a}	100 (89.05)^{a}
T7 – T9	Azoxystrobin 23% SC	54.00 (47.30)^{ef}	55.33 (48.06)^{de}	56.00 (48.70)^{d}
T10- T12	Mancozeb 75% WP	43.55 (41.29)^{h}	48.22 (43.98)^{g}	51.77 (46.01)^{f}
T13-T15	Copper hydroxide 53.8% WP	53.56 (47.04)^{ef}	69.56 (56.53)^{c}	84.22 (66.60)^{b}
T16-T18	Captan (70%) + Hexaconazole (50%) WP	100 (89.05)^{a}	100 (89.05)^{a}	100 (89.05)^{a}
T19-T21	Carbendazim (12%) + Mancozeb (63%) WP	100 (89.05)^{a}	100 (89.05)^{a}	100 (89.05)^{a}
Control		0.00 (0.96)^{i}	0.00 (0.96)^{i}	0.00 (0.96)^{i}
C.D. (0.05)		1.299		
SEm±		1.033		

Mean of four replications

Values followed by similar superscripts are not significantly different at 5% level

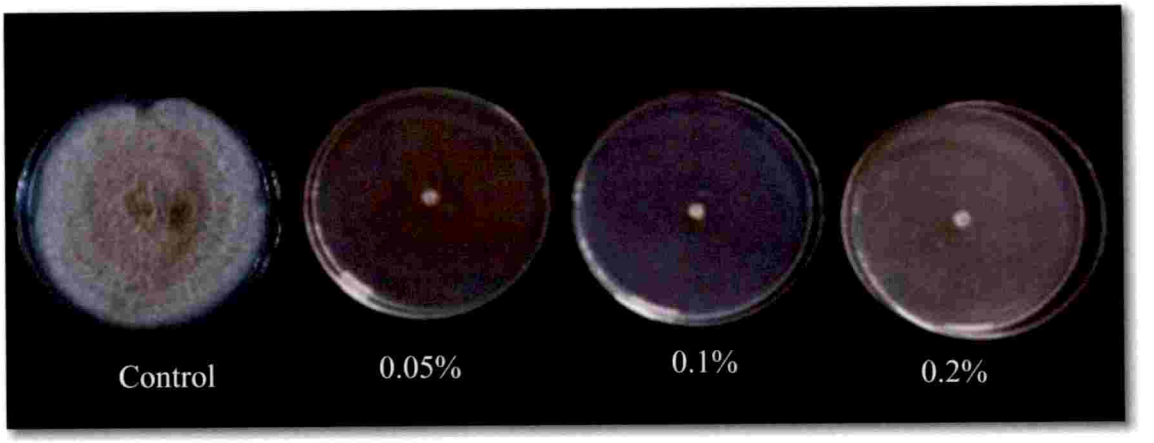
* Values in parenthesis are arc sin transformed

Lower, field and high dose –

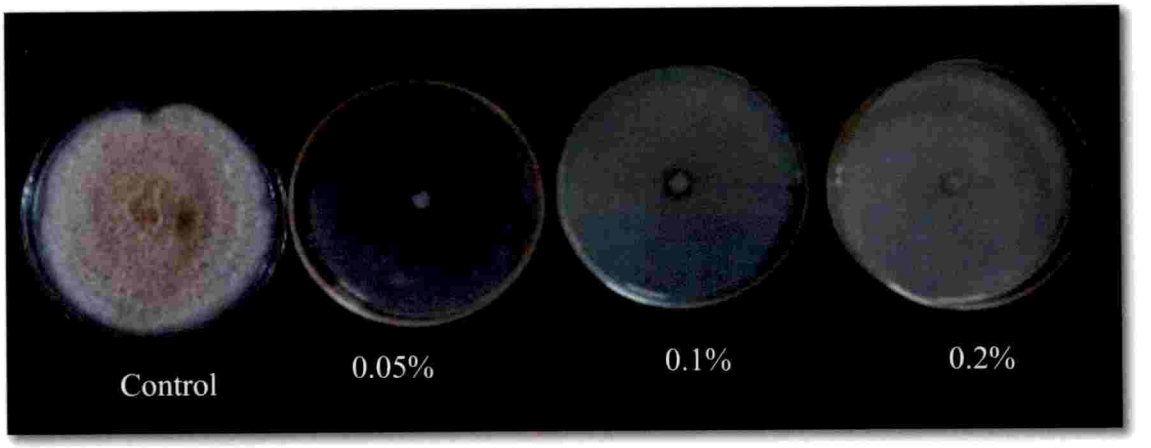
Propiconazole, Tebuconazole, azoxystrobin are 0.05%, 0.1%, 0.2%

Captan + Hexaconazole and Carbendazim + Mancozeb are 0.05%, 0.1%, 0.2%

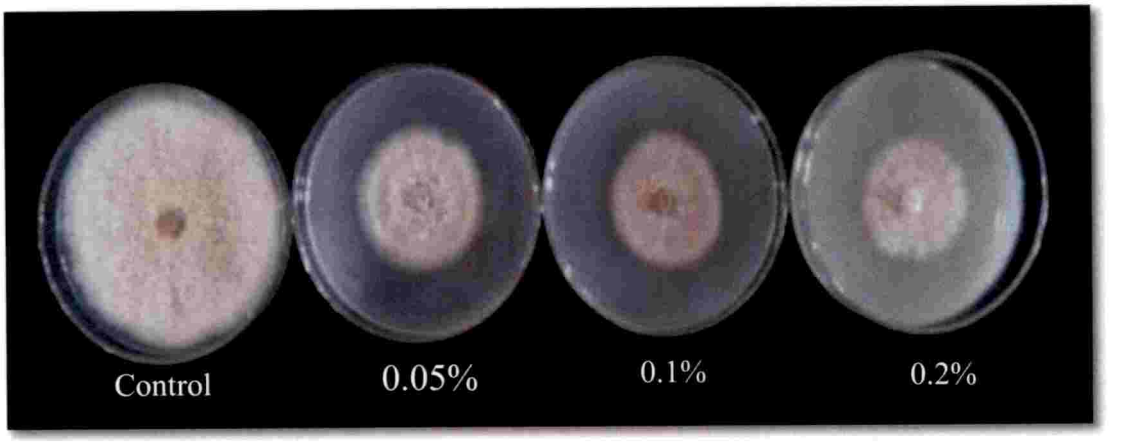
Mancozeb, Copper hydroxide are 0.1%, 0.2% and 0.4%



Propiconazole 25.0% EC

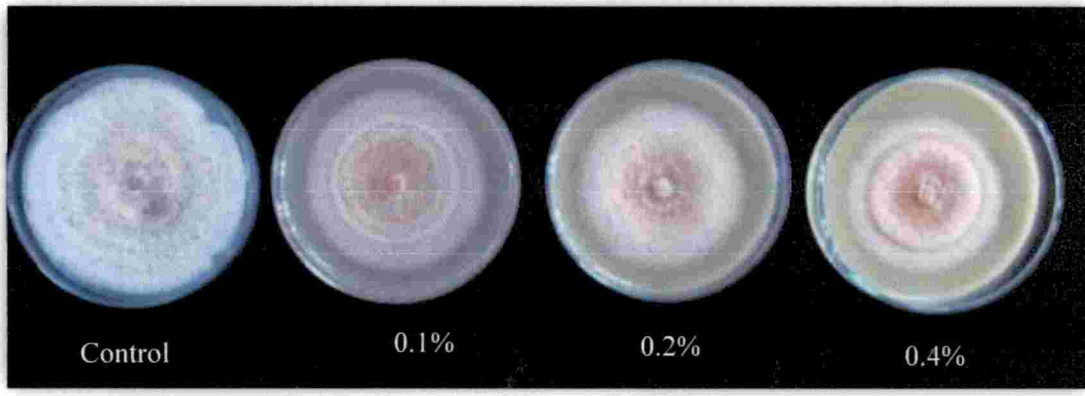


Tebuconazole 25.9% EC

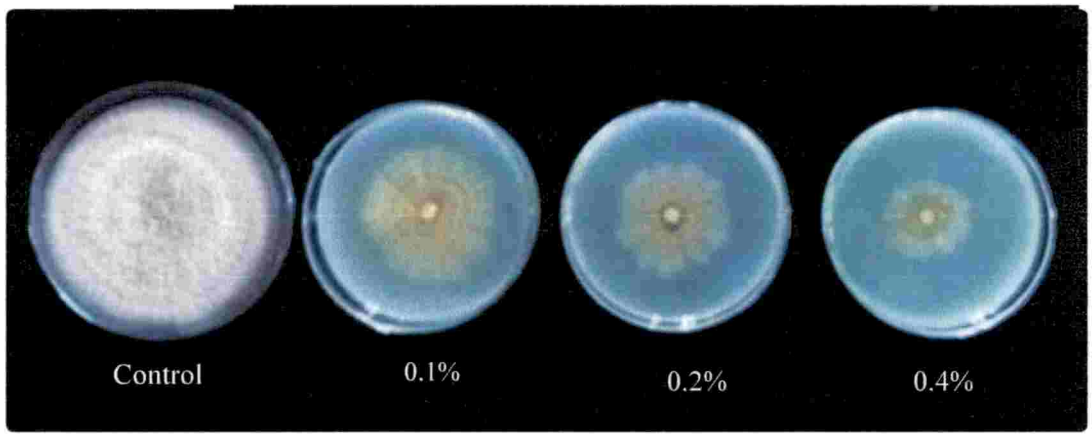


Azoxystrobin 23 % SC

Plate 15. Mycelial growth suppression of *C. gloeosporioides* causing anthracnose of betel vine by systemic fungicides

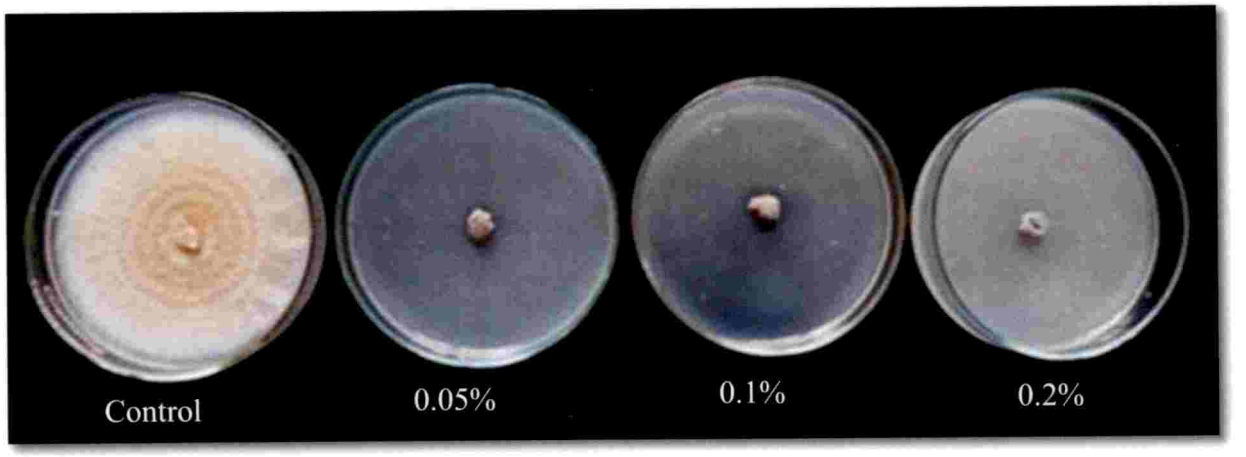


Mancozeb 75% WP

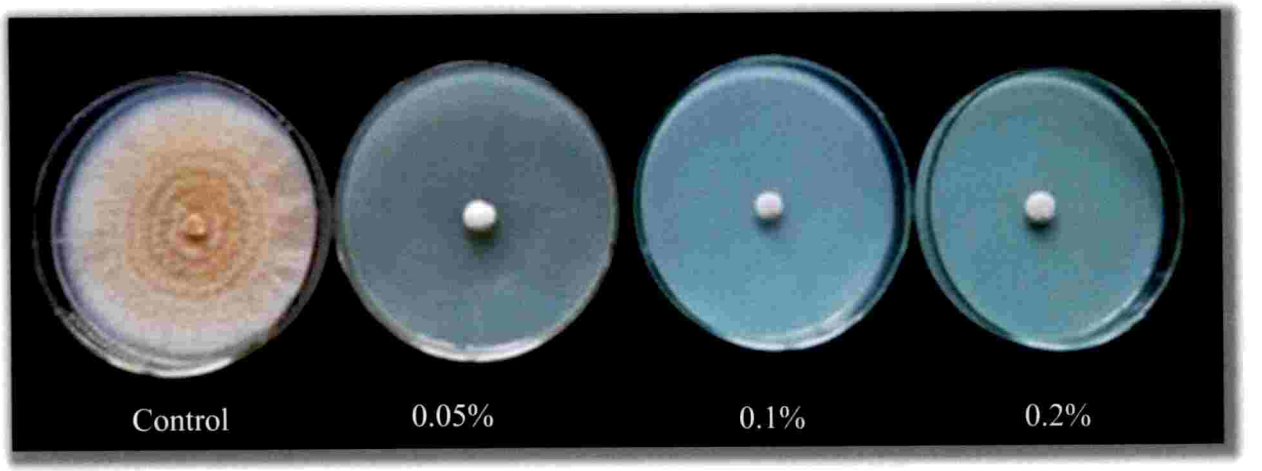


Copper hydroxide 58.3 % WP

Plate 16. Mycelial growth suppression of *C. gloeosporioides* causing anthracnose of betel vine by contact fungicides



Captan (70%) + Hexaconazole (5.0%) WP



Carbendazim (12%) + Mancozeb (63%) WP

Plate 17. Mycelial growth suppression of *C. gloeosporioides* causing anthracnose of betel vine by combination fungicides

At a lower dosage, also the fungicides propiconazole (0.05 per cent), tebuconazole (0.05 per cent), captan + hexaconazole (0.05 per cent) and carbendazim + mancozeb (0.05 per cent) had complete mycelial inhibition. These treatments were statistically superior to other treatments. Azoxystrobin (0.1 per cent) and copper hydroxide (0.1 per cent) with 54 per cent and 53.56 per cent mycelial inhibition of the pathogen respectively and were found as statistically on par. The lowest inhibition of the pathogen was recorded for mancozeb (0.1 per cent) which inhibited 43.55 per cent mycelial growth.

At higher dosage than the recommended field dosage, propiconazole (0.2 per cent), tebuconazole (0.2 per cent), captan + hexaconazole (0.2 per cent) and carbendazim + mancozeb (0.2 per cent) completely suppressed the pathogen *in vitro*. Copper hydroxide (0.4 per cent) recorded 84.22 per cent mycelial inhibition of the pathogen. This was followed by azoxystrobin (0.2 per cent) with 56 per cent mycelial inhibition. Mancozeb (0.4 per cent) was least effective among the higher doses of fungicide tested.

4.5.1. *In vitro* Evaluation of fungicides at Lower Doses Against *C. gloeosporioides*

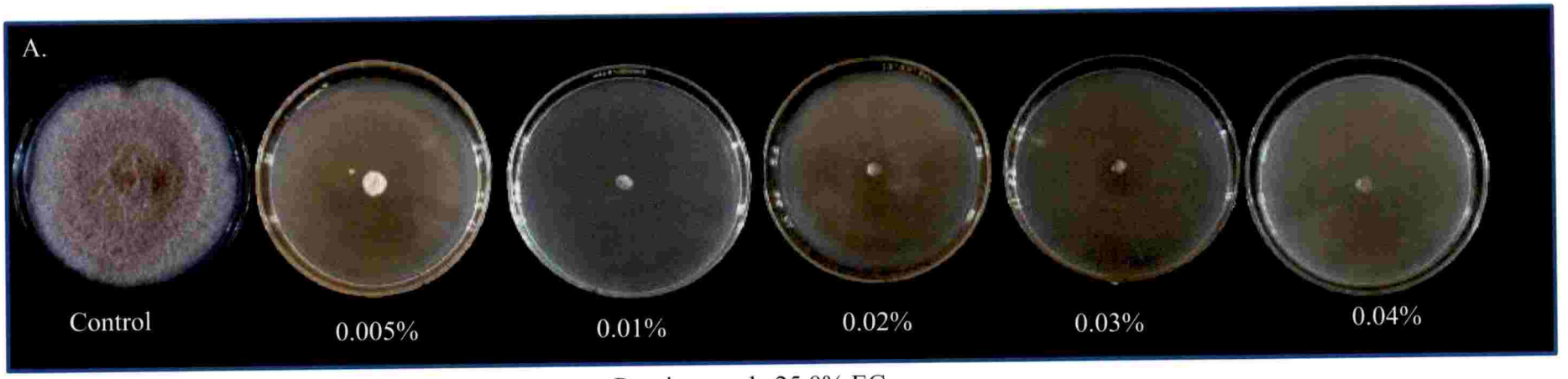
Four fungicides *viz.,* propiconazole, tebuconazole, captan + hexaconazole and carbendazim + mancozeb at 0.05 per cent showed complete growth inhibition at lower concentration were further evaluated at lower concentration *in vitro* (Table 19) (Plate 18 & 19). The lower dosages used for the study were 0.005 per cent (50 ppm), 0.01 per cent (100 ppm), 0.02 per cent (200 ppm),0.03 per cent (300 ppm) and 0.04 per cent (400 ppm). All the fungicides were superior in inhibiting the mycelial growth under *in vitro* conditions.

The *in vitro* study revealed that at 0.005 per cent (50 ppm) concentration propiconazole recorded 95 per cent mycelial inhibition followed by tebuconazole and carbendazim + mancozeb with 84.17 per cent and 82.22 per cent respectively and were on par. The least inhibition was recorded by captan + hexaconazole (41.11 per cent)

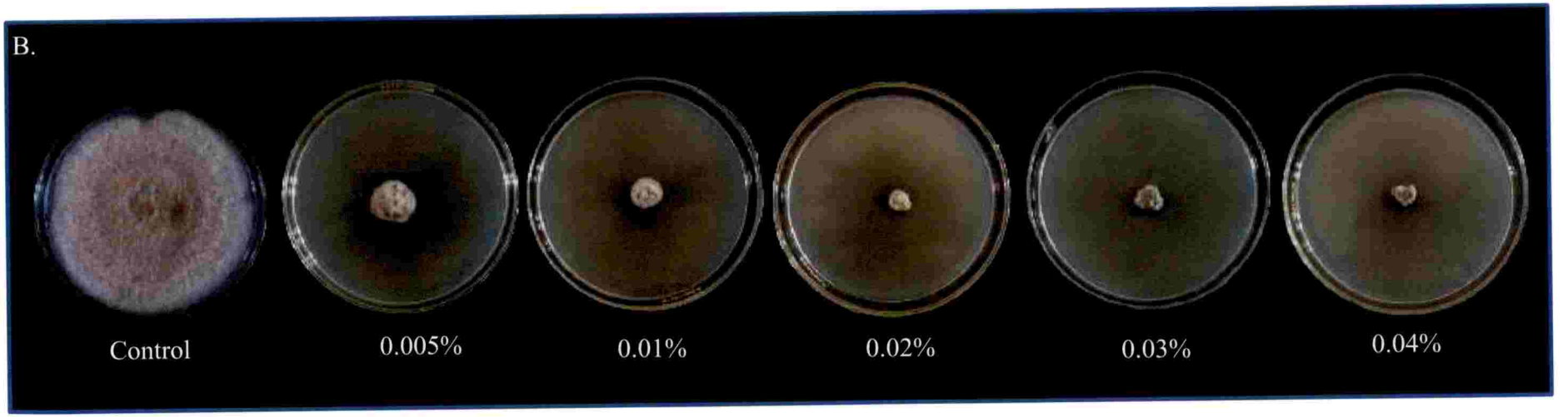
Table 19. Mycelial inhibition of *C. gloeosporioides* causing anthracnose in betel vine by lower doses of test fungicides

Treatments	Fungicides	Percentage mycelial inhibition *				
		0.005%	**0.01%**	**0.02%**	**0.03%**	**0.04%**
T 1	Propiconazole 25.0% EC	95.00^{b} (80.31)	100.00^{a} (89.05)	100.00^{a} (89.05)	100.00^{a} (89.05)	100.00^{a} (89.05)
T 2	Tebuconazole 25.9% EC	84.17^{c} (66.86)	85.28^{c} (67.47)	94.30^{b} (79.66)	94.30^{b} (79.66)	97.36^{ab} (84.54)
T3	Captan (70%) + Hexaconazole (50%) WP	41.11^{f} (39.87)	55.00^{e} (47.86)	67.64^{de} (55.36)	68.48^{d} (55.85)	83.89^{c} (66.34)
T4	Carbendazim (12 %) + Mancozeb (63%) WP	82.22^{c} (65.06)	83.75^{c} (66.27)	84.59^{c} (66.88)	85.13^{c} (67.33)	91.94^{b} (77.69)
T5	Control	0.00^{g} (0.96)	0.00^{g} (0.96)	0.00^{g} (0.96)	0.00^{g} (0.96)	0.00^{g} (0.96)
	CD (0.05)	7.757				
	SEm±	5.490				

*Mean of four replications
Values followed by similar superscripts are not significantly different at 5% level
Values in parenthesis are arc sin transformed

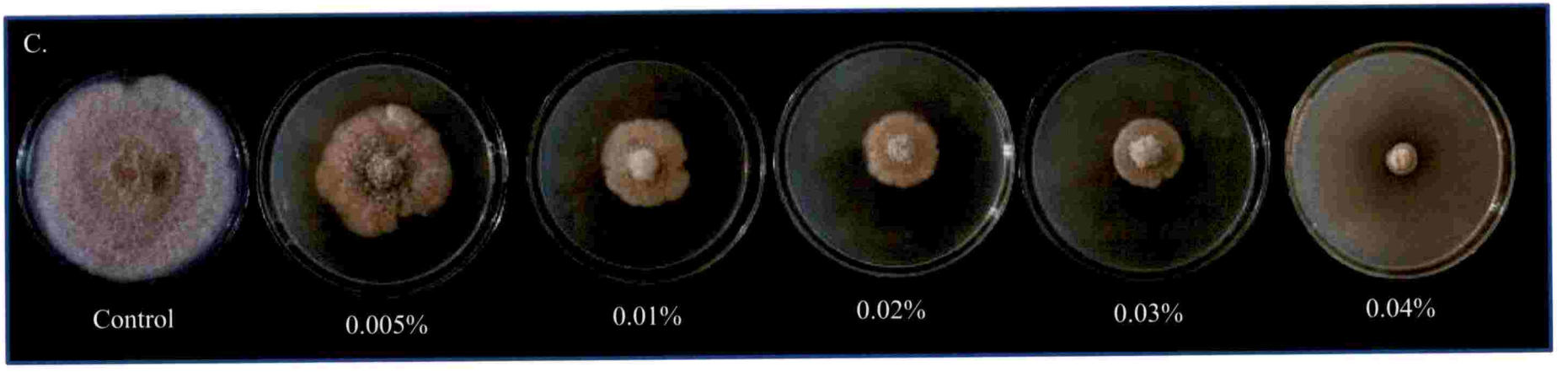


Propiconazole 25.0% EC

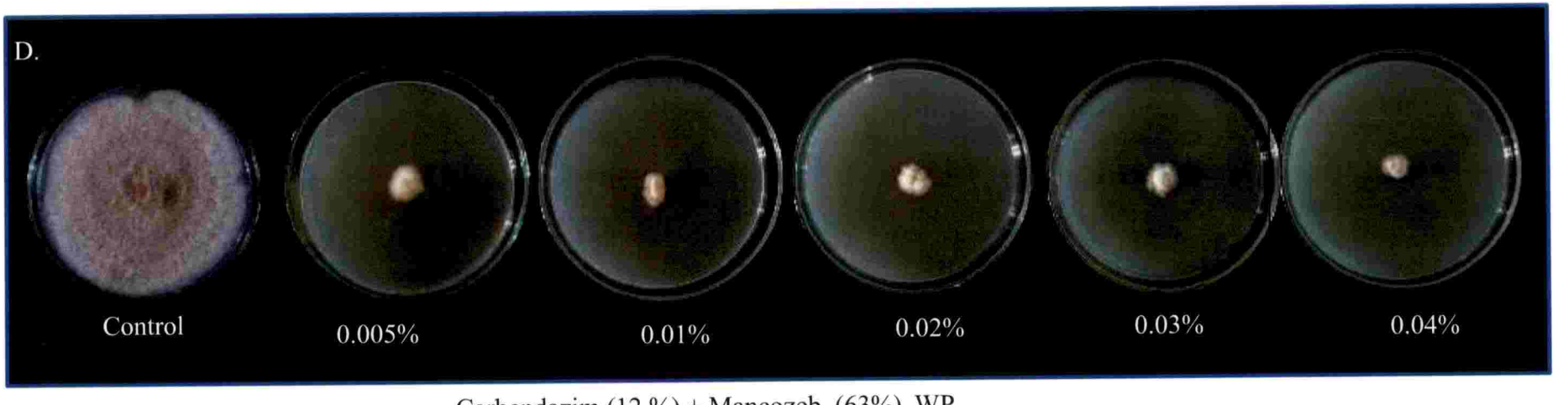


Tebuconazole 25.9% EC

Plate 18 (A-B): *In vitro* inhibition of *C. gloeosporioides* causing anthracnose of betel vine by systemic fungicides at lower dosage



Captan (70%) + Hexaconazole (5.0%) WP



Carbendazim (12 %) + Mancozeb (63%) WP

Plate 19 (C-D): Inhibition of *C. gloeosporioides* causing anthracnose of betel vine by combination fungicides at lower dosage

Propiconazole was the most effective with 100 per cent mycelial inhibition and was statistically superior to other treatments at 0.01 per cent (100 ppm) concentration. Tebuconazole and carbendazim + mancozeb at 0.01 per cent inhibited mycelial growth of the pathogen *i.e.* 85.28 per cent and 83.75 per cent respectively. The lowest inhibition was recorded for captan + hexaconazole with 55 per cent.

Propiconazole and tebuconazole at 0.02 per cent (200 ppm) were effective in inhibiting the pathogen. This was followed by carbendazim + mancozeb with 84.59 per cent inhibition of mycelial growth. The lowest inhibition was observed for captan + hexaconazole (67.64 per cent).

At 0.03 per cent (300 ppm), propiconazole recorded complete growth inhibition of the pathogen and was superior to other treatments. This was followed by tebuconazole (94.30 per cent) and carbendazim + mancozeb (83.88 per cent). The least inhibition was observed for captan + hexaconazole (68.47 per cent).

At 0.04 per cent (400 ppm), propiconazole could also completely inhibit the mycelial growth of the pathogen and was superior to other treatments. This was followed by tebuconazole (97.22 per cent) and carbendazim + mancozeb (91.94 per cent). The lowest inhibition percentage was noted for captan + hexaconazole (83.89 per cent).

4.6. POT CULTURE TRIAL TO DEVELOP INTEGRATED DISEASE MANAGEMENT PACKAGE FOR ANTHRACNOSE OF BETEL VINE

A pot culture study was laid out in Complete Randomised Block Design with 8 treatments and four replications to study the effect of organic preparation, antagonist and chemicals on the suppression of betel vine anthracnose (Plate 20). The best treatments from the above studies *viz.*, panchagavya (10 per cent), bacterial isolate (B5), propiconazole (0.1 per cent), copper hydroxide (0.2 per cent), carbendazim + mancozeb (0.2 per cent) along with chemical check (Bordeaux mixture 0.5 per cent), inoculated and un-inoculated check were

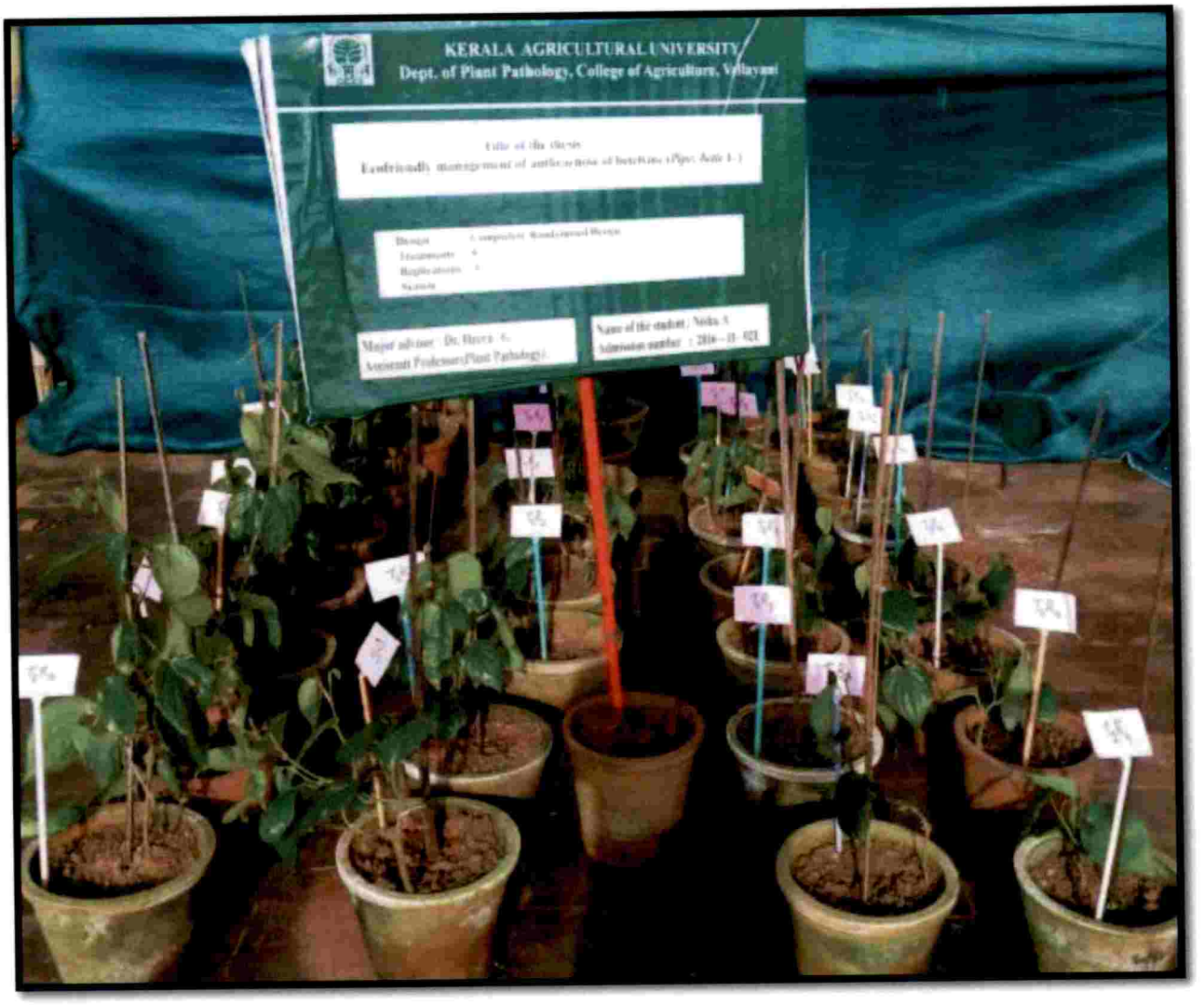


Plate 20. General view of pot culture experiment

compared. The treatments were applied as foliar spray twice at 10 days intervals after 10 days of challenge inoculation of the pathogen. The observations on disease incidence, disease index, parts affected and biometric characters were recorded at 10 days intervals.

The results of the study revealed that foliar spray of the fungicides and bacterial isolate were effective in suppressing the pathogen under *in vivo* when compared to the inoculated control. PDI was minimum for the plants treated with chemicals. Among the chemicals, the lower disease index of 19.99 per cent was recorded for the plants treated with propiconazole (0.1 per cent) at 10 days after second spray with 74.99 per cent disease suppression over inoculated control (Table 20) (Plate 21). The next best treatments were carbendazim + mancozeb and copper hydroxide at 0.2 per cent. The treatments recorded the disease index of 32.87 and 35.71 per cent and a disease suppression of 58.88 and 55.53 per cent respectively over inoculated control. The treatments were statistically on par.

The bacterial isolate B5 was equally effective in suppressing the pathogen with 46.24 per cent disease suppression over inoculated control with a disease index of 42.98. The spraying of Bordeaux mixture recorded 30.88 per cent disease suppression. The maximum disease index (65.71) and minimum disease suppression (17.81) was recorded for the plants treated with panchagavya (10 per cent).

A similar trend was observed for disease incidence. The lower disease incidence (40.46) was recorded for the plants treated with propiconazole (0.1 per cent) at 10 days after second spray. This was followed by treatments carbendazim + mancozeb, copper hydroxide, Bordeaux mixture, bacterial isolate and panchagavya were statistically on par at 10 days after second spray (Table 21).

The biometric character plant height did not show significant difference between the treatments both at 10 days after first and second spraying. There was significant difference between treatments with respect to number of leaves (Table 22). The maximum number of leaves (11) was recorded for propiconazole treated

Table 20. Effect of organic preparation, biocontrol agent and fungicides on disease severity of betel vine anthracnose under *in vivo*

Treatments		PDI (%) *			Disease suppression over control (%)
		Pre treatment	10 DAFT	10 DAST	
T1	Panchagavya (10%)	22.13 (28.05)^{b}	55.69 (48.29)^{a}	65.71(54.36)^{b}	17.81
T2	Bacterial isolate (B5) 10^{8} cfu/ml	22.85 (28.51)^{b}	48.46 (44.11)^{abc}	42.98 (40.96)^{cd}	46.24
T3	Propiconazole 25.0% EC (0.1%)	26.06 (30.70)^{a}	32.12 (34.50)^{e}	19.99 (26.45)^{e}	74.99
T4	Copper hydroxide 53.8% WP (0.2%)	21.07 (27.30)^{b}	42.74(40.82)^{cd}	35.71 (36.66)^{d}	55.53
T5	Carbendazim (12 %) + Mancozeb (63%) WP (0.2%)	23.57 (29.01)^{ab}	37.6 (37.86)^{de}	32.87(34.97)^{d}	58.88
T6	Bordeaux mixture -0.5% (Chemical check)	22.14 (28.03)^{b}	47.14 (43.34)^{bc}	55.26 (48.43)^{bc}	30.88
T7	Inoculated control	21.42 (27.56)^{b}	53.62(47.08)^{ab}	79.95(63.48)^{a}	
T8	Un inoculated control	0.00 (0.29)^{c}	29.99(33.10)^{e}	41.28 (39.95)^{d}	
CD (0.05)		2.112	4.793	7.856	
SEm±		1.447	3.284	5.382	

*Mean of four replications
Values in parenthesis are arc sin transformed values
Values followed by similar superscripts are not significantly different at 5% level
DAFT- Days after first treatment
DAST- Days after second treatment

Table 21. Effect of different treatments on disease incidence (%) of betel vine anthracnose under *in vivo* condition

Treatments		DI (%)		
		Pre treatment	10 DAFT	10 DAST
T1	Panchagavya (10%)	37.63 (37.83)^{b}	92.50 (78.46)^{a}	78.27 (65.36)^{ab}
T2	Bacterial isolate (B5) 10^{8} cfu/ml	38.33 (38.18)^{ab}	72.65 (58.50)^{c}	74.25 (59.54)^{bc}
T3	Propiconazole 25.0% EC	38.50 (38.20)^{ab}	44.58 (40.12)^{f}	40.46 (39.46)^{e}
T4	Copper hydroxide 53.8% WP (0.2%)	42.50 (40.68)^{ab}	58.09 (49.72)^{de}	57.35 (49.23)^{cde}
T5	Carbendazim (12 %) + Mancozeb (63%) WP (0.2%)	45.50 (42.41)^{a}	53.69 (47.18)^{def}	55.57 (48.20)^{de}
T6	Bordeaux mixture -0.5% (Chemical check)	43.25 (41.12)^{ab}	62.68 (52.35)^{cd}	70.75 (57.26)^{bcd}
T7	Inoculated control	42.00 (40.39)^{ab}	84.99 (67.23)^{b}	89.28 (73.17)^{a}
T8	Un inoculated control	00.00 (0.29)^{c}	47.19 (43.37)^{ef}	71.66 (57.88)^{bcd}
CD (0.05)		4.522	8.579	10.595
SEm±		3.098	5.878	7.259

*Mean of four replications
Values in parenthesis are arc sin transformed values
Values followed by similar superscripts are not significantly different at 5% level
DAFT- Days after first treatment
DAST- Days after second treatment

Table 22. Effect of different treatments on plant height and number of leaves under *in vivo* condition

Treatments		Biometric characters					
		Pre treatment	10 DAFT	10 DAST	Pre treatment	10 DAFT	10 DAST
		Plant height (cm)	Plant height (cm)	Plant height (cm)	No. of leaves	No. of leaves	No. of leaves
T1	Panchagavya (10%)	42.12	49.25	57.5	5.25	3.25	7.25^{abcd}
T2	Bacterial isolate (B5)10^8 cfu/ml	44.62	51.50	66.00	7.00	10.50	9.75^{ab}
T3	Propiconazole 25.0% EC	44.37	54.25	60.25	8.75	13.50	11.00^{a}
T4	Copper hydroxide 53.8% WP	44.37	48.12	50.50	8.75	11.75	5.00^{cd}
T5	Carbendazim (12 %) + Mancozeb (63%) WP	44.37	53.75	58.50	10.00	9.75	5.25^{bcd}
T6	Bordeaux mixture -0.5% (Chemical check)	44.62	52.87	54.25	8.50	9.25	9.00^{abc}
T7	Inoculated control	46.06	54.75	58.75	9.50	9.25	3.25^{d}
T8	Un inoculated control	49.50	58.5	61.25	6.75	8.00	6.00^{bcd}
CD (0.05)		NS	NS	NS	NS	NS	4.606

*Mean of four replications
Values in parenthesis are arc sin transformed values
Values followed by similar superscripts are not significantly different at 5% level
DAFT- Days after first treatment
DAST- Days after second treatment

Panchagavya (10%)

Bacterial isolate (B5) 10^8cfu/ml

Propiconazole(0.1%)

Copper hydroxide (0.2%)

Carbendazim + Mancozeb (0.2%)

Bordeaux mixture (0.05%)

Inoculated check

Un inoculated check

Plate 21. Effect of organic preparation, bioagent and fungicides against anthracnose of betel vine in pot culture

plants where as minimum was observed for inoculated control (3.25). The treatments carbendazim + mancozeb, copper hydroxide, panchagavya were also on par with respect to number of leaves.

Discussion

5. DISCUSSION

Betel vine is an important cash crop cultivated for its green heart shaped leaves. Leaves are reported to be cheap source of dietary calcium (Shahzad and Zareen, 1999). The betel leaves are considered as "green ATM" among rural growers and women; and provide immediate continuous earnings. But it's cultivation is threatened by many fungal and bacterial diseases. Among the fungal threats, the leaf spot incited by *Colletotrichum* sp. cause severe damage to the cultivation and the loss was estimated as 10 - 60 per cent (Singh and Joshi, 1971 ; Maiti and Sen, 1982). The disease ranks next to the foot rot disease of betel vine. Anthracnose in severe form, affects the vines and leading to the complete mortality of vines. Old plantations are highly prone to the attack of *Colletotrichum* sp. (Shahzad, 2000). Traditional disease management practices are usually followed by the farmers. It's usage as a masticatory restrict the complete reliance on fungicides for disease management. In this context, the present study investigates to develop an eco-friendly management approach involving indigenous organic preparations, biocontrol agents and fungicides for the management of disease.

A survey was conducted during December 2016 - April 2017 to study the incidence of anthracnose from three Southern districts of Kerala *viz.*, Thiruvananthapuram, Kollam and Alappuzha. In surveyed locations, the disease incidence ranged from 20 to 80 per cent and severity ranged from 5.70 to 20.00. Similar observations were recorded by Haralpatil (2006) in a survey in different parts of Maharashtra during December, 2005 and observed a PDI of 11.0 to 20.0 for anthracnose of betel vine

The highest disease severity and incidence was noticed in Cherthala region of Alappuzha where a minimum temperature of 27.0^0 C and maximum temperature of 33.0^0 C and 84 per cent relative humidity were prevailing during the month of April 2017. High incidence of leaf spot was noticed during March - May when temperature was 26.7^0 C and relative humidity of 88.3 per cent at Barisal region of Bangladesh (Huq, 2011). The minimum disease incidence and

disease index was noted in Kareepra where proper management strategy using Carbendazim + Mancozeb at 0.2% has been adopted by the farmer.

Symptomatology of anthracnose was studied during the survey. The stages of symptom development varied with locations. Symptoms initially appeared as circular necrotic spots surrounded by a yellow halo, mostly seen towards the tip of the leaf. Later, these spots coalesced causing extensive leaf blight especially under high humid conditions. In case of severe infection, defoliation was noticed. Under conducive climatic conditions, the pathogen infects leaf petiole and stem leading in gridiling and drying up of entire vines. Studies by Maiti and Sen, (1979) and Chandra and Sagar, (2004), revealed that symptoms on leaves appeared as irregular spots with black centre having yellow halo. Chattopadhyay and Maiti (1990) observed stem lesions on infected betel vine leading to girdling and death of entire vines.

The pathogen associated with anthracnose of betel vine was isolated from infected samples from three locations by tissue isolation method described by Haralpatil (2006). The culture was purified using single spore isolation technique as described by (Dhingra and Sinclair, 1985).

The pathogenicity of isolated *Colletotrichum* sp. was proved by detached leaf inoculation method. The pathogen produced necrotic spots with a yellow halo on artificial inoculation to leaves. Haralpatil (2006) had similar result in proving the pathogenicity of *C. gloeosporioides* in betel vine. He reported the pathogen produced necrotic black spots which enlarges leading to blighting of leaves.

The days taken for symptom expression after artificial inoculation to betel leaves varied from 2- 4 DAI. Lesion size varied from 1.97- 3.27 mm. Haralpatil (2006) reported that *C. gloeosporioides* from betel vine took 5 days for symptom expression. *C. gloeosporioides* of black pepper took 5-6 days for symptom expression on artificial inoculation to healthy pepper leaves (Chandrakant, 2005). Similarily, Sreeja (2014) reported 4-8 days had taken for symptom expression in

cowpea leaves. Early expression of symptom was due to virulence of the pathogen and also susceptibility of the crop compared to its related species like *Piper nigrum* and *P. longum*.

Variability of *Colletotrichum* sp. was assessed by culture and morphological studies. In the present investigation five isolates obtained varied in their colony and morphological characters. The colony colour of isolated *Colletotrichum* spp. were initially white, light grey to dark grey on the front view and white to pinkish orange on the rear view. The isolates showed pinkish colouration due to spore production and showed sparse to fluffy mycelium with a regular margin. The results of the above study was in confirmation with the findings of Sankar (2002), where *C. gloeosporioides* from black pepper had colony colour ranging from white, light grey to dark grey. Naik and Hiremath (1985) observed smooth greyish black colonies of *C. gloeosporioides* from betel vine. The average growth rate of isolates varied from 1.04 - 1.28 cm day^{-1} and took 7 - 9 days for completion of full growth. Similar results were obtained by Sreeja (2014) where an average growth rate of *C. gloeosporioides* from cowpea ranged from 0.80 to 1.38 cm day^{-1} and took 6 - 10 days for completion of growth. Chacko (2015) reported growth rate of *C.capsici* to be 1.21 - 1.38 cm $day^{-1}$and took 6 - 7 days were needed for growth completion in petridish.

The mycelium of five isolates were hyaline, septate, branched and average mycelial width ranged from 2.65 µm - 3.45 µm and septal distance varied from 9.80 - 25.56 µm.. Sreeja (2014) reported that mycelium was hyaline, septate and branched for *C. gloeosporioides* of cowpea which was similar to result of mycelial studies of *C. gloeosporioides* from betel vine. Mammooty (2003) reported 1.25 - 4 µm mycelial width for the isolate of *C. gloeosporicides* from black pepper whereas Aswani *et al.* (2016) reported it as 2.22 - 3.8 µm of *C. gloeosporioides* from snake gourd. Variation in the mycelial size of *C. gloeosporioides* mycelium may be attributed to host characteristics.

Conidia, the asexual spores were produced in acervuli, were either cylindrical, oblong or dumbbell with an oil globule at centre which was in consonance with the findings of Naik and Hiremath (1986). The coinidial size ranged from 9.6 - 12.2 µm in length and 3.8 - 4.7 µm in breadth. The appressorial size also varied among different isolates from 8.37 - 10.83 µm and 5.02 - 6.28 µm in length and breadth respectively. Sreeja (2014) and Aswani *et al.* (2014) reported 8.6 - 11.3 µm x 3.5 - 4.3 µm and 11 – 15 µm x 4 - 5 µm sized conidia for *C. gloeosporioides* of cowpea and snake gourd respectively.

Pathogenic variability studies were done for all the five isolates of *C. gloeosporioides* on detached leaves. The virulence rating among the different isolates was based on the lesion size, rate of lesion development and time for symptom expression. The isolate C2 produced maximum lesion size of 14.75 mm and initiated symptom expression within 2 DAI (Figure 2). The other isolates C5, C3, C4, C1 produced a lesion size of 5.07 mm, 3.52 mm, 3.00 mm and 2.50 mm and initate symptom within 2 - 4 days. The isolate C2 was found to be most virulent. Pathogenic variability by leaf inoculation method were also conducted by Sankar (2002) and was in concurrence with the result. The different isolates of *C. gloeosporioides* from black pepper produced lesion size between 0.9 to 11.6 mm on 8 DAI. The isolate which produced larger lesion size (C6) was identified as most virulent as in confirmation with the present study. Similarly Joshi (2008) carried out the pathogenic variability studies of *C. gloeosporioides* of mango on different varieties by assessing the virulence index on the basis of lesion size produced following the artificial inoculation of the pathogen.

The ITS - rDNA region was commonly used target sequence and marker for the identification of fungi (Bruns, 2001). The molecular characterization of the virulent isolate was done through DNA barcoding using universal primers. The ITS - rDNA region of the virulent isolate of *C. gloeosporioides* was sequenced for the identification and molecular characterization. The amplified product was approximately 500 bp long using ITS - 1F (forward) and ITS - 4R (reverse) primers. Blast analysis in Gene Bank of NCBI data base using BLAST

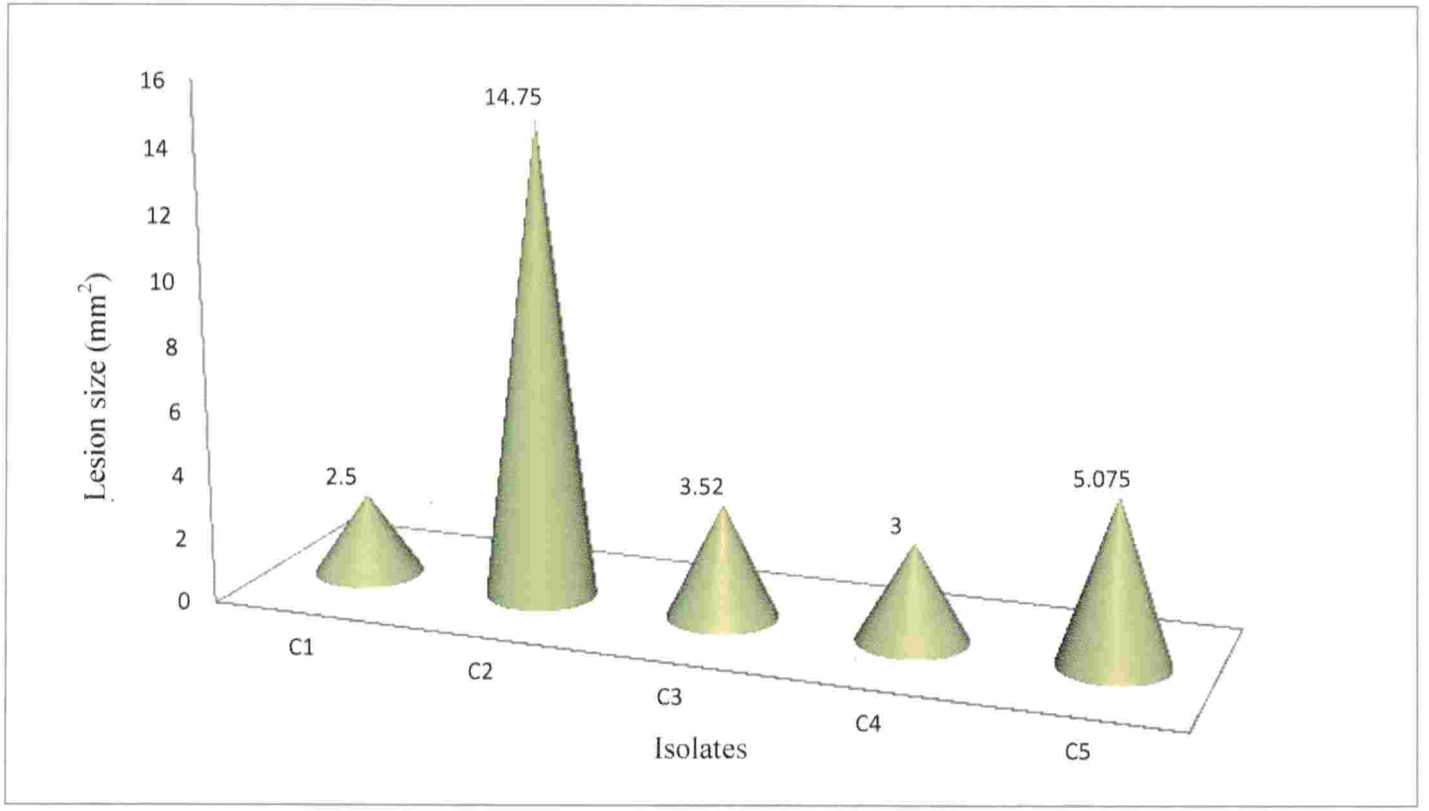


Figure 2. Lesion development of *Colletotrichum* isolates causing anthracnose in betel vine a at 5 DAI

program revealed the C2 isolate showed 99 per cent nucleotide identity with other reported *C. gloeosporioides*. The molecular characterization of *Colletotrichum* spp. using the amplification of ITS - rDNA region followed by BLAST analysis were also reported by Abang *et al.* (2002), Photita *et al.* (2005) and Raj *et al.* (2013).

The use of indigenous organic preparations against plant pathogens is an emerging eco-friendly method for disease management (Parvathy and Girija, 2016). The effectiveness of organic preparations *viz.,* panchagavya, jeevamruth, and vermiwash against the foliar pathogens had been reported by Chadha *et al.* (2012); Parvathy and Girija, (2016) and cow's urine by Rajput, (2011). In the present study the efficacy of indigenous organic preparations *viz.,* panchagavya, jeevamruth, fermented cow's urine and vermiwash at 5 per cent and 10 per cent were evaluated in autoclaved and non-autoclaved extracts against *C. gloeosporioides* of betel vine. The non-autoclaved organic preparations recorded more percentage inhibition when compared to autoclaved ones. The inhibitory action may be attributed to presence of antagonistic microorganisms in non-autoclaved and metabolite in autoclaved ones.

The higher concentration of the organic preparations recorded more inhibition than its lower concentration. Fermented cow's urine at 5 and 10 per cent and panchagavya 10 per cent when used as non-autoclaved extract completely suppressed mycelial growth of *C, gloeosporioides* (Figure 3A). This result is in accordance with the findings of Chacko (2015). The study revealed complete growth inhibition of *C. capsici* in chilli with panchagavya at 2.5 per cent, 5 per cent and 10 per cent concentrations. The anti - fungal effect of cow's urine was also reported against on *C. capsici* of chilli by Chadha *et al.* (2012) and Kambar *et al.* (2013). Similarly in the present study, jeevamruth (5 per cent and 10 per cent) recorded 26.94 per cent and 30.83 per cent of mycelial inhibition of the pathogen respectively. Parvathy and Girija (2016) reported jeevamruth at 5 per cent and 10 per cent as an effective organic preparation against anthracnose pathogen of black pepper.

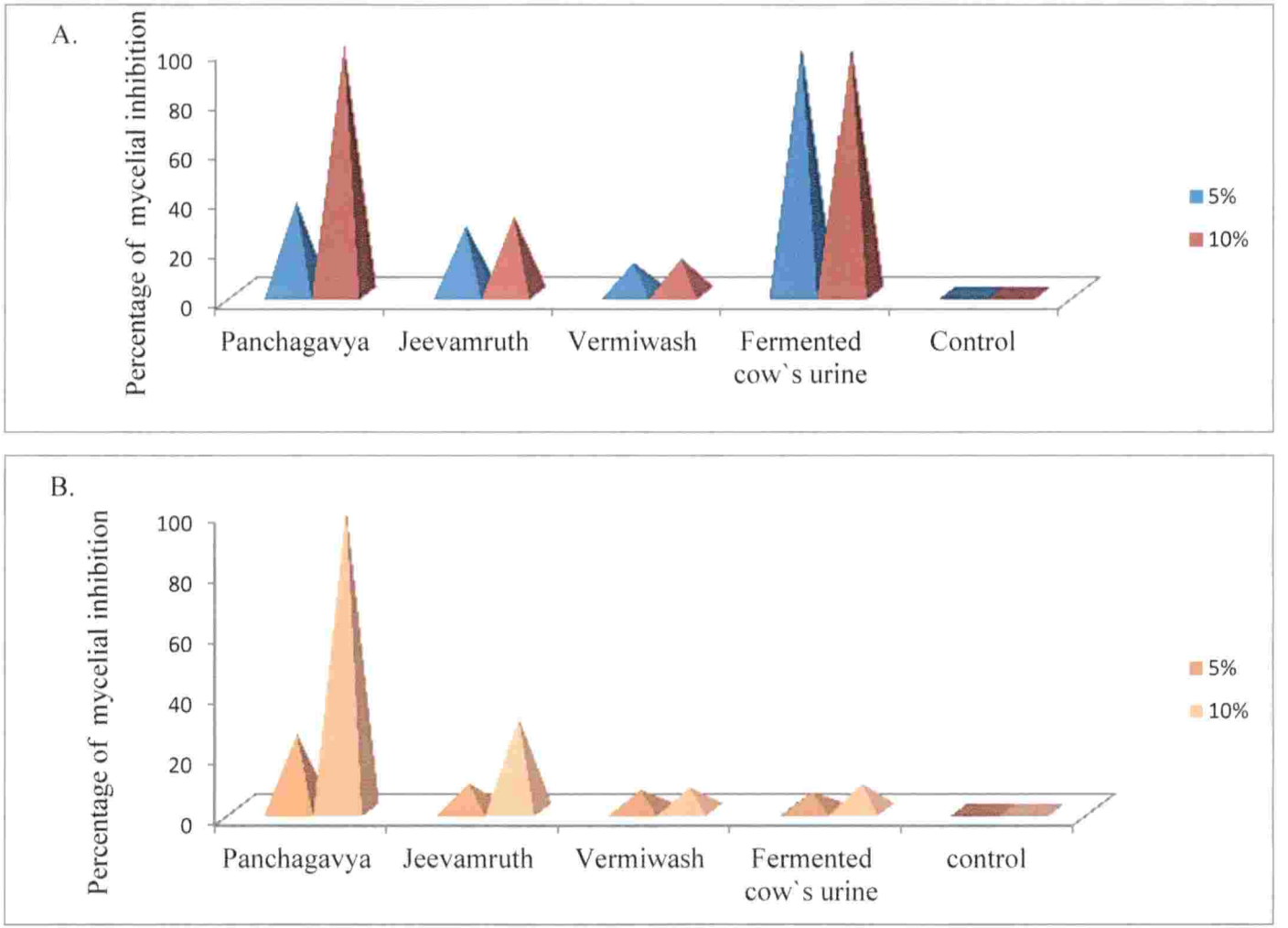


Figure 3. Effect of (A) non- autoclaved and (B) autoclaved organic preparations on mycelial growth of *C. gloeosporioides* causing anthracnose in betel vine

Among the autoclaved organic preparations, panchagavya 10 per cent recorded complete growth inhibition of the pathogen (Figure 3B). George (2015) evaluated autoclaved panchagavya, jeevamruth, vermiwash against *Choaenophora* pod rot of cowpea incited by *Choaenophora cucurbitarum* and found that panchagavya was effective in suppressing the pathogen which is attributed to the thermostable metabolites produced by microorganisms.

Native microflora from the phyllosphere and rhizosphere of healthy betel vine were evaluated for the antagonistic potential against *C. gloeosporioides* incitant of anthracnose of betelvine. The fungal genera *Trichoderma* and *Aspergillus* were predominant in the rhizosphere of betel vine plants. The phyllosphere harboured only few fungal colonies while rhizosphere supported more number of bacterial colonies.

In vitro antagonism of bioagents by dual culture technique revealed that percentage mycelial inhibition of pathogen was 78.89 with *Trichoderma* 1 and 74.72 with *Trichoderma* 2 (Figure 4). Patil *et al.* (2009a) reported *T. viride, T. harzianum* as good biocontrol agents with 66.29 per cent and 58.06 per cent inhibition of *C. gloeosporioides* respectively in betel vine. Similar observations were made by Souza *et al.* (2001) and Haralpatil (2006) against betel vine pathogens attributed to the fast growing nature of *Trichoderma* sp. as one of the factor inhibiting the growth of the pathogen.

Aspergillus sp. was also effective under *in vitro* conditions against *C. gloeosporioides*. Sankar (2002) reported the antagonistic potential of *A. niger* against anthracnose pathogen in black pepper. *Aspergillus* sp. (A1 and A2) recorded 54.08 per cent and 53.06 per cent inhibition of pathogen. The antagonism was due to the disintegration of host hyphae by granulation and vacuolation induced by *A. niger*. Ahmed *et al.* (2014) also reported antagonistic potential of *A. niger* against anthracnose of betel vine.

Deka *et al.* (2008) reported that *Bacillus subtilis* was highly effective in controlling the disease complex of betel vine as it inhibited 79.5 per cent, 68.1 per

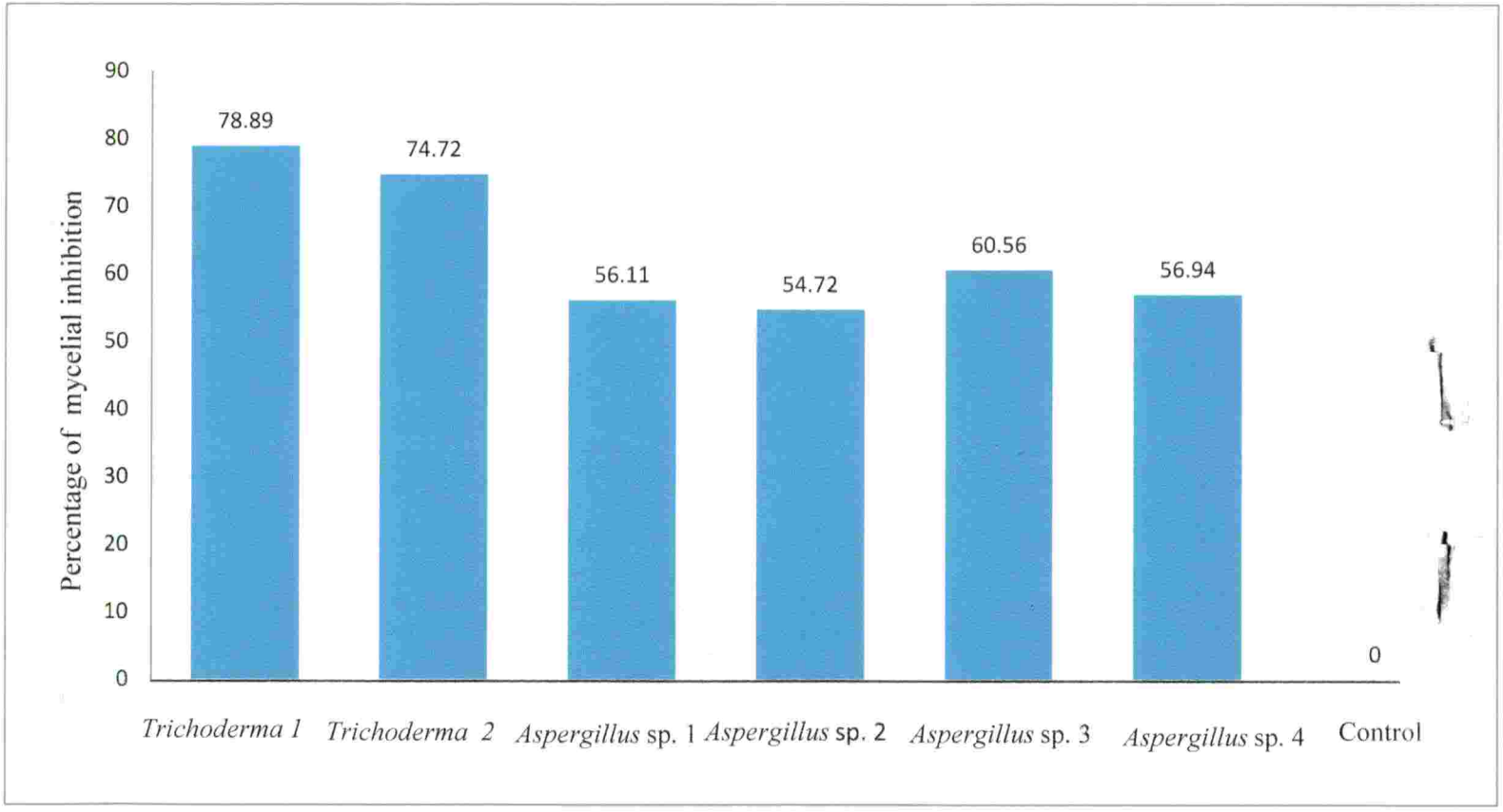


Figure 4. Mycelial inhibition of *C. gloeosporioides* causing anthracnose in betel vine by antagonistic fungi

cent and 76.4 per cent inhibition on growth of *C. capsici, Xanthomonas axonopodis* pv. *betlicola* and their complex respectively under *in vitro* conditions. The study established the effectiveness of bacterial antagonist against the leaf spot disease of betel vine which was in consonance to the present study.

Thirteen antagonistic bacterial isolates were obtained from rhizosphere and phyllosphere of betel vine were evaluated for their antagonistic potential against *C. gloeosporioides* under *in vitro* conditions. The bacterial antagonists recorded 19.72 - 45.00 per cent mycelial inhibition of the pathogen (Figure 5). The bacterial isolate B5 (phyllosphere isolate) was found superior among the different isolates screened and recorded 45 per cent mycelial inhibition under *in vitro* conditions. This isolate was on par with isolate B11 which showed 43.61 per cent mycelial inhibition. This treatment was followed by B14 with 40.27 per cent. The B5 isolate was identified as *Bacillus* sp. Similar findings were also reported by (Ann, 2012) where, 40.4 - 54.2 per cent mycelial growth inhibition of pathogens *C. gloeosporioides, C. capsici, F. solani and Streptobasidium* sp were recorded by *Bacillus* sp. isolated from the rhizosphere of healthy black pepper. The inhibitory mechanism involved was lysing of fungal cell wall by cellulase and protease enzymes. Hyphal malformations *viz.,* hyphal thickening, vacuolar formation and swellings were also reported.

Fungicides are the most reliable, easiest and assured method of plant disease management where proper recovery from disease is ensured. In present study seven commercially available fungicides *viz.,* propiconazole, tebuconazole, azoxystrobin, captan + hexaconazole and carbendazim + mancozeb (0.05 per cent, 0.1 per cent, 0.2 per cent), copper hydroxide and mancozeb (0.1 per cent, 0.2 per cent, 0.4 per cent) were evaluated for *in vitro* pathogen suppression.

In vitro evaluation of seven fungicides revealed that triazole fungicides *viz.,* propiconazole, tebuconazole, and combination fungicide captan + hexaconazole and carbendazim + mancozeb (0.05 per cent, 0.1 per cent, 0.2 per cent) recorded cent percent inhibition of the pathogen (Figure 6). Chandrakant

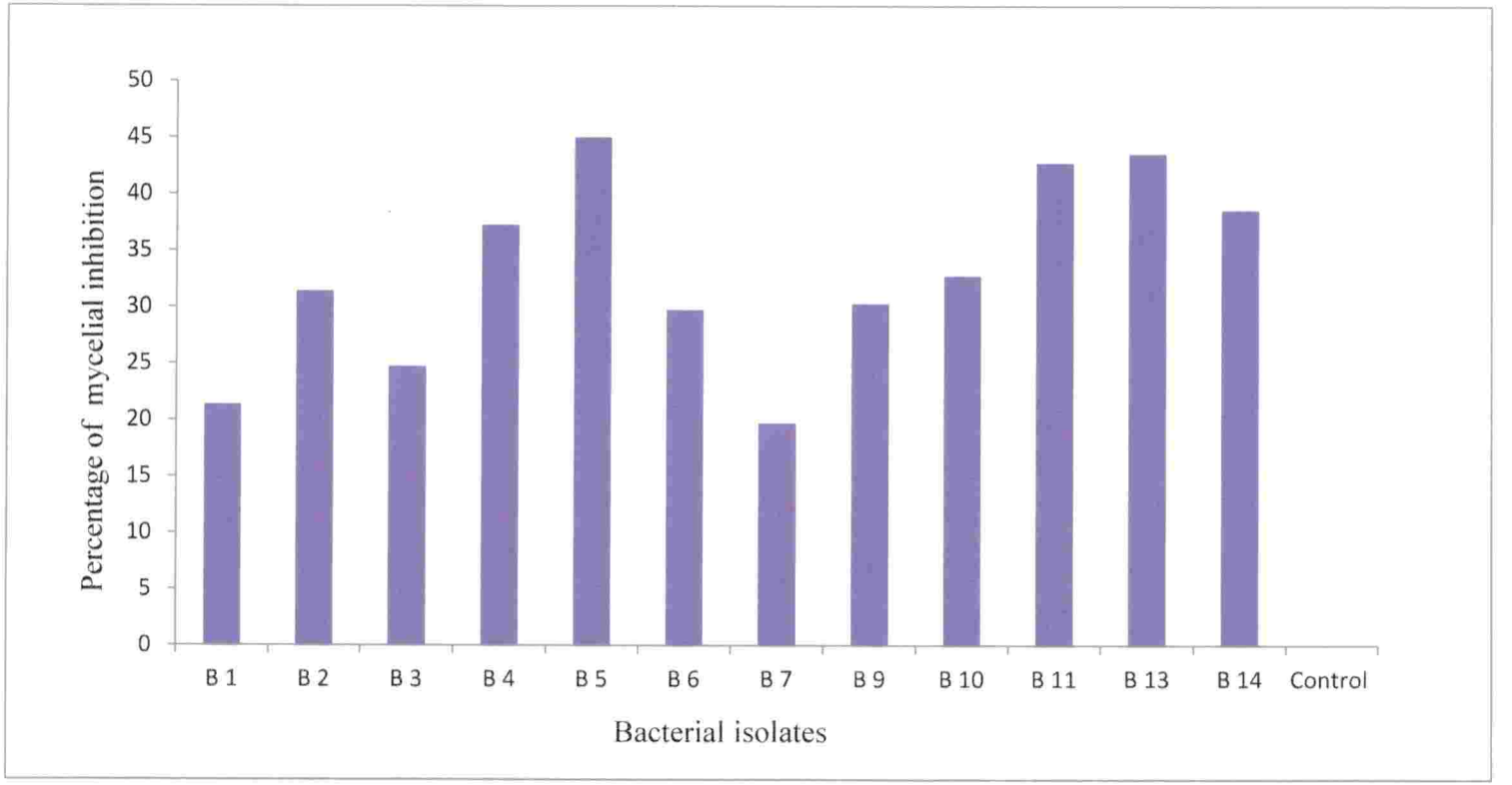


Figure 5. Mycelial inhibition of *C. gloeosporioides* causing anthracnose in betel vine by antagonistic bacteria

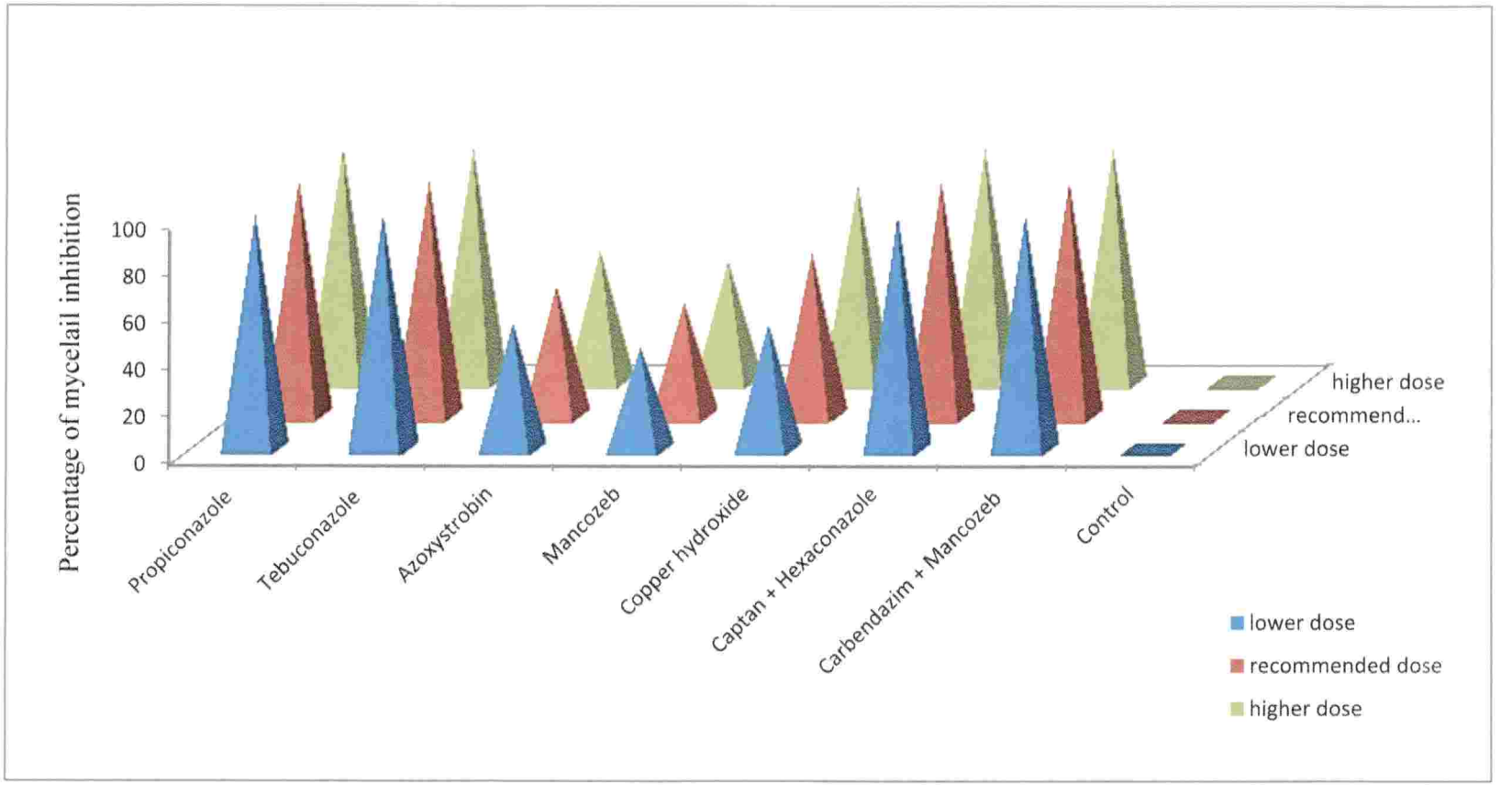


Figure 6. Effect of different fungicides on mycelial growth of *C.gloeosporioides* causing anthracnose in betel vine

(2005) also confirmed the efficacy of propiconazole (0.05 per cent) in inhibiting mycelial growth of black pepper of leaf spot pathogen.

Studies on the lower concentrations of fungicides, revealed that propiconazole was most effective against the pathogen. A complete growth inhibition was recorded from 100 ppm onwards. Ahmed *et al.* (2014), who recorded 100 per cent growth inhibition even at 50ppm of propiconazole against *C. gloeosporioides* of betel vine.

Based on the results of *in vitro* studies, effective treatments *viz,* panchagavya (10 per cent), bacterial isolate (B5), propiconazole (0.1 per cent), copper hydroxide (0.2 per cent), carbendazim + mancozeb (0.2 per cent) along with chemical check (Bordeaux mixture - 0.5 per cent), inoculated and un-inoculated check were evaluated for management of disease under *in vivo* conditions. In pot culture studies, all the treatments were applied twice as foliar spray at 10 days interval.

The results of pot culture trial revealed that foliar application of the fungicides and bacterial isolate were effective in suppressing the pathogen under *in vivo*. The lower disease index of 19.99 per cent was recorded for the treatment propiconazole (0.1 per cent) at 10 days after second spray and disease suppression of 74.99 per cent was recorded over inoculated control (Figure 7). This was followed by foliar application of carbendazim + mancozeb and copper hydroxide at 0.2per cent. The above treatments recorded 32.87 and 35.71 per cent disease index respectively and disease suppression of 58.88 per cent and 55.53 per cent respectively over inoculated control and were statistically on par. Gopinath *et al.* (2006) reported that spraying of propiconazole (0.1 per cent) recorded 16.75 per cent PDI with 70 per cent disease suppression of anthracnose of chilli under greenhouse condition. Three foliar sprays of 0.1 per cent propiconazole recorded 72.79 per cent disease suppression of mango anthracnose (Bhagawat *et al.*, 2016). The monthly spraying of 0.2 per cent carbendazim + mancozeb on thippali provided 33.8 per cent disease suppression over control (Pattil *et al.*, 2009b).

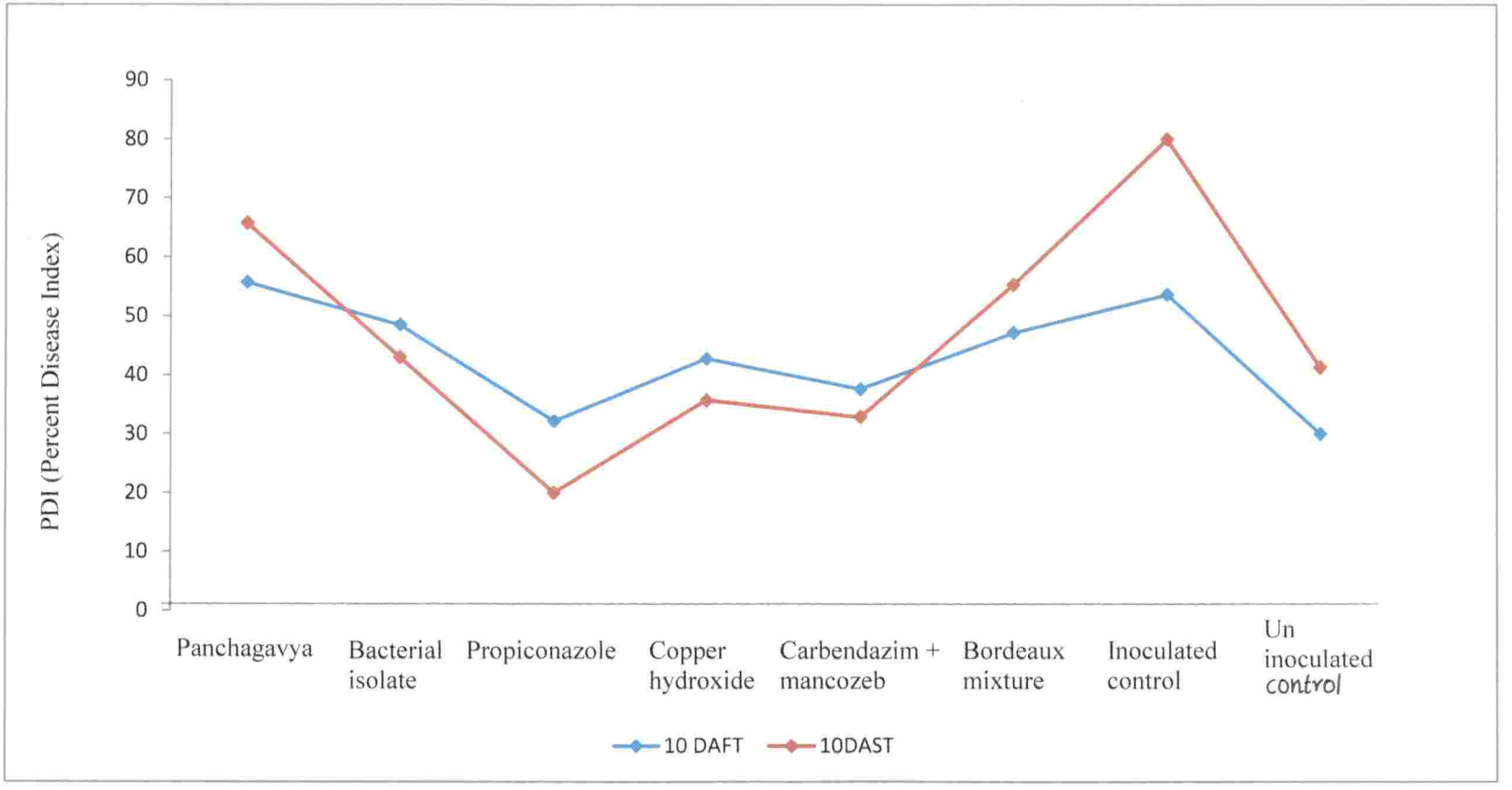


Figure 7. Disease severity of anthracnose of betel vine by different treatments under pot culture study

Similar study by Kumar *et al.* (2018) recorded 73.0 per cent reduction over disease severity by spraying of 0.2 per cent carbendazim + mancozeb at 15 days interval on betel vine.

The present study also revealed that bacterial isolate B5 was equally effective in suppressing the pathogen with 46.24 per cent. The efficacy of *B. subtilis* in managing the leaf spot complex were reported by Deka *et al.* (2008). Chacko (2015) recorded a PDI of 38.59 per cent at three weeks after spraying of *Bacillus* sp. (10^8 cfu ml^{-1}) on the incidence of fruit rot and provided 59.59 per cent disease suppression. The foliar spraying of *Bacillus* sp. reduce the anthracnose of grapes by 25.50 per cent (Sawant, 2016).

A similar trend was observed for disease incidence. The lower disease incidence (40.46) was recorded for the plants treated with propiconazole (0.1per cent) at 10 days after second spray. This was followed by treatments carbendazim + mancozeb, copper hydroxide, Bordeaux mixture, bacterial isolate and panchagavya were statistically on par at 10 days after second spray. The disease incidence were statistically on par for the both the treatments Bordeaux mixture and un inoculated control. Chacko (2015) reported a lower disease incidence of 16.31 per cent of fruit rot of chilli with the plants treated with 0.1per cent propiconazole. was in accordance to the present study. Similarily, Kendre *et al.* (2017) reported a disease index of 9.75 per cent with the thippali plants treated with carbendazim + mancozeb (0.2 per cent) and was recorded the second best treatment in the experiment.

Among the biometric character plant height didn't show significant difference between the different treatments both at 10 days after first and second spraying. There was significant difference between treatments with respect to number of leaves. The highest leaf number of 11.00 was recorded for propiconazole treated plants where as lowest number was observed for inoculated control (3.25). The application of bacterial isolate (B5) was on par with propiconazole treatment. The treatments carbendazim, copper hydroxide,

panchagavya were also on par with respect to number of leaves. Narasimhan and Shivakumar (2014) reported an increase in biometric characters *viz.*, plant height, root and shoot length, fresh and dry weight and yield parameters on the anthracnose affected chilli when treated with *B. subtilis*. Similarily Chacko (2015) recorded a leaf number of 111 and 125 for the anthracnose affected chilli plants treated with propiconazole and panchagavya respectively.

Summary

6. SUMMARY

Betel vine is an important cash crop widely known as "Green Gold". The cultivation of the crop is challenged by many fungal diseases among which anthracnose is a serious disease affecting both the leaves and vines. The disease severely affects young and old plantations. The present study entitled "Ecofriendly management of anthracnose of betel vine (*Piper betle* L.)" was undertaken at College of Agriculture, Vellayani during 2016-2018 with an objective to develop an eco-friendly management strategy involving organic preparations, biocontrol agents and fungicides for the management of the disease.

A survey was conducted in five different locations of three Southern districts of Kerala. The higher disease index (20 per cent) and incidence (80 per cent) was recorded from the Cherthala region of Alappuzha. The disease symptom initiated with small necrotic spots with a yellow halo from the leaf tips coalesced together causing leaf blight and defoliation. Lesions on vines lead to girdling of the stem ultimately resulting in drying and death of the entire vines.

Five isolates of *Colletorichum* sp. were isolated from affected betel leaves collected during survey and were named as C1, C2, C3, C4 and C5. The pathogenicity of isolates were proved by challenge inoculation on leaves and re-isolating the pathogen.

The culture and morphological characters of the different isolates revealed that pathogen isolated was *Colletotrichum* sp. The colony growth of the isolates varied fluffy to sparse with regular margins and took 7 to 9 days to cover petridish. The mycelium of the pathogen was septate, hyaline. The width of mycelium varied between 2.65 - 3.45 µm and septal distance of 8.50 - 25.56 µm. The average conidial and appressorial size varied from 9.6 -12.2 µm x 3.8 - 4.7 µm and 8.37-10.83 µm x 5.02 - 6.28 µm respectively. The pathogenic variability among the isolates were studied by virulence rating on inoculated leaves. The Vellayani isolate (C2) was observed to be the most virulent with minimum period of 2 days to initiate infection on inoculated leaves and produced lesion of 14.75

mm and rate of lesion development as 2.95 mm day^{-1}. The virulent isolate was further confirmed as *C. gloeosporioides* by molecular characterization at Rajiv Gandhi Centre for Biotechnology, Poojappura, Thiruvanthapuram. The molecular characterization of the pathogen was done through ITS sequencing using ITS 1F (forward) and ITS 4R (reverse) primers and revealed that the pathogen showed 99 per cent nucleotide similarity with other reported isolates of *C. gloeosporioides*.

Indigenous organic preparations like Panchagavya, jeevamruth, vermiwash and fermented cow's urine were evaluated for their efficacy in controlling the pathogen under *in vitro* condition. The organic preparations as autoclaved and non-autoclaved extracts were screened against the pathogen at 5 and 10 per cent concentration. The organic preparation panchagavya at 10 per cent completely inhibited the pathogen both in autoclaved and non-autoclaved extracts. Fermented cow's urine at 5 per cent under non-autoclaved condition recorded complete growth inhibition of the pathogen. But under autoclaved condition, the fermented cow's urine failed to inhibit the pathogen growth at both concentrations. The nature and growth of the pathogen in autoclaved and non-autoclaved organic preparations were either fluffy, sparse with regular and irregular pattern.

The bacterial population showed an increasing trend in filtered and non-filtered organic preparations of panchagavya, vermiwash and fermented cow's urine, while the population was decreasing in jeevamruth as the days increased. The fungal colonies were prominent only in vermiwash than other indigenous preparations.

The fungal and bacterial bioagents from the phyllosphere and rhizosphere of healthy betel vine were screened against the pathogen to study their efficacy in suppressing the pathogen under *in vitro* conditions. Six fungal and thirteen bacterial isolates were screened against the pathogen. The fungal bioagents belonging to the genera of *Trichoderma* and *Aspergillus* were predominant in the rhizosphere of betel vine plants. The two *Trichoderma* sp. *viz, Trichoderma* 1 and

Trichoderma 2 were superior in inhibiting the mycelial growth of the pathogen with 78.89 per cent and 74.72 per cent of growth inhibition. The *Aspergillus* sp 3 from the rhizosphere was also equally effective in inhibiting the growth of the pathogen (60.56 per cent).

Among the bacterial bioagents screened, the phyllosphere bacterial isolate B5 was more effective in inhibiting the pathogen (45 per cent). The B5 was on par with the rhizosphere bacterial isolate B11 with a percent inhibition of 43.61. All the bacterial isolates were effective in inhibiting the mycelial growth. The least inhibition of 19.72 per cent was observed for rhizosphere bacterial isolate B7.

Seven commercially available fungicides were evaluated for their efficacy in *in vitro* pathogen suppression. *In vitro* assay of the fungicides revealed that propiconazole, tebuconazole, captan + hexaconazole and carbendazim + mancozeb at 0.05 per cent (lower dose), 0.1 per cent (recommended field dose) and 0.2 per cent (higher dose) completely inhibited mycelial growth of the pathogen (100 per cent). The above treatments were on par but superior to other fungicidal treatments. This was followed by copper hydroxide at 0.4 per cent recorded 84.22 per cent of growth suppression of the pathogen under *in vitro* conditions.

The lower doses of the fungicides like propiconazole, tebuconazole, captan + hexaconazole and carbendazim + mancozeb were evaluated for the *in vitro* suppression of the pathogen as the treatments at 0.05 per cent recorded complete growth inhibition. The lower doses evaluated were 0.005 per cent (50ppm), 0.01 per cent (100 ppm), 0.02 per cent (200 ppm), 0.03 per cent (300 ppm) and 0.04 per cent (400 ppm). The assay revealed that propiconazole was superior to other treatments with complete mycelial inhibition of the pathogen at 0.01 per cent.

A pot culture experiment was conducted to study the effectiveness of indigenous organic preparation, bicontrol agent and fungicides in developing a

management strategy against anthracnose of betel vine. The study revealed that two sprays of propiconazole (0.1 per cent) at 10 days interval was effective in reducing the disease severity to 19.99 and recorded 74.99 per cent disease suppression over control. This was followed by foliar spray of carbendazim + mancozeb (0.2 per cent) and copper hydroxide (0.2 per cent) at 10 days interval recorded the disease suppression of 58.88 per cent. The phyllosphere bacterial isolate (B5) at 10^8 cfu ml^{-1} recorded the next best PDI (42.98) with disease suppression of 46.24 per cent. The fungicidal treatments were superior to the traditional management using Bordeaux mixture (0.05 per cent) giving a disease suppression of 30.88 per cent. The foliar spray of panchagavya at 10 per cent was less effective in managing the disease which recorded minimum disease suppression of 17.81.

The present study reveals that all the fungicides and bacterial isolate from phyllosphere were effective in managing the disease over the traditional management using Bordeaux mixture at 0.05 per cent as per POP of KAU, 2016. Foliar spray of 0.1 per cent propiconazole at 10 days interval can be used for managing the disease.

Betel vine being a masticatory crop, the use of fungicides in disease management will create health hazards. Since the crop is harvested at weekly intervals, the use of chemicals cannot be advocated also. In the present study, spraying of bacterial antagonist reduced the incidence of anthracnose to the level which is comparable with that of the chemicals. For proper disease management approach, the bacterial antagonist may be suggested after conducting further studies on the spray schedule, dosage, compatibility with fungicides and its biosafety.

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Appendices

APPENDIX – 1

COMPOSITION OF MEDIA USED

1. Potato Dextrose Agar

Peeled and sliced potatoes	- 200 g
Dextrose	- 20 g
Agar- agar	- 20 g
Distilled water	- 1000 ml

Potatoes were boiled in 500 ml of distilled water and the extract was collected by filtering through a muslin cloth. Agar- agar was dissolved separately in 500 ml of distilled water. The potato extract was mixed in the molten agar and 20 g of dextrose was dissolved in to the mixture. The volume was made upto 1000 ml with distilled water and medium was sterilised at 15 psi and 121^0 C for 15 min.

2. Martin's Rose Bengal agar

Dextrose	- 10 g
Peptone	- 5 g
KH2PO4	- 1 g
MgSO4. 7H2O	- 0.5 g
Rose Bengal	- 33 mg/l
Agar	- 20 g
Distilled water	- 1000 ml

APPENDIX- II

COMPOSITION OF STAIN USED

1. **Lactophenol – Cotton blue**

   Anhydrous lactophenol - 67.0 ml

   Distilled water - 20.0 ml

   Cotton blue - 0.1 g

   Anhydrous lactophenol prepared by dissolving 20 g phenol in 16 ml lactic acid in 3 ml glycerol

2. **Crystal violet**

   One volume saturated alcohol solution of crystal violet in four volumes one per cent aqueous ammonium oxalate

3. **Gram's iodine**

   Iodine crystals - 1.0 g

   Potassium odide - 2.0 g

   Distilled water - 300 ml

4. **Safranin**

   Ten ml saturated solution of safranin in 100 ml distilled water

ECOFRIENDLY MANAGEMENT OF ANTHRACNOSE OF BETEL VINE (*Piper betle* L.)

by

NISHA A.

(2016-11-021)

Abstract of the Thesis

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COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

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ABSTRACT

The study entitled "Ecofriendly management of anthracnose of betel vine" was conducted during 2016 - 18 at Department of Plant Pathology, College of Agriculture, Vellayani with an objective to evolve an integrated management strategy involving organic preparations, biocontrol agents and new generation fungicides for anthracnose of betel vine.

A survey was conducted in different locations in Southern Kerala *viz.*, Thiruvanathapuram (Kalliyoor, Vellayani and Kattakada), Kollam (Kareepra), and Alappuzha (Cherthala) regions during 2016-18. Anthracnose was the major problem in these areas. The highest percentage disease index of 20.00 was observed from Cherthala region of Alappuzha. The symptoms of the anthracnose appear as small necrotic spots with a yellow halo on the leaf lamina mostly starting from the margin of the leaf. These lesions coalesce together results in leaf blighting and defoliation. Under high humid conditions, stem lesions were also produced which resulted in drying of the entire vines. *Colletotrichum* sp. was found to be associated with the disease in five locations. Isolations were made and five pure cultures of *Colletotrichum* sp. (C1 to C5) were obtained and its pathogenicity was proved using Koch's postulates.

Culture and morphological characters of the five different isolates were studied. The isolated cultures of *Colletotrichum* sp. produced whitish to dark grey, orangish to off white coloured colony having fluffy to sparse mycelial growth with regular margins. The different isolates took 7-9 days for completion of growth in petri dish. The mycelium of the fungus was hyaline, septate and width ranged from 2.65 - 3.45 µm. The septal distance varied from 9.80 - 25.56 µm. The conidia were single celled with an oil globule and the shape varied from cylindrical, oblong to dumbbell among the isolates. The conidial and appressorial size varied from 9.6 - 12.2 µm x 3.8 - 4.7 µm and 8.37 - 10.08 µm x 5.02 - 6.28 µm.

The pathogenic variability among the five different isolates were assessed by virulence rating. The isolate C2 was identified as most virulent which produced lesion size of 14.75 mm at 5 days after inoculation (DAI). The isolate C2 initiated symptom within two days of artificial inoculation having a rate of lesion development of 2.95 mm day^{-1}. The identity of C2 isolate as *C. gloeosporioides* was confirmed by the use molecular technique (DNA barcoding).

The indigenous organic preparations *viz.*, panchagavya, jeevamruth, vermiwash and fermented cow's urine at 5 and 10 per cent (autoclaved and non-autoclaved) were evaluated for *in vitro* pathogen suppression. The microbial population was estimated at 1, 5 and 7 days in the filtered and non-filtered indigenous organic preparations. Microbial population of bacteria was comparatively high in these organic preparations when compared to fungi. The bacterial population showed an increasing trend in non-filtered organic preparations except jeevamruth. The mycelial nature in amended media varied from fluffy to sparse growth with uniform or irregular growth pattern under autoclaved and non-autoclaved extracts of organic preparations.

Panchagavya at 10 per cent and fermented cow's urine at 5 per cent and 10 per cent (non-autoclaved) completely inhibited the pathogen. Autoclaved fermented cow's urine at 5 per cent and 10 per cent failed to inhibit the mycelial growth of the pathogen. The autoclaved and non-autoclaved organic preparation panchagavya at 10 per cent was more effective than other treatments in inhibiting the mycelial growth of the pathogen.

The saprophytic microflora were isolated from the rhizosphere and phyllosphere of healthy betel vine. The fungal and bacterial biocontrol agents from phyllosphere and rhizosphere were screened *in vitro* for pathogen suppression. The fungal antagonist *Trichoderma* 1 was superior in inhibiting the mycelial growth of *C. gloeosporioides*. Among the bacterial isolates screened, the isolate B5 from the phyllosphere of the healthy betel leaves had a higher percentage of mycelial inhibition (45.00 per cent).

Screening of fungicides against the fungal pathogen under *in vitro* condition revealed that systemic fungicides (propiconazole, tebuconazole), combination fungicides (captan + hexaconazole and carbendazim + mancozeb) at 0.05, 0.1 and 0.2 per cent completely inhibited the mycelial growth of the pathogen. The contact fungicide copper hydroxide was also effective in inhibiting the mycelial growth. The fungicide Propiconazole was effective in suppressing complete growth of the pathogen at lower dose of 0.01per cent (100 ppm).

A pot culture study was laid out in CRD with 8 treatments in 4 replications to develop an integrated management package. The effective treatments of the above studies *viz.*, panchagavya (10 per cent), bacterial isolate (B5-10^8cfu/ml), propiconazole (0.1 per cent), copper hydroxide (0.2 per cent), carbendazim and mancozeb (0.2 per cent) and chemical control check (Bordeaux mixture - 0.5per cent) with inoculated and un inoculated control were evaluated for the management of anthracnose of betel vine. The treatment of propiconazole was superior in reducing the percentage disease index and disease incidence. The treatment recorded a disease index of 19.99 per cent at 10 days after second spray with 74 99 per cent disease suppression over inoculated control. The second best treatment was the foliar application of carbendazim + mancozeb (0.2per cent) which was on par with copper hydroxide (0.2per cent) in reducing the severity of the disease. Even though the chemicals were far more effective in managing the disease, the bacterial isolate B5 was equally effective in suppressing the pathogen with 46.24 per cent disease suppression over inoculated control with a disease index of 42.98. The biometric observations did not show significant difference among the different treatments except the number of leaves.

The present study reveals that all the fungicides and bacterial isolate from phyllosphere were effective in managing the disease over the traditional management using Bordeaux mixture at 0.05 per cent POP of KAU, 2016. Since it is a masticatory crop need based application of antagonistic bacteria may be suggested for the anthracnose management in betel vine. For proper disease management approach, the bacterial antagonist may be suggested after conducting

further studies on the spray schedule, dosage, compatibility with fungicides and its biosafety.