CHARACTERIZATION OF FUNGAL PATHOGEN ASSOCIATED WITH LEAF ROT DISEASE OF COCONUT (*Cocos nucifera* L.) AND *IN VITRO* EVALUATION OF PHYLLOPLANE MICROFLORA AS BIOCONTROL AGENTS

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2018-11-038

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VELLAYANI, THIRUVANANTHAPURAM – 695 522

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by

DEENA SEBASTIAN

(2018-11-038)

THESIS

submitted in partial fulfilment of the

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DEPARTMENT OF PLANT PATHOLOGY

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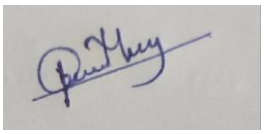
KERALA, INDIA

2020

DECLARATION

I hereby declare that this thesis entitled **"Characterization of fungal pathogen associated with leaf rot disease of coconut (*Cocos nucifera* L.) and *in vitro* evaluation of phylloplane microflora as biocontrol agents"** is a *bonafide* record of research work done by me during the course of research and that 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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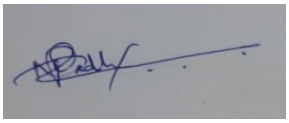

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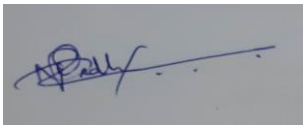

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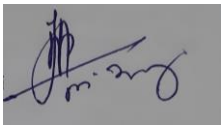

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Deena Sebastian

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND SYMBOLS USED

LRD	Leaf rot disease
RWD	Root(wilt) disease
%	Per cent
^{0}C	Degree Celsius
CD	Critical difference
SE (m) ±	Standard error of mean
Cm	Centimeter
G	Gram
Ha	Hectares
µm	Micrometer
Ml	Millilitre
PDA	Potato Dextrose Agar
PDB	Potato dextrose Broth
KB	King's B
MRBA	Martin's Rose Bengal Agar
DTSA	Days taken for symptom appearance
Viz.	Namely
Et al.	And other co-workers
sp.	Species
spp.	Several species
DAI	Days after inoculation

Introduction

1. INTRODUCTION

Coconut (*Cocos nucifera* L.) is an important plantation crop designated as 'Kalpavriksha', 'Tree of life', 'Tree of abundance' or 'Tree of heaven' due to its multipurpose usage in oil, food and coir industry. Coconut is cultivating in more than 93 countries in the world with total area of 11.90 million hectares which contributes to the production of 67128 million nuts per annum (APCC, 2020).

India shares first in coconut production among the coconut growing countries worldwide with 1.47 million tonnes nuts annually from an area of 2.15 million hectares with a production of 6825 kg nuts /ha (DAC&FW, 2020).

More than 78 per cent of coconut cultivation in India is contributed by the southern states *viz.*, Kerala, Karnataka, Tamil Nadu and Andhra Pradesh. Among the major coconut growing states, Kerala is the leading state in area (0.75 million hectare) and production (7631 million nuts) of coconut with much less productivity (10083 nuts / hectare) compared to the national average (10614 nuts/ hectare) and also with the other states (Andhra Pradesh – 13563 nuts/ha, West Bengal – 12464 nuts/ha, Tamil Nadu – 12144 nuts/ha) (CDB, 2020).

One of the major reasons for the low productivity of coconut in Kerala is attributed to a devastating disease, root (wilt) disease (RWD) caused by phytoplasma which threatened the coconut cultivation for more than a century. The disease has spread widely and caused severe damage in southern districts of Kerala and also in some of the districts of Tamil Nadu and Karnataka. Srinivasan (1991) reported that there was 65 per cent of the RWD affected palms were 'super infected' with leaf rot disease (LRD) and the super infection is due to the easy access of LRD pathogens to the coconut palm which is already weakened by phytoplasma. In this aspect, the management of LRD is a serious concern with regard to the RWD management.

Butler (1908) reported first on incidence of 'leaf rot like' disease on unopened spindle leaves of coconut. The LRD is causing severe damage on the spear leaves and the

infection is much reduced on matured leaves. The disease severity is more on the spindle leaves because of the high moisture content and thin epidermal wall (Lily, 1981). The symptoms were initiated on the distal end and margins of the spindle leaves as minute water soaked brown lesions which are cemented together and later on, the lesions dry up and blown off by wind. When the leaves unfurl, the leaflets on the basal portion of the spindle remain healthy and giving a typical 'fan leaf' like symptom (KAU, 2016).

The LRD is caused by a fungal complex including *Colletotrichum gloeosporioides, Fusarium solani, Fusarium moniliforme, Exserohilum rostratum, Gliocladium vermoeseni, Thielaviopsis paradoxa* (Srinivasan and Gunasekaran, 1996a), and *Scytalidium* sp., *Gliocladium roseum* etc. (Vrinda, 2002).

The disease is more prevalent during the south west monsoon season (June - August) where there is low temperature (less than 27°C) and high relative humidity in the atmosphere (98 %) (Menon and Nair, 1951; Radha *et al.*,1961). *C. gloeosporioides* found to be a major pathogen initiating the symptom development during rainy season and *Fusarium* sp. predominated during the summer season (Srinivasan and Gunasekaran, 1993; Vrinda, 2002).

The fungal-phytoplasma complex causes severe economic losses to the coconut production in Kerala. But it is difficult to calculate the economic losses due to the LRD separately. Even though CPCRI (1985a) estimated an approximate production loss due to LRD as 461 million nuts per annum in Kerala without including the 60 per cent damage to the foliage of the coconut plantations infected with LRD.

The current management strategies to reduce the problems of LRD include cultural, biological and chemical methods. Nowadays, the farmers are seeking for the biological methods of disease management to reduce the environmental and health hazards. So, the isolation of more biocontrol agents from their natural habitat is important to develop potential antagonists against the LRD pathogens.

In this regard, the study aims to tackle the problems of LRD using the natural inhibitors of the disease from the phylloplane of the healthy leaflets of the infected palm and to develop an eco-friendly management strategy for LRD. The main objectives of the study are to identify and characterize the major fungal pathogens associated with the leaf rot disease of coconut and *in vitro* evaluation of phylloplane microflora of coconut against the pathogens.

Review of Literature

2. REVIEW OF LITERATURE

Leaf rot disease is one of the most severe foliar diseases of coconut plantations of Kerala, especially in southern districts.

2.1 ORIGIN AND DISTRIBUTION

Butler (1908) gave the first report on incidence of 'leaf rot like' disease on unopened spindle leaves of coconut. In early 1880's, the disease was predominant in the former Indian princely states of Travancore and Cochin (Varghese, 1934; Menon and Nair, 1951; 1952).

Initially, the disease was observed only in certain regions. But later it spread to new areas and was found prevalently in the central Travancore region with Kollam and Punalur in the south and Oachanthuruthu and Thodupuzha in the north as borders (Menon and Nair, 1948). Gradually, the leaf rot disease (LRD) incidence had risen over years in root (wilt) endemic areas of southern Kerala along with increase in intensity of root (wilt) disease. There was occasional report of LRD in young coconut palms from the areas like Kasaragod where root (wilt) disease was not reported (Radha and Lal, 1968). The LRD was also reported from coconut plantations of Srilanka where Weligam Coconut Leaf Wilt (WCLWD) caused by phytoplasma became a severe problem (Wijesekara *et al.*, 2008).

Even though the LRD was affecting the palms of all age groups from seedling to adult stage, the incidence of LRD was more in palms below the age group of 25 years (Menon and Pandalai, 1958). LRD normally does not affect seedlings in the nursery, but affect the seedlings within ten months of field planting (Koshy, 1999, 2000).

2.2 INTERRELATIONSHIP OF LRD AND RWD

Several early observations by Butler (1908), Menon (1935), Menon and Nair (1952) reported LRD as a part of RWD. But it was not realized at that point of time. Later

findings confirmed the co-occurrence of the diseases and proneness of RWD affected palm to LRD infection.

The simultaneous occurrence of LRD and RWD in the field trial as well as in the inoculation trials was observed by Radha and Lal (1968). They observed the LRD incidence on both RWD affected palm and healthy palm where such palms expressed the RWD symptoms later. They also noticed the variation in LRD intensity in RWD affected areas from 16-40 per cent and also the intensity varied according to the soil types. George and Radha (1973) reported that there was variation in the LRD intensity of the palms with stages of RWD. If the RWD in early stage, there was 40 per cent LRD infection whereas, middle and advanced stage of RWD, the intensity of LRD recorded was 79 and 98 per cent respectively. Mathai (1980) also reported the co-occurrence of both the diseases and the proneness of LRD on RWD affected palm.

LRD was generally confined to the RWD affected area and 65 per cent of the RWD affected palms were 'super infected' with LRD pathogens (Srinivasan, 1991). Srinivasan and Gunasekaran (1996a, 2000) contributed evidence for this by the inoculation of LRD pathogens on two and five-year-old RWD affected coconut palms and they could observe severe leaf rot infection on inoculated seedlings. Severe natural incidence of LRD in RWD affected palms were observed in field condition and it is supposed that, the subsequent infection of RWD affected palm to LRD might be due to the already disrupted defense mechanism of palms due to phytoplasma of RWD. Srinivasan *et al.* (1998) reported that the co-occurrence of these diseases on the same palm is a unique example of phytoplasma-fungal disease complex resulting in substantial crop loss. The LRD reported from Srilanka was also associated with a phytoplasmal disease known as Weligam Coconut Leaf Wilt Disease (WCLWD) with similar symptoms as RWD (Wijesekara *et al.,* 2008).

2.3 SYMPTOMATOLOGY

First report on the symptoms of leaf rot disease was made by Butler (1908). Further descriptions were given by Sundararaman (1925), Varghese (1934) and Menon and Nair (1948; 1952). All these symptomatologic studies revealed that the initial symptom occurred on unopened spindle leaves of coconut. Water soaked minute brown lesions on the distal end and margins of the spindle leaves were the initial symptom recorded by them. Later the lesions extended to interior leading to complete rotting of the leaves. Similar symptoms were observed by Lily (1981) and Srinivasan and Gunasekaran (1992).

High moisture content is an ideal situation for the LRD pathogens to attack. Higher moisture content and comparatively thinner epidermal layer of the spindle leaves made it more susceptible to the disease (Lily, 1981). Further decay of the spindle leaf was aided by maggots, nematodes, earwigs and ants (Menon and Nair, 1951; Koshy, 1999). Menon and Nair (1951) Joseph and Rawther (1991) explained the typical 'fan leaf' like symptom of LRD. The rotted spindle leaflets often cemented together and when such leaflets start unfurls the rotted portion dry up, turn black and are blown off by wind. The basal part of the spindle was healthy, open normally and producing 'fan leaf' like symptom. Severe foliage losses occurred due to the continuous infection of the emerging spindles (Srinivasan and Gunasekaran, 1992). In severe cases, the LRD symptoms were also observed on the midribs and petiole (CPCRI, 1996; Srinivasan and Gunasekaran, 2000; Srinivasan, 2008).

2.4 ETIOLOGY

The early report of salmon coloured *Penicillium* like fungus on the leaf rot disease infected portion of coconut leaves was stated by Mc Rae (1916). He also observed the association of *Gloeosporium* sp. with shoot rot disease of coconut (Mc Rae, 1929), while Sundararaman (1929) noticed *Fusarium* sp. in diseased palms. The various microorganisms such as *Gloeosporium* sp., *Fusarium* sp., *Gliocladium* sp.,

Helminthosporium halodes, *Pestalotia* sp. *Theilaviopsis paradoxa*, *Rhizoctonia solani* etc. were isolated from the LRD infected samples during the detailed investigations on etiology of LRD (DAF, 1940; Menon and Nair, 1948; 1951). Among this, the pathogenicity of the fungi *G. roseum*, *H. halodes*, *Gloeosporium* sp. and *Pestalotia* sp. were proved by developing typical symptoms of LRD (Menon and Nair, 1948; 1951). The most virulent pathogen was *H. halodes* which produced the infection within 12 hours and the rest of them were considered as the secondary parasites and induced the infection within 48 hours. Pathogenicity studies were conducted by using single and mixed inocula of various pathogens. *Helminthosporium* sp. alone caused 84 per cent infection while with mixed inocula, the fungus produced 93 per cent infection (Menon and Nair, 1951). Etiological role of *H. halodes* with LRD was further confirmed by Lily (1963; 1981), Radha and Lal (1968), and CPCRI (1979). *Diplodia* sp. and *Curvularia* sp. were also isolated from LRD affected coconut palm along with the other pathogens (CPCRI, 1985b). The pathogens like *H. halodes, G. roseum, Gloeosporium* sp., *Pestalotia palmarum, Fusarium* sp., *Theilaviopsis paradoxa* and *Diplodia* sp. were isolated from the diseased specimens collected from the southern districts of Kerala by Sathiarajan *et al.* (1988). Similarly, Mishra *et al.* (1989) isolated the pathogens *H. halodes*, *G. roseum*, *Fusarium* spp., *Gloeosporium* sp. and *Macrophomina phaseolina* from the LRD affected palms in Orissa.

Shanmughom (1963) had done a comprehensive morphological study of *H. halodes.* He described that primary spores of the fungus produced secondary spores and the isolate of the fungus infecting coconut was designated as *H. halodes* var. *nuciferae.* Menon and Pandalai (1958) studied the spore characteristics of the fungus *Helminthosporium* sp. and the spores which were air borne enveloped in drops of dew or rain water.

Most of these works were considered *H. halodes* as the major pathogen of LRD of coconut based on their isolations from the infected few palms. The complex nature of the disease needed elaborate studies on their etiology by collecting large number samples

from different locations in different seasons. Srinivasan and Gunasekaran (1993) isolated varied spectra of fungi in varied frequencies from the LRD affected samples. Based on the taxonomic studies they summarized that the fungi isolated earlier as *H. (Bipolaris) halodes, G. roseum* and *Gloeosporium* sp. were *Exserohilum rostratum* (Drechsler) Leonard and Suggs, *Gliocladium vermoeseni* (Biourge) Thom. and *Colletotrichum gloeosporioides* (Penzig and Sacc.) respectively and confirmed pathogenicity of fungal complex in LRD. Srinivasan and Gunasekaran (1994) also reported that all the three pathogens were independently capable of causing leaf rot symptoms and among these *Exserohilum rostratum* developed the disease symptoms faster. Later studies by Srinivasan and Gunasekaran (1995a) and Srinivasan *et al.* (1995) reported the association of fungi *R. solani* Kuhn., *T. paradoxa* (Dade) C. Moreau, *Curvularia* sp., *Mortierella elongata* Linnem, *Acremonium* sp., *Chaetomium brasiliense* Batista and Pont., *Thielavia terricola* (J. Gilman and E.V. Abbott.) Emmons., and *T. microspora* Mouch. The occurrence of *Cylindrocladium scoparium* in LRD was reported by Srinivasan and Gunasekaran (1995b). Srinivasan and Gunasekaran (1996a) identified the pathogens *F. solani* and *F. moniliforme* var. *intermedium* (*Gibberella fujikuroi* var. *intermedia*) from LRD samples of coconut. The frequent isolations and pathogenicity studies showed that the principal pathogen causing LRD was *C. gloeosporioides* (Srinivasan and Gunasekaran, 1996b; 1996c). The role of *Scytalidium* sp. in causing LRD was first reported by Vrinda (2002). She also reported that *C. gloeosporioides* and *Fusarium* sp. were the major fungal species causing LRD in southern Kerala during monsoon season. During the post monsoon season, the population of *C. gloeosporioides* was subsided and *Fusarium* sp. act as major pathogen causing LRD.

The fungi causing LRD (14 different species) were divided into three categories such as group A, B and C based on the relative association with the disease and frequency pattern (CPCRI, 1994). The fungi *C. gloeosporioides, G. vermoeseni, E. rostratum, F. moniliforme* var. *intermedium* and *F. solani* were considered as group A because their association with LRD is predominant, consistent and detectable in definite frequencies in isolation. The fungi included in group B were *T. paradoxa, C. scoparium*

and *R. solani* and their association with disease was inconsistent, discernible and relatively in lesser frequency. The group C fungi included *Curvularia* sp., *M. elongata, T. microspora, T. terricola, C. brasiliense* and *Acremonium* sp. These fungi occur in low frequency in LRD and may have only a secondary role in the disease incidence. The LRD infected leaves were shown intense mycelial growth and spore masses over the lesion and in between the leaflets (Srinivasan *et al.*, 1995; Srinivasan and Gunasekaran, 2000; Srinivasan, 2008).

According to reports by Srinivasan and Gunasekaran (1995a) and Vrinda (2002), the significance of fungal complex in causing severe disease incidence was due to the *in vitro* associative nature of major pathogens of LRD rather than antagonistic activity and also found that the *Fusarium* spp. showed a competitive interaction over certain less frequent fungi.

The studies conducted by Srinivasan and Gunasekaran (1994) reported *E. rostratum* as the most virulent pathogen and *G. vermoeseni* as the least virulent based on the speed of the infection. The disease incidence was found to be higher with combined inoculation of the major LRD pathogens *viz., C. gloeosporioides, E. rostratum* and *G. vermoeseni* rather than the individual inoculation. The artificial inoculation of *C. gloeosporioides* and *E. rostratum* combinedly and individually on RWD affected palm reproduced the similar LRD symptoms (CPCRI, 1996). The healthy seedlings free from RWD produced restricted spots both in pot and field planted palms (Srinivasan and Gunasekaran, 1996a). Vrinda (2002) also observed similar results for the combined inoculation and the results from combined inoculation were found similar to the individual inoculation with *Scytalidium* sp.

2.5 EPIDEMIOLOGY

Severity, incidence and spread of LRD and pathogen composition had direct correlation with the prevailing weather conditions and season. Menon and Nair (1951) reported that the disease incidence was more severe during the seasons when there was

maximum atmospheric humidity. They also noticed that the number of spores in the atmosphere was highest during south-west monsoon (June – August). Radha *et al.* (1961) made similar observations and found that the disease was directly related to low temperature (below 27°C) and high relative humidity (above 98%) prevailed during the monsoon season. These findings were similar to subsequent studies conducted by Radha and Lal (1968). The atmospheric conditions like dew during dry months or rainfall increased the fungal infection and disease occurrence. These atmospheric conditions provided wetness or free water to the leaf surface which become a favourable environment for the pathogen to infect (CCRS, 1962; 1965). Dwivedi *et al.* (1979) reported that the severity of LRD was visually higher in rainy season even though the frequency of LRD necrosis / rotting in spindle leaves of RWD affected palms were at the same level during summer and rainy season. Study conducted by Mathai (1980) revealed that the LRD intensity was minimum during summer months (April-June) and maximum during north east monsoon period (September-October).

The population dynamics of major LRD pathogens accorded with the influence of weather variables were studied by Srinivasan and Gunasekaran (1996c) by monthly sequential isolations of the pathogens from the spindle leaves of the affected palm. They observed that the incidence of *C. gloeosporioides* was higher in frequency and population during the monsoon season, and also implicated *C. gloeosporioides* as the major pathogen causing LRD during monsoon. The population of *E. rostratum* was less correlated with weather pattern, while *Fusarium* spp. were most commonly observed during the dry seasons (January – May) especially on advanced lesions than on early lesion.

The study conducted by Vrinda (2002) also reported that the fungus *C. gloeosporioides* was the major pathogen of LRD during south-west monsoon season and the population decreases towards the end of the season and the *Fusarium* sp. came into action subsequently during the summer season. These observations were made by

collecting the samples from five southern districts of Kerala during the three seasons of the year such as June – July, November – December and March – April.

2.6 PURIFICATION, IDENTIFICATION AND CHARACTERIZATION OF PATHOGENIC MICROFLORA

Srinivasan and Gunasekaran (1994) identified and characterized the major LRD pathogens of coconut such as *C. gloeosporioides* (Penzig) Penzig & Sacc., *E. rostratum* (Drechsler) Leonard & Suggs and *G. vermoeseni* (Biourge) Thom. The *C. gloeosporioides* was characterized by greyish white, abundant aerial mycelium. The rear side of the colony was showing bluish black colour and had concentric growth pattern. The fungus produced fructifications like acervuli with long dark brown setae. Conidia were hyaline, straight to slightly bent, aseptate and dumb bell shaped. *Exserohilum rostratum* (Drechsler) Leonard & Suggs was a slow growing fungus with dark olivaceous, brown velvety appearance on the upper surface and dark colour on rear side. Conidiophore was septate, simple, olivaceous brown and geniculate. Conidia were straight or curved, thick walled, and brown to olivaceous. *G. vermoeseni* was characterized by whitish colonies initially later turns to salmon or pink. Conidiophore was septate, hyaline, simple or penicillate branched, terminating on phialides. Conidia were hyaline, oval to elliptical and unicellular.

Vrinda (2002) characterized the LRD pathogens such as *F. solani, Cephalosporium sacchari* Butler, *Scytalidium* sp. and *Curvularia* sp. along with the above-mentioned pathogens. The *F. solani* was found to be more virulent among the other *Fusarium* spp. causing LRD. *F. solani* produced aerial mycelia, macro conidia and micro conidia where the average size of sickle shaped macro conidia was 25.5 µm x 3.3 µm, while the micro conidia were rod shaped. The chlamydospores were present in chains. *C. sacchari* was initially white in colour later turned to pinkish on the PDA medium. They completely covered the entire Petri plate within 10 days and produced hyaline, single celled and oblong conidia which aggregated together to form ‘heads’.

The pathogenicity of *Scytalidium* sp. causing LRD was first proved by Vrinda (2002). *Scytalidium* sp. was characterized by black colonies with some of the hyphae smooth, narrow, cylindrical and colourless while the others thick, pale to mid-brown with occasional darker swollen cells with thick dark septa. The conidia catenate, smooth and single celled. Two types of conidia were noticed: colourless, thin walled, cylindrical conidia; and broader, dark brown, thick walled, oblong conidia.

2.7 TESTING THE PATHOGENICITY OF ISOLATED MICROFLORA

Pathogenicity of the major LRD pathogens was tested by Srinivasan and Gunasekaran (1994) using leaflet pieces and detached spindle leaves. The leaflet pieces of 7 cm length were cut out from the spindle leaves and were inoculated with drops of spore suspension (approx. 4 X 10^5 spore / ml) with or without the pin prick injury and were placed in sterile Petri plate (9cm) lined with moist filter paper. The inoculated leaflets were incubated at 30°C. Detached spindle leaves were used for pathogenicity studies by keeping the leaf in test tube containing sterile water and the pathogen was spray-inoculated on to the leaf. The mouth of the test tube was plugged with cotton wool to keep the leaf in position. The test tubes were incubated inside the bell jar for symptom development.

Vrinda (2002) studied the pathogenicity of LRD pathogens of coconut by inoculating the pathogens on detached spindle leaves kept inside the conical flask containing sterile water. The mycelial bits from the seven-day old culture plate was cut out and inoculated on the inner side of the leaflet with or without pin prick injury and covered with moist cotton. The whole leaflet was kept inside the polythene tube and incubated. The control was maintained by placing plain agar disc instead of pathogen bit.

2.8. PHYLLOPLANE MICROFLORA IN FOLIAR FUNGAL DISEASE MANAGEMENT

The bacteria and fungi isolated from the phylloplane of coconut leaves were assessed for their ability of antagonism against leaf rot pathogens by dual culture assay and found that *Bacillus*. sp., *Aspergillus* sp. and *Penicillium* sp. were found inhibitory to the mycelial growth of leaf rot pathogens such as *C. gloeosporioides, F. solani* and *E. rostratum* (Srinivasan *et al*., 2006). According to Tembhare *et al.* (2012) the phylloplane fungi showed inhibition to the pathogen *C. capsici* in dual culture assay with higher inhibition by *Aspergillus fumigatus* (61.03 %). Khanna *et al.* (2015) screened phylloplane fungi from the onion leaves against causal agent of purple blotch disease, *Alternaria porri* under *in vitro* condition and found *Trichoderma* isolate showing 89 per cent mycelial inhibition on the pathogen in dual culture assay. 94 per cent mycelial inhibition of taro leaf blight causing *Phytophthora colocasia* by phylloplane fungus *Trichoderma* was observed by Padmaja *et al.* (2015).

The bacterium *Ochrobactrum anthropi* from the phylloplane of tea was elucidated against two major pathogens of tea viz., *Pestalotiopsis theae* and *Exobasidium vexans* (Sowndhararajan *et al.,* 2012). The production of antifungal secondary metabolites was major mode of action of the antagonistic bacteria. The rice associated bacterium *Bacillus amyloliquefaciens* was found effective against *R. solani* in rice (Shrestha *et al.,* 2016). The pink pigmented facultative methylotrophic bacteria, *Methylobacterium populi* was isolated from the phylloplane of rice and tested its antagonism against pathogens *Pyricularia oryzae* causing rice blast and *Rhizoctonia solani* causing sheath blight. By *in vitro* and pot culture studies it was reported that the isolated bacteria had enough potential to manage the disease and also to increase the yield compared to the reference culture (Nysanth *et al.,* 2018).

The antagonistic activity of indigenous phyllosphere actinomycetes from rice plant were tested against *P. oryzae in vitro* and *in planta.* Among the isolates showing

inhibition to the leaf blast pathogen were identified majorly as *Streptomyces* sp. *Saccharothrix, Gordonia*, or *Lentzea* (Harsonowati *et al.,* 2017).

2.9 LRD MANAGEMENT USING BIOCONTROL AGENTS

The idea of management of LRD using the antagonistic microorganisms had initiated as early as in 1952. The bacterium *Bacillus anthracis* found inhibitory effect on the LRD pathogen *H. halodes* (Lily *et al.,* 1952). Later, Lily *et al.* (1955) also reported that the cultural filtrate of the bacterium *B. subtilis* was found inhibitory to the growth of *R. solani, R. bataticola, H. halodes and Botryodiplodia theobromae. The growth of LRD pathogens viz., C. gloeosporioides, F. solani, G. vermoeseni, R. solani, T. paradoxa* and *E. rostratum* were inhibited by the antagonistic bacterium *Pseudomonas fluorescens* (CPCRI, 1998).

Gupta *et al.* (2000) reported two fluorescent *Pseudomonas* bacteria isolated from the rhizosphere of the coconut palm to be effective against the mycelial growth of the most important LRD pathogens *C. gloeosporioides* and *E. rostratum.* They also noticed the assembly of some antifungal substances by the *Pseudomonas* isolates and was meant to be the explanation for antagonism. Gunasekaran *et al.* (2003) observed that the biocontrol agent *Pseudomonas fluorescens* was effective against LRD pathogens both on mycelial growth inhibition and *in vivo* conditions on inoculated leaves.

Srinivasan and Bharathi (2006) observed that the fungal biocontrol agent *Trichoderma viride* was effective against major LRD pathogens compared to the bacterial antagonists *P. fluorescens* and *B. subtilis.* Srinivasan *et al.* (2006) reported that the biocontrol agents *B. subtilis* and *P. fluorescens* independently inhibited the mycelial growth of major LRD pathogens such as *C. gloeosporioides, E. rostratum* and *F. solani*, while the combined application of both bacterial antagonists gave cent per cent inhibition under *in vitro* condition. They also extended the biocontrol of LRD to field level and noticed the decrease in LRD intensity of 55 per cent in palms treated with talc-based formulation of *P. fluorescens* consecutively for three years (twice in year) over the

control plant. Srinivasan *et al.* (2010; 2011) isolated the fungi and bacteria such as *T. viride*, *B. subtilis* and *P. fluorescens* from rhizosphere and found out the isolates among three of them with higher antagonistic activity against major LRD pathogens *in vitro.* The isolates were tested for *in vitro* compatibility and mass multiplied them for the field evaluation.

Materials and Methods

3. MATERIALS AND METHODS

All the laboratory studies were conducted in the Department of Plant Pathology, College of Agriculture, Vellayani.

3.1 ISOLATION OF LEAF ROT DISEASE PATHOGENS OF COCONUT

Leaf rot samples from the disease affected coconut palms were collected from six taluks of Thiruvananthapuram district during south-west monsoon. The taluks selected for sample collection were Thiruvananthapuram, Neyyattinkara, Chirayinkeezhu, Varkala, Kattakada and Nedumangadu. The microorganisms associated with leaf rot disease were isolated from the infected portion by routine isolation method.

The samples were thoroughly washed in tap water and allowed to dry. The further steps were proceeded inside the laminar air flow chamber. The small bits of leaves were cut out from the disease advancing region of the infected leaf. The bits were then surface sterilized in 0.1 per cent mercuric chloride solution for one minute followed by three sterile water washes by three sequential transfers of the bits in three Petri dishes. The chemical residues that attaches to leaf bits were removed by this sterile water washes. The bits were plated on solidified Potato Dextrose Agar (PDA) medium (Rahimloo and Ghosta, 2015). The plates were kept for incubation at room temperature.

3.2 PURIFICATION, IDENTIFICATION AND CHARACTERIZATION OF PATHOGENIC MICROFLORA

3.2.1 Purification

Pure culturing was done when the mycelial growth starts from the leaf bits (2 to 3 days). All the fungal pathogens isolated from infected leaves were purified by continuous sub-culturing by hyphal tip culture (Brown, 1924) or single spore method.

3.2.1.1 Hyphal tip culture

The distal end of the growing mycelia was cut out using a cork borer and transferred to a sterile Petri plate containing solidified PDA medium. The Petri plates were incubated at room temperature (28 ± 2°C). After obtaining the pure cultures of the fungi, they were transferred to PDA slants and kept for further studies.

3.2.1.2 Single spore isolation

Single spore isolation technique was used for the purification of fungi, which had spores. The spore suspension was prepared by mixing spore mass in 9 ml sterile water under laminar air flow chamber. One ml of the spore suspension was transferred to the sterile Petri plate using micropipette. Melted and cooled 2 per cent plain agar medium was poured to the Petri plate, sealed using cling film and kept for incubation. After 24 hours, the Petri plate was observed under microscope and a single germinated spore was marked for pure culturing.

3.2.2 Identification and characterization

Morphological characters of the pathogens were studied by examining the cultural as well as the microscopic observations. The pathogens were inoculated in Petri plates containing PDA medium and mycelial growth was observed subsequently. The days taken by pathogens to completely cover 9mm Petri plates were observed. The cultural characters like colony colour, colony morphology, mycelial colour and radial growth (cm) on third, fifth, and and seventh day after culturing were also observed.

To study the morphological characters under microscope, slide culture technique (Riddel, 1950) was adopted. The slides were observed on third day. The microscopic slides were prepared using the stain lactophenol cotton blue. The slides were observed under compound microscope and recorded the morphological characters such as colour of mycelia, spore colour, spore shape and spore size.

3.3 TESTING PATHOGENICITY OF ISOLATED MICROFLORA

The pure culture of the isolated fungi were subjected to pathogenicity tests. For pathogenicity test, healthy unopened spindle leaves of coconut were collected from the field. The leaves were inoculated with the pathogens by placing the mycelial bits from seven-day old cultures. The bits of 5 mm diameter were cut and were placed on the inner surface of the leaflet with pin pricks. Moistened cotton lining was given over the inoculated region to ensure proper moisture and humidity. The inoculated leaflets were completely covered with polythene cover moistened with water, labelled, tied and incubated at room temperature (28 ± 2^0C).

The leaflets showing disease symptoms were subjected to re-isolation of pathogen and compared the characters of those re-isolated pathogens with that of isolated pathogens in order to prove Koch's postulates.

To check the virulence of the major LRD pathogen isolates, the seven-day old cultures of the isolates were inoculated on the detached spindle leaflets. Each isolate was inoculated on the healthy leaf let and three replications were maintained. To identify the virulent isolate among the major pathogens, the days taken for symptom initiation, the length and breadth of the lesion produced by each isolate on fifth, seventh and tenth day after inoculation were noted. The virulent isolate identified among the major pathogens were used for further studies.

3.4 SYMPTOMATOLOGY

The nature and sequence of symptom development were studied based on artificial inoculation of a spectrum of pathogens on to the detached spindle leaflets.

The pathogens were cultured in PDA medium and the seven-day old cultures were used for inoculation. The inoculation procedure is same as explained in 3.3. Three replications of each of the pathogens were maintained. The nature and the colour of the lesion produced by the pathogens were observed.

3.5 COMBINED INOCULATION OF MAJOR LEAF ROT PATHOGENS ON DETACHED SPINDLE LEAVES AND RE-ISOLATION OF PATHOGEN AND ITS CONFIRMATION

The major pathogens of LRD identified were used for further studies. These major pathogens were inoculated in combinations on to the unopened spindle leaflets by following the same inoculation procedure as explained in 3.3. The virulent isolates of major pathogens identified were inoculated on a single leaflet by pin pricking. If the major pathogens were A and B, then A+B combination of pathogens were used for the study. The mycelial bits were covered by moistened cotton and were kept inside a polythene cover for incubation. The control was maintained by inoculating the detached leaflet with 2 per cent agar bit. The individual inoculations were also done with major pathogens. The time taken for the symptom initiation and the lesion size were measured. The lesion size were measured on fifth, seventh and tenth day after inoculation and compared the results of combined inoculation with that of the individual inoculation. The pathogens were re-isolated from those infected leaves by routine isolation method and compared the characteristics of those re-isolated pathogen with that of isolated pathogens.

3.6 ISOLATION OF PHYLLOPLANE MICROFLORA OF COCONUT

In order to identify the effective antagonists from the phylloplane of coconut, healthy leaflets were collected from the LRD infected palm. The leaf lets were taken to the laboratory and were subjected to serial dilution its plating and pure culturing of microflora (Rodriguez-Kabana, 1967).

The healthy leaflets were taken inside the laminar air flow chamber and washed with sterile water and made up to 100 ml. This will serve as stock with 10^{-2} dilution. The stock solution was gently vortexed for proper mixing and 1 ml of the stock was pipetted out and transferred into a test tube containing 9 ml sterile water and vortexed thoroughly. This will give 10^{-3} dilution. Then 1 ml from this test tube was transferred into another test tube with 9 ml sterile water and vortexed to give 10^{-4} dilution.

After performing the serial dilution, the diluted stock was transferred to the Petri plate using pour plate method. The medium MRBA (Martin's Rose Bengal Agar) was used for the isolation of phylloplane fungi from the healthy spindle leaflets. One ml of the solution was pipetted out from 10^{-3} and 10^{-4}dilutions and transferred to sterile Petri plates inside the laminar air flow chamber. 15-20 ml of melted and cooled medium was poured to the Petri plates carrying 1 ml solution. 10^{-3} and 10^{-4} dilutions were used for isolating fungi in MRBA. The plates were wrapped with cling film and kept for incubation at room temperature. Three replications of each treatment were kept. The plates were observed regularly from 24 hours after incubation. The colonies developed were sub-cultured to a sterile Petri plate with PDA medium before the colony merge with other. The colonies were pure cultured.

The colony characters of the phylloplane fungi were observed and the pathogenicity of the isolates were tested by inoculating seven-day old cultures of the phylloplane fungi on healthy spindle leaves by the same inoculation procedure explained in 3.3. The isolates which are found to be non-pathogenic to coconut leaves were selected for further *in vitro* evaluation.

3.7 *IN VITRO* EVALUATION OF ANTAGONISTIC POTENTIAL OF ISOLATED PHYLLOPLANE MICROFLORA AGAINST MAJOR LRD PATHOGENS

The isolated microfloras were tested for their antagonistic potential against major pathogens of LRD under *in vitro* condition by dual culture technique (Dennis and Webster, 1971) and also by detached spindle leaf assay. The effects of the phylloplane fungi were also compared to the existing biocontrol agent *P. fluorescens* (PN026) and also to Copper oxy chloride (COC) (0.2%).

3.7.1 Direct Antagonism

The direct antagonistic effect of phylloplane fungi against LRD pathogens were tested by dual culture technique. The seven-day old pathogens and phylloplane fungi were used for the assay. For testing the antagonistic effect of fungal microflora against

pathogen, culture discs of 5 mm size were cut out from the actively growing tip of mycelia and were placed opposite to each other with 2 cm distance from the periphery of the Petri plate. The culture disc of pathogen alone at 2 cm away from the periphery of the sterile Petri plate was served as the control. The procedure was followed for all isolated non-pathogenic phylloplane fungi against major leaf rot pathogens.

The same procedure was followed to compare the effect of *P. fluorescens* (PN026) against phylloplane fungi. Instead of PDA medium, 1:1 mixture of PDA and KB (King's B) media were used. Moreover, instead of placing culture disc in the place of fungal antagonist, a bacterial streak was given 2 cm from the periphery of the Petri plate opposite to the pathogen disc. The observations were taken when the control plate of the pathogen attained full growth.

The poisoned food technique was used to compare the dual culture effect of phylloplane fungi with COC. The poisoned food technique was done by mixing 0.2 gram of COC into 50 ml sterile water and mixing the suspension with melted and cooled 50 ml of double strength PDA medium. The poisoned medium was poured into sterile Petri plate and the major pathogens of LRD identified were placed 2 cm away from one of the sides of the Petri plate. The growth of the pathogens was noticed when the pathogen in the control plate achieve full growth and calculated the per cent mycelial inhibition. The per cent mycelial inhibition of pathogens by *P. fluorescens* (PN026) and COC (0.2 %) with that of phylloplane fungi was compared. For each treatment, three replicates were maintained. The plates were kept for incubation at room temperature (30°C). The observations were taken when the growth in the control plate became full. The inhibition zone developed between the pathogen and treatments were measured. The radial growth of pathogen in control and treated plates were recorded to find out the per cent inhibition of pathogen by the phylloplane fungi. The per cent inhibition was calculated using the formula given by Vincent (1927) to find out the effective antagonist.

$$\text{Per cent inhibition} = \frac{(C - T)}{C} \times 100$$

C - Radial mycelial growth of pathogen in control plate (cm);

T - Radial mycelial growth of pathogen in the presence of respective treatment (cm)

3.7.2 Detached spindle leaf assay

The seven-day old cultures of non-pathogenic fungal antagonists were inoculated into the Potato Dextrose broth. The spore count of the one-week old culture broth was adjusted approximately to 10^8 spores / ml using haemocytometer. The healthy spindle leaflets were wiped with 70 per cent ethyl alcohol and the culture broth was sprayed on to the leaflet using a sprayer and allowed to dry. The antagonist sprayed leaflets were inoculated with major pathogens of LRD. The same procedure was followed for the *P. fluorescens* (PN026) with difference in the broth used for inoculating the culture. The King's B broth was used for inoculating *P. fluorescens* (PN026). Copper oxy chloride @ 0.2 per cent was also sprayed on to the leaflets to compare the results of phylloplane fungi. The days taken for symptom initiation, lesion development on fifth, seventh and tenth day were measured. Disease suppression was calculated based on the lesion size measured on 10 DAI using the formula:

$$\text{Per cent disease suppression} = \frac{(C - T)}{C} \times 100$$

C- Lesion length in cm on leaflet 10 DAI (Control);

T- Lesion length in cm on leaflet 10 DAI (Treatment).

The best treatments were found out by comparing the results from dual culture assay as well as detached spindle leaf assay through statistical analysis.

Results

4. RESULTS

The study entitled 'Characterization of fungal pathogen associated with leaf rot disease of coconut (*Cocos nucifera* L.) and *in vitro* evaluation of phylloplane microflora as biocontrol agents' was conducted in the Department of Plant Pathology, College of Agriculture, Vellayani during the year 2018-2020, with the objective to identify and characterize the major fungal pathogens associated with the LRD of coconut and *in vitro* evaluation of phylloplane microflora of coconut against the pathogens. The results of the study are described below:

4.1 ISOLATION OF LEAF ROT DISEASE PATHOGENS OF COCONUT

The pathogens associated with leaf rot disease (LRD) of coconut were isolated from six taluks of Thiruvananthapuram district such as Thiruvananthapuram, Neyyattinkara, Nedumangad, Chirayinkeezhu, Kattakada and Varkala during south-west monsoon of the year 2019 (Plate 1). The samples were collected from three different locations in each taluk and a total of eighteen samples were collected from the district.

The symptoms of the LRD samples were observed as the water soaked brown lesions on the distal end of the spindle leaves which were cemented together whereas the symptoms on the matured leaves as dried leaf rot. In case of the samples collected from the severely infected palm, the dried lesions from the margin of the leaves were blown off by the wind and reduced leaf size was also observed (Plate 2).

The locations from which samples collected and the pathogens isolated from the infected leaves collected from those locations were enlisted in Table 1.

4.1.1 Thiruvananthapuram Taluk

The LRD samples were collected from Kowdiar, Vellayani and Anayara. The infected specimen collected from Kowdiar and Vellayani were harbored the pathogens such as *Colletotrichum gloeosporioides* and *Fusarium* sp. while the leaf rot disease symptom from Anayara was caused by a single pathogen *C. gloeosporioides.*

4.1.2 Neyyattinkara Taluk

The LRD on sample collected from Neyyattinkara was caused by *Fusarium* sp. along with one unidentified pathogen and the sample collected from Balaramapuram was harbored by *C. gloeosporioides* and an unidentified pathogen. *Gliocladium* sp. was the major pathogen isolated from the sample collected from Kanjiramkulam and that was the only *Gliocladium* sp. isolate identified during the study.

4.1.3 Nedumangad Taluk

The LRD samples were collected from three different places Peringamala, Palode and Nedumangad. *C. gloeosporioides* was the pathogen found to be associated with the samples collected from Peringamala and Palode. The sample collected from Nedumangad was harbored by *C. gloeosporioides* and *Fusarium* sp.

4.1.4 Chirayinkeezhu Taluk

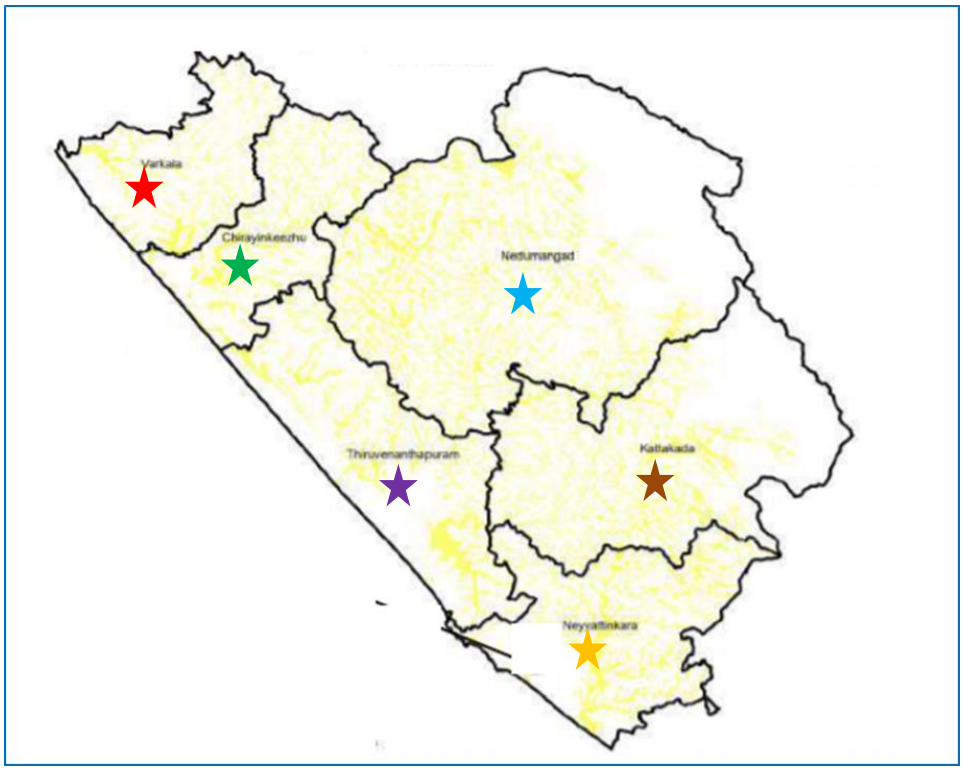
The LRD samples collected from Attingal and Kilimanoor were infected by the pathogen *C. gloeosporioides* and the sample from Alamcode was harbored by *Fusarium* sp.

4.1.5 Varkala Taluk

The sample collected from Varkala was harbored by pathogen *C. gloeosporioides* alone and that of the sample from Akathumuri was combinely infected with *C. gloeosporioides* and *Fusarium* sp. The sample from Kavalayoor was infected with the pathogen *Scytalidium* sp. and that was the only *Scytalidium* sp. identified from the LRD sample collected during the study.

4.1.6 Kattakada Taluk

The samples were collected from Kattakada, Malayinkeezhu and Keezharoor under Kattakada taluk. The sample from Kattakada was harbored with a combination of



Taluks

Thiruvananthapuram

Neyyattinkara

Nedumangad

Chirayinkeezhu

Varkala

Kattakada

Plate 1. District map of Thiruvananthapuram showing taluks from which the LRD samples collected

pathogens *C. gloeosporioides* and *Fusarium* sp. while the LRD in sample collected from Malayinkeezhu was caused by *C. gloeosporioides* alone and the sample from Keezharoor was harbored by *Fusarium* sp. alone.

The observations showed that, the LRD in Thiruvananthapuram district during south-west monsoon was caused by wide spectrum of pathogens such as *C. gloeosporioides*, *Fusarium* spp., *Gliocladium* sp., and *Scytalidium* sp. The disease can occur either by a single pathogen or with multiple pathogens.

The cultural characters of the isolates were observed and the details of *C. gloeosporioides* isolates were given in Table 2 (Plate 3), *Fusarium* spp. isolates in table 3 (Plate 4) and the minor pathogens in table 4 (Plate 5).

Table 1. Fungal pathogens found to be associated with leaf rot disease of coconut in Thiruvananthapuram district

Taluk	Place	Pathogens
Thiruvananthapuram	Kowdiar	*Colletotrichum gloeosporioides* (C1) *Fusarium* sp. (F1)
	Vellayani	*Colletotrichum gloeosporioides* (C2) *Fusarium* sp. (F2)
	Anayara	*Colletotrichum gloeosporioides* (C3)
Neyyattinkara	Neyyattinkara	*Fusarium* sp. (F3) Unidentified (U1)
	Balaramapuram	*Colletotrichum gloeosporioides* (C4) Unidentified (U2)
	Kanjiramkulam	*Gliocladium* sp. (G1)
Nedumangad	Peringamala	*Colletotrichum gloeosporioides* (C5)
	Palode	*Colletotrichum gloeosporioides* (C6)
	Nedumangad	*Colletotrichum gloeosporioides* (C7) *Fusarium* sp. (F4)
Chirayinkeezhu	Attingal	*Colletotrichum gloeosporioides* (C8)
	Alamcode	*Fusarium* sp. (F5)
	Kilimanoor	*Colletotrichum gloeosporioides* (C9)
Varkala	Varkala	*Colletotrichum gloeosporioides* (C10)
	Akathumuri	*Colletotrichum gloeosporioides* (C11) *Fusarium* sp. (F6)
	Kavalayoor	*Scytalidium* sp. (S1)
Kattakada	Kattakada	*Colletotrichum gloeosporioides* (C12) *Fusarium* sp. (F7)
	Malayinkeezhu	*Colletotrichum gloeosporioides* (C13)
	Keezharoor	*Fusarium* sp. (F8)

a)

Kowdiar

Vellayani

Anayara

b)

Neyyattinkara

Balaramapuram

Kanjiramkulam

c)

Peringamala

Palode

Nedumangad

Plate 2. LRD samples collected from different taluks of Thiruvananthapuram district a) Thiruvananthapuram b) Neyyattinkara c) Nedumangad

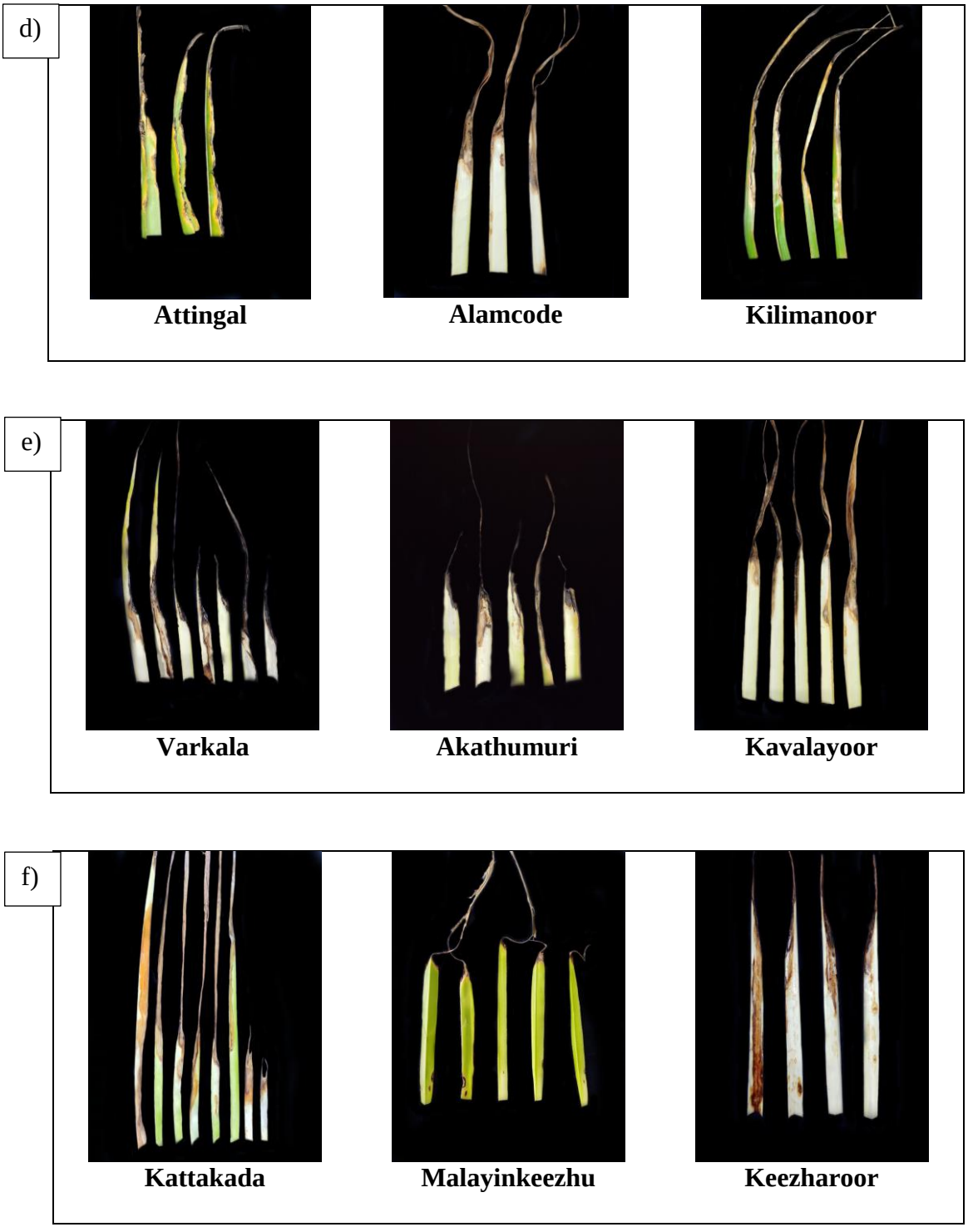


Plate 2. LRD samples collected from different taluks of Thiruvananthapuram district d) Chirayinkeezhu e) Varkala f) Kattakada

Table 2. Cultural characters of *C. gloeosporioides* isolated from LRD samples

Isolates	Colony morphology	Colony colour		Colour of mycelium
		Front view	Rear view	
C1	Dense cottony appearance	Off white	White to off white	Greyish white
C2	Dense cottony appearance	Off white to grey	Grey	White
C3	Dense cottony appearance	White	Off white to grey	Greyish white
C4	Dense cottony appearance	Grey	Grey	Greyish white
C5	Dense cottony appearance	Grey	Dark green	Greyish white
C6	Dense cottony appearance	Off white to grey	White to grey	Greyish white
C7	Dense cottony appearance	White	Off white to dark green	White
C8	Dense cottony appearance	Off white to grey	Grey	Greyish white
C9	Dense cottony appearance	Grey	Grey to dark green	Greyish white
C10	Dense cottony appearance	Grey	Grey to dark green	Greyish white
C11	Dense cottony appearance	Off white to grey	Grey	White
C12	Dense cottony appearance	White to off white	White to off white	White
C13	Dense cottony appearance	Off white	Off white to grey	Greyish white

Table 3. Cultural characters of *Fusarium* spp. isolated from LRD samples

Isolates	Colony morphology	Colony colour		Colour of mycelium
		Front view	Rear view	
F1	Sparse	White	Pink	White
F2	Dense cottony appearance	White	Off white	White
F3	Dense cottony appearance	White	Light yellow	White
F4	Dense cottony appearance	White	Light yellow	White
F5	Sparse	Off white	Off white	White
F6	Sparse	Off white	Off white	White
F7	Sparse	White	Off white	White
F8	Dense cottony appearance	White	Light yellow	White

Table 4. Cultural characters of minor pathogens isolated from LRD samples

Isolates	Colony morphology	Colony colour		Colour of mycelium
		Front view	Rear view	
G1	Fluffy	White to salmon	White to yellow	White
S1	Sparse	Black	Black	Black and white
U1	Fluffy	Off white	Off white	White
U2	Fluffy	Off white	Off white	White

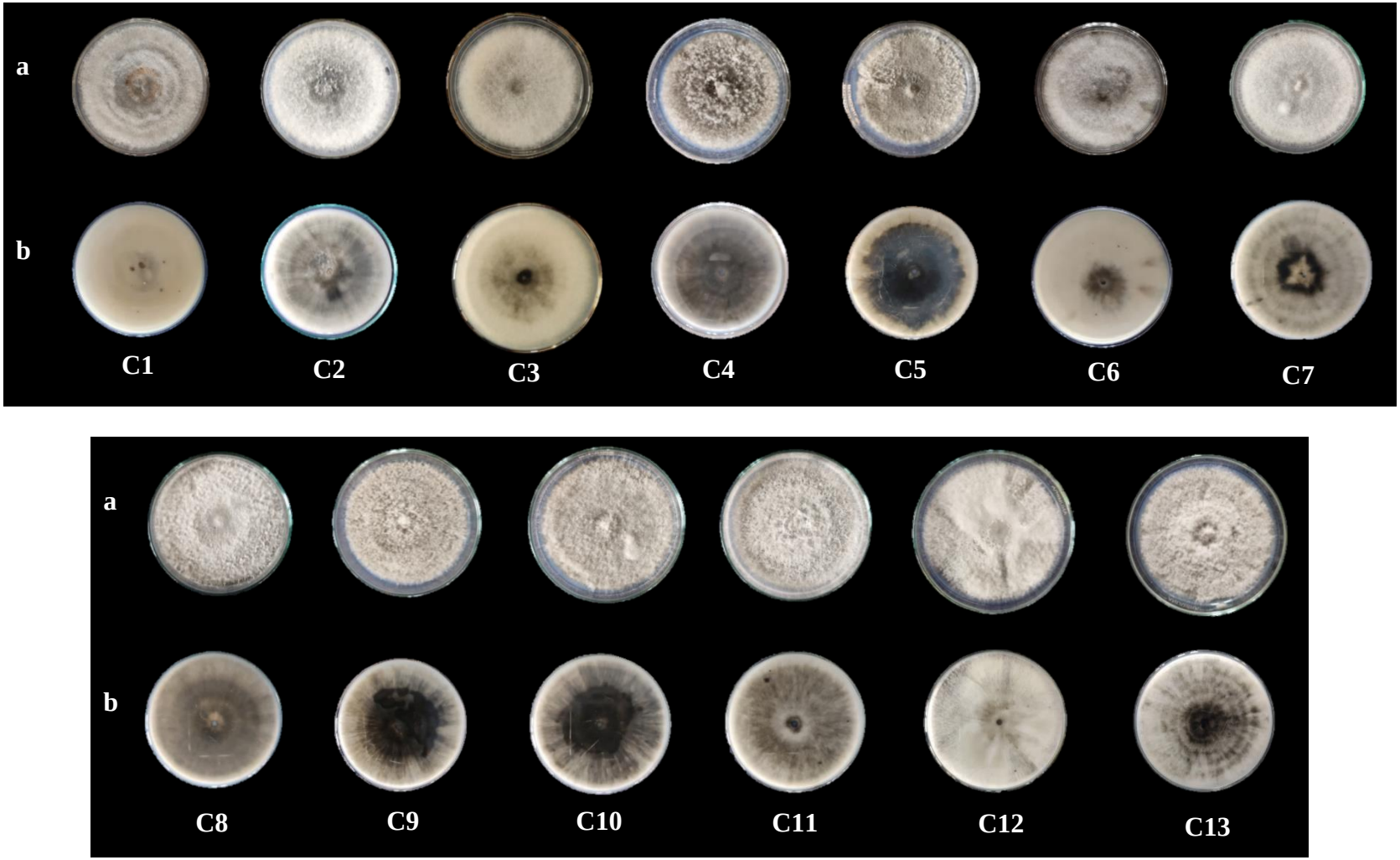


Plate 3. Cultural characters of *C. gloeosporioides* isolated from LRD samples (a) Front view (b) Rear view of isolates C1 –C13

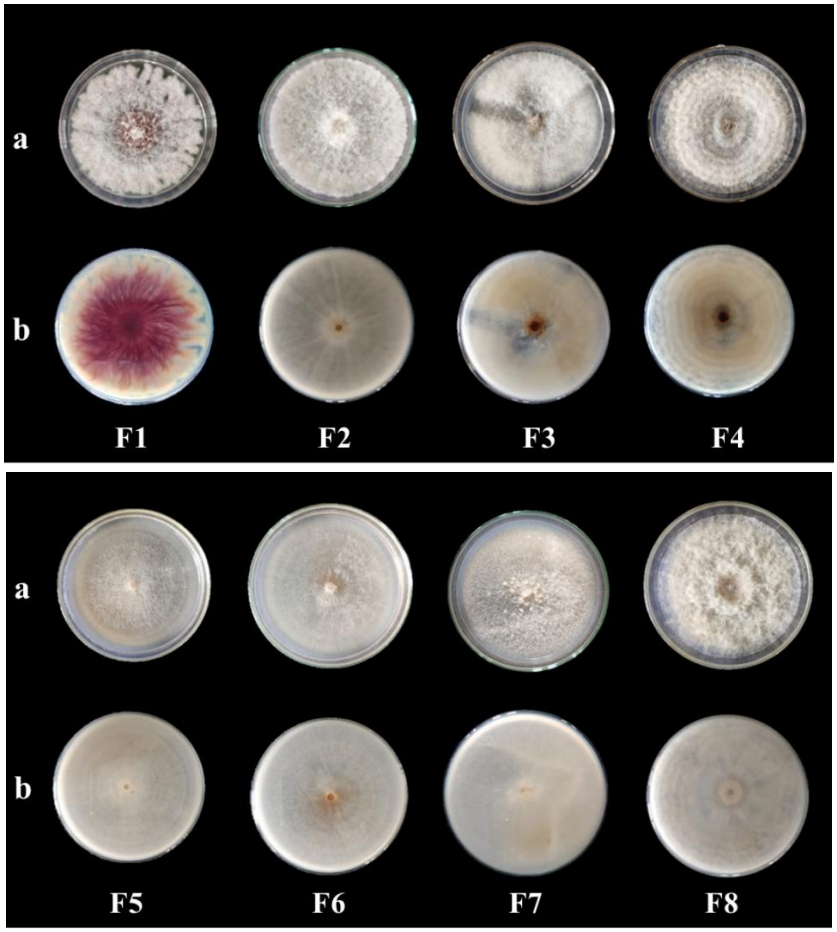


Plate 4. Cultural characters of *Fusarium* spp. isolated from LRD samples (a) Front view (b) Rear view of isolates F1-F8.

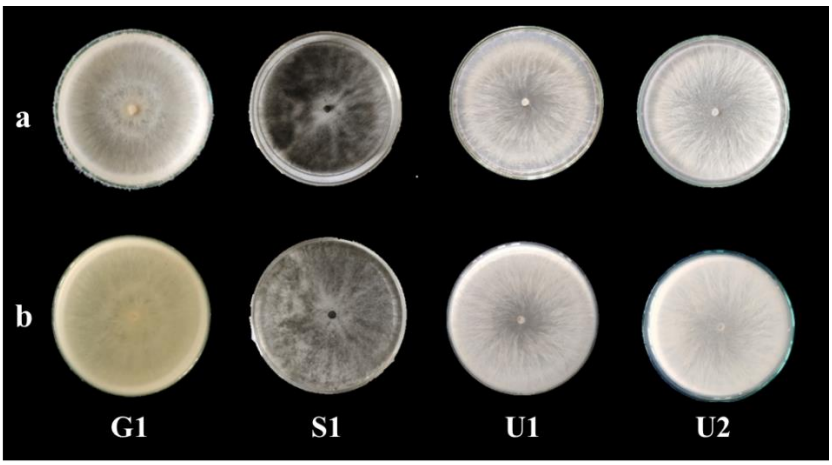


Plate 5. Cultural characters of minor pathogens isolated from LRD samples (a) Front view (b) Rear view of isolates G1, S1, U1 and U2

4.2 PURIFICATION, IDENTIFICATION AND CHARACTERIZATION OF PATHOGENIC MICROFLORA

Cultures of *C. gloeosporioides, Fusarium* spp., *Scytalidium* sp. and *Gliocladium* sp. were purified by hyphal tip culture method and were maintained on PDA slants for further study. The isolated pathogens were identified and characterized based on cultural characters (Table 2, Table 3, Table 4) and microscopic characters. The spore characters of *C. gloeosporioides* isolates, *Fusarium* spp. isolates and minor pathogens of LRD were described in Table 5, Table 6 and Table 7 respectively. The radial growth of pathogens on the PDA medium was observed on third, fifth and seventh day after culturing till the complete growth occur and the details of *C. gloeosporioides*, *Fusarium* spp. and other minor pathogens were shown in Table 8, Table 9 and Table 10 respectively. Based on all these observations, the identified LRD pathogens were described as below:

4.2.1 *Colletotrichum gloeosporioides* (Penzig) Penzig and Sacc.

The culture of *C. gloeosporioides* had dense cottony appearance on PDA medium. The colour of the colony was varied from white to grey colour on front view and white to dark green colour on the rear side based on the isolate. The aerial mycelia are white to greyish white with hyaline, septate and branched hyphae. Individually the conidia are hyaline, straight, single celled, slightly narrower at the middle with rounded ends/ cylindrical and oil globule at the centre (Plate 6) but they appeared as orange coloured spore masses on Petri plate. They produced melanised appressoria with irregular shape. The size of the conidia varied from 12.23 X 3.07 µm to 16.76 X 4.36 µm (Table 5). All the *C. gloeosporioides* isolates were found to be covering entire Petri plate within 10 days after culturing (Table 8).

Table 5. Spore characters of *C. gloeosporioides* isolates

Isolates	Spore shape	Spore size (µm)*
C1	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	16.76 ± 0.08 X 4.36 ± 0.04
C2	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	14.20 ± 0.86 X 4.12 ± 0.04
C3	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	14.96 ± 0.62 X 3.46 ± 0.17
C4	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	12.80 ± 0.15 X 3.96 ± 0.09
C5	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	16.33 ± 0.23 X 4.08 ± 0.14
C6	Conidia hyaline, aseptate, single celled, straight, cylindrical shape and oil globule at the centre	13.93 ± 0.08 X 3.50 ± 0.14
C7	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	15.46 ± 0.45 X 3.32 ± 0.12
C8	Conidia hyaline, aseptate, single celled, straight, cylindrical shape and oil globule at the centre	12.40 ± 0.26 X 3.12 ± 0.11
C9	Conidia hyaline, aseptate, single celled, straight, cylindrical shape and oil globule at the centre	15.10 ± 0.02 X 3.95 ± 0.06
C10	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	12.23 ± 0.35 X 3.07 ± 0.10
C11	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	13.20 ± 0.50 X 3.02 ± 0.16
C12	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	15.26 ± 0.52 X 3.87 ± 0.05
C13	Conidia hyaline, aseptate, straight, slightly narrower in the middle with rounded ends and oil globule at the centre	13.23 ± 0.32 X 3.64 ± 0.07

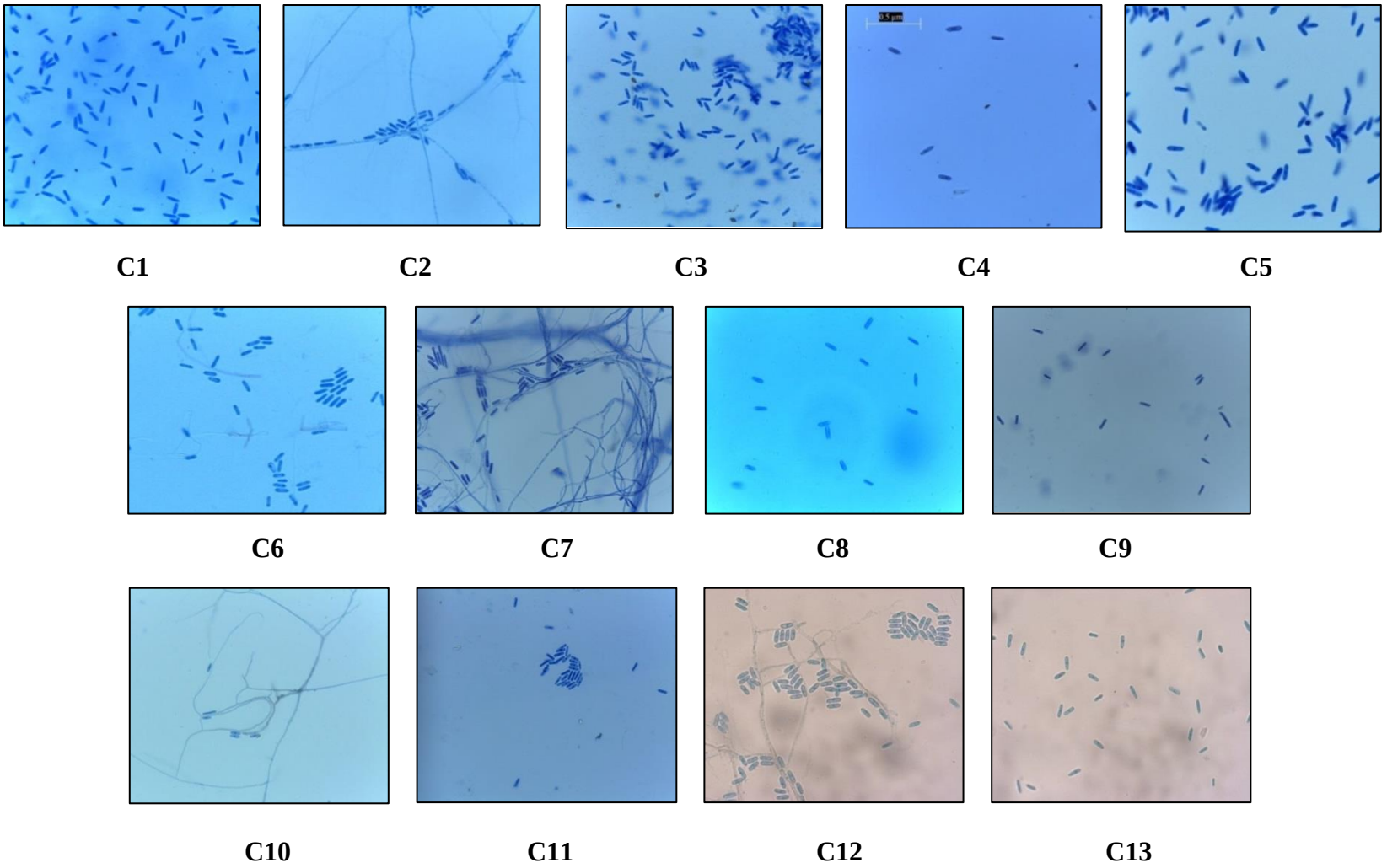


Plate 6. Spore characters of *C. gloeosporioides* isolates C1 – C13

4.2.2 *Fusarium* spp.

Some of the isolates of *Fusarium* spp. had sparse growth and others had dense cottony appearance on the PDA medium. The colony colour varied from white to off white on upper side of the Petri plate and off white to light yellow and pink on the rear side of the culture plate. They produced hyaline, septate and branched mycelia with macro and micro spores. The macro conidia are hyaline, septate, sickle shaped and 2-4 celled while the micro conidia are hyaline, single celled with clavate shape (Plate 7). The length of macro conidia varied from 14.80 µm to 24.33 µm and the length of micro conidia varied from 6.12 µm to 7.84 µm (Table 6). All the isolates except F1 covered the entire Petri plate within 10 DAI (Table 9).

4.2.3 *Gliocladium* sp.

Initially the upper side of the Petri plate showed the whitish growth of the pathogen later turns to salmon colour while the rear side of the plate showed light yellow colour. The fungus was a fast grower with fluffy appearance on PDA medium and completely covered the 9 cm Petri plate within 5 days after culturing (Table 10). The fungus consists of distinct conidiophores which are hyaline and septate with penicillate branches and ends with specialized structures known as phialides. Conidia are hyaline, single celled, oval to round in shape and appeared as chains from the phialides (Plate 8). The average size of the conidia is 1.09 µm (Table 7).

4.2.4 *Scytalidium* sp.

The fungal culture was observed as sparse, black colonies on both the sides of the Petri plate. They produced two types of mycelia; one is brown coloured, thick and septate; and the other is narrow, hyaline and septate. The pathogen was a fast grower which completed the full growth in 9 cm Petri plate within 4 days after culturing (Table 10). The fungus produced two types of conidia with catenate arrangement. Cylindrical, thin walled and hyaline conidia with 10.4 µm X 4.75 µm size and brown, thick walled

and broader conidia with 11.0 µm X 6.61 µm size (Table 7). The morphological and microscopic characters of the *Scytalidium* sp. were given in Plate 8.

Table 6. Spore characters of *Fusarium* spp. isolates

Isolates	Spore shape	Spore size (µm)*
F1	Single celled hyaline conidia with clavate shape	6.12 ± 0.01 X 1.69 ± 0.09
F2	Two celled and four celled macroconidia with pointed ends	15.10 ± 0.26 X 1.95 ± 0.16
F3	Four celled macroconidia with pointed ends	24.33 ± 0.54 X 4.04 ± 0.11
F4	Four celled macroconidia with pointed ends	16.70 ± 0.06 X 2.75 ± 0.07
F5	Single celled conidia with pointed ends	7.84 ± 0.13 X 2.33 ± 0.06
F6	Single celled conidia with pointed ends	6.13 ± 0.47 X 2.17 ± 0.12
F7	Two celled conidia with pointed ends	14.8 ± 0.28 X 2.53 ± 0.05
F8	Two celled and four celled macroconidia with pointed ends	19.00 ± 0.15 X 2.63 ±0.07

*Mean ± SD of three replications

Table 7. Spore characters of minor pathogens of LRD

Isolates	Spore characters	Spore size (µm)*
G1	Round shaped conidia with *Penicillium* like conidiogenous cells	1.06 ± 0.09
S1	Catenate arrangement of ovular shaped spores alternate with brown coloured and hyaline spores	11.0 X 6.61 10.4 X 4.75
U1	No spore	-
U2	No spore	-

*Mean ± SD of three replications

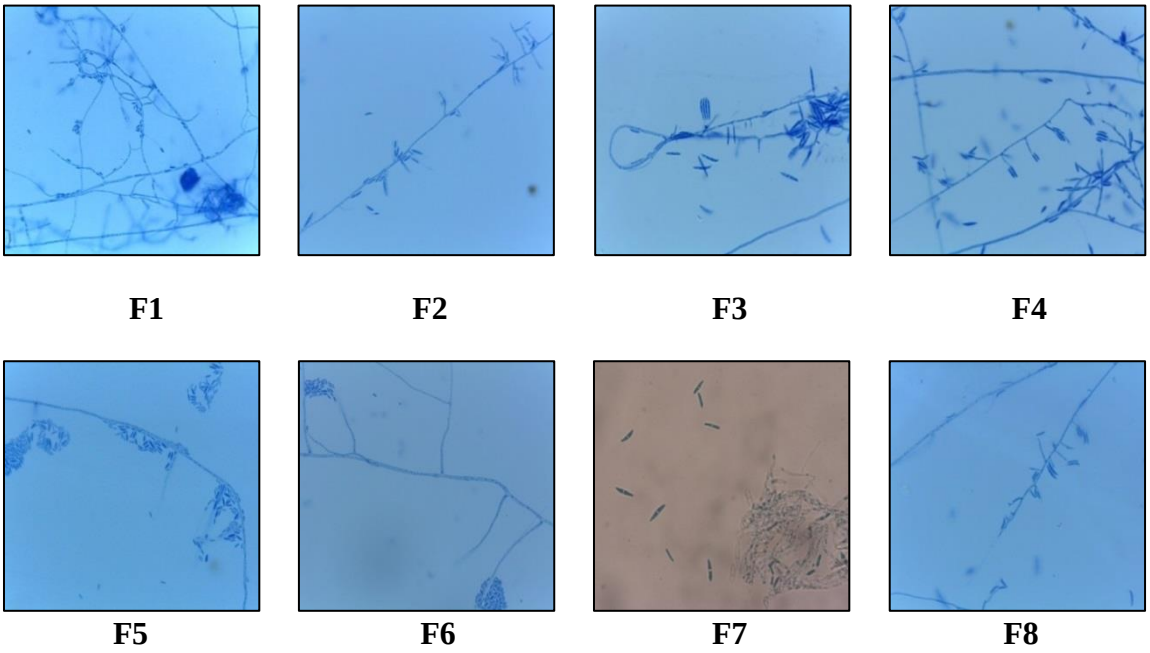


Plate 7. Spore characters of *Fusarium* spp. isolates: F1 –F8

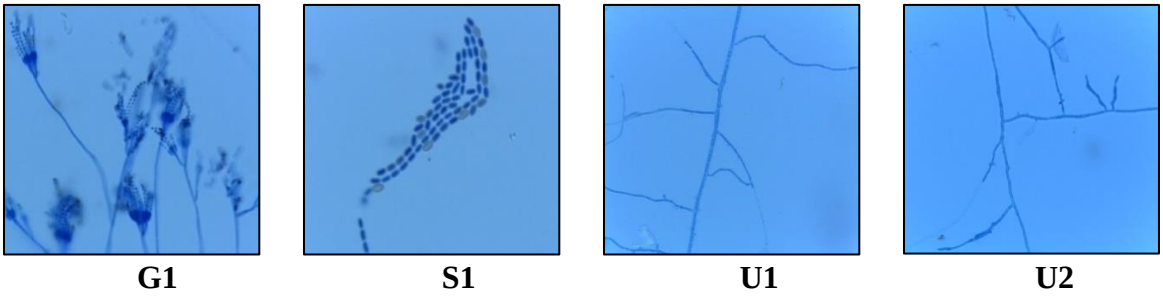


Plate 8. Microscopic characters of minor LRD pathogens

Table 8. Growth of different *C. gloeosporioides* isolates on PDA medium

Isolates	Radial growth (cm)*			Growth rate on 7th day (cm/day)
	3rd day	5th day	7th day	
C1	3.50 ± 0.06	5.56 ± 0.03	8.46 ± 0.08	1.20
C2	3.13 ± 0.03	5.06 ± 0.03	7.60 ± 0.17	1.08
C3	3.83 ± 0.03	5.93 ± 0.09	8.73 ± 0.06	1.24
C4	3.60 ± 0.06	5.66 ± 0.03	8.36 ± 0.03	1.19
C5	3.33 ± 0.03	5.40 ± 0.06	7.70 ± 0.12	1.10
C6	3.66 ± 0.03	5.70 ± 0.06	8.46 ± 0.08	1.20
C7	2.93 ± 0.03	5.06 ± 0.07	7.93 ± 0.09	1.13
C8	2.96 ± 0.07	5.06 ± 0.03	7.73 ± 0.08	1.10
C9	3.46 ± 0.07	5.46 ± 0.08	8.30 ± 0.10	1.18
C10	3.26 ± 0.09	5.40 ± 0.06	8.13 ± 0.08	1.16
C11	3.73 ± 0.03	5.73 ± 0.03	8.56 ± 0.08	1.22
C12	3.26 ± 0.08	5.03 ± 0.09	7.36 ± 0.09	1.05
C13	2.93 ± 0.09	5.10 ± 0.12	7.60 ± 0.06	1.08

*Mean ± SD of three replications

Table 9. Growth of different *Fusarium* spp. isolates on PDA medium

Isolates	Radial growth (cm)*			Growth rate on 7th day (cm/day)
	3rd day	5th day	7th day	
F1	2.06 ± 0.03	4.36 ± 0.03	5.73 ± 0.03	0.82
F2	2.53 ± 0.08	5.36 ± 0.03	7.13 ± 0.03	1.01
F3	2.76 ± 0.03	5.73 ± 0.03	7.23 ± 0.03	1.03
F4	2.93 ± 0.03	5.00 ± 0.06	6.60 ± 0.06	0.94
F5	3.23 ± 0.03	6.13 ± 0.08	8.26 ± 0.03	1.18
F6	3.03 ± 0.03	5.80 ± 0.06	7.93 ± 0.03	1.13
F7	3.10 ± 0.06	5.76 ± 0.06	7.40 ± 0.06	1.05
F8	2.53 ± 0.03	4.70 ± 0.06	7.23 ± 0.08	1.03

*Mean ± SD of three replications

Table 10. Growth of minor pathogens on PDA medium

Isolates	Radial growth (cm)*			Growth rate on 3rd day (cm/day)
	3rd day	5th day	7th day	
U1	6.88 ± 0.04	9.0	9.0	2.29
U2	6.88 ± 0.06	9.0	9.0	2.29
G1	6.65 ± 0.02	9.0	9.0	2.21
S1	7.42 ± 0.04	9.0	9.0	2.47

*Mean ± SD of three replications

4.3 TESTING THE PATHOGENICITY OF ISOLATED MICROFLORA

The pathogenicity was proved by Koch's postulates. Altogether five different fungi isolated from various locations of Thiruvananthapuram district were found to be pathogenic in causing leaf rot disease on healthy spindle leaflets of coconut. The average time taken for initiation of symptoms by pathogens varied from one to four days when inoculated by pin pricking. The time taken for symptom initiation was least for the pathogen *Scytalidium* sp. and more for *Gliocladium* sp. which took one and five days respectively for symptom initiation. Average time taken for symptom initiation for *C. gloeosporioides* and *Fusarium* varied from two to three days. All those pathogens were re-isolated from the artificially inoculated leaflets and the pathogenicity was proved. The virulent isolates among the major pathogens *C. gloeosporioides* and *Fusarium* spp. were identified by observing the lesion size developed by each isolate on 5^{th}, 7^{th} and 10^{th} day after inoculation (DAI).

4.3.1 Virulence testing of *C. gloeosporioides* isolates on healthy spindle leaves

Altogether thirteen *C. gloeosporioides* isolates were obtained from the LRD infected coconut palms from different regions of Thiruvananthapuram district (Table 1). The relative virulence of those isolates was assessed by inoculating them on the unopened healthy spindle leaflets by pin pricking.

The observation on the days taken for the initiation of symptom and the lesion size developed by each *C. gloeosporioides* isolate on 5^{th}, 7^{th} and 10^{th} DAI were described in Table 11. This data indicated that most of the isolates of *C. gloeosporioides* started infection on 2^{nd} DAI while the isolates C5, C7 and C12 started infection on 3^{rd} and the isolate C10 on 4^{th} DAI.

There was variation in the size of lesion produced on spindle leaflets of coconut by different isolates of *C. gloeosporioides* (Plate 9). Maximum lesion size of 6.43 cm X 1.53 cm (10 DAI) produced by isolate C3 and the least lesion size was observed for

isolate C5 (4.06 cm X 1.16 cm). All other isolates produced lesion size ranging from 4.0 cm to 6.0 cm on inoculated healthy spindle leaflet.

Based on the virulence studies, the isolate C3 from Anayara (Thiruvananthapuram taluk) was chosen as the test pathogen of *C. gloeosporioides* for further studies. So it was also inferred from this experiment that the spore size hasn't any significance in virulence of the pathogen, where greater spore size among *C. gloeosporioides* was for isolate C1 from Kowdiar (Thiruvananthapuram taluk).

Table 11. Lesion size produced by *C. gloeosporioides* isolates on healthy spindle leaflets

Isolates	DTSA*		Lesion size (cm)*		
			5 DAI	7 DAI	10 DAI
C1	2	Length (cm)	1.96 ± 0.06^{ab}	2.93 ± 0.12^{bcde}	5.23 ± 0.14^{cde}
		Breadth (cm)	0.53 ± 0.01	0.70 ± 0.07	1.40 ± 0.06
C2	2	Length (cm)	1.26 ± 0.08^{ef}	2.33 ± 0.12^{f}	4.23 ± 0.18^{f}
		Breadth (cm)	0.43 ± 0.03	0.93 ± 0.08	1.26 ± 0.14
C3	2	Length (cm)	2.00 ± 0.04^{ab}	3.40 ± 0.12^{a}	6.43 ± 0.12^{a}
		Breadth (cm)	0.53 ± 0.03	0.78 ± 0.02	1.53 ± 0.12
C4	2	Length (cm)	1.56 ± 0.03^{cde}	3.23 ± 0.14^{abc}	5.03 ± 0.08^{de}
		Breadth (cm)	0.53 ± 0.03	1.10 ± 0.15	1.56 ± 0.17
C5	3	Length (cm)	0.96 ± 0.08^{fg}	1.93 ± 0.08^{f}	4.06 ± 0.12^{f}
		Breadth (cm)	0.35 ± 0.05	0.46 ± 0.03	1.16 ± 0.17
C6	2	Length (cm)	1.93 ± 0.12^{ab}	3.26 ± 0.12^{bcd}	5.50 ± 0.32^{bcd}
		Breadth (cm)	0.46 ± 0.03	0.62 ± 0.07	1.36 ± 0.18

C7	3	Length (cm)	0.96 ± 0.20^{fg}	1.90 ± 0.17^{e}	4.86 ± 0.18^{e}
		Breadth (cm)	0.43 ± 0.03	0.50 ± 0.06	1.10 ± 0.12
C8	2	Length (cm)	1.90 ± 0.23^{abc}	2.83 ± 0.17^{de}	5.46 ± 0.12^{bcd}
		Breadth (cm)	0.40 ± 0.02	0.78 ± 0.04	1.43 ± 0.17
C9	2	Length (cm)	1.90 ± 0.12^{abc}	3.23 ± 0.12^{abc}	5.76 ± 0.14^{b}
		Breadth (cm)	0.55 ± 0.02	1.13 ± 0.20	2.00 ± 0.20
C10	4	Length (cm)	0.8 ± 0.12^{g}	1.63 ± 0.13^{g}	4.93 ± 0.14^{e}
		Breadth (cm)	0.31 ± 0.01	0.43 ± 0.02	0.83 ± 0.18
C11	2	Length (cm)	1.66 ± 0.14^{bcd}	2.7 ± 0.12^{b}	5.73 ± 0.08^{b}
		Breadth (cm)	0.45 ± 0.02	0.83 ± 0.08	1.46 ± 0.08
C12	3	Length (cm)	2.03 ± 0.08^{a}	3.20 ± 0.12^{abcd}	5.66 ± 0.06^{bc}
		Breadth (cm)	0.58 ± 0.04	1.43 ± 0.12	1.90 ± 0.15
C13	2	Length (cm)	1.36 ± 0.08^{de}	2.86 ± 0.14^{cde}	4.10 ± 0.20^{f}
		Breadth (cm)	0.43 ± 0.03	0.90 ± 0.15	1.00 ± 0.12
SE (m)			0.045	0.052	0.079
CD(0.05)			0.358	0.384	0.472

*Mean ± SD of three replications

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

*DTSA- Days taken for symptom appearance

4.3.2 Virulence testing of *Fusarium* spp. isolates on healthy spindle leaves

The data on days taken for symptom initiation of *Fusarium* spp. isolates on detached spindle leaves and the lesion developed by each isolate on 5^{th}, 7^{th} and 10^{th} DAI are shown in Table 12. This data indicated that majority of the *Fusarium* spp. isolates initiated the symptom development at 2 DAI and while the rest of the isolates F1 and F8 initiated the symptom development at 3 DAI. There was variation in the size of lesion produced by each isolate of *Fusarium* spp. (Plate 10). The maximum lesion size developed on inoculated leaflet at 10 DAI was observed in the case of F5 isolate with lesion size 7.06 cm X 2.16 cm and the least lesion size was produced by the isolate F1 with 5.03 cm X 1.76 cm.

Based on the virulence studies, the isolate F5 from Alamcode (Chiraayinkeezhu) was chosen as the test pathogen for *Fusarium* spp. for further experiments. While comparing the cultural characters and virulence of the pathogens, it was also inferred that the pigmentation of the pathogen hasn't any significance in virulence of the culture.

The data on lesion size developed by minor pathogens on healthy spindle leaves of coconut (Table 13) gave an inference that the most virulent pathogen among the five different pathogen isolates was isolate S1, *Scytalidium* sp. from Kavalayoor with average lesion size of 13.0 cm X 2.0 cm at 10 DAI and least virulent isolate was isolate G1, *Gliocladium* sp. from Kanjiramkulam with average lesion size of 3.0 cm X 0.7 cm at 10 DAI (Plate 11).

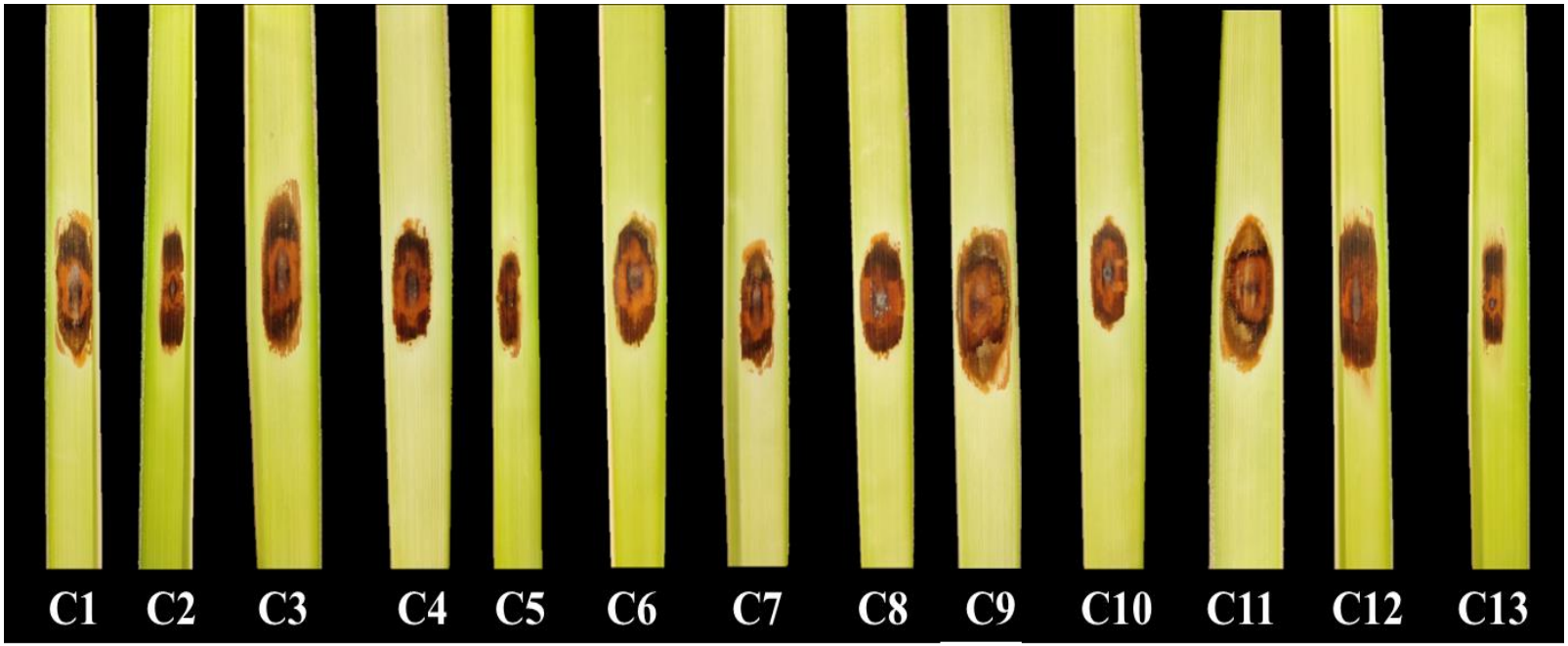


Plate 9. Symptom appearance on artificial inoculation of different isolates of *C. gloeosporioides* on coconut leaves 10 days after inoculation (10DAI)

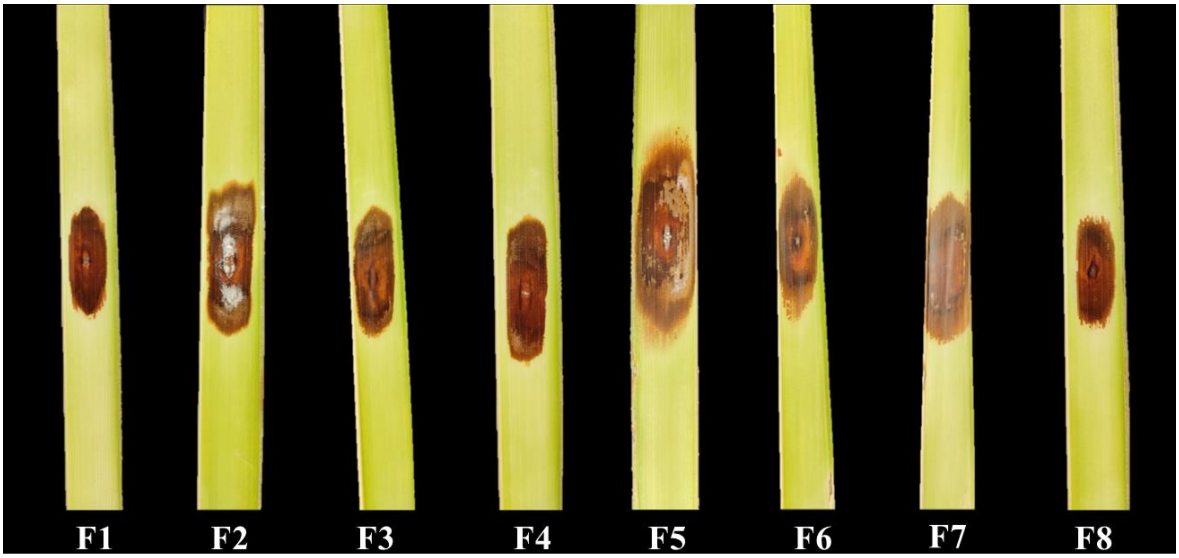


Plate 10. Symptom appearance on artificial inoculation of different isolates of *Fusarium* on coconut leaves 10 days after inoculation (10DAI)

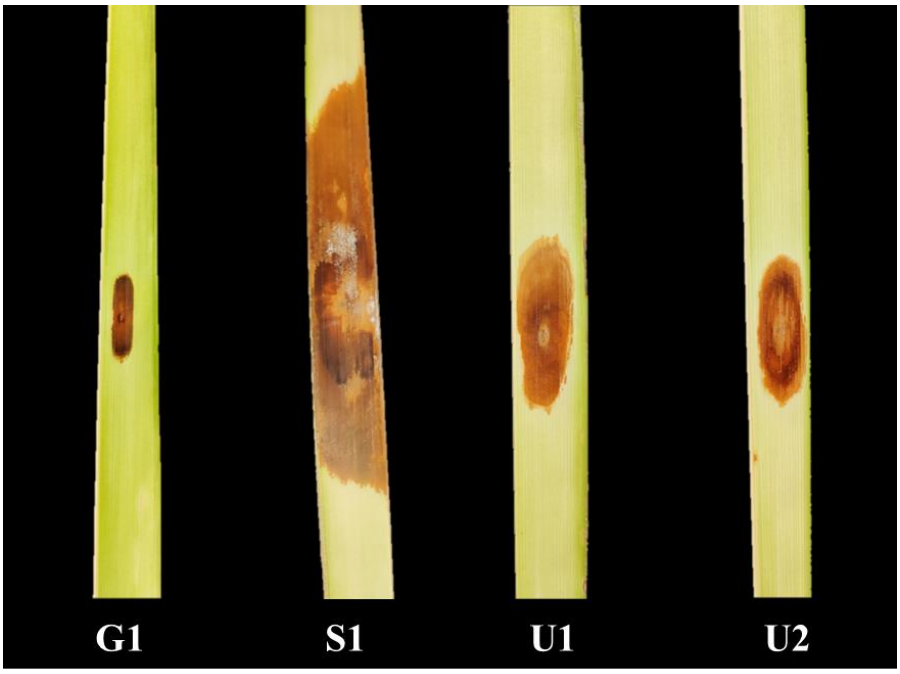


Plate 11. Symptom appearance on artificial inoculation of minor pathogens on healthy spindle leaflets 10 DAI

Table 12. Lesion size produced by *Fusarium* spp. isolates on healthy spindle leaflets

Isolates	DTSA*		Lesion size (cm)*		
			5 DAI	7 DAI	10 DAI
F1	3	Length (cm)	1.66 ± 0.16^{c}	3.30 ± 0.15^{b}	5.03 ± 0.26^{d}
		Breadth(cm)	0.48 ± 0.04	0.90 ± 0.06	1.76 ± 0.08
F2	2	Length (cm)	2.13 ± 0.03^{ab}	4.33 ± 0.18^{a}	6.76 ± 0.17^{a}
		Breadth(cm)	0.55 ± 0.03	1.16 ± 0.08	2.03 ± 0.12
F3	2	Length (cm)	2.10 ± 0.10^{ab}	4.33 ± 0.14^{a}	6.13 ± 0.22^{bc}
		Breadth(cm)	0.56 ± 0.06	1.40 ± 0.06	1.93 ± 0.08
F4	2	Length (cm)	2.10 ± 0.12^{ab}	3.10 ± 0.06^{b}	6.63 ± 0.18^{ab}
		Breadth(cm)	0.60 ± 0.06	1.33 ± 0.08	1.90 ± 0.12
F5	2	Length (cm)	2.36 ± 0.08^{a}	4.60 ± 0.17^{a}	7.06 ± 0.17^{a}
		Breadth(cm)	0.66 ± 0.03	1.60 ± 0.15	2.16 ± 0.23
F6	2	Length (cm)	2.00 ± 0.06^{bc}	2.63 ± 0.08^{c}	6.06 ± 0.24^{bc}
		Breadth(cm)	0.53 ± 0.03	1.03 ± 0.08	1.80 ± 0.10
F7	2	Length (cm)	0.96 ± 0.14^{d}	3.43 ± 0.14^{b}	6.16 ± 0.23^{bc}
		Breadth(cm)	0.35 ± 0.03	0.83 ± 0.08	1.56 ± 0.12
F8	3	Length (cm)	1.66 ± 0.16^{c}	3.40 ± 0.10^{b}	5.93 ± 0.08^{c}
		Breadth(cm)	0.45 ± 0.03	1.16 ± 0.12	1.70 ± 0.06
SE (m)			0.042	0.057	0.12
CD(0.05)			0.355	0.412	0.599

*Mean ± SD of three replications

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

*DTSA- Days taken for symptom appearance

Table 13. Lesion size produced by minor pathogens on healthy spindle leaflets

Isolates	DTSA*		Lesion size (cm)*		
			5 DAI	7 DAI	10 DAI
G1	5	Length (cm)	0.31 ± 0.03	1.05 ± 0.09	3.00 ± 0.13
		Breadth(cm)	0.18 ± 0.03	0.75 ± 0.41	0.70 ± 0.04
S1	1	Length (cm)	4.27 ± 0.10	7.55 ± 0.10	13.0 ± 0.15
		Breadth(cm)	1.25 ± 0.08	1.88 ± 0.10	2.00 ± 0.07
U1	2	Length (cm)	1.65 ± 0.08	3.37 ± 0.08	4.52 ± 0.06
		Breadth(cm)	0.51 ± 0.04	1.02 ± 0.08	1.45 ± 0.08
U2	2	Length (cm)	2.17 ± 0.12	4.05 ± 0.08	6.02 ± 0.15
		Breadth(cm)	1.00 ± 0.07	1.22 ± 0.08	2.00 ± 0.15

*Mean ± SD of four replications

*DTSA- Days taken for symptom appearance

4.4 SYMPTOMATOLOGY

The symptomatology was studied by artificial inoculation of pathogens on the detached spindle leaflets. Initially the symptom appeared as water soaked brown tiny lesion which expanded in length and breadth wise; gradually turned to dark brown and covered large area of the leaf surface. All the isolates produced similar type of symptom with alteration in the size of the lesion and time taken for symptom development. The symptom produced by different pathogens such as *C. gloeosporioides, Fusarium* sp., *Gliocladium* sp., *Scytalidium* sp. and unidentified pathogen were shown in Plate 12.

In case of *Fusarium* isolates, the lesion was covered by plenty of white mycelia of the pathogen and in case of *C. gloeosporioides* isolates, orange coloured spore masses of the pathogen was observed on the lesion in addition to the white mycelial growth (Table 14).

Table 14. Nature of symptom developed by different pathogens on artificial inoculation

Pathogens	Nature of lesion	Colour of lesion
C. gloeosporioides	Appearance of orange coloured spore masses and white mycelial growth over the water soaked lesion	Brown
Fusarium sp.	Water soaked lesion with white mycelial growth	Brown
Gliocladium sp.	Water soaked lesion with white mycelial growth	Brown
Scytalidium sp.	Spindle shaped water soaked lesion later spread length and breadth wise	Brown
Unidentified pathogen	Water soaked lesion	Brown

4.5 COMBINED INOCULATION OF MAJOR LRD PATHOGENS ON DETACHED SPINDLE LEAVES AND RE-ISOLATION OF PATHOGEN TO CONFIRM THE PATHOGENECITY

The observations on the combined inoculation of the major pathogens identified (*C. gloeosporioides* - C3 isolate and *Fusarium* sp. - F5 isolate) on detached spindle leaf lets indicated that the symptom development initiated 24 hours after inoculation itself and the size of the lesion produced during combined inoculation was found to be larger than that of the individual inoculation (Plate 13) and the inoculated leaf became completely rot at 10 DAI (Table 15). The re-isolation of the inoculated sample indicated that the same pathogens were found to be associated with the symptom development.

Table 15. Comparison of combined inoculation of major pathogens with individual inoculation

Isolates	DTSA*		Lesion size (cm)*		
			5 DAI	7 DAI	10 DAI
C3	2	Length (cm)	2.00 ± 0.04	3.42 ± 0.08	6.43 ± 0.12
		Breadth(cm)	0.55 ± 0.03	0.76 ± 0.02	1.53 ± 0.12
F5	2	Length (cm)	2.35 ± 0.06	4.62 ± 0.12	7.06 ± 0.17
		Breadth(cm)	0.62 ± 0.04	1.70 ± 0.10	2.16 ± 0.23
Combined inoculation of C3 and F5	1	Length (cm)	6.40 ± 0.14	13.22 ± 0.1	Complete rot
		Breadth(cm)	1.90 ± 0.04	2.55 ± 0.06	Complete rot
Control	-	Length (cm)	0.00	0.00	0.00
		Breadth(cm)	0.00	0.00	0.00

*Mean ± SD of four replications

*DTSA- Days taken for symptom appearance

Plate 12. Symptom produced by different pathogens by artificial inoculation on detached spindle leaflets

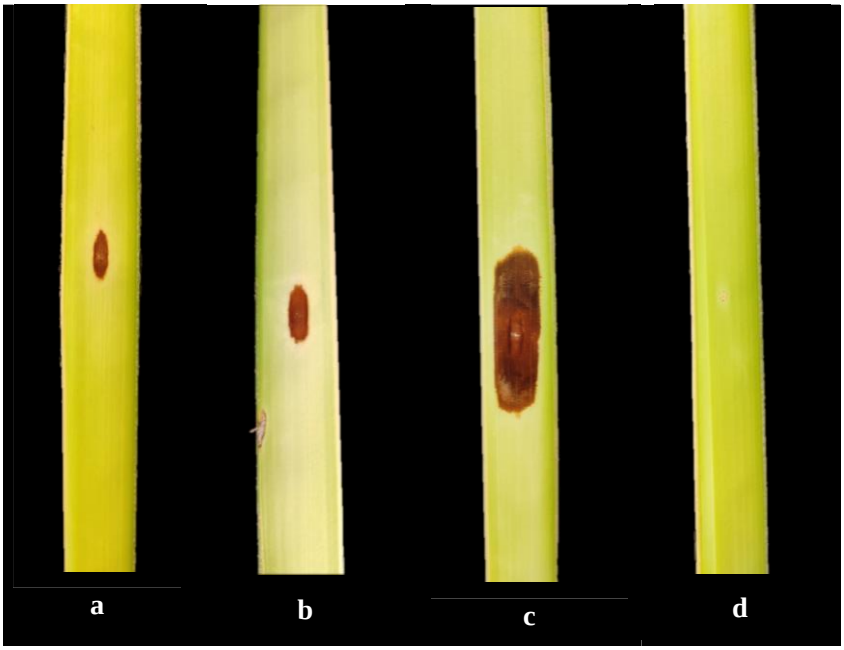


Plate 13. Symptom developed by major pathogens by artificial inoculation on detached spindle leaflets 5 DAI a) C3 b) F5 c) Combined inoculation of C3 and F5 d) Control.

4.6 ISOLATION OF PHYLLOPLANE MICROFLORA OF COCONUT

Phylloplane fungi were isolated from the healthy leaves of the infected palm by serial dilution and plating method. The morphological characters of the isolated five phylloplane fungi were described in Table 16 and the isolates were shown in Plate 14.

All the five phylloplane fungi isolated were tested for their pathogenicity on the detached spindle leaflets and it was found that all the fungal isolates except isolate PF3 were found non-pathogenic.

Table 16. Cultural characters of phylloplane fungi isolated from healthy leaves of coconut

Fungal biocontrol agents	Colony appearance		Colour of Mycelium
	Upside view	Rear view	
PF1	Dark green	Yellow	White
PF2	Olive green	Yellow	White
PF3*	White	White	White
PF4	Brown	White	White
PF5	Brown	White	White

* Pathogenic to coconut

4.7 *IN VITRO* EVALUATION OF ANTAGONISTIC POTENTIAL OF ISOLATED PHYLLOPLANE MICROFLORA AGAINST MAJOR LRD PATHOGENS

The isolated four non-pathogenic phylloplane fungi were tested to find out their antagonistic activity against the identified major LRD pathogens such as *C. gloeosporioides* (C3 isolate) and *Fusarium* sp. (F5 isolate) by dual culture and detached spindle leaf assays. The effects of the phylloplane fungi was also compared with the

existing management practices such as *Pseudomonas fluorescens* (PN026) and Copper oxy chloride (COC- 0.2 %).

4.7.1 DIRECT ANTAGONISM

The direct antagonistic effect of phylloplane fungi against the LRD pathogens were tested by dual culture assay.

The suppression of mycelial growth of *C. gloeosporioides* (C3 isolate) by non-pathogenic phylloplane fungi in dual culture assay were shown in Plate 15. The isolates PF1 and PF2 developed a small inhibition zone between the mycelia of pathogen and phylloplane fungi and the inhibition zone was found to be higher in case of *P. fluorescens* (PN026) (Table 17). The results showed that there was maximum inhibition of mycelial growth of *C. gloeosporioides* by the isolate PF5 with 54.44 per cent inhibition and the result was statistically on par with the effect of COC - 0.2% (53.33 %). The least effective antagonist was isolate PF2 with 30.00 per cent inhibition to mycelial growth.

The suppression of mycelial growth of *Fusarium* sp. (F5 isolate) by non-pathogenic phylloplane fungi in dual culture assay was shown in Plate 16. An inhibition zone was developed between the mycelia of the pathogen and treatments by the isolate PF1, PF2 and *P. fluorescens* (PN026). Per cent inhibition of mycelial growth of *Fusarium* sp. by phylloplane fungi varied from 36.6 to 64.4 per cent (Table 18). Maximum inhibition of mycelial growth was shown by isolate PF4 (64.4 %) and this result was significant than the effect of COC - 0.2 % (57.7 %) and *P. fluorescens* (PN026) (41.1 %). The least inhibitory effect of 36.66 per cent was shown by the isolate PF2.

Both of these dual culture assays indicated that the isolates PF4 and PF5 were giving satisfactorily higher results compared to the isolates PF1 and PF2 which in turn reflected that, the inhibition zone had not much significance in inhibition of mycelial growth of the pathogen.

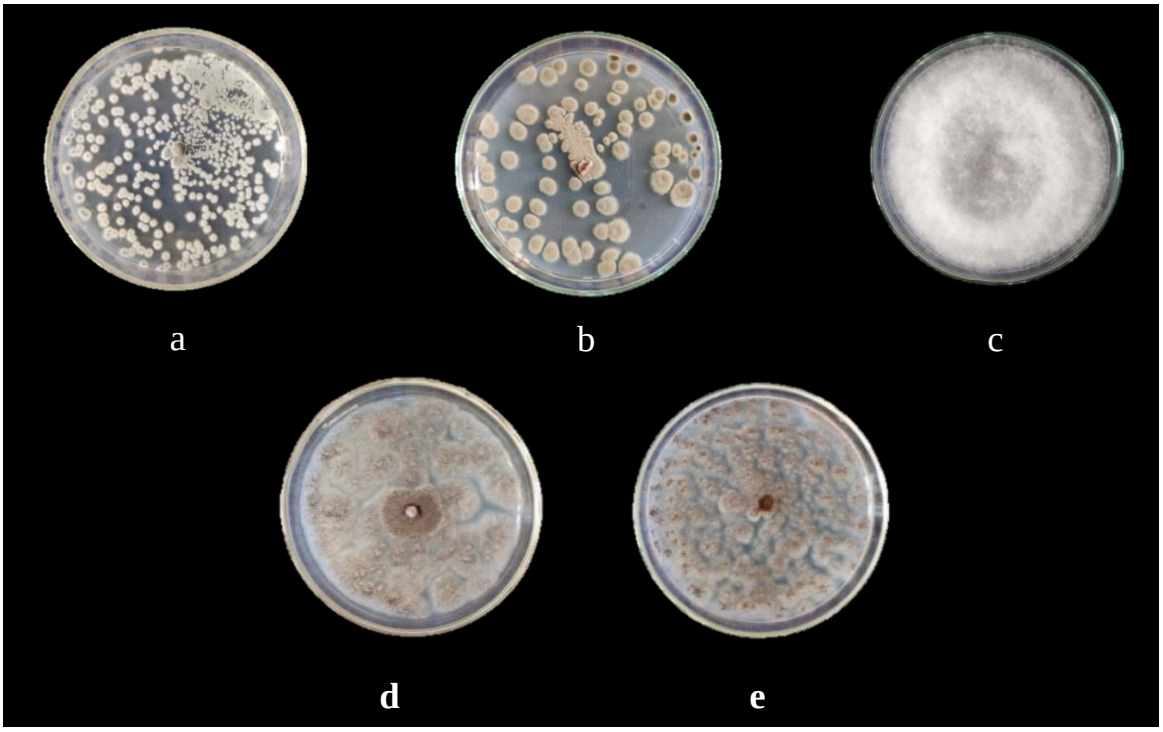


Plate 14. Phylloplane fungi isolated from healthy leaves of coconut (a) Isolate PF1 (b) Isolate PF2 (c) Isolate PF3 (d) Isolate PF4 (e) Isolate PF5

Table 17. Suppression of mycelial growth of *C. gloeosporioides* by phylloplane fungi in dual culture assay

Treatments	Inhibition zone (cm)	Per cent inhibition of mycelial growth (%)
PF1	0.5	35.56 (36.60)^{c}
PF2	0.1	30.00 (33.21)^{e}
PF4	0.0	43.33 (41.16)^{b}
PF5	0.0	54.44 (47.54)^{a}
P. fluorescens (PN026)	0.9	32.22 (34.58)^{d}
COC (0.2%)	-	53.33 (46.91)^{a}
Pathogen alone - C3 (control)	0.0	0.00 (0.955)^{f}
CD (0.05)		1.015
SEm±		0.580

*Values followed by similar superscripts are not significantly different at 5% level
Values in parenthesis are arc sine transformed values

Table 18. Suppression of mycelial growth of *Fusarium* sp. by phylloplane fungi in dual culture assay

Treatments	Inhibition zone (cm)	Per cent inhibition of mycelial growth (%)
PF1	0.2	38.88 (38.57)^{e}
PF2	0.3	36.66 (37.26)^{f}
PF4	0.0	64.40 (53.37)^{a}
PF5	0.0	45.55 (42.44)^{c}
P. fluorescens (PN026)	1.6	41.1 (39.87)^{d}
COC (0.2%)	-	57.7 (49.43)^{b}
Pathogen alone – F5 (control)	0.0	0.00 (0.955)^{g}
CD (0.05)		0.731
SEm±		0.844

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

Values in parenthesis are arc sine transformed values

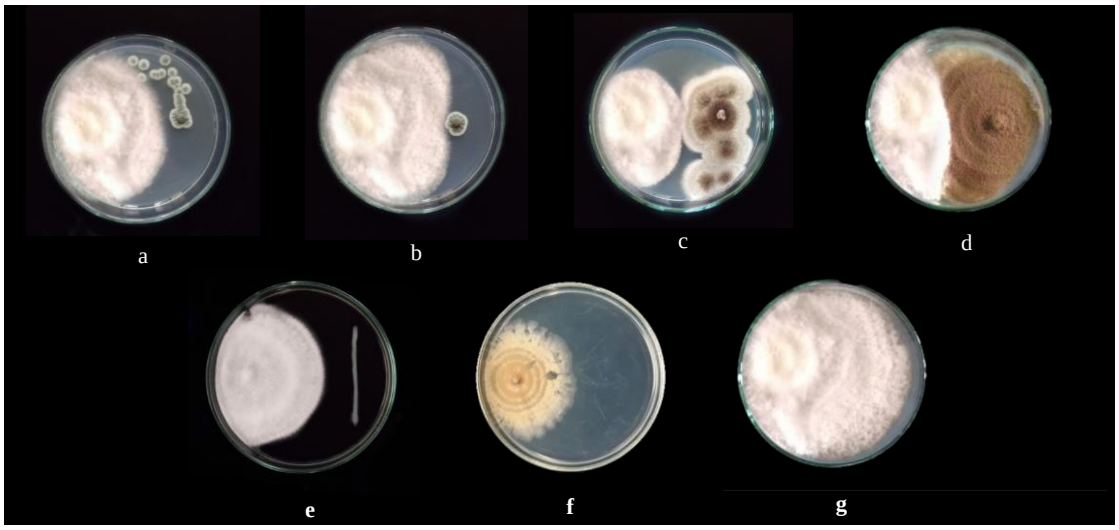


Plate 15. Inhibition of mycelial growth of *C. gloeosporioides* (C3) by different phylloplane fungi (a) Isolate PF1 (b) Isolate PF 2 (c) Isolate PF 4 (d) Isolate PF 5 (e) *P. fluorescens* (KAU isolate) (f) COC (0.2 %) and (g) *C. gloeosporioides* (C3) control

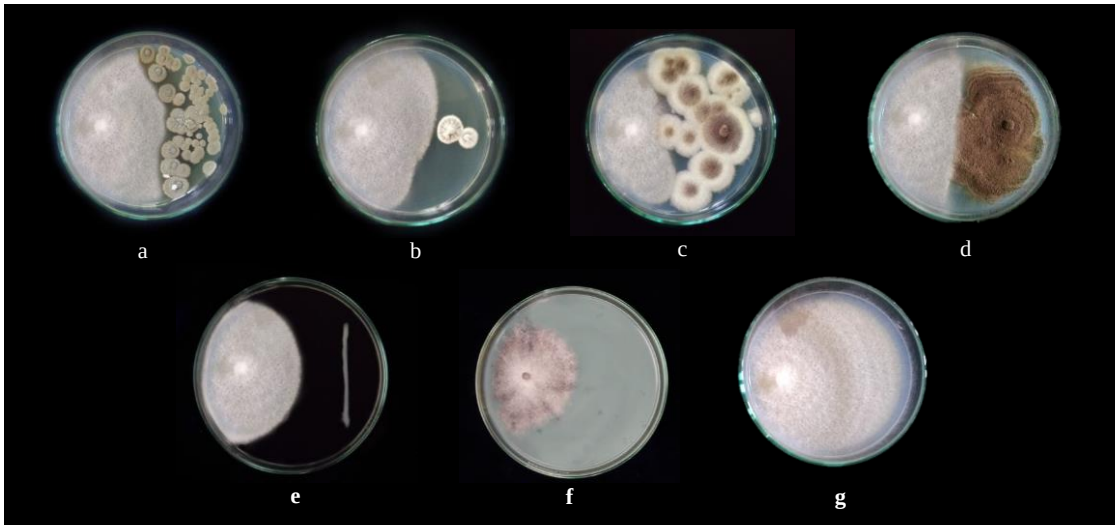


Plate 16. Inhibition of mycelial growth of *Fusarium* sp. (F5) by different phylloplane fungi (a) Isolate PF1 (b) Isolate PF 2 (c) Isolate PF 4 (d) Isolate PF 5 (e) *P. fluorescens* (KAU isolate) (f) COC (0.2 %) and (g) *Fusarium* sp. (F5) control.

4.7.2 DETACHED SPINDLE LEAF ASSAY

The results from detached spindle leaf assay also followed the similar trend as dual culture assay with slight decline in the per cent inhibition.

The results of *in vitro* assay of phylloplane fungi against *C. gloeosporioides* (C3 isolate) by detached spindle leaf assay were enlisted in Table 19. The treatments did not delay the symptom initiation but there was suppression of the disease spread afterwards. The detached spindle leaf assay also followed the similar trend as dual culture assay with greater suppression of the disease by the phylloplane fungal isolate PF5 (28.77 %) followed by the isolate PF4 (23.6 %), but the effect was inferior to that of the COC - 0.2 per cent (50.31 %). The least inhibition was shown by isolate PF1 (12.60 %) (Plate 17).

The observations on detached spindle leaf assay of phylloplane fungi against *Fusarium* sp. (F5 isolate) were shown in Table 19. None of the treatments delayed the symptom initiation. The results showed that the maximum suppression of the disease caused by *Fusarium* sp. was by isolate PF4 (34.57 %) and the least inhibition by isolate PF1 (12.6 %) which was on par with the effect of *P. fluorescens* (13.05 %) (Plate 18).

The comparison of two *in vitro* studies revealed that, there was reduction in the antagonistic effect of the treatments in detached spindle leaf assay than that of dual culture assay; and this might be due to the involvement of host factors in the pathogen-biocontrol interaction.

Table 19. *In vitro* evaluation of phylloplane fungi against *C. gloeosporioides* on detached spindle leaves

Treatments	DTSA*	Lesion size (cm)*			Per cent suppression of disease (%)*
		5 DAI	7 DAI	10 DAI	
PF1	2	1.83 ± 0.03	3.26 ± 0.08	5.56 ± 0.09	12.60 (20.78)^{e}
PF2	2	1.66 ± 0.12	3.10 ± 0.12	5.40 ± 0.06	15.09 (22.85)^{d}
PF4	2	1.43 ± 0.12	2.56 ± 0.06	4.86 ± 0.08	23.60 (29.06)^{c}
PF5	2	1.30 ± 0.06	2.33 ± 0.08	4.53 ± 0.14	28.77 (32.43)^{b}
P. fluorescens (PN026)	2	1.73 ± 0.03	3.03 ± 0.08	5.53 ± 0.12	13.05 (21.17)^{e}
COC (0.2%)	2	1.13 ± 0.08	2.03 ± 0.08	3.16 ± 0.08	50.31 (45.18)^{a}
Pathogen alone – C3 (control)	2	2.06 ± 0.14	3.53 ± 0.03	6.36 ± 0.07	0.00 (1.136)^{f}
CD (0.05)					0.875
SEm±					0.500

*Mean ± SD of three replications

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

Values in parenthesis are arc sine transformed values

*DTSA- Days taken for symptom appearance

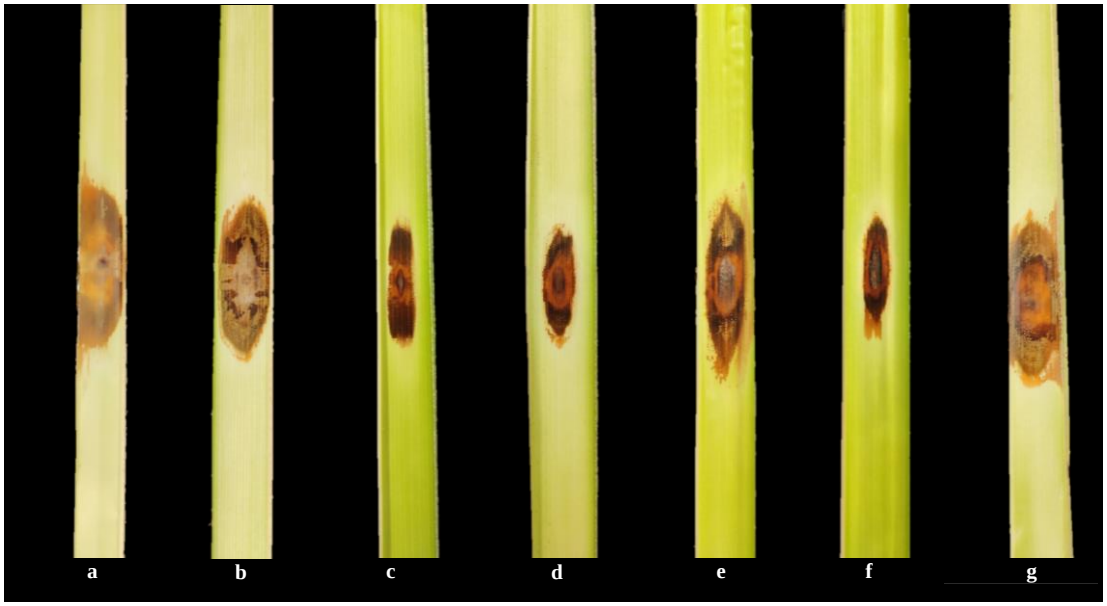


Plate 17. Lesions formed on leaves after spraying with phylloplane fungi and subsequently inoculated with *C. gloeosporioides,* (a) Isolate PF1 (b) Isolate PF 2 (c) Isolate PF 4 (d) Isolate PF 5 (e) *P. fluorescens* (f) COC-0.2 % (g) *C. gloeosporioides* (Control)

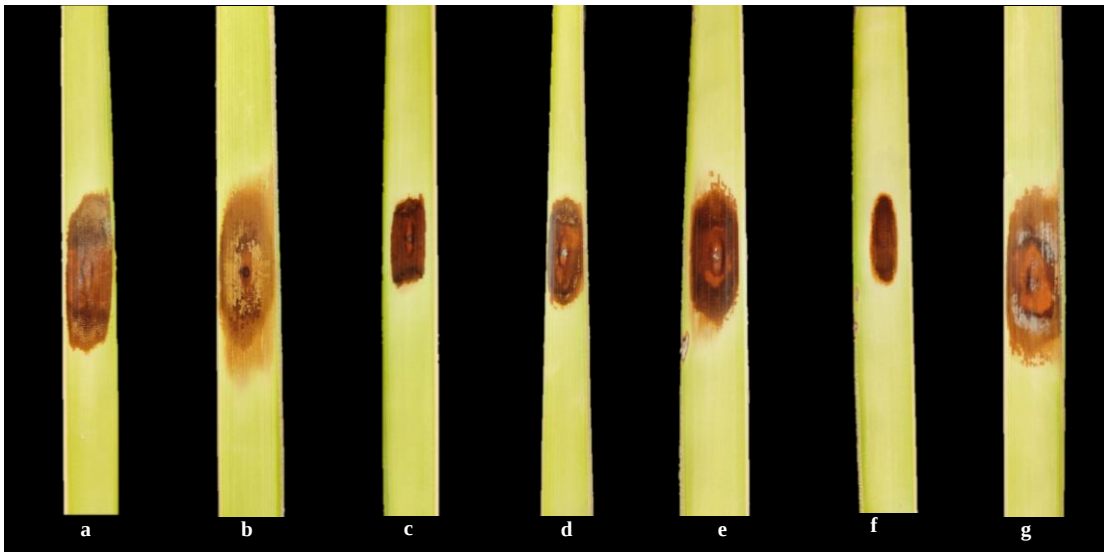


Plate 18. Lesions formed on leaves after spraying with phylloplane fungi and subsequently inoculated with *Fusarium* sp., (a) Isolate PF1 (b) Isolate PF 2 (c) Isolate PF 4 (d) Isolate PF 5 (e) *P. fluorescens* (f) COC-0.2 % (g) *Fusarium* sp. (Control)

Table 20. *In vitro* evaluation of phylloplane fungi against *Fusarium* sp. on detached spindle leaves

Treatments	DTSA*	Lesion size (cm)*			Per cent suppression of disease (%)*
		5 DAI	7 DAI	10 DAI	
PF1	2	2.06 ± 0.03	3.96 ± 0.12	5.76 ± 0.08	18.06 (25.14)^{e}
PF2	2	2.16 ± 0.08	4.13 ± 0.14	5.96 ± 0.09	15.22 (22.95)^{f}
PF4	2	1.50 ± 0.06	3.46 ± 0.08	4.60 ± 0.06	34.56 (36.00)^{b}
PF5	2	1.36 ± 0.09	4.00 ± 0.06	5.00 ± 0.12	28.87 (32.49)^{c}
P. fluorescens (PN026)	2	1.93 ± 0.03	3.96 ± 0.09	5.56 ± 0.06	20.91 (27.20)^{d}
COC (0.2%)	2	1.16 ± 0.08	2.46 ± 0.08	3.80 ± 0.12	45.94 (42.67)^{a}
Pathogen alone – F5 (control)	2	2.33 ± 0.09	4.63 ± 0.09	7.03 ± 0.08	0.00 (1.08)^{g}
CD (0.05)					1.050
SEm±					0.600

*Mean ± SD of three replications

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

Values in parenthesis are arc sine transformed values

*DTSA- Days taken for symptom appearance

Discussion

5. DISCUSSION

Leaf Rot Disease (LRD) is one of the major foliar disease affecting coconut plantations of Kerala in association with Root (Wilt) Disease (RWD), especially in southern districts. The present study dealt with the characterization of fungal pathogens associated with LRD in six taluks of Thiruvananthapuram district during south-west monsoon season and the eco-friendly management using phylloplane microflora as biocontrol agents. In this chapter, comparing the results with earlier reports is given here under.

5.1 ISOLATION OF LEAF ROT DISEASE PATHOGENS OF COCONUT

The LRD pathogens were isolated from six taluks of Thiruvananthapuram district *viz.*, Thiruvananthapuram, Neyyattinkara, Nedumangad, Chirayinkeezhu, Kattakada and Varkala during south-west monsoon. The samples collected were showing varied symptoms like water soaked brown lesions on the spindle leaves which were cemented together and on matured leaves the dry leaf rot symptoms on the distal end of the leaves. The rotted distal ends of the severely infected leaves were blown off by wind and reduced the leaf length (Plate 2). Similar symptoms of LRD were observed by Srinivasan and Gunasekaran (2000) and Srinivasan (2008) with water soaked brown lesions on the spindle leaves which coalesced together and the reduced disease severity was observed comparatively to the matured leaves which restricted the symptoms on the distal ends.

A spectrum of five different fungi was found to be associated with the LRD in Thiruvananthapuram district such as *C. gloeosporioides*, *Fusarium* spp., *Gliocladium* sp., and *Scytalidium* sp. during the south-west monsoon (Table 1). The major pathogens found to be associated with LRD in this district was considered as *C. gloeosporioides* and *Fusarium* spp. based on the frequency of isolation. 52 per cent of the LRD pathogens isolated were found to be *C. gloeosporioides* and 32 per cent of them were reported as *Fusarium* spp. Earlier reports of Srinivasan and Gunasekaran (1993; 1996b; 2000) also described that the LRD symptom initiation during the rainy season was mainly due to the

pathogen *C. gloeosporioides* in the presence or absence of other pathogens. This also indicated the occurrence of LRD by a single pathogen and with multiple pathogens. Vrinda (2002) also reported that the major pathogens found associated with LRD in Thiruvananthapuram district were *C. gloeosporioides* and *Fusarium* spp. during south-west monsoon season.

There is only a single isolate of *Scytalidium* sp. identified during the study and it was from the LRD samples collected from Kavalayoor in Varkala taluk. The pathogen was not found to be in association with any of the other fungus. Vrinda (2002) also reported the occurrence of *Scytalidium* sp. from the samples collected from Moncompu (Alappuzha) and Changanassery (Kottayam) during the rainy season. Their report also pointed to the same fact that the pathogen is able to initiate the symptoms during the rainy season without any association of other fungus.

5.2 PURIFICATION, IDENTIFICATION AND CHARACTERIZATION OF PATHOGENIC MICROFLORA

The five different fungal isolates were described by observing the morphological and microscopic characters. The culture of *C. gloeosporioides* had dense cottony appearance on PDA medium. The cultural characters of the *C. gloeosporioides* were found to be similar to those given by Udhayakumar *et al.* (2019) where they observed white, greyish white, greenish grey to pink colour of the colony and mycelia based on the isolate. The conidia of *C. gloeosporioides* was found to be hyaline, single celled, dumbbell / cylindrical shaped with size ranges from 12 to 17 µm length and 3.0 to 4.5 µm breadth. These microscopic characters of *C. gloeosporioides* were found similar to the observation by Sharma (2012) where he identified that the conidia are hyaline, single celled and size of the conidia varied from 10 to 20 µm length to 3.8 to 6.7 µm width. All the *C. gloeosporioides* isolates were found to be covering entire Petri plate (9 cm) within 10 days after culturing (Table 8); while Udhayakumar *et al.* (2019) observed that some of the *C. gloeosporioides* isolates completed 9 cm growth in 7 days and other isolates completed 4.5 cm to 8.8 cm in 7 days.

Some of the isolates of *Fusarium* spp. had observed sparse growth and others had dense cottony appearance on the PDA medium. They produced hyaline, septate and branched mycelia; with macro and micro spores. The macro conidia were hyaline, septate, sickle shaped and 2-4 celled while the micro conidia were hyaline, single celled with clavate shape (Plate 7). The length of macro conidia varied from 14.80 µm to 24.33 µm and the length of micro conidia varied from 6.12 µm to 7.84 µm (Table 6). The characters of *Fusarium* spp. were found similar to the characters described by Vrinda (2002) for *F. solani.* Chandran and Kumar (2012) divided *F. solani* isolates into three categories such as fast growers, moderate growers and slow growers based on the radial growth on PDA medium. According to this classification, none of the isolates of *Fusarium* spp. from LRD samples were classified under fast growers (grow > 8.5 cm in seven days), while most of the isolates F2, F3, F5, F6, F7 and F8 were classified as moderate growers (grow 7.0 to 8.5 cm in seven days) and the isolates F1 and F4 considered as slow growers (grow < 7.0 cm in seven days) (Table 9).

Gliocladium sp. showed the whitish growth on the upper side of the Petri plate which later turns to salmon colour while the rear side of the plate showed light yellow colour. The fungus consisted of distinct conidiophores which were hyaline and septate with penicillate braches and ends with specialized structures known as phialides. These characters of *Gliocladium* sp. were similar to the report of Srinivasan and Gunasekaran about *G. vermoeseni* (1994). The fungus was a fast grower with fluffy appearance on PDA medium and completely covered the Petri plate (9 cm) within 5 days after culturing (Table 10). Conidia were hyaline, single celled, oval to round in shape and appeared as chains from the phialides (Plate 8). The average size of the conidia is 1.09 µm (Table 7). The microscopic characters of *Gliocladium* sp. were comparable with that of the report of Vrinda (2002) on *G. roseum.*

The pathogenicity of *Scytalidium* sp. causing LRD was first reported by Vrinda (2002) and the characters identified for the *Scytalidium* sp. in present study was similar to the report of Vrinda (2002). The culture observed as sparse, black colonies on both the

sides of the Petri plate. They produced two types of mycelium; one was brown coloured, thick and septate mycelia and the other one was narrow, hyaline and septate mycelia. The pathogen was a fast grower which completed the full growth in 9 cm Petri plate within 4 days after culturing (Table 10). The fungus produced two types of conidia with catenate arrangement. Cylindrical, thin walled and hyaline conidia with 10.4 µm X 4.75 µm size and brown, thick walled and broader conidia with 11.0 µm X 6.61 µm size (Table 7).

5.3 TESTING THE PATHOGENICITY OF ISOLATED MICROFLORA

The pathogenicity of the fungal isolates was proved by Koch's postulates. All the isolated fungi such as *C. gloeosporioides*, *Fusarium* spp., *Gliocladium* sp., and *Scytalidium* sp. were proved the pathogenicity by inoculating the pathogens on healthy spindle leaves and the characters of re-isolated pathogens were found to be similar to the isolated pathogens. The pathogenicity of the fungi *C. gloeosporioides, Fusarium* spp. and *G. vermoeseni* in causing LRD were proved by Srinivasan and Gunasekaran (1996a; 1996c) by *in vitro* and *in vivo* studies. The pathogenic role of fungus *Scytalidium* sp. in causing LRD was first proved by Vrinda (2002) by detached spindle leaf assay.

The virulence was compared among the thirteen isolates of *C. gloeosporioides* (Table 11) and eight isolates of *Fusarium* spp. (Table 12) based on the days taken for symptom initiation and lesion size developed at 5^{th}, 7^{th} and 10^{th} DAI. The isolates of same pathogen collected from different location showed variation in the size of the lesion produced, time taken for symptom initiation, cultural characters and spore characters. The variation in cultural characters, pathogenicity and virulence of each isolate was due to the regional differences (Pande *et al.*, 1991). Udhayakumar *et al.* (2019) observed the variation in pathogenicity of the isolates of *C. gloeosporioides* and inferred that this may be due to the variation in the locations from where the samples were collected. The isolate C3 from Anayara (Thiruvananthapuram taluk) was found to be more virulent among *C. gloeosporioides* isolates which showed maximum lesion size on artificial inoculation and a slightly higher mycelial growth rate than other isolates.

The *Gliocladium* sp. found to be least virulent among all the pathogen isolates with delayed symptom initiation and lower lesion size developed on subsequent days. Srinivasan and Gunasekaran (1994) also reported that the least virulent pathogen of LRD found to be *G. vermoeseni.* Among the different pathogens isolated from LRD of Thiruvananthapuram district, the *Scytalidium* sp. was found to be more virulent which initiated the symptom development on one day after inoculation itself and higher lesion size produced on subsequent days. Vrinda (2002) also observed the higher virulence of the pathogen *Scytalidium* sp. than that of major pathogens *C. gloeosporioides* and *Fusarium* spp.

5.4 SYMPTOMATOLOGY

The nature of symptom developed by isolated five fungal species was studied by inoculating the pathogens on unopened detached spindle leaves. It was revealed from the study that, whatever the pathogen causing LRD on coconut leaves, the symptom developed by each of them was found similar with slight differences in the size of the lesion developed. The initiation of water soaked, brown lesion was noticed as the characteristic symptom of LRD caused by each of the identified pathogen. The *C. gloeosporioides* isolates showed orange spore masses over the lesion while the *Fusarium* sp. produced plenty of mycelial growth over the lesion (Plate 12). The earlier reports also mentioned the appearance of mycelial growth and spore masses over the symptoms produced by LRD pathogens (Srinivasan and Gunasekaran, 2000; Srinivasan, 2008). According to Li and Zhang (2007) the appearance of spore masses and acervuli over the lesions produced by *C. gloeosporioides* was due to the high relative humidity. Abundant mycelial growth over the lesion produced by the *F. solani* isolates on strawberry was observed by Mehmood *et al*. (2017).

5.5 COMBINED INOCULATION OF MAJOR LRD PATHOGENS ON DETACHED SPINDLE LEAVES AND RE-ISOLATION OF PATHOGEN TO CONFIRM THE PATHOGENICITY

By the combined inoculation of major LRD pathogens such as *C. gloeosporioides* and *Fusarium* spp. on detached spindle leaflets, it was observed that the symptom initiated 24 hours after inoculation itself and this was comparable with that of individual inoculation by *Scytalidium* sp. The lesion size developed on combined inoculation was more compared to the individual inoculation by *C. gloeosporioides* and *Fusarium* spp. (Table 14; Plate 13), but the progress of lesion development was more compared to inoculation by *Scytalidium* sp. alone. The increased disease incidence and earlier initiation of symptom development due to combined inoculation gave a clear insight into the role of fungal complex in increasing disease severity and initiating symptom development. Vrinda (2002) also gave similar reports by combined inoculation of all the isolated LRD pathogens such as *C. gloeosporioides*, *E. rostratum, F. solani*, *Scytalidium* sp., *C. sacchari*, *G. roseum* and *Curvularia* sp. in all possible combinations. All the combined inoculation observed greater lesion size and was confirmed by dual culturing of the isolates in combination. The lesion size developed on combined inoculation was noticed similar to that of the individual inoculation by *Scytalidium* sp. The dual culturing of *C. gloeosporioides* and *F. solani* showed no inhibition to the growth with free merging of the fungi (CPCRI, 1994; Vrinda, 2002). This might be the reason for the co-existence of LRD pathogens on the leaves and higher disease incidence.

5.6 ISOLATION OF PHYLLOPLANE MICROFLORA OF COCONUT

The phylloplane fungi were isolated from the healthy leaves of infected palm by serial dilution and plating method. Among the five phylloplane fungi isolated, the isolate PF3 was found pathogenic to coconut leaves by artificial inoculation on detached spindle leaves (Table 15). According to Kohl *et al.* (2011) the microorganisms used as biocontrol agents must be non-pathogenic to the host plant. So, the isolate PF3 was eliminated from further studies.

The two of the non-pathogenic fungal isolate PF4 and PF5 resembled to *Aspergillus* sp. and the other two (Isolate PF1 and PF2) to *Penicillium* sp. by observing the morphological and cultural characters of the phylloplane fungi (Plate 14). The phylloplane fungi isolated from healthy coconut leaves by Srinivasan *et al*. (2006) found to be associated with coconut leaves were *Aspergillus* sp. and *Penicillium* sp.

5.7 *IN VITRO* EVALUATION OF ANTAGONISTIC POTENTIAL OF ISOLATED PHYLLOPLANE MICROFLORA AGAINST MAJOR LRD PATHOGENS

The antagonistic activity of isolated four non-pathogenic phylloplane fungi were tested against the identified major LRD pathogens such as *C. gloeosporioides* (C3 isolate) and *Fusarium* sp. (F5 isolate) by dual culture assay and detached spindle leaf assay. The effects of the phylloplane fungi were also compared to the existing management practices such as *Pseudomonas fluorescens* (PN026) and Copper oxy chloride (COC- 0.2 %).

5.7.1 DIRECT ANTAGONISM

The direct antagonistic effect of phylloplane fungi against major pathogens of LRD were tested by dual culture assay by calculating the per cent inhibition to mycelial growth.

The suppression of mycelial growth of *C. gloeosporioides* (C3 isolate) by non-pathogenic phylloplane fungi in dual culture assay (Plate 15) showed that there was maximum inhibition of mycelial growth of *C. gloeosporioides* by the isolate PF5 with 54.44 per cent (Table 17; Figure 1) and the result was statistically on par with the effect of COC - 0.2% (53.33 %). The least effective antagonist was isolate PF2 with 30.00 per cent inhibition of mycelial growth. Srinivasan *et al.* (2006) reported that the antagonistic effect of phylloplane fungi against *C. gloeosporioides* varied from 9.0 to 64 per cent mycelial inhibition in dual culture assay. According to Tembhare *et al.* (2012) the phylloplane fungi showed inhibition to the pathogen *C. capsici* in dual culture assay with higher inhibition by *Aspergillus fumigatus* (61.03 %).

The suppression of mycelial growth of *Fusarium* sp. (F5 isolate) by non-pathogenic phylloplane fungi in dual culture assay were shown in Plate 16. Per cent inhibition of mycelial growth of *Fusarium* sp. by phylloplane fungi varied from 36.6 to 64.4 per cent (Table 18). Maximum inhibition of mycelial growth was shown by isolate PF4 (64.4 %) and this result was significant than the effect of COC - 0.2 % (57.7 %) and *P. fluorescens* (PN026) (41.1 %). 36.66 per cent inhibition given by isolate PF2 was the least (Figure 2). The phylloplane fungi isolated from healthy coconut leaves were reported wide variation in the antagonistic effect in dual culture assay against *F. solani* with 6.0 to 89 per cent mycelial growth inhibition (Srinivasan *et al.,* 2006)

Both of these dual culture assays indicated that the isolates PF4 and PF5 were giving satisfactorily higher results compared to the isolates PF1 and PF2. Srinivasan *et al.* (2006) tested the antagonistic effect of phylloplane fungi against major LRD pathogens such as *C. gloeosporioides, E. rostratum* and *F. solani* in dual culture assay; and found that the phylloplane fungi *Aspergillus* sp. was giving higher inhibition to pathogens in dual culture assay than that of *Penicillium* sp. The mode of action of the phylloplane fungus *Aspergillus* sp. against *C. gloeosporioides* showed that *Aspergillus* sp. lysed the hyphae of the pathogen at the point of contact between the pathogen and antagonist in the dual culture assay and could not re-isolate the pathogen from this point of contact (Evueh and Ogbebor, 2008).

5.7.2 DETACHED SPINDLE LEAF ASSAY

The results of *in vitro* assay of phylloplane fungi against *C. gloeosporioides* (C3 isolate) by detached spindle leaf assay (Table 19) showed that there was no delay in the symptom initiation but there was suppression of the disease spread. The detached spindle leaf assay also followed the similar trend as dual culture assay with greater suppression of the disease by the phylloplane fungal isolate PF5 (28.77 %) followed by the isolate PF4 (23.6 %), but the effect was inferior to that of the COC - 0.2 % (50.31 %). The least inhibition was shown by isolate PF1 (12.60 %) (Plate 17).

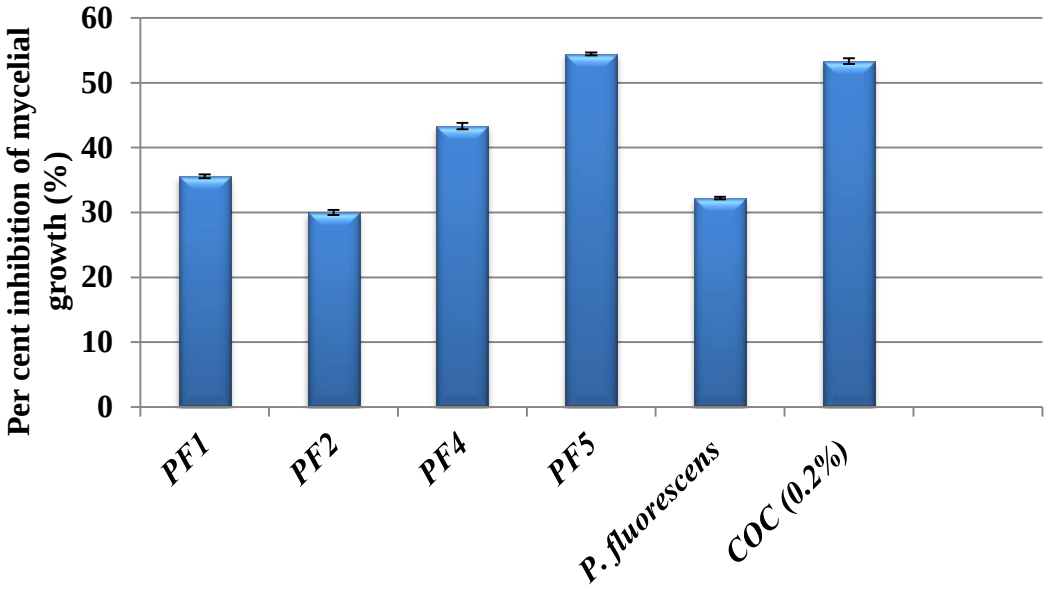


Figure 1. Per cent inhibition of mycelial growth of *C. gloeosporioides* by phylloplane fungi

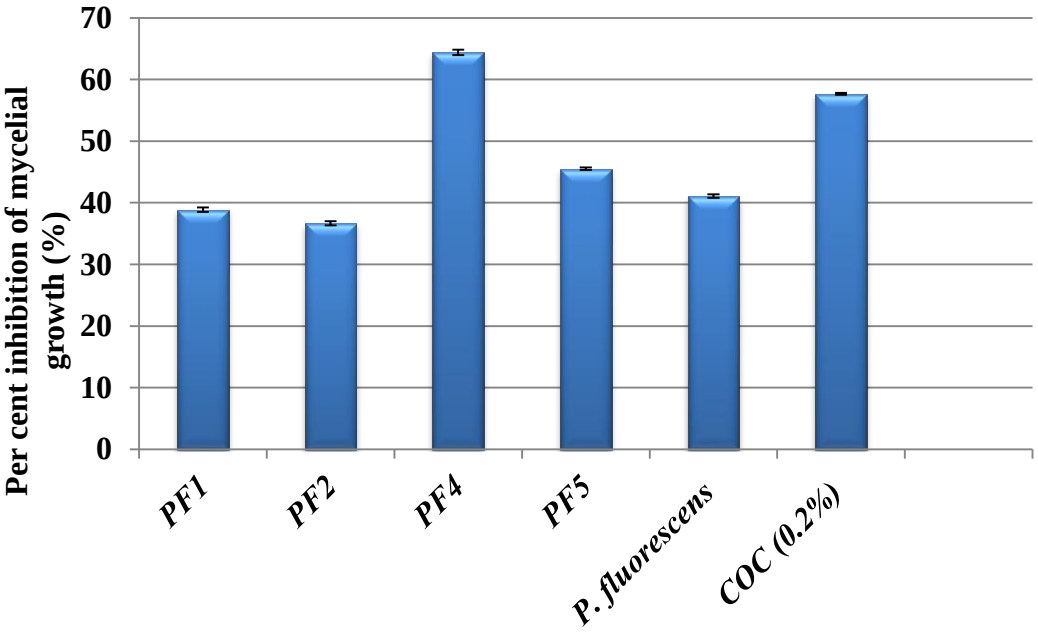


Figure 2. Per cent inhibition of mycelial growth of *Fusarium* sp. by phylloplane fungi

The observations on detached spindle leaf assay of phylloplane fungi against *Fusarium* sp. (F5 isolate) (Table 19) were noted that none of the treatments showed delay in symptom initiation. The results also showed that the maximum suppression of the disease caused by *Fusarium* sp. was by isolate PF4 (34.57 %) and the least inhibition by isolate PF1 (12.6 %) which was on par with the effect of *P. fluorescens* (13.05 %) (Plate 18).

The comparison of the *in vitro* effects of phylloplane fungi against *C. gloeosporioides* (C3 isolate) were exhibited in Figure 3. The results revealed that even though there was reduction in the antagonistic effect of phylloplane fungi on detached spindle leaf assay, the isolate PF5 was persistently giving higher inhibition to the pathogen with 54.44 per cent inhibition to mycelial growth and 28.77 per cent suppression of the disease *in vitro.* While comparing the *in vitro* antagonistic effects of phylloplane fungi against *Fusarium* sp. (F5 isolate), the isolate PF4 was constantly showing higher inhibition to the pathogen with 64.4 per cent inhibition to mycelial growth of *Fusarium* sp. and 34.57 per cent suppression to the disease caused by pathogen (Figure 4).

The comparison of two *in vitro* studies revealed that, there was reduction in the antagonistic effect of the treatments in detached spindle leaf assay than that of dual culture assay; and this might be due to the involvement of host factors in the pathogen-biocontrol interaction. Pliego *et al.* (2011) observed that the dual culture assay like *in vitro* assays gave more antagonistic effect against the pathogens than that of the real *in vivo* field situation because of the exclusion of host factors interacting with the antagonistic effect of biocontrol agents. So it is necessary to evaluate the effects of selected biocontrol agents in the field to ensure its antagonistic effect in the field and also to ensure safety to environment, host and other animals.

Thus, the present study revealed that the isolate PF4 and PF5 can be further tested for *in vivo* biocontrol potential before going for the development of a formulated product.

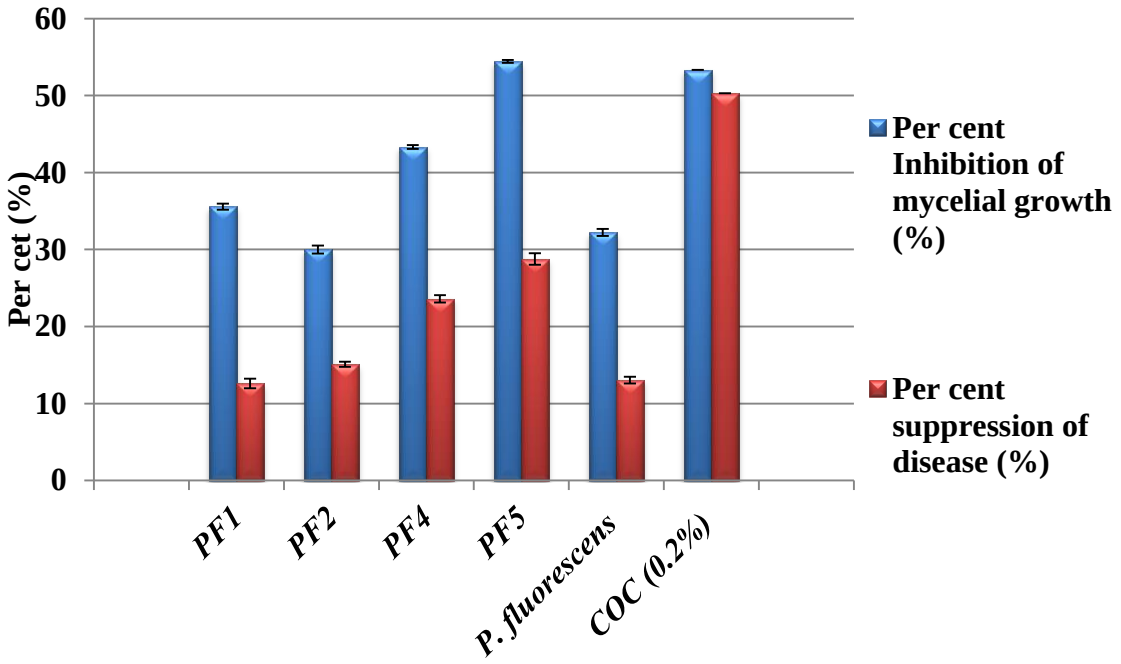


Figure 3. Comparison of effect of phylloplane fungi against *C. gloeosporioides*

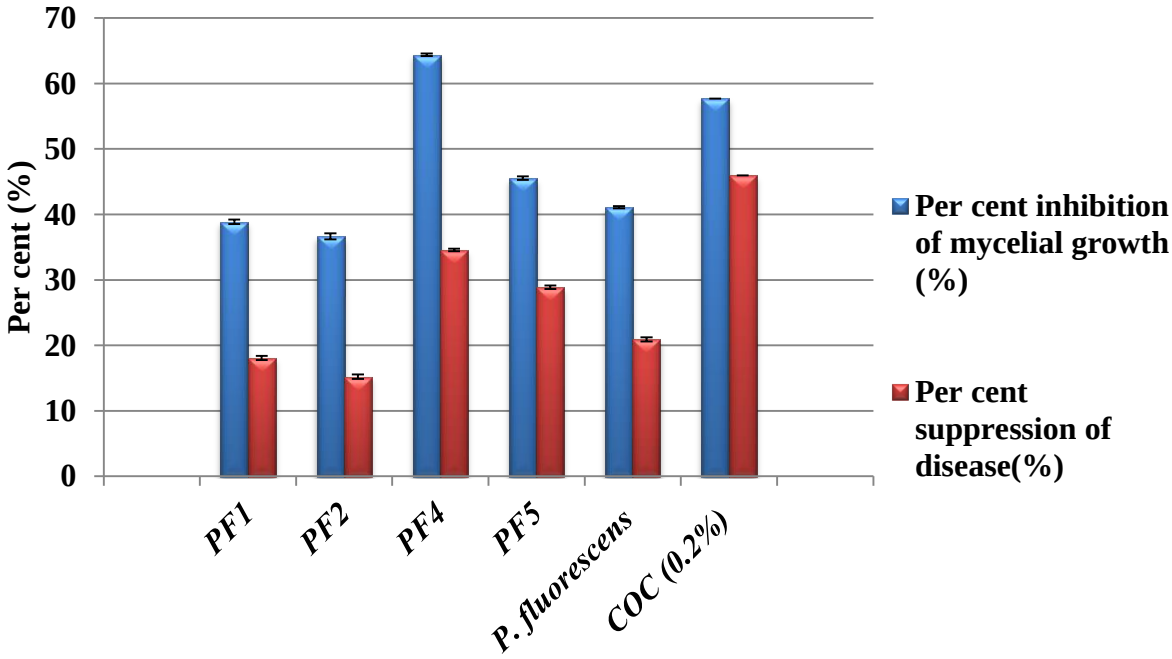


Figure 4. Comparison of effect of phylloplane fungi against *Fusarium* sp.

Summary

6. SUMMARY

Leaf rot disease (LRD) is a major foliar disease affecting coconut plantations of Southern Kerala especially in root (wilt) endemic areas. Approximately 65 per cent of the RWD affected palms were 'super infected' with LRD. In this context, the management of LRD is very much essential in the RWD management. With this, the study entitled 'Characterization of fungal pathogen associated with leaf rot disease of coconut (*Cocos nucifera* L.) and *in vitro* evaluation of phylloplane microflora as biocontrol agents' was conducted in the Department of Plant Pathology, College of Agriculture, Vellayani during the year 2018-2020, with the objective to identify and characterize the major fungal pathogens associated with the LRD of coconut and *in vitro* evaluation of phylloplane microflora of coconut against the pathogens.

The isolation of LRD pathogens was carried out from six taluks of Thiruvananthpuram district such as Thiruvananthapuram, Neyyattinkara, Nedumangad, Chirayinkeezhu, Kattakada and Varkala. Three locations were selected from each taluk and a total of eighteen samples were collected during the study. The results revealed that the disease in Thiruvananthapuram district was caused by a spectrum of pathogens such as *C. gloeosporioides*, *Fusarium* spp., *Gliocladium* sp., and *Scytalidium* sp. during the south-west monsoon.

The symptoms of the LRD samples were observed as the water soaked brown lesions on the distal end of the spindle leaves which were cemented together while the symptoms on the matured leaves as dried leaf rot. In case of the samples collected from the severely infected palm, the dried lesions from the margin of the leaves were blown off by the wind and reduced the leaf length.

The LRD was caused either by a single pathogen or by combinations of pathogens. *C. gloeosporioides* and *Fusarium* spp. were found to be the major pathogens of LRD based on the frequency of isolation. Thirteen isolates out of the twenty five pathogen isolates were identified as *C. gloeosporioides* and eight as *Fusarium* spp. The minor pathogens observed were *Gliocladium* sp., and *Scytalidium* sp.

The pathogenicity studies proved that, all the isolated fungi from the LRD samples were pathogenic to coconut leaves. Each and every isolate of the same pathogen differed from one another in cultural characters and virulence. All the pathogens produced water soaked brown lesion on artificial inoculation on detached spindle leaves; though the days taken for symptom initiation and size of the lesion developed varied. The isolate C3 (Isolate from Anayara, Thiruvananthapuram taluk) was found to be more virulent among the *C. gloeosporioides* isolates. Among the *Fusarium* spp. isolates, the isolate F5 (Isolate from Alamcode, Chirayinkeezhu taluk) was found to be more virulent.

The cultural characters and spore characters of pathogens revealed that *C. gloeosporioides* had dense cottony appearance on PDA medium. The colour of the colony was varied from white to grey colour on front view and white to dark green colour on the rear side based on the isolate with white to greyish white mycelia. The pathogen produced hyaline, straight, aseptate, single celled conidia with dumb-bell shape / cylindrical shape with oil globule at the centre. But the size of the conidia varied with the isolates and greater spore size for the isolate C1 with an average spore size of 16.76 X 4.36 µm. Some of the isolates of *Fusarium* spp. had sparse growth and others had dense cottony appearance on the PDA medium. The colony colour varied from white to off white on upper side of the Petri plate and off white to light yellow and pink on the rear side of the culture plate with white aerial mycelia. The *Fusarium* spp. produced two kinds of conidia such as macro conidia and micro conidia. The micro conidia were hyaline, single celled and clavate shaped with average spore size ranging from 6.12 X 1.69 to 7.84 X 2.33 µm. The macro conidia were hyaline, two to four celled with sickle shape and the size varied from 14.8 X 2.53 to 24.33 X 4.04 µm. The *Fusarium* spp. isolates exhibited varied pigmentation in the culture plate with more pigmentation by the isolate F1. By observing the cultural and spore characters of the isolates, it was found that the spore size and pigmentation of the culture haven’t any significance to the virulence of the pathogen.

Combined inoculation of the major pathogens on detached spindle leaves caused severe incidence of the disease compared to the individual inoculation of the pathogens.

This result indicated that the LRD caused by fungal complex is more severe than that caused by individual fungal isolates.

There are phylloplane fungi existing on healthy leaves of the infected palm with enough inhibition potential to LRD. The cultural characters of the phylloplane fungi were studied and the pathogenicity test indicated that the isolate PF3 initiated the symptom development on the healthy spindle leaves of coconut and all the other isolates were found to be non-pathogenic. The isolate PF3 was eliminated from further studies because the biocontrol agents should be non-pathogenic.

The *in vitro* assays were carried out to find out the antagonistic activity of phylloplane fungi against the major LRD pathogens and the effects were compared with the existing management practices such as COC (0.2%) and *Pseudomonas fluorescens* PN026. The *in vitro* assays included dual culture assay and detached spindle leaf assay.

The phylloplane fungal isolate PF5 showed more per cent inhibition to mycelial growth of *C. gloeosporioides* (54.44 %) and the result was statistically on par with the effect of COC (0.2 %) poisoned medium (53.33 %). The second best treatment was isolate PF4 (43.33 %); though there was no inhibition zone development in case of these two isolates. The isolate PF4 showed more inhibition to mycelial growth of *Fusarium* sp. (64.44 %) followed by the isolate PF5 (45.55 %) in the dual culture assay. The effect of isolate PF4 was significant than that of COC (0.2 %).

The detached spindle leaf assay also supported the same fact that the isolate PF5 was observed to be more suppressive to the disease caused by *C. gloeosporioides* (28.77 %) and the isolate PF4 was reported to have more suppression to the disease caused by *Fusarium* sp. (34.56 %). These pre-treatment effects are more promising than *Pseudomonas fluorescens* PN026, but inferior to copper oxy chloride (0.2 %). None of the treatments showed any delay in the symptom initiation, but there was subsequent inhibition on the lesion size produced by the isolates.

Thus, the present study revealed that the LRD of coconut in Thiruvananthapuram district is caused by a combination of pathogenic fungi *viz., C. gloeosporioides*, *Fusarium*

spp., *Gliocladium* sp., and *Scytalidium* sp. during the south-west monsoon. Prophylactic application of the phylloplane fungal isolates PF4 and PF5 could reduce the LRD severity *in vitro* to a promising level and these isolates can be further tested for compatibility and *in vivo* biocontrol potential before going for the development of a formulated product.

References

7. REFERENCES

APCC [Asian and Pacific Coconut Community].2020. APCC Statistical year book, 2017 [online]. Available: https://www.apccsec.org/ [25 April 2020].

Brown, W. 1924. Two mycological methods. I. A simple method of freeing fungal cultures from bacteria. *Ann. Bot.* 2: 401- 404.

Butler, E.J. 1908. *Report on coconut palm disease in Travancore.* Bulletin No.9, Agricultural Research Institute, Pusa, 23 p.

CCRS [Central Coconut Research Station]. 1962. *Annual Report 1960-1961*. Central Coconut Research Station, Kayamkulam, 166p.

CCRS [Central Coconut Research Station]. 1965. *Annual Report 1963-1964.* Central Coconut Research Station, Kayamkulam, 218p.

CDB [Coconut Development Board]. 2020. CDB home page [online]. Available: http://agricoop.gov.in/coconut-development-board-kochi-kerala [18 April 2020].

Chandran, M.R. and Kumar, M.R. 2012. Studies on cultural, morphological variability in isolates of *Fusarium solani* (Mart.) Sacc., incitant of dry root-rot of Citrus. *Current Biotica* 6(2): 152-162.

CPCRI [Central Plantation Crops Research Institute]. 1979. *Annual Report 1978*. Central Plantation Crops Research Institute, Kasargod, 204p.

CPCRI [Central Plantation Crops Research Institute]. 1985a. *A survey report – Coconut root (wilt) disease-intensity, production, loss and future strategy.* Central Plantation Crops Research Institute, Kasargod, 45p.

CPCRI [Central Plantation Crops Research Institute]. 1985b. *Annual Report 1984*. Central Plantation Crops Research Institute, Kasargod, 211p.

CPCRI [Central Plantation Crops Research Institute]. 1994. *Annual Report 1993-1994*. Central Plantation Crops Research Institute, Kasargod, 193p.

CPCRI [Central Plantation Crops Research Institute]. 1996. *Annual Report 1995-1996*. Central Plantation Crops Research Institute, Kasargod, 220p.

CPCRI [Central Plantation Crops Research Institute]. 1998. *Annual Report 1997-1998*. Central Plantation Crops Research Institute, Kasargod, 158p.

DAC & FW [Directorate of Agriculture, Cooperation and Farmers Welfare]. 2020. DAC&FW home page [online]. Available: http://agricoop.nic.in/divisiontype/horticulture [14 September 2020].

DAF [Department of Agriculture and Fisheries]. 1940. *Annual report of the scheme of investigations on the diseases of coconuts in South India for 1939.* Department of Agriculture and Fisheries, Travancore, 16p.

Dennis, C. and Webster, J. 1971. Antagonistic properties of species-groups of *Trichoderma*: II. Production of volatile antibiotics. *Transactions of the British Mycological Society* 57(1) : 41- 144.

Dwivedi, R.S., Mathew, C., Ray, P.K., Amma, B.S.K., and Ninan, S. 1979. Certain closely associated morphological symptoms of root (wilt) disease of coconut (*Cocos nucifera*). *Pl. Dis. Reptr.* 63: 461-463.

Evueh, G.A. and Ogbebor, N.O. 2008. Use of phylloplane fungi as biocontrol agent against *Colletotrichum* leaf disease of rubber (*Hevea brasiliensis* Muell. Arg.). *Afr. J. Biotechnol.* 7(15): 2569-2572.

George, M. V. and Radha, K. 1973. Computation of disease index of root (wilt) disease of coconut. *Indian J. Agric. Sci.* 43: 366- 370.

Gunasekaran, M., Gupta, A., and Srinivasan, N. 2003. *In vitro* biocontrol of coconut leaf rot pathogens with *Pseudomonas fluorescens. J. Plantn Crops* 31 (2): 52-54.

Gupta, A., Gunasekaran, M., and Srinivasan, N. 2000. Isolation of bacterial antagonists from rhizosphere and their *in vitro* evaluation against pathogens of leaf rot disease. In: Zeng, S., Zhou, G., and Li, H. (eds), *Plant Diseases and their Control.* Proceedings of First Asian Conference of Plant Pathology, Beijing, China. Chinese Agricultural Science and Technology Press, Beijing, pp.261.

Harsonowati, W., Astuti, R.I., and Wahyudi, A.T. 2017. Leaf blast disease reduction by rice phyllosphere actinomycetes producing bioactive compounds. *J. Gen. Plant Pathol.* 83: 98-108.

Joseph, T. and Rawther, T.S.S. 1991. Leaf rot disease. In: Nair, M.K., Nambiar, K.K.N., Koshy, P.K., and Jayasankar, N.P. (eds.), *Coconut root (wilt) disease.* Cord Word Process and Printers, Mangalore, pp. 92-98.

KAU [Kerala Agricultural University]. 2016. *Package of Practices Recommendations: Crops* (15th Ed.). Kerala Agricultural University, Thrissur, 393p.

Khanna, M.R., Devi, G.U., Sagar, B.V., and Sankar, A.S. 2015. Screening of phylloplane microflora against *Alternaria porri* under *in vitro* condition. *Progressive Res. Int. J.* 10: 1477-1479.

Kohl, J., Postma, J., Nicot, P., Ruocco, M., and Blum, B. 2011. Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. *Biol. control* 57: 1-12.

Koshy, P. K. 1999. Root (wilt) disease of coconut. *Indian Phytopath.* 52: 335 - 353.

Koshy, P. K. 2000. Leaf rot disease of coconut. *Indian Cocon. J.* 31(2): 4-10.

Li, H.Y. and Zhang, Z.F. 2007. First report of *Colletotrichum gloeosporioides* causing anthracnose fruit rot of *Trichosanthes kirilowii* in China. *Plant disease* 91(5): 636-636.

Lily, V. G. 1963. Host parasite relations of *Helminthosporium halodes* (Drechs.) Shoemaker on the coconut palm. *Indian Cocon. J.* 16(4): 149 - 153.

Lily, V. G. 1981. Host parasite relations of *Bipolaris halodes* (Drechs.) Shoemaker on the coconut palm. *Indian Cocon. J.* 11(11): 1 - 4.

Lily, V.G., Nair, U.K., Pandalai, K.M., and Menon, K.P.V. 1952. Observations on the inhibitory activity of a species of a bacterium on some fungi parasite on the coconut palm. *Indian Cocon. J.* 5(4): 162-170.

Lily, V.G., Radha, K., Pandalai, K.M., and Menon, K.P.V. 1955. Observations on the inhibitory activity of a species of a bacterium. Activity of the antifungal substance produced by the bacterium *Bacillus subtilis*. *Indian Cocon. J.* 8(3): 137-149.

Mathai, G. 1980. Seasonal and varietal variations on the incidence of leaf rot of coconut in the root (wilt) affected tracts. *Indian Cocon. J.* 11(6): 1-3.

Mc Rae, W. 1916. Communication to Dewan of cochin. In: Menon, K.P.V. and Nair, U.K. 1948. The leaf rot disease of coconut, in Travancore and Cochin. *Indian Cocon. J.* 1(2): 33-39.

Mc Rae, W. 1929. India: New diseases reported during the year 1928. *Intern. Bull. Pl. Prot.* 3: 21-22.

Mehmood, N., Riaz, A., Jabeen, N., Anwaar, S., Hussain, S.Z., Abbas, M.F., Zhang, X., Rosli, H., and Gleason, M.L. 2017. First report of *Fusarium solani* causing fruit rot of strawberry in Pakistan. *Plant Disease* 101(9): 1681-1681.

Menon, M.S. 1935. *The Coconut Leaf Disease.* Bulletin No. 1 Botanical series. Department of Agriculture, Cochin State, 20 p.

Menon K.P.V. and Nair, U.K. 1948. The leaf rot disease of coconut, in Travancore and Cochin. *Indian Cocon. J.* 1(2): 33-39.

Menon K.P.V. and Nair, U.K. 1951. Scheme for the investigation of the root and leaf disease of the coconut palms in South India- Consolidated final report of the work done from 8 March 1937 to 31 March 1948. *Indian Cocon. J.* 5(1): 5-19.

Menon K.P.V. and Nair, U.K. 1952. Scheme for the investigation of the root and leaf diseases of the coconut palms in south India- Consolidated final report of the work done from 8 March 1937 to 31 March 1948. *Indian Cocon. J.* 5(2): 81-100.

Menon K.P.V. and Pandalai, K. M. 1958. *The coconut palm – a monograph.* Indian Central Coconut Committee, Ernakulam, 384 p.

Mishra, D., Singh, N., Hota, A.K., and Sahoo, S. C. 1989. A survey of important diseases of coconut palm in Orissa. *Orissa J. Hort.* 17: 10-14.

Nysanth, N. S., Meenakumari1, K. S., Syriac, E. K., and Subha, P. 2018. Isolation, characterization and evaluation of Pink Pigmented Facultative Methylotrophs (PPFM) associated with paddy. *Int.J. Curr. Microbiol. App. Sci.* 7(7): 2187- 2210.

Padmaja, G., Devi, U.G., Mahalakshmi, K., and Sridevi, D. 2015. Compatability of phylloplane microflora of taro and fungicides in managing *Phytophthora colocasiae*. *Progressive Res. Int. J.* 10: 1437-1440.

Pande, S., Mughogho, L.K., Bandyopadhyay, R., and Karunakar, R.I. 1991. Variation in pathogenicity and cultural characteristics of sorghum isolates of *Colletotrichum graminicola* in India. *Plant Disease 75*(8): 778-783.

Pliego, C., Ramos, C., de Vicente, A., and Cazorla, F.M. 2011. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. *Plant and soil* 340(1-2): 505-520.

Radha, K., Sukumaran, C.K., and Prasannakumari, T.O. 1961. Studies on the leaf rot disease of coconut fungal infection in relation to environmental conditions. *Indian Cocon. J.* 15(1): 1-11.

Radha, K. and Lal, S.B. 1968. Some observations on the occurrence of leaf rot disease of coconut and associated factors. *Third session, FAO Technical Working Party.* Coconut Production Protection and Processing, Jakarta, pp. 1-5.

Rahimloo, T. and Ghosta, Y. 2015. The occurrence of *Alternaria* species on cabbage in Iran. *Zemdirbyste-Agric.* 102(3): 343-350.

Riddel, R.W. 1950. Slide cultures. *Mycologia* 42: 265-270.

Rodriguez-Kabana, R. 1967. An improved method for assessing soil-fungus population density. *Plant and Soil* 26(2): 393-396.

Sathiarajan, P. K., Rajan, K. M., Govindan, M., Radhakrishnan, T. C., Philip, S., Rehmatuniza, T. J., and Mathew, J. 1988. Diseases and their management. In: Aravindakshan, M. and Nair, R. R. (eds), *Six decades of Coconut Research.* Kerala Agricultural University, Thrissur, pp. 133-135.

Shanmughom, S.N. 1963. Morphological and physiological studies on *Helminthosporium halodes* Drechs. The leaf rot fungus of coconut. M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 87p.

Sharma, S. K. 2012. Studies on fruit rot of pomegranate caused by *Colletotrichum gloeosporioides* and its management. Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan, 70p.

Shrestha, B. K., Karki, H. S., Groth, D. E., Jungkhun, N., and Ham, J. H. 2016. Biological control activities of rice-associated *Bacillus* sp. strains against sheath blight and bacterial panicle blight of rice. *PLoSOne* 11(1): 21-38.

Sowndhararajan, K., Marimuthu, S., and Manian, S. 2012. Biocontrol potential of phylloplane bacterium *Ochrobactrum anthropi* BMO-111 against blister blight disease of tea. *J. Appl. Microbiol.* 114: 209-218.

Srinivasan, N. 1991. Occurrence of coconut leaf rot in relation to root (wilt) disease. *Indian Cocon. J.* 21(10): 14-18.

Srinivasan, N. 2008. *Management of Coconut Leaf Rot Disease.* CPCRI Technical Bullettin Series No. 58, Niseema printers and publishers, Ernakulam, 10p.

Srinivasan, N. and Bharathi, R. 2006. Biocontrol agents against pathogens of coconut leaf rot disease. *J. Plantn Crops* 34(3): 494-499.

Srinivasan, N. and Gunasekaran, M. 1992. An appraisal of symptom expression in coconut due to leaf rot disease. *Indian Cocon. J.* 23(7): 2-6.

Srinivasan, N. and Gunasekaran, M. 1993. Fungi associated with leaf rot disease of coconut. *Indian Cocon. J.* 23(10): 2-7.

Srinivasan, N. and Gunasekaran, M. 1994. Identification of *Colletotrichum gloeosporioides, Exserohilum rostratum* and *Gliocladium vermoeseni* associated with leaf rot disease of coconut in India. *Cord* 10: 34-50.

Srinivasan, N. and Gunasekaran, M. 1995a. *Cylindrocladium scoparium* in relation to leaf rot disease of coconut. *Indian Phytopathol.* 48: 196-198.

Srinivasan, N. and Gunasekaran, M. 1995b. Additional fungi associated with leaf rot disease of coconut in India (A research note). *Philipp. J. Coconut Stud.* 19: 26 - 27.

Srinivasan, N. and Gunasekaran, M. 1996a. Pathogenicity of preponderant fungi associated with leaf rot disease of coconut. *Indian Cocon. J.* 27(3): 24.

Srinivasan, N. and Gunasekaran, M. 1996b. Coconut leaf rot intensity and fungal incidence. *Indian Cocon. J.* 26(9): 10-13.

Srinivasan, N. and Gunasekaran, M. 1996c. Incidence of fungal species associated with leaf rot disease of coconut palms in relation to weather and the stage of lesion development. *Ann. Appl. Biol.* 129(3): 433-449.

Srinivasan, N. and Gunasekaran, M. 2000. *Leaf Rot Disease of Coconut.* CPCRI Technical Bulletin Series No. 38, Niseema printers and publishers, Ernakulam, 14p.

Srinivasan, N., Chandramohanan, R., Bharathi, R., Radhika, N.S., and Issak, S. 2010. Mass production and use of biocontrol agents in the integrated management of coconut leaf rot disease–technology popularization through participatory programmes in disease affected regions. In: Thomas, G. V., Krishnakumar, V., and Jerard, B. A. (eds), *Improving Productivity and Profitability in Coconut farming.* Proceedings of international conference on coconut biodiversity for prosperity, 25-28 October 2010, Kasargod. Central Plantation Crops Research Institute, Kasargod, pp.424- 431.

Srinivasan, N., Chandramohanan, R., Bharathi, R., Radhika, N.S., and Issak, S. 2011. Development of Technology and Popularization of Biological Control Agents for Integrated Management of Coconut Leaf Rot Disease. *CORD* 27(1): 48-60.

Srinivasan, N., Gunasekaran, M., Iyer, R., Chandramohanan, R., and Babu, K.M. 1995. Mycoflora of leaf rot affected coconut palms. *Indian Phytopathol.* 48: 93-95.

Srinivasan, N., Gunasekaran, M., Joseph T., and Rawther T.S.S. 1998. Leaf rot disease. In: Nampoothiri, K.U.K. and Koshy, P.K. (eds), *Coconut root (wilt) disease.* Codeword process and printers, Mangalore, pp: 93 – 95.

Srinivasan, N., Rahna, J.S., and Anishkumar, V.K. 2006. Evaluation of fungicides and antagonistic organism against major pathogens of leaf rot disease of coconut and their eco-friendly management. *Cord* 22(1): 27-50.

Sundararaman, S. 1925. Preliminary note on coconut leaf rot on Cochin. *Year Book.* Madras Agricultural Department, pp. 6-8.

Sundararaman, S. 1929. *Administration Report of the Mycologist.* Government Press, Coimbatore, 27p.

Tembhare, N.R., Mate, G.D., and Gajbhiye, P.R. 2012. Phyllosphere fungi of chilli and antagonism against *Colletotrichum Capsici*. *J. Plant Dis. Sci. 7*(1): 45-47.

Udhayakumar, R., Usharani, S., and Muthukumar, A. 2019. Pathogenicity variation, morphological and cultural characteristic of *Colletotrichum gloeosporioides* isolates. *Plant Arch.*19(1): 425-430.

Varghese, M.K. 1934. *Disease of the coconut palm*. Department of Agriculture and fisheries, Travancore, 105 p.

Vincent, J.M. 1927. Distortion of fungal hyphae in the presence of certain inhibitors. *Nat.*159: 850.

Vrinda, T.S. 2002. Integrated management of leaf rot disease of coconut. M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 93p.

Wijesekara, H.T.R., Perera, L., Wickramananda, I.W., Herath, I., Meegahakumbura, M.K., Fernando, W.B.S., and De Silva, P.H.P.R. 2008. Preliminary investigation on Weligama coconut leaf Wilt disease: a new disease in Southern Sri Lanka. In: N.P.A.D. and Everard, J.M.D.T. (eds), *Proceedings of the Second Symposium on Plantation Crop Research—Export competitiveness through quality improvements,* Coconut Research Institute, Lunuwila. Samayawardana Printers, Sri Lanka. pp.336–341.

Appendices

APPENDIX – I

COMPOSITION OF MEDIA USED

Potato Dextrose Agar (PDA) Medium

Potato	: 200 g
Dextrose	: 20 g
Agar	: 20 g
Distilled water	: 1 L

King's B Medium (KB) Medium

Proteose peptone	: 20 g
Glycerol	: 10 ml
K_2HPO_4	: 1.5 g
$MgSO_4$	: 1.5 g
Agar	: 15 g
Distilled water	: 1 L

Martin's Rose Bengal Agar (MRBA)

Glucose	: 10 g
Peptone	: 5 g
K2HPO4	: 0.5 g
KH2PO4	: 0.5 g
MgSO4. 7 H2O	: 0.5g
Rose Begal	: 30 mg
Yeast extract	: 0.5 g
Streptomycin sulphate	: 30 mg
Distilled water	: 1 L

APPENDIX – II

COMPOSITION OF STAIN USED

Lactophenol Cotton Blue

Anhydrous lactophenol	: 67 ml
Distilled water	: 20 ml
Cotton blue	: 0.1 g

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

CHARACTERIZATION OF FUNGAL PATHOGEN ASSOCIATED WITH LEAF ROT DISEASE OF COCONUT (*Cocos nucifera* L.) AND *IN VITRO* EVALUATION OF PHYLLOPLANE MICROFLORA AS BIOCONTROL AGENTS

by

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Abstract of Thesis

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ABSTRACT

Leaf rot disease (LRD) is a major foliar disease affecting coconut plantations of Southern Kerala especially in root (wilt) affected areas. In this context, the study entitled 'Characterization of fungal pathogen associated with leaf rot disease of coconut (*Cocos nucifera* L.) and *in vitro* evaluation of phylloplane microflora as biocontrol agents' was conducted in the Department of Plant Pathology, College of Agriculture, Vellayani during the year 2018-2020, with the objective to identify and characterize the major fungal pathogens associated with the LRD of coconut and *in vitro* evaluation of phylloplane microflora of coconut against the pathogens.

The isolation of LRD pathogens was carried out from six taluks of Thiruvananthpuram district such as Thiruvananthapuram, Neyyattinkara, Nedumangad, Chirayinkeezhu, Kattakada and Varkala. Three locations were selected from each taluk and a total of eighteen samples were collected during the study. The results revealed that the disease in Thiruvananthapuram district was caused by a spectrum of pathogens such as *Colletotrichum gloeosporioides*, *Fusarium* spp., *Gliocladium* sp., and *Scytalidium* sp.

The LRD was caused either by a single pathogen or by combinations of pathogens. *C. gloeosporioides* and *Fusarium* spp. were found as the major pathogens of LRD based on the frequency of isolation. Each and every isolate of the same pathogen differed from one another in cultural characters and virulence. All the pathogens produced water soaked brown lesion on artificial inoculation on detached spindle leaves; though the days taken for symptom initiation and size of the lesion developed varied. The isolate C3 (Isolate from Anayara, Thiruvananthapuram taluk) was found to be more virulent among the *C. gloeosporioides* isolates; and among the *Fusarium* spp. isolates, the isolate F5 (Isolate from Alamkode, Chirayinkeezhu taluk) was found to be more virulent. By observing the spore characters of the isolates, it was found that the spore size and pigmentation of the culture haven't any significance to the virulence of the pathogen.

Dual inoculation of the major pathogens on detached spindle leaves caused severe incidence of the disease compared to the individual inoculation of the pathogens. This

result indicated that the LRD caused by fungal complex is more severe than that caused by individual fungal isolates.

There are phylloplane fungi existing on healthy leaves of the infected palm with enough inhibition potential to LRD. The phylloplane fungal isolate PF5 showed more per cent inhibition to mycelial growth of *C. gloeosporioides* (54.44%) followed by the isolate PF4 (43.33%); and the isolate PF4 showed more inhibition to *Fusarium* sp. (64.44%) followed by the isolate PF5 (45.55%) in the dual culture assay.

The detached spindle leaf assay also supported the same fact that the isolate PF5 was observed to be more suppressive to the disease caused by *C. gloeosporioides* (28.77%) and the isolate PF4 was reported to have more suppression to the disease caused by *Fusarium* sp. (34.56%). These pre-treatment effects are more promising than *Pseudomonas fluorescens* PN026, but inferior to copper oxy chloride (0.2%).

Thus, the present study revealed that the LRD of coconut in Thiruvananthapuram district is caused by a combination of pathogenic fungi *viz., C. gloeosporioides*, *Fusarium* spp., *Gliocladium* sp., and *Scytalidium* sp. Prophylactic application of the phylloplane fungal isolates PF4 and PF5 could reduce the LRD severity *in vitro* to a promising level and these isolate can be further tested for *in vivo* biocontrol potential before going for the development of a formulated product.

സംഗ്രഹം

ദക്ഷിണ കേരളത്തിലെ തെങ്ങിൻതോപ്പുകളിൽ, പ്രത്യേകിച്ച് കാറ്റുവീഴ്ച ബാധിത പ്രദേശങ്ങളിൽ കണ്ടുവരുന്ന ഒരു പ്രധാന രോഗമാണ് ഇലകരിച്ചിൽ. വെള്ളായണി കാർഷിക കോളേജിലെ സസ്യരോഗ വിഭാഗത്തിൽ 'തെങ്ങിന്റെ ഇലകരിച്ചിൽ രോഗത്തിന് കാരണമായ കുമിളുകളും തെങ്ങോലകളിലെ മിത്രകുമിളുകൾ ഉപയോഗിച്ച് രോഗകാരികളായ കുമിളുകളുടെ നിയന്ത്രണവും' എന്ന വിഷയത്തിൽ 2018-2020 കാലയളവിൽ നടത്തിയ ഗവേഷണത്തിന്റെ ഫലങ്ങൾ സംക്ഷിപ്ത രൂപത്തിൽ ചേർക്കുന്നു. ഇലകരിച്ചിൽ രോഗത്തിന് കാരണമായ കുമിളുകളെ കണ്ടെത്തുക, തെങ്ങോലകളിൽ നിന്നും വേർതിരിച്ചെടുത്ത മിത്രകുമിളുകളെ ഉപയോഗപ്പെടുത്തി രോഗകാരികളായ കുമിളുകളെ നിയന്ത്രിക്കുക എന്നിവയായിരുന്നു പഠനത്തിന്റെ മുഖ്യ ലക്ഷ്യങ്ങൾ.

തിരുവനന്തപുരം ജില്ലയിലെ ആറ് താലൂക്കുകളായ തിരുവനന്തപുരം, നെയ്യാറ്റിൻകര, നെടുമങ്ങാട്, ചിറയിൻകീഴ്, കാട്ടാക്കട, വർക്കല എന്നിവ കേന്ദ്രീകരിച്ചായിരുന്നു പഠനം. ഓരോ താലൂക്കിൽ നിന്നും മൂന്ന് തെങ്ങിൻ തോട്ടങ്ങൾ തിരഞ്ഞെടുക്കുകയും മൊത്തം പതിനെട്ട് ഇലകരിച്ചിൽ ബാധിത സാമ്പിളുകൾ പഠന സമയത്ത് ശേഖരിക്കുകയും ചെയ്തു. തിരുവനന്തപുരം ജില്ലയിൽ ഇലകരിച്ചിൽ രോഗകാരികളായി കണ്ടെത്തിയ കുമിളുകൾ കൊളറ്റോട്രിക്കം ഗ്ലിയോസ്പോറിയോയിഡ്സ്, ഫ്യൂസേറിയം സ്പീഷിസ്, ഗ്ലിയോക്ളാഡിയം സ്പീഷിസ്, സ്കൈറ്റാലിഡിയം സ്പീഷിസ് എന്നിവയായിരുന്നു.

രോഗകാരിയായ ഒരു കുമിളോ, അല്ലെങ്കിൽ ഒന്നിലധികം കുമിളുകളോ ആണ് ഇലകരിച്ചിൽ രോഗമുണ്ടാക്കുന്നത്. പ്രധാനമായും കൊളറ്റോട്രിക്കം ഗ്ലിയോസ്പോറിയോയിഡ്സ്, ഫ്യൂസേറിയം സ്പീഷിസ് എന്നിവയാണ് തിരുവനന്തപുരത്ത് ഇലകരിച്ചിൽ രോഗമുണ്ടാക്കുന്നതെന്നു കണ്ടെത്തി. പ്രാദേശിക വ്യത്യാസമനുസരിച്ച് ഒരേ വർഗത്തിൽപ്പെട്ട കുമിളുകൾക്ക് രോഗമുണ്ടാക്കാനുള്ള കഴിവ് വ്യത്യാസപ്പെട്ടിരിക്കുന്നു. കുരുത്തോലയുടെ ഇലകളിൽ കൃത്രിമമായി കുമിളുകൾ ഇനോക്കുലേറ്റ് ചെയ്യുമ്പോൾ രോഗലക്ഷണങ്ങൾ ആരംഭിക്കാൻ അവ എടുക്കുന്ന സമയവും, പുള്ളികളുടെ വലിപ്പവും വ്യത്യാസപ്പെട്ടിരിക്കുന്നുവെങ്കിലും എല്ലാ കുമിളുകളും പൊതുവെ കുതിർന്ന

തവിട്ടു നിറത്തിലുള്ള പുള്ളികളാണ് ഇലകളിൽ ഉണ്ടാക്കുന്നത്. ഈ വ്യത്യാസങ്ങളെ ആസ്പദമാക്കി രോഗലക്ഷണങ്ങൾ ഉണ്ടാക്കാൻ കൂടുതൽ കഴിവുള്ള കൊളറ്റോട്രിക്കം ഗ്ലിയോസ്പോറിയോയിഡ്സ് വിഭാഗത്തിൽപെട്ട കുമിളുകളിൽ ഐസൊലേറ്റ് സി 3 ഉം (ആനയറ, തിരുവനന്തപുരം താലൂക്ക്) ഫ്യൂസേറിയം സ്പീഷിസ് വിഭാഗത്തിൽ ഐസൊലേറ്റ് എഫ് 5 ഉം (ആലംകോട്, ചിറയിൻകീഴ് താലൂക്ക്) ആണെന്ന് തെളിയിച്ചു.

കുരുത്തോലയുടെ ഇലകളിൽ രോഗകാരികളായ കുമിളുകൾ ഒരുമിച്ച് ഇനോക്കുലേറ്റ് ചെയ്യുമ്പോൾ രോഗലക്ഷണങ്ങൾ മൂർച്ചരിക്കുന്നതിനു കാരണമായി. ഒന്നിൽ കൂടുതൽ കുമിളുകളുടെ ആക്രമണം ഇലകരിച്ചിൽ രോഗത്തിന്റെ തീവ്രത വർധിപ്പിക്കുന്നു.

ഇലകരിച്ചിൽ രോഗമുള്ള തെങ്ങുകളുടെ രോഗബാധയില്ലാത്ത തെങ്ങോലകളിൽ ധാരാളം ഫില്ലോപ്ലെയ്ൻ മിത്ര കുമിളുകളുണ്ടായിരുന്നു. ഫില്ലോപ്ലെയ്ൻ മിത്ര കുമിൾ ഐസൊലേറ്റ് പി.എഫ്. 5 ഉം, പി.എഫ്. 4 ഉം കൊളറ്റോട്രിക്കം ഗ്ലിയോസ്പോറിയോയിഡ്സ് കുമിളിന്റെ തന്തുക്കളുടെ വളർച്ചയെ യഥാക്രമം 54.44 % ഉം 43.33 % ഉം പ്രതിരോധിക്കുന്നു. ഫില്ലോപ്ലെയ്ൻ മിത്ര കുമിൾ ഐസൊലേറ്റ് പി.എഫ്. 4 ഉം പി.എഫ്. 5 ഉം ഫ്യൂസേറിയം സ്പീഷിസുകളുടെ തന്തുക്കളുടെ വളർച്ചയെ യഥാക്രമം 64.44 % ഉം 45.55 % ഉം പ്രതിരോധിക്കുന്നു.

കൃത്രിമമായി രോഗകാരികൾ ഇനോകുലേറ്റ് ചെയ്ത കുരുത്തോലയുടെ ഇലകളിൽ ഫില്ലോപ്ലെയ്ൻ മിത്ര കുമിളുകൾ തളിച്ചു നടത്തിയ പഠനത്തിൽ കൊളറ്റോട്രിക്കം ഗ്ലിയോസ്പോറിയോയിഡ്സ് ഉണ്ടാക്കുന്ന ഇലകരിച്ചിൽ രോഗത്തെ ഐസൊലേറ്റ് പി.എഫ്. 5, 28.77% ഉം ഫ്യൂസേറിയം സ്പീഷിസ് ഉണ്ടാക്കുന്ന ഇലകരിച്ചിൽ രോഗത്തെ ഐസൊലേറ്റ് പി.എഫ്. 4, 34.56% ഉം കൂടുതൽ പ്രതിരോധിക്കുന്നു.

പരീക്ഷണ ശാലയിൽ പി.എഫ്. 4, പി.എഫ്. 5 എന്നീ മിത്രകുമിളുകൾ രോഗകാരികളായ കുമിളുകൾക്കെതിരെ ഫലപ്രദമാണെന്നതിനാൽ ഇവയുടെ സാധ്യതകളെ തെങ്ങിൻ തോട്ടങ്ങളിൽ വിലയിരുത്തിയതിനു ശേഷം ഒരു ഉല്പന്നമാക്കി വിപണിയിലിറക്കാം.