HOST RANGE STUDIES AND MANAGEMENT OF ANTHRACNOSE OF NUTMEG CAUSED BY *COLLETOTRICHUM* SPP.

BOMMANA DIVYA

(2018-11-096)

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE,

VELLAYANI, THIRUVANANTHAPURAM-695522

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by

BOMMANA DIVYA

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THESIS

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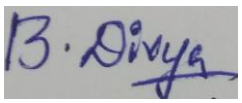
KERALA, INDIA

2020

DECLARATION

I, hereby declare that this thesis entitled "**Host range studies and management of anthracnose of nutmeg caused by *Colletotrichum* spp."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.



Vellayani **Bommana Divya**

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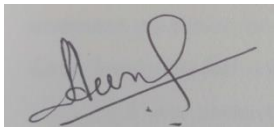


Vellayani

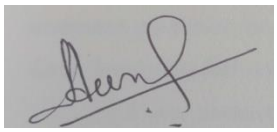
Date: 6/03/2021

Dr. Heera G.

(Major Advisor, Advisory Committee)
Assistant Professor
Department of Plant Pathology
College of Agriculture, Vellayani

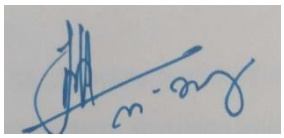
CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Bommana Divya, a candidate for the degree of Master of Science in Agriculture with major in Plant Pathology, agree that the thesis entitled "**Host range studies and management of anthracnose of nutmeg caused by *Colletotrichum* spp.**" may be submitted by Bommana Divya, in partial fulfillment of the requirement for the degree.



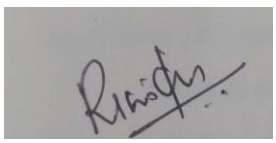
Dr. Heera G.

(Chairman, Advisory committee)
Assistant Professor
Department of Plant Pathology
College of Agriculture Vellayani



Dr. Joy M.

(Member, Advisory committee)
Associate Professor and Head
Department of Plant Pathology
College of Agriculture, Vellayani



Dr. Radhika N.S.

(Member, Advisory committee)
Assistant Professor
Department of Plant Pathology
College of Agriculture, Padanakkad

Dr. Sreekala G.S.

(Member, Advisory committee)
Assistant Professor
Dept. of Plantation Crops and Spices
College of Agriculture, Vellayani

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LIST OF ABBREVIATIONS USED

et al.,	And other co workers
Viz.,	Namely
Spp.	Species
PDA	Potato dextrose agar
PDI	Per cent disease index
DTS	Days taken for symptom appearance
DAI	Days after inoculation
DAG	Days after growth
CD	Critical difference

LIST OF SYMBOLS USED

cm	Centimetre
µm	Micrometer
%	Per cent
^{0}C	Degree Celsius

Introduction

1. Introduction

Nutmeg (*Myristica fragrans* Houtt.) a perennial evergreen tree spice belonging to Myristicacea family is native of Moluccas islands in Indonesia which is popularly known as a twin spice as it yields two products *i.e*, nutmeg from seed and mace from seed covering in which both of them are used for spice purpose due to its flavour, aroma, and fragrance properties. Other names of nutmeg are jaatipatree, jatiphalam, muskatnuss, muscade and jatiphal.

Nutmeg has immense applications as a conventional ingredient in eggnog, beverage, wine, cider and is also used as a flavouring agent in making of sweets, puddings, cakes, cookies etc. It is used for the extraction of essential oils, sabinene and safrole which have a potential role in pharmaceuticals and perfumery industries. It has medicinal properties widely used in the treatment of diarrhaea, vomiting, dizziness etc. Nutmeg is well known for its high market value due to its value-added products *i.e.*, nutmeg syrup, nutmeg jelly and nutmeg candy. As a spice, nutmeg also helps in relieving pain, stress, depression, and boosts up the digestion. It also helps in regulating blood cholesterol and blood pressure levels. Nutmeg oils containing eugenols, phenolic compounds and mace lignans have antioxidant properties (Dorman *et al*., 2000: Kwon and Jeong, 2008). The leaf or aril crude extracts were found to possess inhibitory effect against *Helicobacter pylori* at concentration of 12.5 µg ml^{-1} (Bhamarapravati *et al.,* 2003).

India is the largest exporter of nutmeg, in which Kerala, Karnataka, Tamil Nadu and Goa are the leading producers. The production of nutmeg in India during 2016–17 was about 16,000 MT from an area of 23,000 ha (GOI, 2017*).* Kerala contributes to a production of 13,746 tons from an area of 22,065 ha (GOK, 2017).

The crop is affected by various diseases *viz.* leaf spot, thread blight, fruit rot, dieback, twig blight, of which leaf spot / anthracnose / fruit rot caused by *Colletotrichum gloeosporioides* is one of the major pathogens that causes huge economic loss as it can even affects fruit.

Anthracnose of nutmeg was reported for the first time from Kerala in 1961 by an unknown species of *Colletotrichum* later confirmed as *C. gloeosporioides* by Nair *et al.*

(1978). The disease was characterised by central necrotic region surrounded by reddish brown or yellow halo on the foliage. The advanced infection shows the shredding of the central necrotic portion resulting in shot hole symptom.

C. gloeosporioides has wide host range which can occur in different crops *viz.*, cereals (maize, sorghum), legumes (cowpea, bengal gram, redgram, groundnut etc.), grasses, perennial fruit crops (citrus, mango, guava, pomegranate, jackfruit etc.), spice crops (chilli, black pepper, nutmeg), plantation crops (tea, coffee, coconut) and ornamentals (orchids, dracaena, pansy). Information on cross infectivity of *C. gloeosporioides* isolate from nutmeg in infecting the other spice crops *i.e.,* clove, cinnamon, betel vine, black pepper and all spice.

Current management strategy of leaf spot / shot hole / anthracnose disease includes spraying of one per cent Bordeaux mixture, biocontrol practices of applying *Pseudomonas flourescens* and *Trichoderma*. in which the complete management is not achieved. The information on practical application of new generation fungicides *i.e*, triazoles and strobilurins in the perennial tree spice crops is limited and thus there is a need for developing a new management strategy with new generation fungicides.

In this background, the present study entitled "Host range studies and management of anthracnose of nutmeg caused by *Colletotrichum* spp." is aimed at achieving the following objectives:

- Collection of diseased samples from nutmeg growing tracts of Kerala *i.e.,* Thiruvananthapuram, Kottayam, Ernakulam and Idukki.
- Morphological and pathogenic variability of *Colletotrichum* isolates
- Study of the host range of the virulent isolate of nutmeg in other tree spices *i.e.,* clove, cinnamon, black pepper, allspice, betelvine, along with coconut.
- *In vitro* screening of the new generation fungicides *i.e.,* triazoles, strobilurins and combination fungicides

Review of literature

2. REVIEW OF LITERATURE

Nutmeg (*Myristica fragrans* Houtt.) belonging to family Myristicaceae is unique among spices as the donor of two distinct spices, nutmeg from its seed, and mace from the seed covering. Both mace and nutmeg have immense applications as a spice in flavoring food and medicine for different stomachic disorders. The value-added products *viz.,* nutmeg syrup, nutmeg jelly and candy are having high market value. It is also a commercial source of nutmeg butter and essential volatile oils such as sabinene and safrole.

Nutmeg is a perennial tree spice which grows to a height of 25m, widely distributed in India in Western Ghat region (Challadurai and Ramalingam, 2017). It belongs to the Kingdom - Plantae, Super division - Angiosperms, Phylum - Tracheophyta, Class - Magnoliopsida, Order - Mangoliales, Family - Myristicaceae, Genus- Myristica and Species - malabarica, fragrans (Challadurai and Ramalingam, 2017) (Kumar *et al*., 2016). The term anthracnose was first coined by Fabra and Dunal (1883) which means coal like symptoms.

2.1 HISTORY, PREVALENCE AND YIELD LOSS

Tode (1790) has placed *Colletotrichum* under *Vermicularia*. Later Corda (1837) re-established it as *Colletotrichum* with a single species - *C. sublineolum.* Penzing (1882) described the fungus *C. gloeosporioides* Penz., as *Vermicularia gloeosporioides* and the *Colletotrichum* was later placed as a separate genus in 1887.

Taxonomic classification of *Colletotrichum* known as Von Arxian classification was first proposed by Arx and Muller (1954) based on the conidial size and shape. Based on this classification, the genus was established with 11 species whose number varied over the course of evolution (Arx, 1957). Sutton (1980) has done species delimitation to 22 species based on conidial size and shape, colony characteristics, presence of sclerotia, appressorial morphology and host specificity. Sutton (1992) classified the genus *Colletotrichum* with 40 species. Kirk *et al*. (2008) reported 60 species in the book Dictionary of fungi.

Stoneman (1898) identified *Colletotrichum* sexual morphs under the genus *Gnomoniopsis*. Shear and Wood (1907) for the first time identified *Glomerella* as teleomorph stage of *Colletotrichum* spp. The sexual stage of *C. gloesporioides* was later confirmed as *Glomerella cingulata* by Small (1926).

C. gloeosporioides was first reported in India in coffee as causal agent of leaf spot disease by Butler (1918).

Colletotrichum spp. was ranked as eighth most important pathogen and was described as facultative parasite. Also, all the crops were affected by at least one or more species of *Colletotrichum* among which *C. gloeosporioides* is one of the most important pathogens. *Colletotrichum* genus has a unique infection strategy due to its hemibiotrophic life style and thus possessing pathogenic variability even within the species (Dean *et al*., 2012).

Colletotrichum gloeosporioides belongs to the Kingdom: Fungi, Phylum: Imperfect fungi, Genus: *Colletotrichum*, Species: *gloeosporioides*, and the teleomorph of the fungus belongs to Kingdom: Fungi, Phylum: Ascomycota, Subphylum: Pezizomycotina, Class: Sordariomycetes, Order: Glomerellales, Family: Glomerellaceae Genus: *Glomerell*a, Species: *cingulate* (Kirk *et al*., 2008).

Menon and Rema Devi (1967) reported the anthracnose disease of nutmeg for the first time from Kerala and found incited by unknown *Colletotrichum* sp. Nair *et al.* (1978) confirmed the anthracnose disease in nutmeg which had been incited by *C. gloeosporioides.*

Colletotrichum spp. cause disease in wide range of crops and extensively distributed in tropical and subtropical regions. *C. gloeosporioides* was found all over the world *viz.*, Philippines, Trinidad, Florida, India, Pakistan, Indonesia, Argentina, South Africa, Peru, and many other countries and was the most prevalent in the regions having low temperature and high relative humidity (Waller,1992).

More than 50 per cent of losses of fresh fruits and vegetables are caused by *Colletotrichum* species (Paull *et al*., 1997; Awang *et al*., 2011). Up to cent per cent losses in stored fruit can be incited by *Colletotrichum* disease (Prusky, 1996). Die back of mango caused by *C. gloeosporioides* is the major problem in Konkan region of Maharashtra (Sawant and Raut, 2000). Cent per cent fruit loss was reported in apple by Shane and Sutton, (1981) from USA, whereas 28 to 34 per cent yield loss due to the *Colletotrichum* infection was reported in black pepper (Nair *et al*., 1987) and 100 per cent yield loss if berries were infected (Santhakumari and Rajagopalan, 2000). Fifty per cent yield loss of citrus due to soft brown decay caused by *Colletotrichum* spp. was reported in Canada by Osorio (2000). Fruit rot of mango caused by *C. gloeosporioides* resulted in yield loss of 39 per cent, 32 per cent, 64.6 per cent, 100 per cent, 20-30 per cent and 29 per cent, in Philippines (Clara, 1927), South Africa (Sanders *et al*., 2000), Costarica (Arauz *et al*., 1994), Indonesia (Arauz *et al*., 2000), Hyderabad (Prakash *et al*., 1989), Bangalore (Sohi *et al*., 1973) respectively. In

Ethiopia, yield loss due to *Colletotrichum* spp. infection was 24 -30 per cent (Derso, 1997) and 100 per cent yield loss in case of favourable epidemic conditions (Derso, 2000).

Survey conducted to estimate severity of anthracnose disease in pomegranate caused by *C. gloeosporioides* revealed that it was common in pomegranate growing regions of north Karnataka with a severity of 15-25 per cent (Benagai *et al*.,2009). Patil *et al*. (2009) reported the incidence of the betel vine blight caused by *Colletotrichum s*pp. was severe during the months of August to December in Anjangaon and Akot of Maharashtra.

Black pepper anthracnose caused by *C. gloeosporioides* was severe when the environmental conditions *viz.,* minimum temperature is 18^0C and high rainfall of 306.6 mm. The disease was less severe in the case of higher temperature of 29.1 - 32.23^0C and the severity of the disease ranged between 7.5 – 39.5 per cent (Biju *et al.,* 2013).

Ahmed *et al*. (2014) reported the disease incidence of betelvine anthracnose was found to be severe in July month with 80.50 per cent and the lowest disease severity was recorded during the December month with 10.87 per cent.

Kumar *et al*. (2016) reported the humid tropical condition prevailing in the Kerala state was favourable for the development of many fungal pathogens in nutmeg. *Colletotrichum* leaf spot disease and Phytophthora leaf fall disease were severe affecting the production and productivity by reducing the quality and quantity of the nutmeg.

2.2. SYMPTOMATOLOGY, ISOLATION OF PATHOGEN, PROVING PATHOGENICITY OF *Colleotrichum* spp. CAUSING ANTHRACNOSE DISEASE OF NUTMEG

2.2.1 Symptomatology

The infection starts as small brown spots, enlarges to form oval spots which are irregular in shape with a well-defined margin. A brown halo on the periphery of the necrotic spots was present as thick band. Appearance of shot hole in advanced stage of infection was the characteristic symptom of the disease. The central necrotic regions surrounded by reddish-brown bands get detached and resulted in shot hole formation (Chattopadhyay and Maiti, 1990).

The symptoms of anthracnose caused by *C. gloeosporioides* in papaya were as circular to irregular lesions with greyish centre which coalesce resulting in the blighting and

shot hole symptoms on the foliage. The symptoms of necrotic lesions were also observed in petiole and fruits (Dickman and Alwarez,1983)

Betel vine anthracnose was more common in older plantations than new plantations. The symptoms of anthracnose were initially noticed as small black spots on the stem which coalesce to form black streaks leading to splitting up of the stem (Shahzad and Zareen, 2000).

In cashew symptoms of anthracnose appeared as water-soaked lesions both on young seedlings and adult plants, which later turned to orange brown or reddish-brown colour. In severe cases leaves, twigs, inflorescence and fruits developed showed complete blighting and drop off. The disease resulted in yield loss upto 50 per cent in the north eastern region of Brazil (Freire *et al*., 2002)

Gosh *et al*. (2003) reported the symptoms of anthracnose in mango as brown spots initially which coalesce leading to leaf blight, shot hole and also fruit rotting symptoms in the later stage of infection. They also stated environmental conditions *viz.*, high temperature and dry climate were not favourable for the growth of the fungus.

Colletotrichum spp. affects the various plant parts *viz.*, twigs, leaves, blooms and fruit, producing symptom, leaf spot, leaf blight, shot hole, die back, crown root rot, defoliation, bloom blight and fruit rot (Lubbe *et al*., 2006).

2.2.2. Isolation of the pathogen and maintenance of pure culture

Kumar *et al*., 2016 used 1% sodium hypochlorite solution for surface sterilization of samples for isolating the pathogen from different locations. The surface sterilized bits were then inoculated on the solidified PDA medium in petridish and incubated at a room temperature of 26 ± 2^0C. The pure culture obtained by hyphal tip method was maintained in PDA slants.

Savani and Rajashekar (2016) isolated *C. gloeosporioides* from leaf spot and twig blight disease of clove obtained from different parts of Kerala. The procedure followed was similar to that done by Kumar *et al.* (2016).

Behera *et al.*, 2019 isolated anthracnose pathogen of black pepper from the infected leaves by treating with 0.1 per cent mercuric chloride (Hgcl2) followed by three washings with sterile distilled water. The surface sterilized bits were transferred to petri dish with PDA medium.

2.2.3. Proving pathogenicity

The pathogenicity of *C. gloeosporioides* on black pepper was proved by inoculating the fungus by pin prick method after making the aberrations. The pricked portions of the leaf were inoculated with bits of mycelium along with the spore mass of seven-day old culture. The mycelial bits placed on the leaf were covered with a thin layer of moist cotton for providing the humidity to develop the symptom (Sankar, 2002). Artificial inoculation by spraying the spore suspension of *C. gloeosporioides* on the wounded leaves of arecanut produced symptoms as brown lesions within 4-5 DAI (Ashoka, 2003).

Wasanatha (2004) proved the pathogenicity of *C. gloeosporioides* obtained from papaya by artificial inoculation of spore suspension @ 1.0×10^7 conidia ml $^{-1}$ on two-week-old seedlings of papaya. The pathogenicity of *C. gloeosporioides* in black pepper was proved on the cuttings by making the aberrations on the leaves with sterilized sand paper. The mycelial bits of the fungus along with the conidia were placed on the abberated surface and the cuttings are incubated in the humid chamber for the development of symptoms (Chandrakant, 2005).

In anthracnose of betel vine, the pathogenicity was proved by the following technique. The leaves to be inoculated were surface-sterilized in 70 per cent alcohol followed by 3-4 washes in sterile distilled water and injured by dusting the carborundum powder over the leaves. The leaves were artificially inoculated by spore suspension along with fungal mycelial bits and incubated at room temperature (26 ± 2^0C) for symptom development (Haralpatil, 2006).

Pathogenicity of *C. gloeosporioides* causing pomegranate anthracnose was proved by artificial inoculation of the spore suspension and mycelial bits. The inoculation produced brown to black coloured lesions at 4 DAI and the lesions later turned to brownish surrounded by a chlorotic halo from 6 DAI (Jayalakshmi, 2010).

Wound drop technique was used to prove the pathogenicity of ten strains of *Colletotrichum* spp. in various crops *viz.*, guava, rose apple, mango, papaya, chilli and orange (Phouhvong *et al*., 2012). Gautam (2014) studied the taxonomy, biology and pathogenicity of *C. gloeosporioides* infecting mango, coffee, papaya and vanilla**.** Studies on pathogenic variability of *C. gloeosporioides* isolate on the detached leaves of mango, guava, kiwi, apple and peach revealed that the pathogen expressed the symptoms within 5-7 DAI on the various hosts with a maximum and minimum lesion size on kiwi and mango respectively. Sudha and

Narendrappa (2016) detected a highly virulent strain of *C. gloeosporioides* from mango which developed symptoms within two days of inoculation on artificially inoculated fruits.

2.3. MORPHOLOGICAL AND PATHOGENIC VARIABILITY OF *COLLETOTRICHUM* ISOLATES AND IDENTIFICATION OF VIRULENT ISOLATE

Conventionally, the *Colletotrichum* species were identified and characterised based on the variation in morphological features *viz.,* colour of the colony, size and shape of conidia and appressoria, (Arx, 1957; Smith and Black, 1990; Gunnell and Gubler, 1992; Sutton, 1992).

2.3.1. Morphological characterisation of different isolates

Yee and Sarriah (1993) studied the morphology of *C. gloeosporioides* isolated from cocoa, the conidia were cylindrical in shape with a size ranging 5-2 µm X 2 -6 µm. Conidia of *Colletotrichum gloeosporioides* obtained from nutmeg were hyaline, single celled with round edges and the size ranged from 11.45 - 23.62 µm x 3.58 - 5.72 µm (Kumar *et al*., 2016).

Sankar (2002) reported the variation in the conidial size from isolates of *C. gloeosporioides* obtained from black pepper.The conidial size varied between 13.10 - 16.85 µm length and 3.28 - 5.43 µm width. Mamooty (2003) observed the mycelial width of the *C. gloeosporioides* from black pepper ranged from 1.25 - 4.00 µm and the size of the conidia ranged from 13.45 - 16.45 µm length and 3.86 - 5.36 µm width.

The mycelium of *C. gloeosporioides* isolates obtained from papaya were brown to black and septate; and the conidia were cylindrical with a size ranging 13.2-14.8 µm x 4.6-5.1 µm (Bag, 2004) The conidia of *C. gloeosporioides* isolates obtained from *Cymbidium sinense* were either straight or cylindrical or oblong in shape and the size of conidia varied from 14.0 -19.5 µm x 4-6 µm (Huang *et al*., 2012). The conidia of *C. gloeosporioides* isolate obtained from citrus were either straight or cylindrical with its size varying from 8.4 – 16.8 µm x 3.5 – 4.2 µm (Jiang *et al*., 2012)

Sudha and Narendrappa (2016) reported the conidia of the isolates *C. gloeosporioides* obtained from mango varied in their size ranging from 11.45- 16.87 µm length and 4.23-6.30 µm width. *C. gloeosporioides* causing clove leaf spot disease produced hypae which were coloured, septate and branched. Conidia were hyaline, single celled with round ends and had a size of 11.45 - o 23.62 µm length and 3.58 - 5.72 µm width (Savani and Rajashekar, 2016)

Chavan *et al*. (2017) reported *C. gloeosporioides* isolates from different fruit crops produced hyaline and cylindrical conidia; and its size ranged from 7.00 -18.00 µm in length, 3.32- 8.13 µm in width. Dharbale *et al*. (2019) reported that the *C. gloeosporioides* isolates causing orange anthracnose varied in conidial size ranging from 18.21 to 26.62 µm.

2.3.2 Cultural characterisation of different isolates

The colony colour of *C. gloeosporioides* isolates from cocoa varied from white to olive grey in potato dextrose agar medium. The rear view of colony showed smoky greyish colouration (Yee and Sarriah, 1993). The isolates of *C. gloeosporioides* of black pepper had different mycelial growth *i.e*., cottony to suppressed with dark grey to black colony. The colony characteristics showed either white with light grey or dark greyish centre (Sankar, 2002). Mammooty (2003) reported isolates of *C. gloeosporioides* from black pepper with colony colour of light grey or dark grey. The colony of *C. gloeosporioides* isolates from orchid showed a colour variation from greyish white or pinkish grey to orange colouration in the front view whereas whitish, dark grey to orange in the rear view (Chowdappa *et al*., 2012). The colony colour of *C. gloeosporioides* isolates obtained from *Cymbidium sinense* were white coloured at first later turning to greyish white to grey and pink to brownish red. (Huang *et al*., 2012)

C. gloeosporioides isolated from nutmeg leaf spot completed full growth in the Petridish containing PDA medium within six DAI (Kumar *et al.* 2016). Savani and Rajashekar (2016) also reported *C. gloeosporioides* from clove took six days for complete growth in Petridish. The mycelium was pinkish white initially later turning to brown and black.

Sudha and Narendrappa (2016) reported the isolates of *C. gloeosporioides* causing mango anthracnose produced greyish to dark grey colonies in the front view and in the rear view, orange to black pigmentation. The colonies of isolates exhibited raised fluffy to flat mycelial growth with smooth, regular to irregular margins. These cultures of Colletotrichum produced orange or salmon coloured conidial mass and acervuli as black dots in the culture when grown on PDA medium.

Chavan *et al*. (2017) reported the isolates of *C. gloeosporioides* from different fruit crops *viz.*, pomegranate, papaya, banana, chikoo, mango, citrus, custard apple and guava showed variation in colony colour *viz.*, greyish pink, greyish black, off white, cottony white, creamy white and dull white with smooth, raised or fluffy raised margins.

Dharbale *et al*. (2019) reported the isolates of *C. gloeosporioides* obtained from sweet orange showed black coloured colonies with regular margin.

Udhaykumar *et al*. (2019) reported the isolates of *C. gloeosporioides* causing mango anthracnose showed difference in colony colour varying from pinkish brown, normal white to light grey, greyish white, greyish brown, and greenish grey.

2.3.3 Pathogenic variability of *C. gloeosporioides*

Freeman and Shabi (1996) isolated forty-two strains of *C. gloeosporioides* from different plants *viz.,* mango, apple, pecan, almond, and avocado. The different isolates showed a significant difference with respect to the lesion size. In case of isolates obtained from mango and avocado when artificially inoculated onto the hosts, it was found the isolates obtained from apple were not virulent. Pathogenic variability studies for virulence rating conducted by artificial inoculation of the pathogen classified the pathogen as most virulent, moderately virulent and less virulent based on the lesion size at 7DAI (Sankar 2002). Martinez *et al*. (2009) inoculated thirty *C. gloeosporioides* isolates from mango to the healthy mango fruits. They found that 19 isolates were able to produce symptoms at four DAI whereas other isolates failed to develop any symptoms. Figueiredo *et al*. (2012) observed that eighteen isolates of *C. gloeosporioides* from cashew when inoculated on to healthy leaves developed symptoms within 3-5 days. The variability of *C. gloeosporioides* isolates from mango was expressed as most virulent, moderately virulent and less virulent based on the per cent disease index and it ranged from 12.55 to 76.30 per cent (Devamma *et al*., 2012). Virulence of the various *C. gloeosporioides* isolates obtained from cowpea were subjected to pathogenic variability studies and the most virulent isolate was selected based on the days taken for symptom appearance (Sreeja, 2014)

2.4 HOST RANGE STUDIES OF *C. gloeosporioides*

Sanders and Korsten (2003) reported the cross-inoculation potential of three hundred and eight isolates of *C. gloeosporioides* obtained from avocado and mango which showed bigger lesions on their original hosts and also produced lesions on all other hosts *viz.,* strawberry, guava, papaya, and chilli except citrus. They also reported that a single host plant can be affected by many *Colletotrichum* species strains and in the same way one strain of *Colletotrichum* spp. can affect more than one host.

Isolates of *C. gloeosporioides* obtained from papaya were reported to produce symptoms on banana and mango which were more susceptible. Cross inoculation of isolates from banana and mango produced symptoms. These isolates from banana and mango developed a lesser sized lesion on grapes (Kumara and Rawal, 2004).

C. gloeosporioides isolates obtained from different plant parts of custard apple *viz.*, stems, branches, leaves, flowers, twigs and buds developed symptoms of blackening of fruits when cross inoculated on various hosts *viz.*, curry leaves, chilli, papaya, mango, grape and guava which also resulted in yield loss of 60-70 per cent due to the disease (Gaikwad *et al*., 2005)

C. gloeosporioides isolate obtained from turmeric was subjected to host range studies which revealed that the isolate showed symptoms when artificially inoculated onto the healthy detached mango, chilli, mung bean, sadafuli and citrus leaves; and also on fruit which could produce fruit rot symptom (Patel and Joshi, 2005).` In Sri Lanka, *C. gloeosporioides* had a wide host range with infectivity in 23 fruit crops such as ambarella, avocado, beli, cashew, citrus, syzygium, durian, guava, katu, mango, mangosteen papaya, passion fruit, pini, jambu, pomegranate, rambutan, rata, sapota, seeni, tomato, ugurassa, weralu and wood apple (Alahakoon and Brown, 1994). *C. gloeosporioides* isolates from papaya and mango produced bigger lesions in the original hosts; and smaller lesion on the other hosts in cross inoculation studies (Wijeratnam *et al*., 2008)

Lakshmi *et al.* (2011) studied and proved cross inoculation potential of *C. gloeosporioides* in subtropical fruit crops *viz.*, mango, papaya, guava, custard apple and pomegranate. The isolates of *C. gloeosporioides* obtained from mango developed anthracnose symptoms on all hosts except papaya; being more aggressive on the guava custard apple and cashew with PDI of 60.4, 86.7 and 69.3 respectively and lowest per cent disease index of 8.4 was recorded in acid fruits.

Bandgar *et al*. (2018) reported a wide host range of *C. gloeosporioides* including chilli, mango, papaya, turmeric, jasmine, onion, garlic pomegranate, sweet orange and guava. The 14 isolates obtained from the above-mentioned crops were cross inoculated to study the host range and all the crops developed symptoms within 7-10 DAI. Symptoms of sunken necrotic tissues with concentric rings of acervuli on the chilli fruits, dark brownish to blackish spots which later coalesced to form sunken patches on the mango fruits, dark brown lesions with grey centre on papaya fruit, brown spots with white or grey centre in case of

turmeric, irregularly shaped dark grey lesions on the leaves of onion, pomegranate and sweet orange, and dark sunken necrotic tissues in garlic were observed.

2.5 *IN VITRO* SCREENING OF FUNGICIDES AGAINST MOST VIRULENT ISOLATE OF *C. gloeosporioides*

In vitro studies on various fungicides against turmeric leaf spot caused by *C. gloeosporioides* revealed that systemic fungicides like propiconazole, hexaconazole and tricyclazole completely inhibited the growth of mycelium at 100 ppm (Patel and Joshi, 2005).

In vitro evaluation of various systemic and non-systemic fungicides against *C. gloeosporioides* isolate obtained from vanilla revealed that systemic fungicides carbendazim + mancozeb and benomyl showed cent per cent mycelial inhibition even at 0.025 per cent (Ashoka,2005). Chandrakrant (2005) observed triazole fungicides *viz.*, propiconazole, difenoconazole and hexaconazole at 0.05 % per cent completely inhibited the mycelial growth of *C. gloeosporioides* isolate of black pepper under *in vitro* conditions

Nithyameenakshi *et al.*, (2006) reported the efficacy of stobilurin fungicides azoxystrobin and difenoconazole at 0.05 percent concentration in managing grapevine anthracnose disease.

Sundravadana *et al. (*2007) evaluated the efficacy of the azoxystrobin under both *in vitro* and *in vivo* conditions against *C. gloeosporioides* from mango. Among the different concentration of azoxystrobin evaluated against the pathogen, one ppm and above concentrations gave complete mycelial inhibition. *In vivo* evaluation indicated that application of azoxystrobin at 2 and 4 ml l^{-1} reduced the incidence of anthracnose on both leaves and panicles; and increased the yield by 40 kg per plant compared to the control (13.8 kg per plant).

Kurian *et al*. (2008) reported that the anthracnose disease incidence of black pepper caused by *C. gloeosporioides* was significantly less when treated with carbendazim + mancozeb at 0.1 per cent in Panniyur 1. Prashanth *et al*. (2008) conducted studies on *in vitro* evaluation of various systemic and non-systemic fungicides against pomegranate anthracnose caused by *C. gloeosporioides*. They found that combination fungicide carbendazim + mancozeb at 0.1 per cent concentration showed highest per cent inhibition of 89.23; and propiconazole and difenoconazole which were on par among systemic fungicides showed maximum inhibition of 90.78 and 90.78 per cent respectively at 0.1 per cent concentration.

Among the nine fungicides tested under *in vitro*, carbendazim + mancozeb at 0.25 per cent, propiconazole at 0.1 per cent and tricyclazole at 0.15 per cent showed cent per cent inhibition of mycelial growth of *C. gloeosporioides* (Jadhav *et al*., 2008)

Patil *et al.* (2009) reported that the fungicides carbendazim + mancozeb and propiconazole at 0.2 per cent and 0.1 per cent respectively were most effective against *C. gloeosporioides* obtained from betel vine. Watve *et al*. (2009) reported both propiconazole and difenconazole at 0.1 per cent completely inhibited the mycelial growth and sporulation of *C. gloeosporioides* isolated from jatropha.

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

Jayalakshmi (2010) evaluated systemic, non-systemic and combination fungicides against anthracnose caused by *C. gloeosporioides* of pomegranate. The study revealed that mancozeb and carbendazim + mancozeb at 0.3 per cent showed 22.99 per cent and 81.88 per cent inhibition of mycelial growth of the pathogen respectively. Azoxystrobin and propiconazole at 0.15 per cent recorded 62.77 per cent and 87.10 per cent of mycelial inhibition under *in vitro* conditions respectively.

In vitro studies of various fungicides against *C. gloeosporioides* causing leaf blight of sapota was conducted by Patil *et al*. (2010). They noted 100 per cent inhibition of mycelial growth under *in vitro* condition with propiconazole, difenoconazole, hexaconazole at 0.1 per cent and copper oxy chloride at 0.15 per cent. Basalingappa (2011) reported that the systemic fungicides propiconazole and tebuconazole at 400 ppm effectively inhibited the mycelial growth of *C. gloeosporioides* of mango by 83.11 per cent and 80.33 per cent respectively. Kenny *et al.* (2012) reported that the triazole fungicide propiconazole as an effective one against coffee berry anthracnose with a mycelial growth inhibition of 91 per cent at 100 µg mL^{-1} a.i. Adhikary *et al*. (2013) evaluated the efficacy of azoxystrobin under *in vitro* conditions at 100 ppm and observed 99.69 per cent mycelial inhibition and inhibition of conidial germination by 97.83 per cent. The incidence of the disease on the leaves continued to decline from 100, 200, 300 and 400 ppm concentrations in which the optimum rate was 100 ppm which resulted in increased fruit yield of 39.88 kg per tree under field conditions.

Ahmed *et al*. (2014) reported the complete mycelial inhibition of *C. gloeosporioides* of betel vine *at* 50, 100, 200, 300, 400 and 500 ppm by systemic fungicides *viz.*, propiconazole, tricyclazole and tebuconazole. *In vitro* studies on different fungicides on *C. gloeosporioides*, causal agent of turmeric anthracnose revealed that propiconazole, hexaconazole, tricyclazole and carbendazim + mancozeb fungicides at 0.1 per cent gave 100 per cent mycelial inhibition (Kadam *et al*., 2014).

Dev and Narendrappa (2016) tested *in vitro* efficacy of fungicides against anthracnose disease of pomegranate caused by *C. gloeosporioides*. They reported that trifloxystrobin + tebuconazole 75 WG at 100 ppm, 250 ppm, 500 ppm and 1000 ppm and triazole fungicide propiconazole at 500 ppm, 1000 ppm and 2000 ppm showed 100 per cent mycelial inhibition. They also reported that another triazole fungicide difenoconazole 25 SC at 500 ppm, 1000 ppm and 2000 ppm showed mycelial growth inhibition of 79.24, 85.85 and 89.43 per cent respectively. The strobilurin fungicide azoxystrobin 25 SC was found to inhibit the mycelial growth by 52.64, 52.64 and 50.64 per cent at concentrations of 500 ppm, 1000 ppm and 2000 ppm respectively.

Parvathy and Girija (2016) evaluated the efficacy of various fungicides *in vitro viz.*, propiconazole (0.1 %), tebuconazole (0.1 %), azoxystrobin (0.1 %) and combination fungicides carbendazim + mancozeb (0.1 %), captan + hexaconazole (0.1 %) against *C. gloeosporiodes* from black pepper. The study revealed that the above fungicides completely inhibited the mycelial growth.

Trifloxystrobin + tebuconazole at 0.1 g l^{-1}, 0.2 g l^{-1} and 0.4 g l^{-1} showed mycelial inhibition of 39.36, 84.62 and 100 per cent respectively against *C. gloeosporioides* causing anthracnose in black pepper under *in vitro* conditions. In field studies, 0.2 g l^{-1} showed 100 per cent reduction of disease on both leaves and blackberries (Ann and Mercer, 2017)

Behera *et al.* (2019) reported that the *in vitro* evaluation of combination fungicide carbendazim + mancozeb at a concentration of 0.1 per cent showed a mycelial growth inhibition of 97.26 per cent over control and was superior to all the tested fungicides in inhibiting the black pepper anthracnose caused by *C. gloeosporioides*. Trifloxystrobin + tebuconazole at 0.15 + 0.3 g a.i. L^{-1} reduced pomegranate anthracnose and also increased the fruit yield by 12.54 t ha^{-1} (Ekabote and Narayanswamy, 2019). *In vitro* study of various fungicides against *C. gloeosporioides* isolated from mango revealed that propiconazole 25% WP at 0.1, 0.2 and 0.3 per cent gave 100 per cent mycelial inhibition whereas Difenconazole

25% EC at 0.1, 0.2 and 0.3 per cent gave a 83.22, 86.68, 86.60 per cent mycelial inhibition respectively. Tebuconazole + Trifloxystrobin 75% WG gave mycelial inhibition of 92.05, 96.27 and 96.27 per cent at 0.1, 0.2 and 0.3 per cent respectively (Ranjitha *et al*., 2019).

Materials and methods

3. MATERIALS AND METHODS

The present study entitled with “Host range studies and management of anthracnose of nutmeg caused by *Colletotrichum* spp.” was conducted during 2018-2020 at the Department of Plant Pathology, College of Agriculture, Vellayani.

3.1 COLLECTION OF ANTHRACNOSE INFECTED SAMPLES FROM NUTMEG GROWING AREAS OF KERALA,

3.1.1 Diseased sample collection

The disease was severe during the post monsoon period. Nutmeg growing areas were identified and survey was conducted. Purposive samplings were done from the four nutmeg growing tracts, *viz.*, Thiruvananthapuram, Kottayam, Ernakulam and Idukki during August 2019 to January 2020. Three locations from Thiruvananthapuram district (Vellayani, Karamana and Palode), two locations from Kottayam district (Vaikom and Kumarakom), one location from Ernakulam district (Kadungaloor), six locations from Idukki district (Pampadumpara, Myladumpara, Adimali, Panickankudy, Kamblikandam and Kattappana) were identified for collecting the diseased samples.

To measure the disease incidence and per cent disease index, ten plants from each location were selected randomly. The grading of the leaves was done by random selection of ten leaves from each plant.

Disease incidence was calculated by using the formula given by Singh (2002)

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

Per centage disease index was calculated by the formula given by Mayee and Datar (1986)

$$\text{PDI} = \frac{\text{Sum of grades of all the leaves}}{\text{Total no. of leaves observed}} \text{ X } \frac{100}{\text{Maximum grade use}}$$

Table1. Grading for anthracnose disease in nutmeg (Score chart)

Grade of the leaf	% of the infected are of leaf
0	**No infection**
1	**1-10%**
3	**11-25%**
5	**26-50%**
7	**>50%**

3.2. SYMPTOMATOLOGY, ISOLATION OF PATHOGEN, PROVING PATHOGENICITY OF *Colletotrichum sp.* CAUSING ANTHRACNOSE.

3.2.1 Symptomatology

The symptoms produced by *C. gloeosporioides* include leaf spot, leaf blight, twig blight, dieback, shot hole and fruit rot. The plant parts *viz.*, leaves, twigs and fruits were observed for symptoms and variation in the symptoms from different locations were recorded.

3.2.2 Isolation of the pathogen, purification and maintenance

The diseased samples collected from different locations were washed thoroughly under running tap water for the removal adhering particles. The small bits containing the diseased portion along with the healthy portion were cut using the blade. The cut bits were then surface sterilized with 0.1 per cent mercuric chloride for one min followed by washing in sterile water for three times. The surface sterilized bits were placed on to the sterile tissue paper for the removal of excess moisture from the leaf bits.

Using the flame sterilised forceps, the leaf bits were placed on to the PDA medium in Petri dishes. Four bits of the surface sterilised tissue were placed in each plate. The plates were then incubated at room temperature of 26 ± 3^0C for four to five days. The tip of the growing mycelium from each bit was sub cultured on to the PDA medium containing Petri dishes. The purified cultures were maintained on PDA slants. The same procedure was repeated for isolation of infected samples from different locations.

Single spore isolation technique (Dhingra and Sinclair, 1985) was used for the purification of the isolates of *C. gloeosporioides*. The conidial suspension was prepared by

placing a small mycelial bit along with the spore mass into test tube containing sterile water. Serial dilution was then performed until the desired concentrations of spores in the suspension were obtained. Using a micropipette, 1 ml of conidial suspension was taken and poured in the sterile Petri dish. Molten 2 per cent plain agar was plated over the previously poured conidial suspension in a uniform thin layer and allowed to solidify. The plates were then observed under the microscope. The spores were marked by using the fine marker. The plain agar media with the identified spores were cut by using a sterile cork borer and placed on to the solidified PDA medium. The plates were then incubated at room temperature.

The purified isolates were repeatedly sub cultured on to PDA slants every month for maintenance. The cultures were incubated at room temperature of 27 ± 3^0C and further maintenances was carried out in the refrigerator at a temperature of 4^0C (Nakasone *et al.*, 2004). The virulence of the isolates was maintained by inoculating the host plant and reisolation was further done to obtain the virulent culture.

3.2.3. Pathogenic variability studies

Pathogenicity assays were performed on the excised leaves of nutmeg. The freshly harvested twigs were thoroughly washed under running tap water and allowed to air dry. The twigs were rinsed with a swab dipped in 70 per cent ethanol. Pin pricks were made on the surface of the leaves by using the needle in order to make aberrations for the easy infection of the pathogen. The small mycelial discs of about 5 mm diameter were cut from the seven-day old culture and placed on pinpricked area of the foliage. The mycelial discs on the leaves were then covered with a thin layer of moistened cotton. The twigs with leaves inoculated with the pathogen were then incubated in the moist chamber until the symptoms appeared. The observations on the days taken for symptom development and nature of symptoms developed were recorded. The leaves which developed the symptoms were further reisolated by following the same procedure as that of isolation of the pathogen. The reisolated culture was then compared with the original culture for proving the pathogenicity.

3.3 MORPHOLOGICAL AND PATHOGENIC VARIABILITY OF *Colletotrichum* ISOLATES AND IDENTIFICATION

3.3.1. Morphological characterisation of different isolates obtained from different locations

The morphological characterisation of the various isolates was done by using slide culture technique. The slide culture unit contains two small glass rods, a blotting paper of Petri plate size, a glass slide and a cover slip which were sterilised. Under aseptic conditions, the slide culture unit was arranged in such a way that the glass slide will rest on glass rods which were placed at the two edges of the Petri dish. In another Petri dish, molten 2 per cent plain agar was poured in the form of a thin layer and allowed to solidify. Using a sterile needle, the solidified 2 per cent agar were cut into a small block of size similar to that of the cover slip. The agar block was placed over the glass. With the help of the heat flamed inoculation needle, the conidial mass from the seven-day old culture was picked up and inoculated on to the four corners of the agar block under aseptic conditions. The coverslip was then placed carefully over the agar block and the moisture was maintained by wetting the blotting paper with sterile water to provide enough humidity for incubation of the inoculated fungi. The cover slips were then lifted carefully by using a sterile forceps and placed over a slide containing a drop of lactophenol cotton blue stain. The slide was mounted with DPX mount and observed under the microscope by using the LEICA software with magnification of 400x and 1000x. The observations were made on the size of the mycelium and conidia etc.

3.3.2 Cultural characterisation of *Colletotrichum* isolates obtained from different locations.

The different isolates obtained were subjected to cultural characterisation for variations in terms of colony colour, colony growth pattern, rate of growth of mycelium and days taken to cover Petri dish. PDA medium was melted, poured and allowed to solidify in Petri dish. Small mycelial discs of 5 mm diameter were cut from the seven-day old culture. The PDA plates were then inoculated with the mycelial disc by placing at the centre of the Petri plate. The plates were then wrapped and incubated at room temperature of 26 ± 3^{0}C. Three replications for each isolate were maintained. The same procedure was repeated for all the isolates and the observations were recorded on colony colour, nature of growth of the mycelium, radial growth of the mycelium and days taken for covering the entire Petri dish.

3.3.3 Screening for pathogenic variability of different isolates of *Colletotrichum* spp.

The different isolates were screened for the pathogenic variability by using virulence rating. The virulence rating was performed on detached leaves of the excised twigs. The healthy twigs of uniform size were collected from a single plant in order to maintain the germplasm uniformity. The twigs were washed under running tap water and surface cleaned

with a cotton swab dipped in 70 per cent ethanol. After drying, pinpricks were made on the surface of the leaves in order to create wounds for the easy infection of the pathogen. The small mycelial discs of 5 mm diameter were taken from the seven-day old culture, placed over the pin pricked areas of the leaves. The mycelial discs were then covered with a thin layer of moist cotton and the inoculated twigs were incubated in a moist chamber. Three replications were maintained for each isolate. The uninoculated twig served as control. The same procedure was repeated for all the isolates and the observations were made on the nature of symptom developed, days taken for symptom development and lesion size.

3.4 HOST RANGE OF MOST VIRULENT ISOLATE OF *Colletotrichum* spp.

The most virulent isolate of *Colletotrichum* spp. obtained after the virulence rating was subjected for the host range studies in other tree spices *viz.,* black pepper, clove, cinnamon, all spice, betelvine and coconut (Table 2). For carrying out the experiment, the healthy twigs of the perennial spices were collected fresh from the field.

The twigs were washed under running tap water and allowed to dry. The surface of the leaves was sterilised by swabbing the leaves with 70 per cent ethanol. By using a sterile needle small aberrations or wounds were made in the form of pin pricks. The mycelial discs of five mm diameter using a sterile cork borer under aseptic conditions were taken from the most virulent culture. The twigs of all other tree spices along with the coconut were then inoculated with the pathogen by placing the mycelial discs over the pinpricks on the surface of the leaves. The mycelial discs were then covered by placing the moist cotton in the form of a thin layer. Three replications for each host were maintained. The natural host *i.e* nutmeg inoculated with virulent isolate was kept as control for comparison. The inoculated twigs were incubated at room temperature of 26 $\pm$ 3^0C in a moist chamber. Observations *viz.,* size of the lesions, nature of symptom developed in comparison with the normal host and days taken for symptom development were made on every alternate day *i.e* 3rd, 5th 7th and 9th days.

Table 2: List of plants used for host range studies

Sl. No.	Host plant		Family
	Common name	Scientific name	
1	Allspice	*Pimenta dioica*	Myrtaceae
2	Black pepper	*Piper nigrum*	Piperaceae
3	Betel vine	*Piper betel*	Piperaceae

4	Cinnamon	*Cinnamomum zeylanicum*	Lauraceae
5	Clove	*Syzygium aromaticum*	Myrtaceae
6	Coconut	*Cocos nucifera*	Arecaceae

3.5 *IN VITRO* SCREENING OF FUNGICIDES AGAINST MOST VIRULENT ISOLATE OF *Colletotrichum spp.*

The most virulent isolate obtained from the screening of the different isolates from different localities was tested with different commercially available new generation fungicides *i.e.,* two triazoles (propiconazole and difenoconazole), one strobilurin (azoxystrobin,) and three combination fungicides (carbendazim + mancozeb, trifloxystrobin + tebuconazole, captan + hexaconazole) by using poisoned food technique (Nene and Thapliyal, 1993).

C. gloeosporioides isolate was allowed to grow in the sterile Petri dishes containing PDA medium for seven days. Double strength PDA medium of 50 ml and 50 ml of sterile water were prepared for the respective fungicides of different concentrations and autoclaved. Under aseptic conditions, pre-weighed fungicides of different concentrations (Table 3) were dispersed into the conical flasks containing 50 ml sterile water and shaken thoroughly for the complete dispersion of the fungicide. In case of soluble concentrate fungicides, the desired concentrations were taken out by using the micropipettes and allowed to dissolve in the sterile water by thorough shaking. Melted medium was mixed with sterile water containing fungicide. The amended molten medium was poured into the sterile Petri dishes and allowed to solidify. This method was repeated for the different concentrations of each treatment.

After the solidification of the medium, mycelial discs of 5 mm diameter were cut by using the sterile cork borer from the seven-day old culture plate. The mycelial discs were taken by flame sterilized inoculation needle and placed on the centre of the solidified PDA medium amended with the fungicide. Three replications for each concentration of different fungicides were maintained. A control plate inoculated with the pathogen alone and without the fungicide was maintained as control. The Petri dishes were then wrapped and incubated at the room temperature of 28 ± 3^0C. The radial growth of the pathogen was recorded when the pathogen in the control plate was fully grown and the per cent inhibition by the fungicide was calculated.

Per cent inhibition of the pathogen by the fungicide over the control was calculated by the formula:

Per cent inhibition = C-T/C *100

Where C= Radial growth of the pathogen in control plate in cm

T= Radial growth of the pathogen in the treatment plate in cm

Table 3. Fungicides used for the assay

Sl. No.	**Fungicide**		**Formulation**	**Concentration used**
	Chemical name	**Trade Name**		
1	Propiconazole	Tilt	25 EC	10, 25, 50, 100 ppm
2	Difenoconazole	Score	25 EC	10, 25, 50, 100 ppm
3	Azoxystrobin	Amistar	23 SC	10, 25, 50, 100 ppm
4	Pyraclostrobin		20 EC	10, 25, 50, 100 ppm
5	Captan 50% WP + Hexaconazole 5% WP	Taaqat	50% WP + 5% WP	10, 25, 50, 100 ppm
6	Trifloxystrobin 25% + Tebuconazole 55% WP	Nativo	25% + 55% WP	10, 25, 50, 100 ppm
7	Carbendazim 12% WP + Mancozeb 63% WP	Saaf	12% WP + 63% WP	10, 25, 50, 100 ppm

Results

4. RESULTS

The current study entitled "Host range studies and management of anthracnose of nutmeg caused by *Colletotrichum* spp." was conducted during 2018- 2020 at Department of Plant Pathology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala with the objectives to characterize the causal agent, study the host range of the anthracnose causing pathogen of nutmeg, *Colletotrichum* spp. and to evolve the best management strategy for the control of the disease by using the new generation fungicides. The results obtained through the experimental studies are mentioned below.

4.1 PREVALENCE, OCCURRENCE AND COLLECTION OF ANTHRACNOSE INFECTED SAMPLES

Survey for the collection of diseased samples was conducted during August – September, 2019 in four agro ecological zones. *viz.*, Thiruvananthapuram district from the southern zone, Kottayam from special problem zone, Ernakulam district (Kadungalloor) from central zone, Idukki district from the high range zone. For the infected sample collections, three locations from Thiruvananthapuram (Vellayani, Karamana and Palode), two locations from Kottayam (Kumarakom and Vaikom), six locations from Idukki (Myladumpara, Pampadumpara, Adimali, Kambilikandam, Panickankudy and Kattapana) and one location from Ernakulam (Kadungalloor) were surveyed (Plate 1). The local varieties were widely cultivated in the sample collected areas. The disease incidence at the surveyed locations varied between 20-90%. The maximum DI was noticed at Kadungalloor (90%) followed by Kumarakom (80%). The lowest DI was noticed at Myladumpara of Idukki (20%). The disease incidence in different locations of Thiruvananthapuram ranged between 30-50%; while 30, 60, 60, 50 and 40% incidence were observed at Pampadumpara, Adimali, Panickankudy, Kambilikandam and Kattapana respectively. The disease severity (DS) ranged between 14.80-56.40%. Maximum severity of anthracnose was noticed at Kadungalloor (56.40) followed by Adimali (38.40). The disease severity or per centage disease index (PDI) was minimum at Thiruvananthapuram while locations at Kottayam showed a higher PDI (Table 4). The infected samples were collected from these areas for the isolation of the pathogen.

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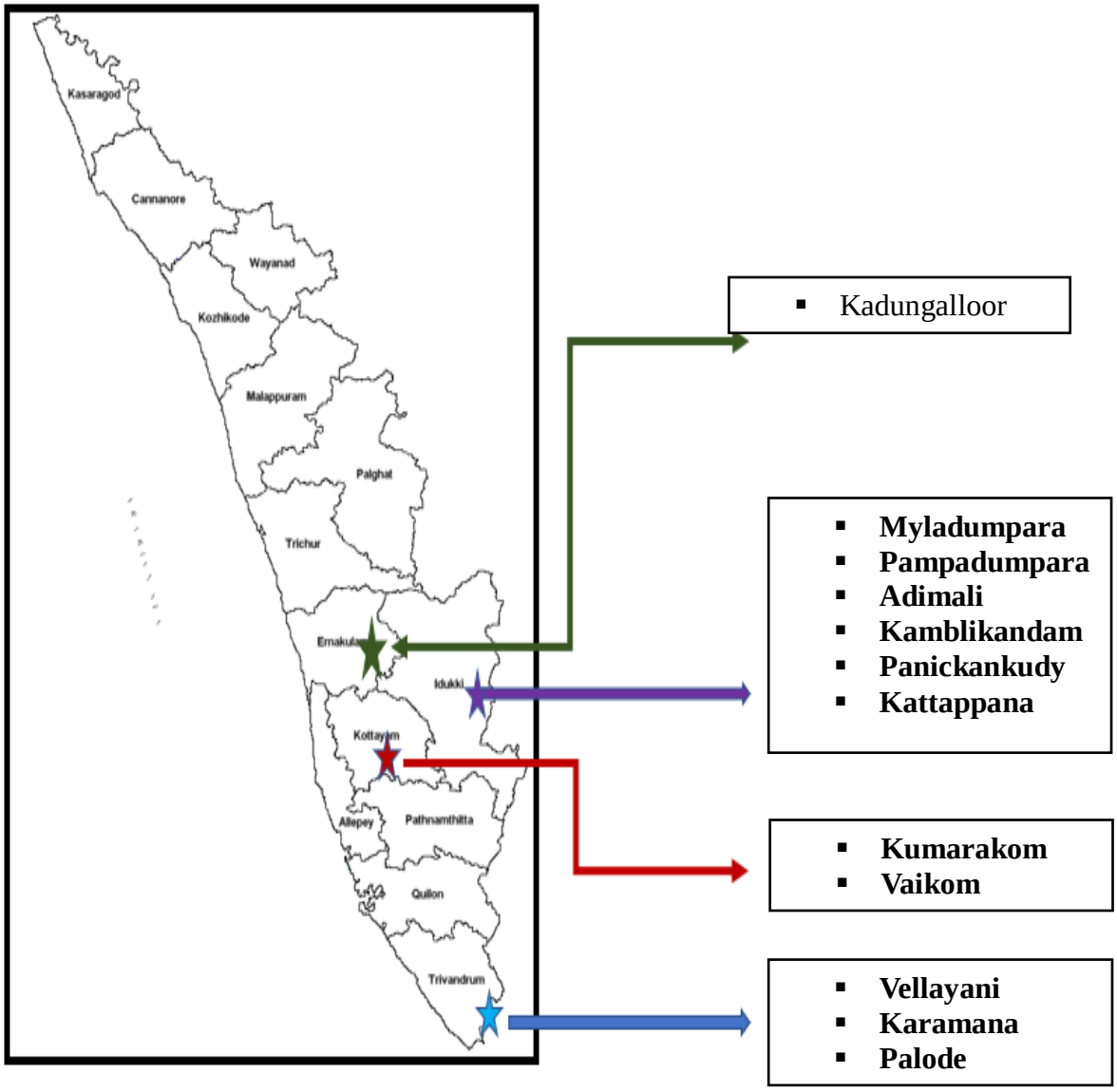


Plate 1: Map of Kerala showing locations of collection of anthracnose infected nutmeg

4.2. SYMPTOMATOLOGY, ISOLATION OF PATHOGEN, PROVING PATHOGENICITY OF *Colleotrichum* spp. CAUSING ANTHRACNOSE DISEASE OF NUTMEG

.4.2.1. Symptomatology

Anthracnose disease of nutmeg was most severe during the post rainy season and the disease was favoured by the high relative humidity and recurrent rains. Under field conditions, the various symptoms of the disease observed on the leaves were leaf spot; leaf blight, shot hole and fruit rot (Plate 2 and 3). The leaf spot showed dark brown necrotic lesion which was typically surrounded by the yellow halo. In the case of leaf blight, several spots coalesce together and the leaves start up drying from the tip to the entire lamina. Shot hole symptom was the characteristic one with the necrotic tissue being blown away. Fruit symptom was expressed as water-soaked brown necrotic lesions on fruits leading to the rot resulted in severe economic loss as the fruit is the economical part (Table 5).

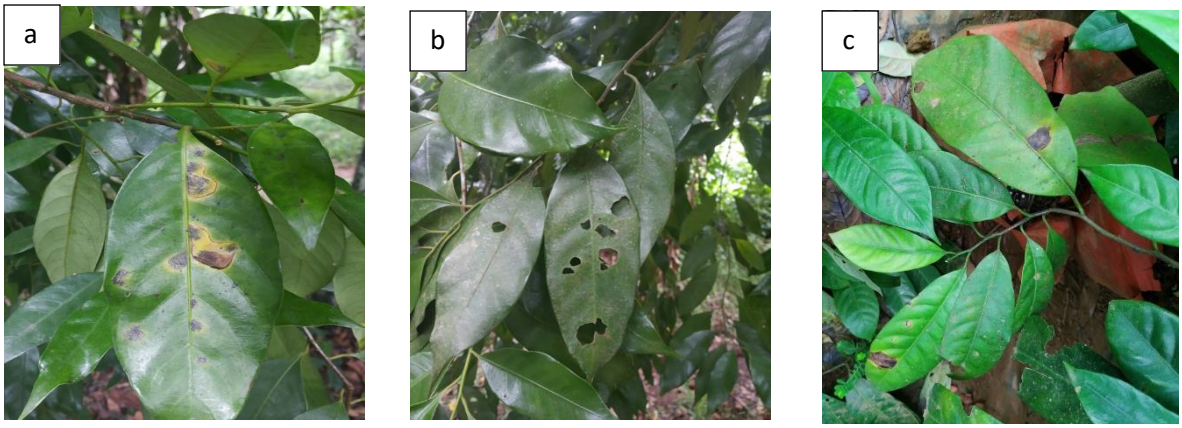
4.2.1.1. Stages in symptom development

The disease was initially seen as the brown necrotic spots surrounded by a yellow halo (Stage 1); followed by the coalescing of the spots leading to the blighting of the leaf (Stage 2); and ultimately leading to the shredding of the necrotic part seen as shot hole (Stage 3). The blighting of the entire twig and dieback (Stage 4) followed the shot hole (Plate 4). The final stage of the disease was seen on the economical part of the nutmeg as fruit rot (Stage 5)

4.2.2. Isolation of the pathogen, purification and maintenance

The leaves and fruits that showed typical anthracnose symptoms collected from different locations during the survey period were cut into small bits for isolation of the fungus using the standard procedure as described in the 3.1. The growth initiated as mycelium within two to three days in PDA medium. All the isolates obtained from different locations were identified as *Colletotrichum* sp. The isolates were purified by hyphal tip method and maintained in PDA slants for further studies

The isolates from Trivandrum, Kottayam, Ernakulam and Idukki were labelled as C1, 2, 3….C18; of which 1-3 isolates were from Thiruvananthapuram, 4-12 isolates from Kottayam, 13-15 isolates from Idukki and 16-18 isolates from Ernakulam (Plate 5 and 6).



Thiruvananthapuram:

a) Vellayani **b) Karamana** **c) Palode**

Kottayam:

a and b) Kumarakom **c)Vaikom**

Ernakulam: **a and b) Kadangalloor**

Plate 2. Symptoms of anthracnose on nutmeg foliage observed at different locations of Thiruvananthapuram, Kottayam and Ernakulam districts

a) Pampadumpara

b) Myladumpara

c) Adimali

d) Panickankudy

e) Kamblikandam

f) Kattappana

Plate 3: Symptoms of anthracnose on the nutmeg foliage and fruits observed at different locations of Idukki district

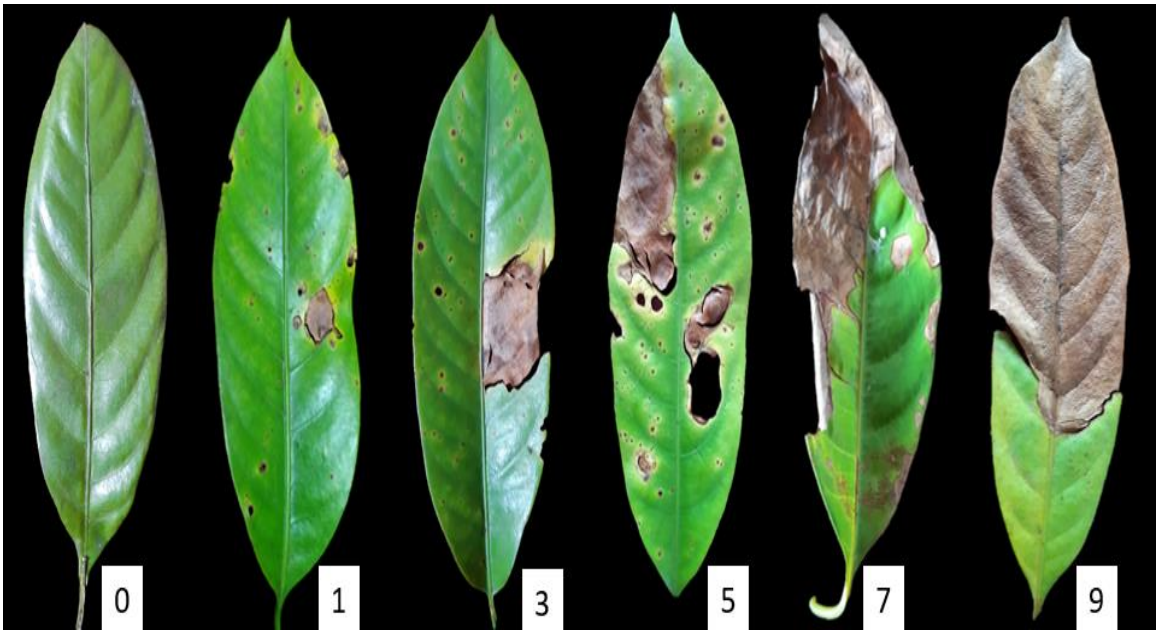


Plate 4: Score chart for anthracnose disease caused by *Colletotrichum* spp. in nutmeg

Table 4. Disease incidence and severity of anthracnose of nutmeg caused by *Colletotrichum* spp. in surveyed locations

Locations	Variety	Period of sample collection	Disease incidence (%)	Per cent disease index
Vellayani	Local	September, 2019	40.00	14.80
Karamana	Local	September, 2019	30.00	19.86
Palode	Local	September, 2019	50.00	18.50
Kumarakom	Acc2, Acc10	August, 2019	80.00	41.33
Vaikom	Local	August, 2019	70.00	33.33
Kadangalloor	Local	October, 2019	90.00	56.40
Pampadumpara	Local	October, 2019	30.00	18.62
Myladumpara	Local	October, 2019	20.00	15.53
Adimali	Local	October, 2019	60.00	38.40
Panickankudy	Local	October, 2019	60.00	21.33
Kambilikandam	Local	October, 2019	50.00	27.30
Kattapana	Local	October, 2019	40.00	17.50

Table 5. Nature of symptoms of anthracnose observed in nutmeg at different surveyed locations

District	Sl. No.	Locations	Variety	Nature of symptoms
Thiruvananthapuram	1.	Vellayani	Local	Necrotic spots with prominent yellow halo
	2	Karamana	Local	Shot hole
	3	Palode	Local	Necrotic spots
Kottayam	4.	Kumarakom	ACC2, ACC10, Local	Leaf blight and shot hole
	5.	Vaikom	Local	Necrotic spot with a prominent yellow halo
Ernakulam	6	Kadungalloor	Local	Leaf spot and blight
Idukki	7.	Pampadumpara	Local	Leaf spot and leaf blight
	8.	Myladumpara	Local	Leaf spot and leaf blight
	9.	Adimali	Local	Leaf blight and shot hole
	10.	Panickankudy	Local	Shot hole and fruit rot
	11.	Kamblikandam	Local	Shot hole and fruit rot
	12.	Kattappana	Local	Leaf blight and shot hole

Table 6. Days taken for symptom development, nature of symptom developed, lesion size and PDI in nutmeg by artificial inoculation of *Colletotrichum* isolates

Isolates		DTS*	Nature of symptom developed	Lesion size (cm)**			PDI (9 DAI)
				5th day	7th day	9th day	
Thiruvana-nthapuram	C1	2	Brown necrotic lesion	0.70	1.56	1.80	11.11
	C2	3	Brown necrotic lesion	1.23	2.06	2.66	24.44
	C3	3	Brown necrotic lesion with yellow halo	0.70	0.76	0.90	8.33
Kottayam	C4	3	Brown necrotic lesion	1.50	2.40	3.53	33.33
	C5	4	Brown necrotic lesion	0.66	0.90	1.10	25.92
	C6	4	Brown necrotic lesion with distinct yellow halo	0.43	0.53	0.60	11.11
	C7	5	Brown necrotic lesion with yellow halo	0.36	0.50	0.60	11.11
	C8	5	Brown necrotic lesion with distinct yellow halo	0.60	0.66	0.80	16.66
	C9	2	Brown necrotic lesion spread over the entire leaf	1.93	3.76	4.13	77.77
	C10	4	Brown necrotic lesion	0.63	0.80	0.90	8.88
	C11	4	Brown necrotic lesion	0.63	0.80	0.90	11.11

*DTS- Days taken for symptom development; ** Mean of 3 replications;

PDI - Per cent disease index

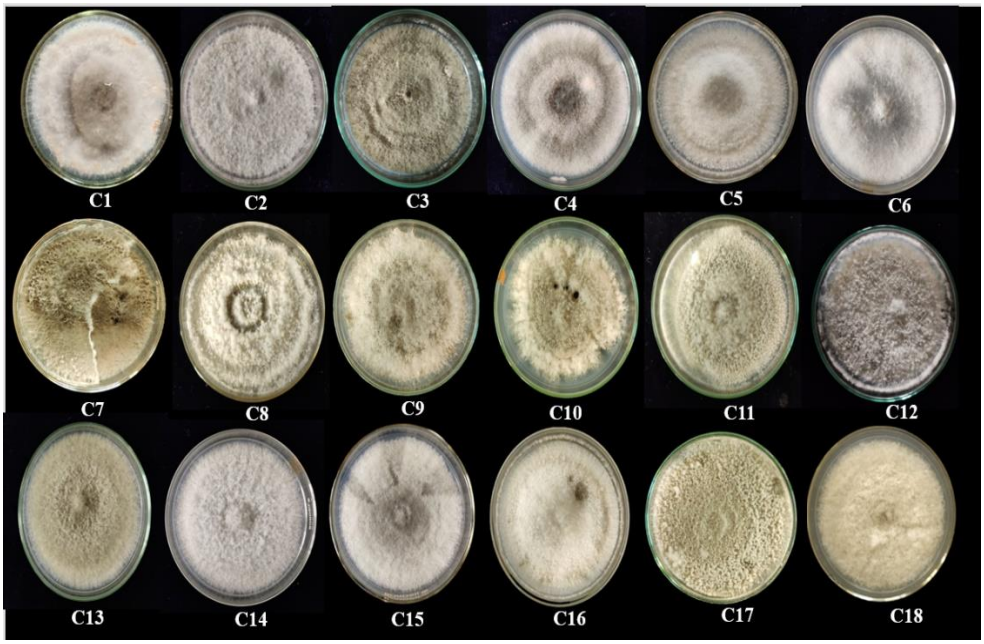
Table 7. Days taken for symptom development, nature of symptom developed, lesion size and PDI in nutmeg by artificial inoculation of *Colletotrichum* isolates

Isolates		DTS*	Nature of symptom developed	Lesion size (cm)**			PDI (9DAI)
				5th day	7th day	9th day	
Idukki	C12	3	Brown necrotic lesion with distinct yellow halo. Acervuli seen at the centre	0.60	1.90	2.10	33.33
	C13	3	Brown necrotic lesion with yellow halo	0.40	0.53	0.70	6.66
	C14	3	Brown necrotic lesion with distinct yellow halo and shot hole	1.06	2.40	3.13	44.44
	C15	3	Brown necrotic lesion with light yellow halo	0.60	0.70	0.80	8.33
Ernakulam	C16	4	Brown necrotic lesion with clear yellow halo	0.33	0.50	0.70	8.88
	C17	4	Brown necrotic lesion with light yellow halo	0.36	0.53	0.70	5.55
	C18	3	Brown necrotic lesion with distinct yellow halo	0.80	1.30	1.76	24.44
CD (0.05)				0.193	0.182	0.169	
SEm				0.067	0.063	0.059	

* DTS- Days taken for symptom development; * Mean of 3 replications;

PDI - Per cent disease index

(a) Front view



(b) Rear view

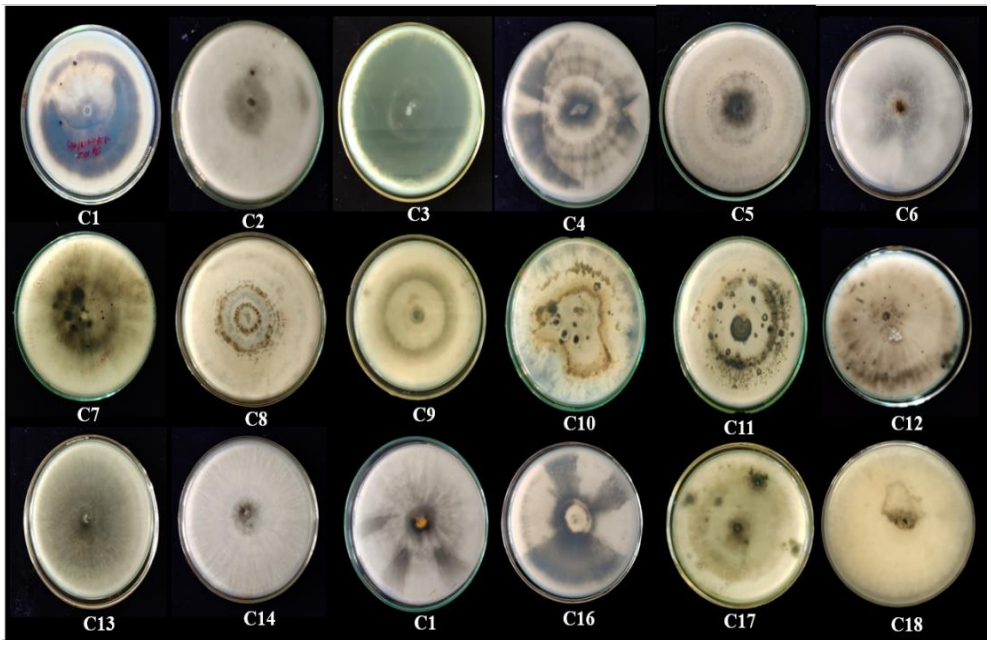


Plate 5. *Colletotrichum* sp. isolates from different locations.

1-3: Thiruvananthapuram, 4-12 Kottayam, 13-15: Idukki, 16-18: Ernakulam

(a) Front view

(b) Rear view

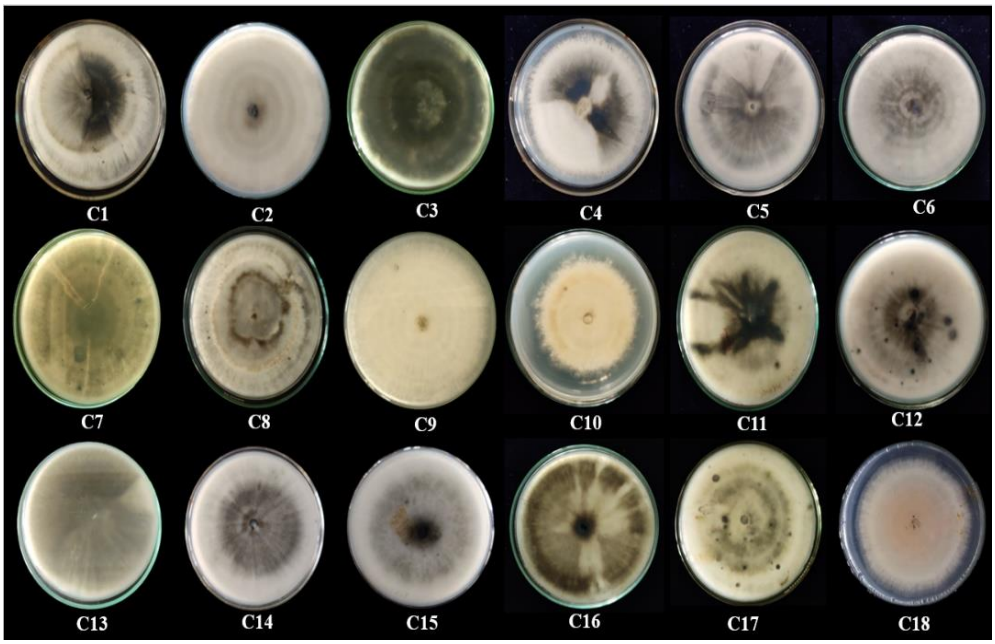


Plate 6. Re-isolated cultures of *Colletotrichum* sp. from different locations.

1-3: Thiruvananthapuram, 4-12 Kottayam, 13-15: Idukki, 16-18: Ernakulam

4.2.3. Proving pathogenicity of *Colletotrichum* spp.

All the isolates of *Colletotrichum* obtained from the surveyed locations were inoculated on to the healthy leaves of the nutmeg. Pin prick method was used for inoculation of the pathogen. The leaves inoculated with different *Colletotrichum* isolates showed symptoms of brown lesions or brown lesion typically surrounded by a yellow halo and shot hole. The days taken for symptom appearance varied between 2-5, while the isolates C1 and C9 developed symptoms within 2 days after inoculation. The isolates C2, C3, C4, C12 C13, C14 and C15 took three days for symptom appearance. Four days for symptom appearance was observed with respect to C5, C6, C10, C11 and C16 isolates. The isolates C7 and C8 developed symptoms after 5 days. The lesion size produced by various isolates at 5 DAI ranged between 0.33 - 1.9 cm. The results revealed that the lesion size produced by the various isolates varied from 0.50-2.4 cm at 7 DAI with a maximum lesion size produced by C4 and C14 isolates which were on par. At 10 DAI, lesion size produced varied between 0.60 - 4.13 cm. The isolate C9 produced a maximum lesion size of 4.13 cm. The isolates C4 and C14 were on par with respect to the size of lesion developed (Plate 7 and 8). The minimum sized lesions were produced by the isolates C6, C7, C13, C15, C16 and C17. The PDI ranged between 5.55- 77.77. Maximum PDI was recorded by the isolate C9 (77.77) and minimum for C17. The isolates C4, C5, C2, C12, C18 and C14 produced a PDI between 24.44 to 44.44 (Table 6 and 7)

Of the 18 isolates obtained from different locations, screening was done to shortlist to seven isolates based on the days taken for symptom development and spread of the disease. In some isolates there was production of the acervuli and spore mass on the underside of the leaves. The seven isolates obtained after screening (C1, C2, C4, C9, C12, C14, C18) were purified using single spore isolation technique and labelled as C1 (Vellayani), C2 (Palode), C3 (Kumarakom), C4 (Kumarakom), C5 (Vaikom), C6 (Panickankudy) and C7 (Kadungalloor) respectively. These isolates produced PDI between 11.11 to 77.77. The isolate C4 was the most virulent followed by C6 (Table 8, Plate 9).

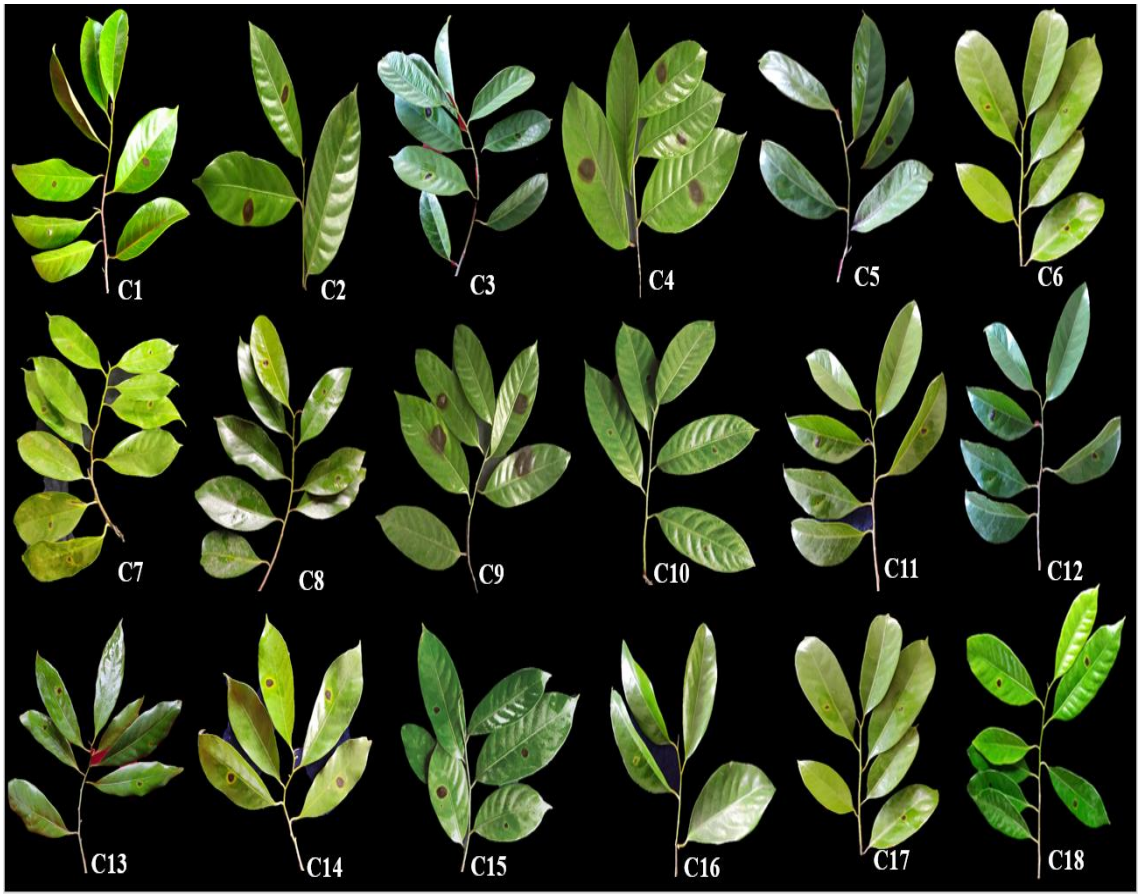


Plate 7. Symptom appearance on artificial inoculation of *Colletotrichum* spp. isolates on nutmeg leaves at 5 DAI. 1-3: Thiruvananthapuram, 4-12: Kottayam, 13-15: Idukki, 16-18: Ernakulam

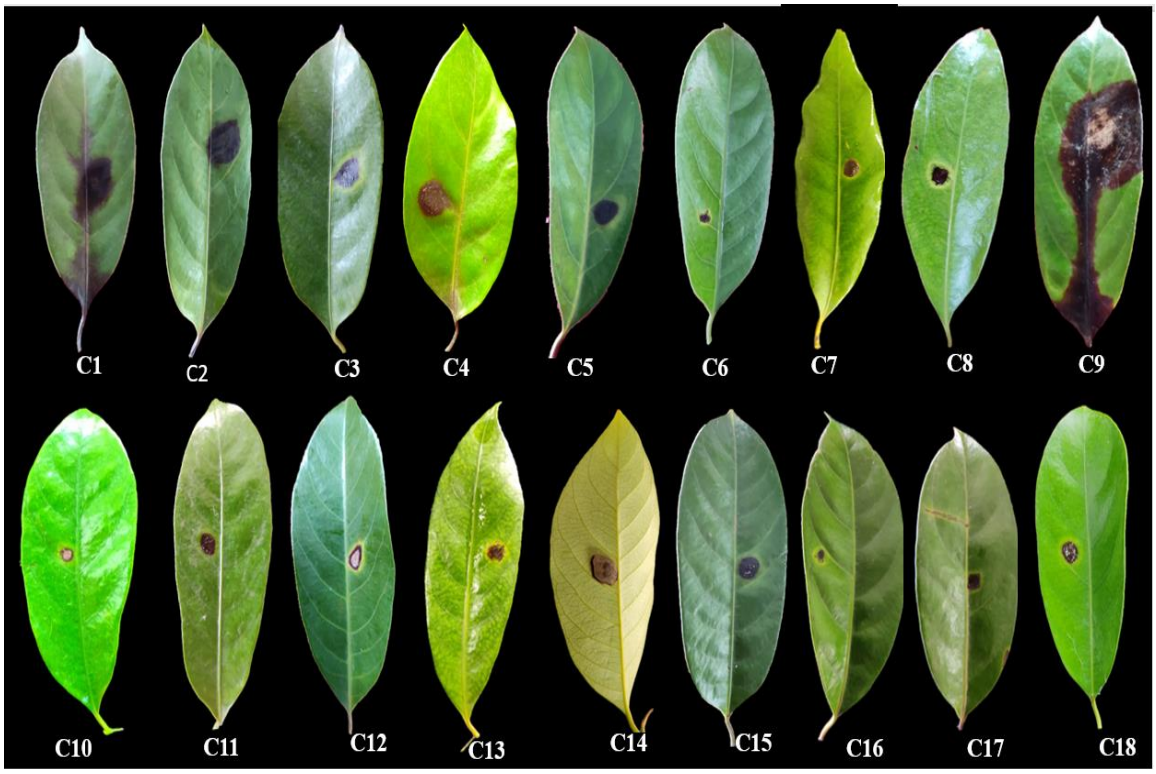


Plate 8. Comparison of symptoms produced of different isolates of *Colletotrichum* spp. on artificial inoculation at 5DAI.

1-3: Thiruvananthapuram, 4 -12 Kottayam, 13 -15: Idukki, 16 -18: Ernakulam

Table 8: Isolates of *Colletotrichum* spp. selected for further studies

Isolate	Renamed isolate no.	Locality	PDI (%)
C1	C1	Vellayani, Tvpm	11.11
C2	C2	Palode, Tvpm	24.44
C4	C3	Kumarakom, Kottayam	33.33
C9	C4	Kumarakom, Kottayam	77.77
C12	C5	Vaikom, Kottayam	33.33
C14	C6	Panickankudy, Idukki	44.44
C18	C7	Kadungalloor, Ekm	24.44

4.3 MORPHOLOGICAL AND PATHOGENIC VARIABILITY OF *Colletotrichum* ISOLATES, FOR SCREENING THE MOST VIRULENT ISOLATE

4.3.1 Morphological characterisation of different isolates

The morphological characters of seven *Colletotrichum* isolates were evaluated by slide culture technique as described in 3.2.1

On microscopic observation, the mycelium of the fungus was hyaline, septate, branched and slender. The mycelial width of different Colletotrichum isolates ranged from 0.46-2.48 µm. The isolate C4 had the maximum width of 2.48 µm followed by C3, C7, C6, C1, C2 and C5 with 2.34, 1.88, 1.59, 1.23, 1.14 and 0.46 µm respectively. The growth patterns of different isolates were either fluffy or sparse. Appearance of the mycelia was either white to grey with concentric or radiating zones and regular margin (Table 9, Plate 10). Among the seven isolates, C1, C3, C5 and C6 showed fluffy growth whereas C2, C4 and C7 isolates had sparse growth with a regular margin. C1 isolate was white to greyish colour in the front view and white to greyish concentric zonation on the rear side. C2 showed white with concentric zones in the front view and white in the rear view. C3 isolate appeared whitish to grey in the upper side and greyish in the rare view. C4 isolate showed full whitish pattern in the front view and creamy white with greyish concentric zones in the rear view. C5 isolate varied from white with grey radiating zones in the upside view and in the rear view pinkish white colouration was noticed. C6 isolate varied from greyish centre with white

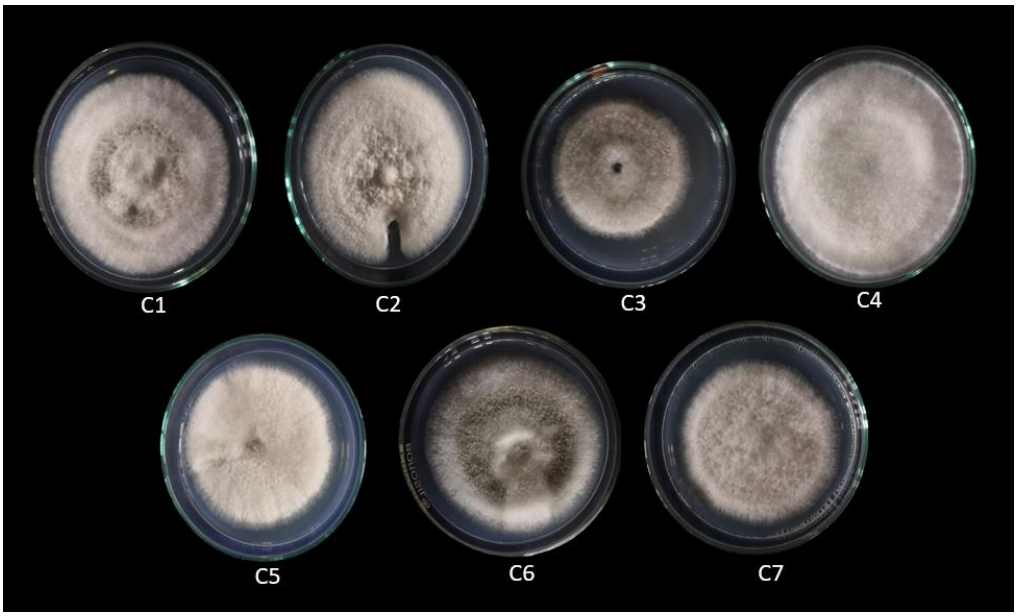
margin in the upside view and in the rear-view white with greyish centre. C7 isolate showed greyish white in the upside view and white with pinkish centre in the rear view

4.3.2. Cultural characterisation of different isolates

Among all the isolates, C4 and C2 were fast growing with the average growth rate of 1.32 cm day^{-1} and 1.31 cm day^{-1} and took 7 and 8 days to complete the full growth of 9 cm in the Petri dish respectively. C1, C3, C5, C6 and C7 took 9, 10, 9, 9, and 9 days respectively to complete the full growth in the Petri dish with the average growth rate of 1.26, 0.98, 0.80, 1.30, and 1.00 cm day^{-1} respectively. The isolates showed a growth ranging from 4.53 to 7.96 cm at 5DAI. The isolate C4 completed 7.96 cm growth in 7 days. The isolates C4 and C5 were on par with respect to growth at 5 DAI. The isolate C4 completed 8.96 cm growth at 7 DAI. The isolate C4 had minimum growth of 5.46 cm at 7 DAI. The rate of growth ranged from 0.80-1.23 cm day^{-1}, with a maximum growth rate in isolate C4 and minimum in C3. The other isolates had a rate of growth between 0.98-1.30 cm day^{-1}(Table 10).

The conidial shape of the various isolates was dumbbell or oblong. The size of the conidia varied between 7.87 µm - 19.97 µm x 3.26 µm- 5.68 µm. Maximum conidial size of 19.97 µm x 4.26 µm was observed for C7 isolate followed by 18.78 µm - x 5.68 µm for C5 isolate and the least conidial size of recorded 7.87 µm - x 3.68 for C2 isolate (Table 11, Plate 11).

(a) Front view



(b) Rear view

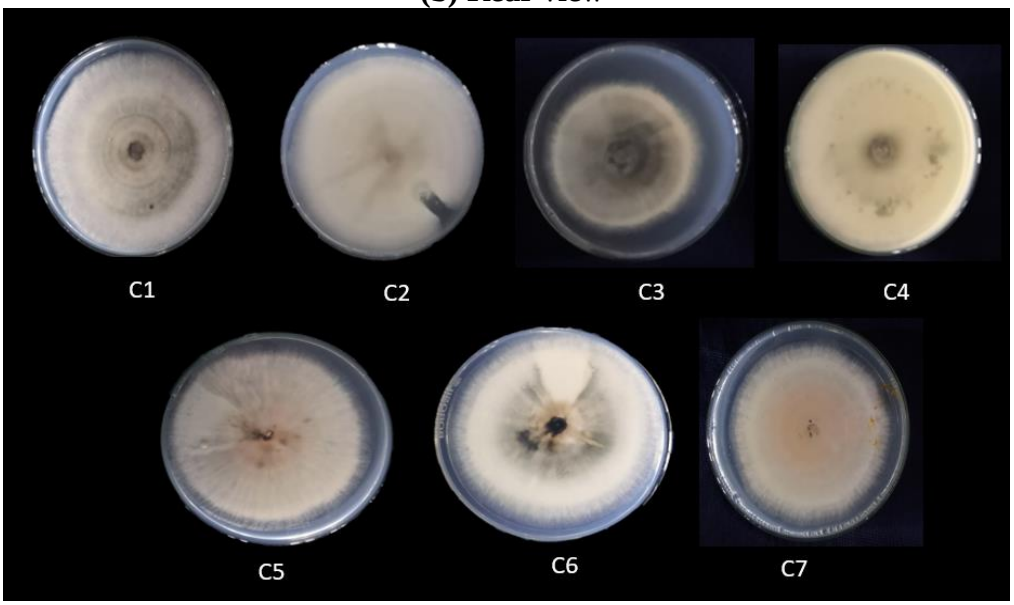


Plate 9. Colour, appearance and growth of selected *Colletotrichum* isolates: C1-C2 (Thiruvananthapuram), C3-C5 (Kottayam), C6-Idukki, C7- Ernakulam.

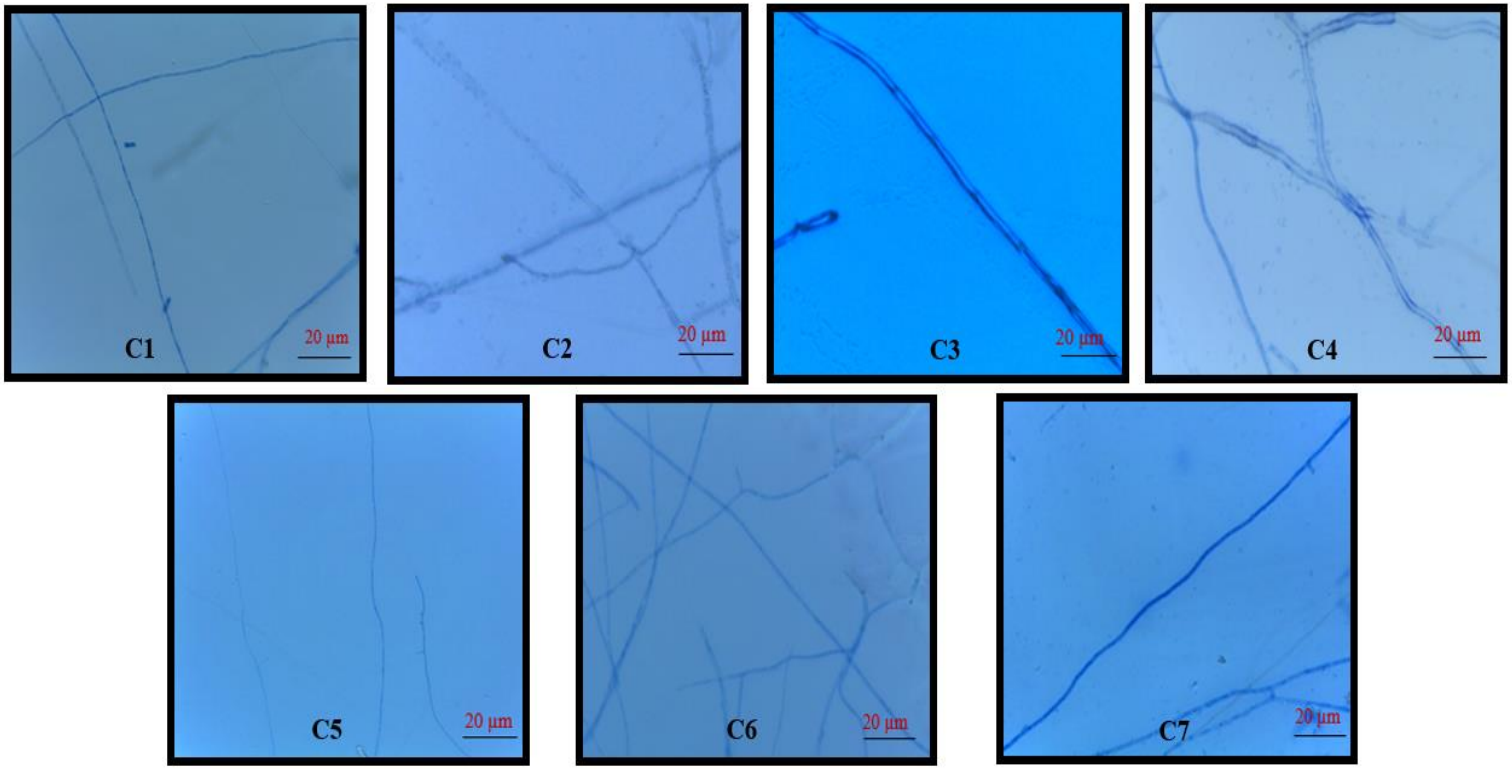


Plate 10. Mycelial characteristics of different *Colletotrichum* isolates from nutmeg at 7 DAG

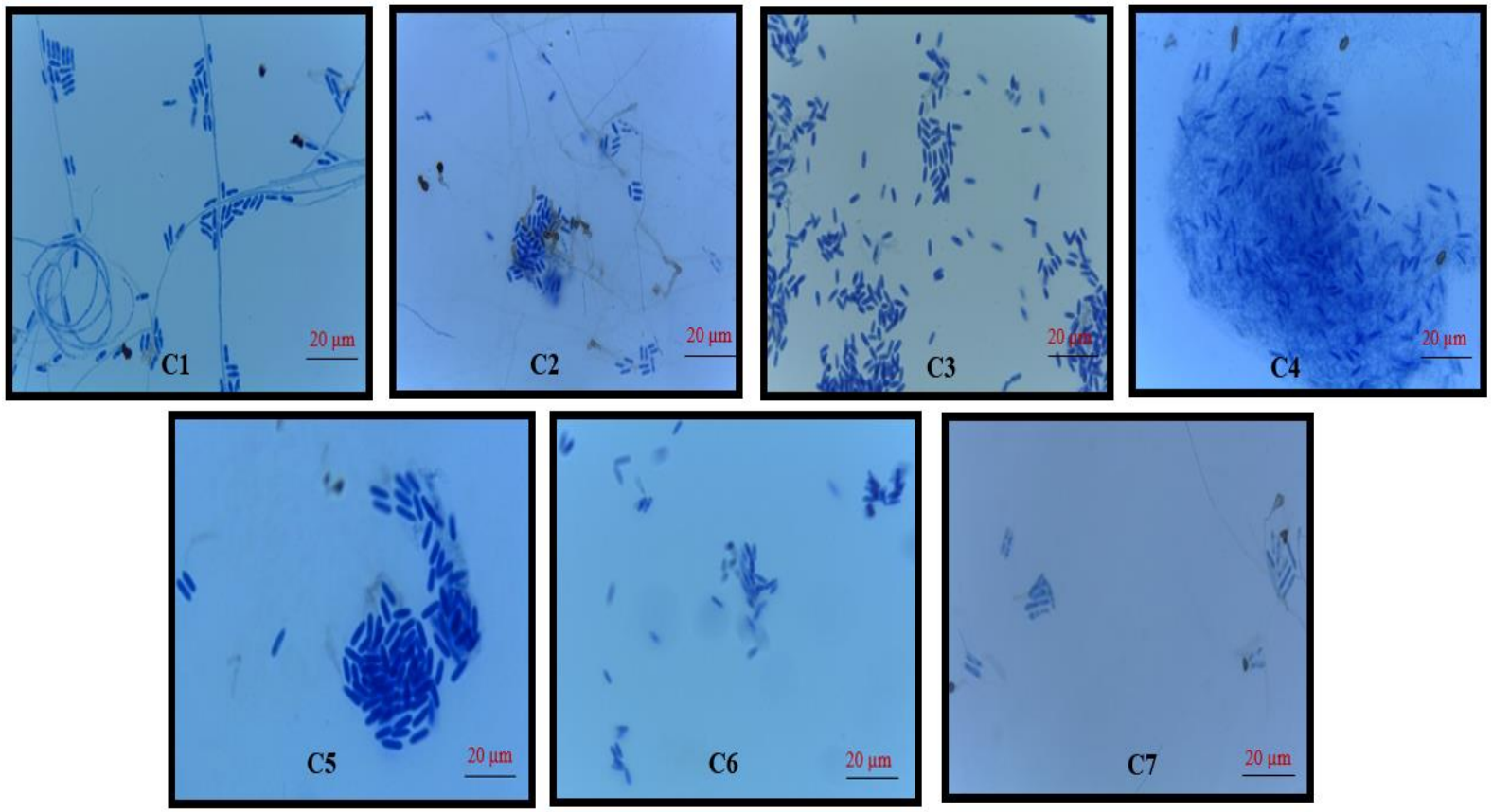


Plate 11. Microscopic view of conidia of different Colletotrichum isolates at 7 DAG

Table 9. Appearance and growth pattern of mycelia of different isolates of *Colletotrichum* spp. causing anthracnose of nutmeg in PDA medium at 7 days after growth

Isolates	Growth pattern	Appearance of the mycelium			Nature of mycelium	Mycelial width (µm)
		Front view	Rear view	Margin		
C1	Fluffy	White to greyish	White with grey concentric zonation	Regular	Septate	1.23
C2	Sparse	White with concentric zones	White	Regular	Septate	1.12
C3	Fluffy	White to grey	Greyish	Regular	Septate	2.34
C4	Sparse	White	Creamy white with grey zones	Regular	Septate	2.48
C5	Fluffy	White with grey radiating zones	Pinkish white	Regular	Septate	0.46
C6	Fluffy	Greyish centre with white margin	White with grey centre	Regular	Septate	1.59
C7	Sparse	Greyish white	White with pink centre	Regular	Septate	1.88

Table 10. Growth characters of the selected isolates of *Colletotrichum* sp. causing anthracnose in nutmeg in PDA medium at different interval

Isolates	Radial growth of mycelium in Petri dish (DAI)*				Rate of growth (cm day^{-1})	DTCP**
	3^{rd}	5^{th}	7^{th}	9^{th}		
C1	4.73 (2.39)^{a}	5.70 (2.58)^{c}	8.30 (3.04)^{b}	8.93 (3.15)^{a}	1.26	9
C2	4.60 (2.36)^{b}	6.06 (2.65)^{b}	8.73 (3.12)^{b}	9.00 (3.16)^{a}	1.30	8
C3	3.40 (2.09)^{c}	4.53 (2.35)^{d}	5.46 (2.54)^{c}	8.60(3.09)^{c}	0.98	10
C4	4.70 (2.38)^{a}	7.96 (2.99)^{a}	8.96 (3.15)^{a}	9.00 (3.16)^{a}	1.32	7
C5	4.10 (2.25)^{b}	5.93 (2.63)^{b}	8.43 (3.07)^{b}	9.00 (3.16)^{a}	0.80	9
C6	4.43 (2.33)^{a}	6.20 (2.68)^{b}	8.70 (3.11)^{b}	9.00 (3.16)^{a}	1.30	9
C7	4.03 (2.24)^{b}	5.70 (2.58)^{c}	7.90 (2.98)^{c}	8.93 (3.15)^{b}	1.00	9
CD (0.05)	0.066	0.085	0.037	0.025		
SEm	0.022	0.028	0.012	0.008		

*DAI- Day after inoculation; Mean of three replication; **DTCP-Days taken to cover 9cm petridish

Table 11. Conidial characteristics of the different *Colletotrichum isolates* causing anthracnose in nutmeg

Isolates	Conidia	
	Size (µm)	Shape
C1	16.48 x 3.26	Dumbell
C2	7.87 x 3.68	Oblong
C3	9.98 x 3.84	Oblong
C4	11.84 x 3.42	Oblong
C5	18.78 x 5.68	Dumbell
C6	10.49 x 3.79	Oblong
C7	19.97 x 4.26	Dumbbell

*Mean of three replications

4.3.3 Pathogenic variability of *Colletotrichum* isolates for screening the most virulent isolate

To identify the most virulent isolate, the seven isolates screened from the pathogenicity studies were subjected to virulence rating by artificially inoculating the seven isolates simultaneously on uniform and healthy nutmeg leaves. The observations on the days for symptom appearance, lesion size, rate of lesion development and PDI were noted. All the isolates produced symptoms within 2- 4 days. The isolate C4 produced symptom within 2 DAI while isolates C1, C2, C5 and C6 developed symptom within 3 days. The isolates C3 and C7 produced symptoms after four days of inoculation. The lesion size produced by the *Colletotrichum* isolates varied from 0.36- 1.41 cm. The isolate C4 produced the maximum lesion size of 1.31 cm followed by C7 (1.09cm). The isolates C1, C2 and C5 produced lesion size of 0.83, 0.84 and 0.91 cm respectively. Seven days after inoculation, isolate C4 showed a rapid increase in lesion size to11.88 cm. All the other isolates showed a lesion size ranging between 0.61-2.74 cm. The isolate C4 showed an increase in lesion size to 16.81 cm at 9 DAI (Fig. 4). The lowest lesion size was expressed by C7 isolate. The isolates C5 and C6 produced a lesion size of 3.15 and 3.16 cm respectively. The isolates C1, C2 and C3 produced lesion size ranging 4.94 - 6.89 cm. The rate of lesion development ranged between 0.18 – 4.12 cm day^{-1}, highest for the C4 isolate with average growth rate of 4.12 cm day^{-1} followed by C2 and C3 isolates with 1.86 and 3.26 cm day^{-1} and lowest mean average growth rate of 0.18 cm day^{-1} was recorded for C7 isolate (Table 12). Per cent disease index on 9^{th} DAI ranged between 11.11 - 96.29 and highest PDI was recorded for C4 isolate with 96.29% and lowest was for C1 and C7 isolates (11.11%). The most virulent isolate was C4 from Kumarakom based on the days taken for symptom development, lesion size and rate of lesion development (Plate 12). C4 isolate was used for further studies.

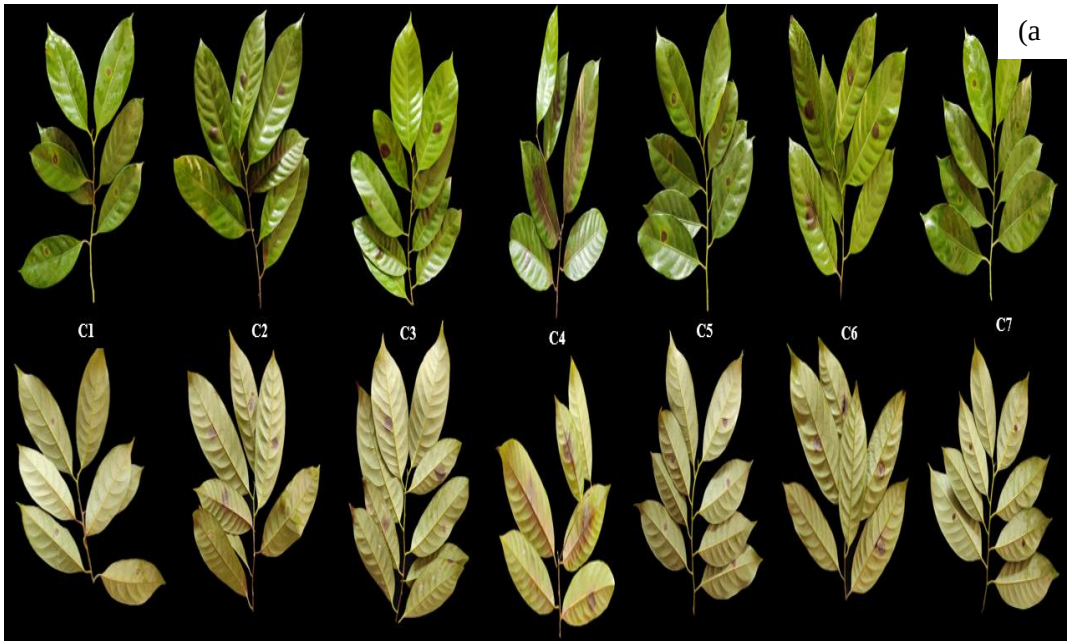


Plate 12. Lesion development on artificial inoculation of *C. gloeosporioides* isolates on detached leaf of nutmeg twig at 7DAI (a) and 9DAI (b)

Table 12. Virulence rating of *C. gloeosporioides* isolates causing anthracnose in nutmeg on detached leaf assay

Isolates	Days taken for symptom development	Lesion size (cm) (DAI)*				Rate of development of lesion (cm day^{-1})	PDI (9 DAI)
		3rd	5th	7th	9th		
C1	3	0.20 (1.09)^{b}	0.83 (1.35)^{b}	2.33 (1.80)^{b}	4.94 (2.28)^{b}	1.19	11.11
C2	3	0.31 (1.14)^{a}	0.84 (1.34)^{b}	2.31 (1.75)^{b}	5.91 (2.62)^{b}	1.86	33.33
C3	4	0.00 (1.00)^{c}	0.36 (1.15)^{d}	1.30 (1.47)^{c}	6.89 (2.54)^{b}	3.26	22.22
C4	2	0.33 (1.15)^{a}	1.31 (1.51)^{a}	11.89 (3.30)^{a}	16.81 (4.22)^{a}	4.12	96.29
C5	3	0.35 (1.16)^{a}	0.91 (1.37)^{b}	1.16 (1.46)^{c}	3.16 (2.01)^{c}	0.93	22.11
C6	3	0.11 (1.05)^{b}	1.09 (1.44)^{b}	2.74 (1.93)^{b}	3.15 (2.03)^{c}	1.01	33.33
C7	4	0.00 (1.00)^{c}	0.47 (1.21)^{c}	0.61 (1.27)^{d}	0.84 (1.35)^{d}	0.18	11.11
CD(0.05)		0.047	0.146	0.760	0.737		
SEm		0.016	0.051	0.264	0.256		

*Mean of six replication; DAI – days after inoculation; Values in parenthesis are square root transformed values

4.4 . HOST RANGE OF MOST VIRULENT ISOLATE OF *Colletotrichum* spp.

An experiment was carried out to find the host range of the most virulent nutmeg isolate of *Colletotrichum spp.* in other perennial trees. The experiment revealed that the selected perennial spices *viz.*, clove (*Syzygium aromaticum*) cinnamon (*Cinnamomum zeylanicum*), betelvine (*Piper betel*), allspice (*Pimenta dioica*), blackpepper (*Pipernigrum*) along with the coconut (*Cocos nucifera*) had developed related characteristic symptoms when inoculated with the nutmeg isolate of *C. gloeosporioides* (Plate 13).

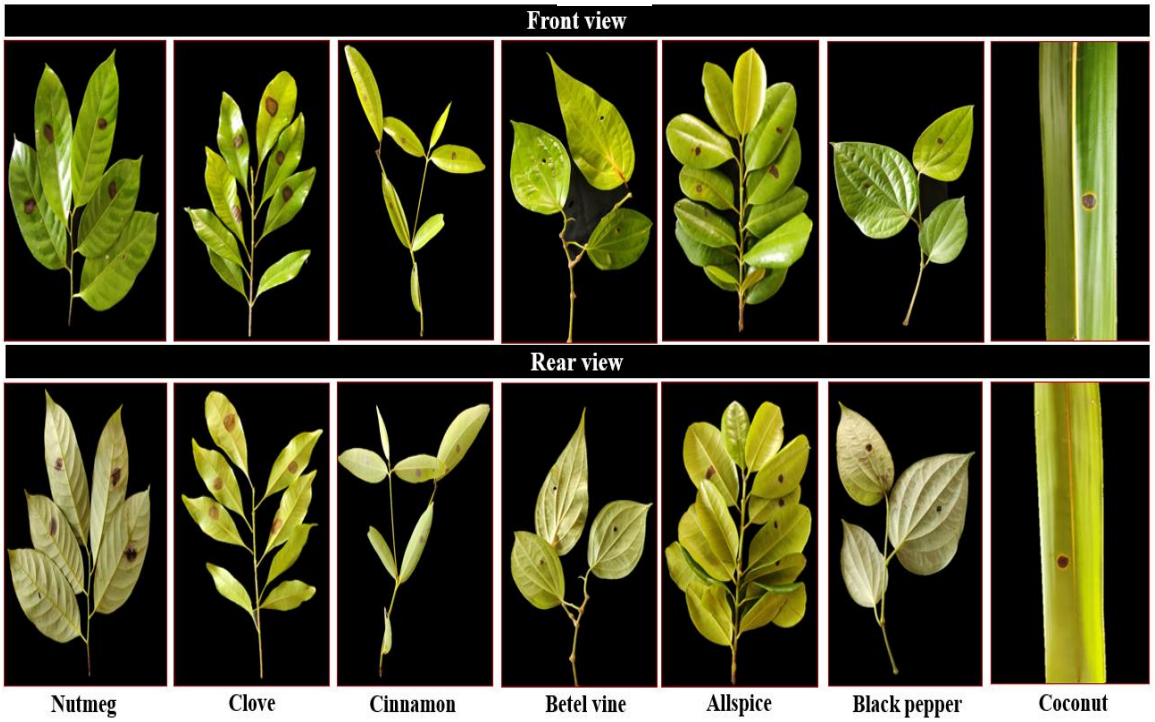
Days taken for symptom development varied between 2-4 days and symptoms produced varied from brown necrotic lesions alone to brown lesions surrounded by yellow halo, black lesions and shot hole symptom (Table 13).

The lesion size was recorded at 3, 5, 7, 9 and 15 days after inoculation. On 3rd day after inoculation, maximum lesion size of 0.66 cm and 0.63 cm was recorded in the clove and betel vine followed by black pepper, coconut, cinnamon, allspice with the lesion size of 0.52 cm, 0.47 cm, 0.41 cm, 0.37 cm respectively.

Nine days after inoculation, maximum lesion of 5.0 cm was recorded in clove followed by 1.8 cm in betelvine and 1.1 cm in cinnamon. The lesion size of 0.94 cm, 0.93 cm and 0.76 cm were recorded in coconut, black pepper and allspice respectively (Table 11).

Per cent disease index in the hosts ranged between 11.11 - 37.77. Highest PDI of 37.77 was recorded in clove followed by cinnamon with 27.77 compared to the control (nutmeg) which showed 33.33 per cent disease severity. Lowest PDI of 11.11 was recorded in betelvine, black pepper and coconut (Table).

(a)



(b)

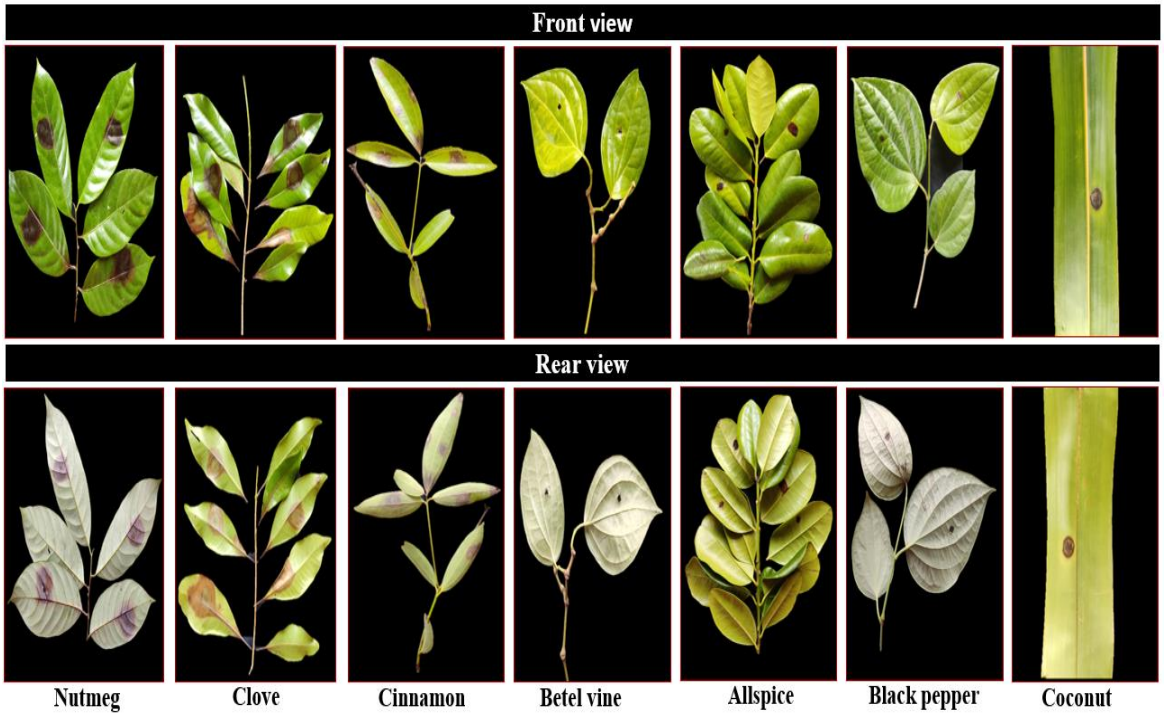


Plate 13. Lesion development by the virulent isolate from nutmeg inoculated on to the detached twigs of different hosts at 7 DAI (a) and 9DAI (b)

Table 13. Time taken for symptom development, symptom produced, lesion size and Disease severity in other host by virulent isolate of *C. gloeosporioides* (C4)

Host	DTS*	Symptoms produced	Lesion size (cm)** at different days after inoculation				PDI (9 DAI)
			3rd	5th	7th	9th	
Clove	3	Brown lesions	0.66 (1.28)[b]	1.23 (1.48)[b]	2.22 (1.79)[b]	5.00 (2.43)[b]	37.77
Cinnamon	4	Brown lesions	0.41 (1.18)[c]	0.46 (1.21)[c]	0.64 (1.28)[d]	1.11 (1.44)[c]	27.77
Betelvine	2	Brown lesions with shot hole	0.63 (1.26)[b]	1.30 (1.42)[b]	1.89 (1.51)[c]	1.94 (1.53)[c]	11.11
Allspice	4	Necrotic spot with yellow halo	0.37 (1.17)[c]	0.41 (1.18)[d]	0.51 (1.23)[d]	0.73 (1.31)[d]	16.66
Black pepper	2	Black lesions	0.52 (1.23)[b]	0.66 (1.28)[c]	0.72 (1.31)[d]	0.93 (1.38)[d]	11.11
Coconut	2	Necrotic spot with yellow halo	0.47 (1.21)[b]	0.61 (1.26)[c]	0.76 (1.32)[d]	0.94 (1.39)[d]	11.11
Control	2	Brown lesion	1.31 (1.51)[a]	3.21 (2.04)[a]	4.52 (2.33)[a]	9.04 (3.14)[a]	33.33
CD(0.05)			0.140	0.282	0.398	0.472	
SEm			0.049	0.098	0.138	0.164	

*DTS- Days taken for symptom development; PDI- Per cent disease index; **Mean of six replications; Values in parenthesis are square root transformed values; DAI – days after inoculation

4.5 *IN VITRO* SCREENING OF FUNGICIDES AGAINST MOST VIRULENT ISOLATE of *Colletotrichum* spp.

An *in vitro* screening was carried out for finding the most effective fungicide against the mycelial growth of *C. gloeosporioides* by using the poisoned food technique. Different fungicides used in the experiment are Propiconazole 25 EC, difenoconazole 25 EC, azoxystrobin 23 SC, captan 50% WP + hexaconazole, 5% WP, trifloxystrobin 25% + tebuconazole 55% WP and carbendazim 12% WP + mancozeb 63% WP. These fungicides were evaluated at four different concentrations of 10, 25, 50 and 100 ppm.

Nature of mycelial growth in fungicides amended medium varied in all the fungicides compared to control which had white fluffy pattern in the front view and whitish with grey radiating zones in the rear view. Propiconazole 25 EC amended medium showed whitish fluffy colony growth in front view and orange colour in the rear view. In difenoconazole 25 EC amended medium, the fungal growth was white cottony in front view and greyish centre with white margin in rear view (Table 14, Plate 14). Azoxystrobin 23 SC and carbendazim 12% WP + mancozeb 63% WP, amended medium fungus showed white cottony growth in front view and whitish with orange centre in rear view. In captan 50% WP + hexaconazole, 5% WP amended medium the fungal growth was white fluffy in front view and white with greyish centre in rear view, whereas in trifloxystrobin 25% + tebuconazole 55% WP amended medium the fungus growth pattern was greyish cottony in front view and off white with orange centre in rear view (Table 15). Propiconazole @100ppm showed the least mycelial growth on 3, 5, 7 and 9 DAI. Carbendazim + mancozeb did not produce mycelial growth @25, 50 and 100ppm at different intervals (Table 16 and 17)

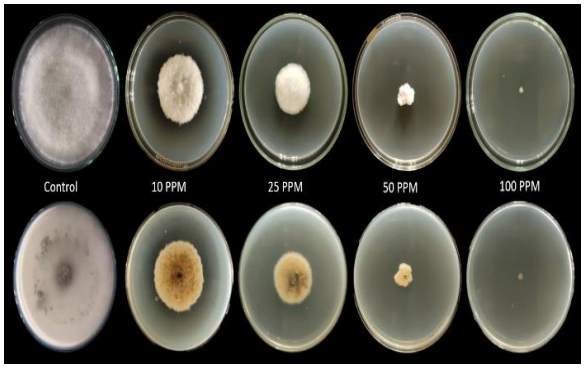
At 10 ppm concentration, per cent mycelial growth inhibition ranged between 39.11 - 76.22. The maximum inhibition was recorded for trifloxystrobin 25% + tebuconazole 55% WP with 76.22 per cent followed by propiconazole with 60.81 per cent. The least per cent mycelial growth inhibition of 39.11 was observed with captan 50% WP + hexaconazole. The other fungicides were on par with respect to the per cent inhibition.

At 25 ppm concentration, per cent mycelial growth inhibition ranged from 48.21 to 100. Cent per cent inhibition was observed by carbendazim 12% WP + mancozeb 63% WP followed by Propiconazole 25 EC with 80.22 which was on par to trifloxystrobin 25% + tebuconazole 55% WP with 79.33 per cent inhibition. The least per cent inhibition of 48.22 was observed with captan 50% WP + hexaconazole.

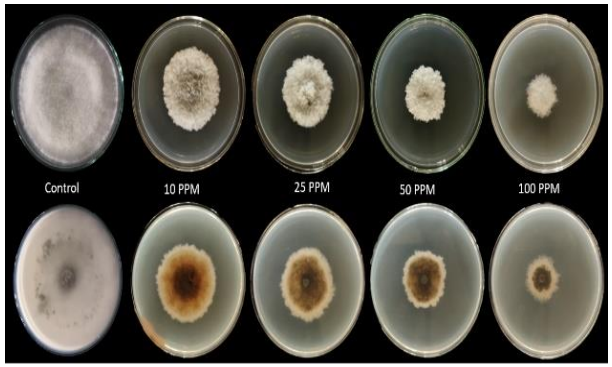
At 50 ppm concentration, 100 per cent inhibition was observed by carbendazim 12% WP + mancozeb 63% WP followed by propiconazole 25 EC with 85.55 which was on par to the trifloxystrobin 25% + tebuconazole 55% WP with 84.00 per cent inhibition. The least per cent inhibition of 57.55 was observed with captan 50% WP + hexaconazole.

At higher concentration of 100 ppm, 100 per cent mycelial inhibition was observed with the three fungicides *viz.,* carbendazim 12% WP + mancozeb 63% WP, propiconazole 25 EC, trifloxystrobin 25% + tebuconazole 55% WP which was followed by captan 50% WP + hexaconazole with per cent inhibition of 79.11. The least per cent inhibition was observed by two fungicides *viz.,* difenoconazole 25 EC, azoxystrobin 23 SC with 69.33 and 73.33 respectively (Table 18, Plate 15).

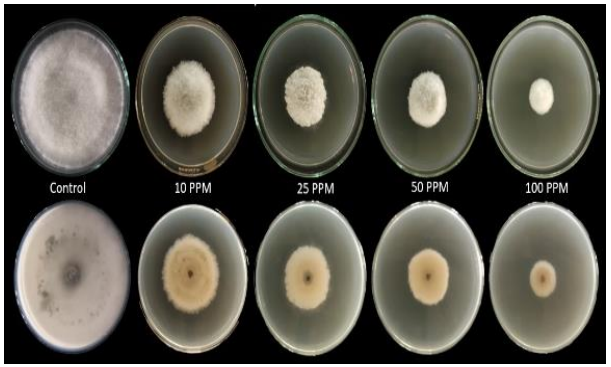
Among the six new generation fungicides used for screening, carbendazim 12% WP + mancozeb 63% WP was effective in inhibiting the growth of the mycelium by 100 per cent even at a lower concentration of 25 ppm and was significantly superior to all the other fungicides used for evaluation. From this experiment, carbendazim 12% WP + mancozeb 63% WP among three combination fungicides group tested, Propiconazole 25 EC among the triazoles group tested were effective in managing the anthracnose disease of nutmeg.



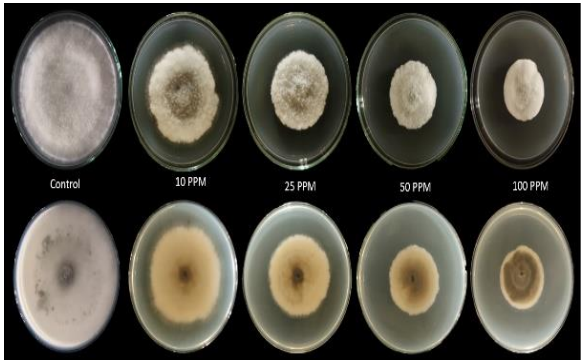
T1- T4: Propiconazole 25 EC



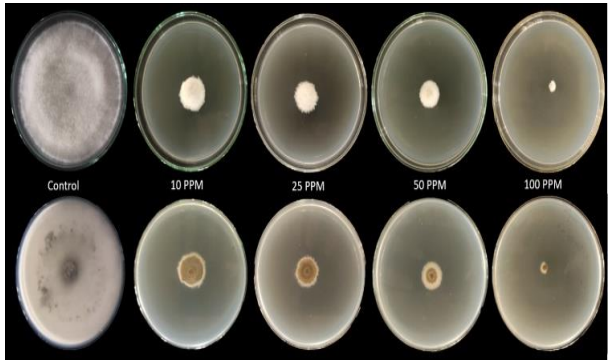
T5- T8: Difenoconazole 25 EC



T9- T12 : Azoxystrobin 23 SC



T13- T16: Captan 50% WP + Hexaconazole 5% WP



T17-T20 : Trifloxystrobin25% + Tebuconazole 55% WP

T21- T24: Carbendazim 12% WP + Mancozeb 63% WP

Plate 14. Nature of mycelial growth *C. gloeosporioide*s (C4)in PDA medium amended with various fungicides at 7 DAI

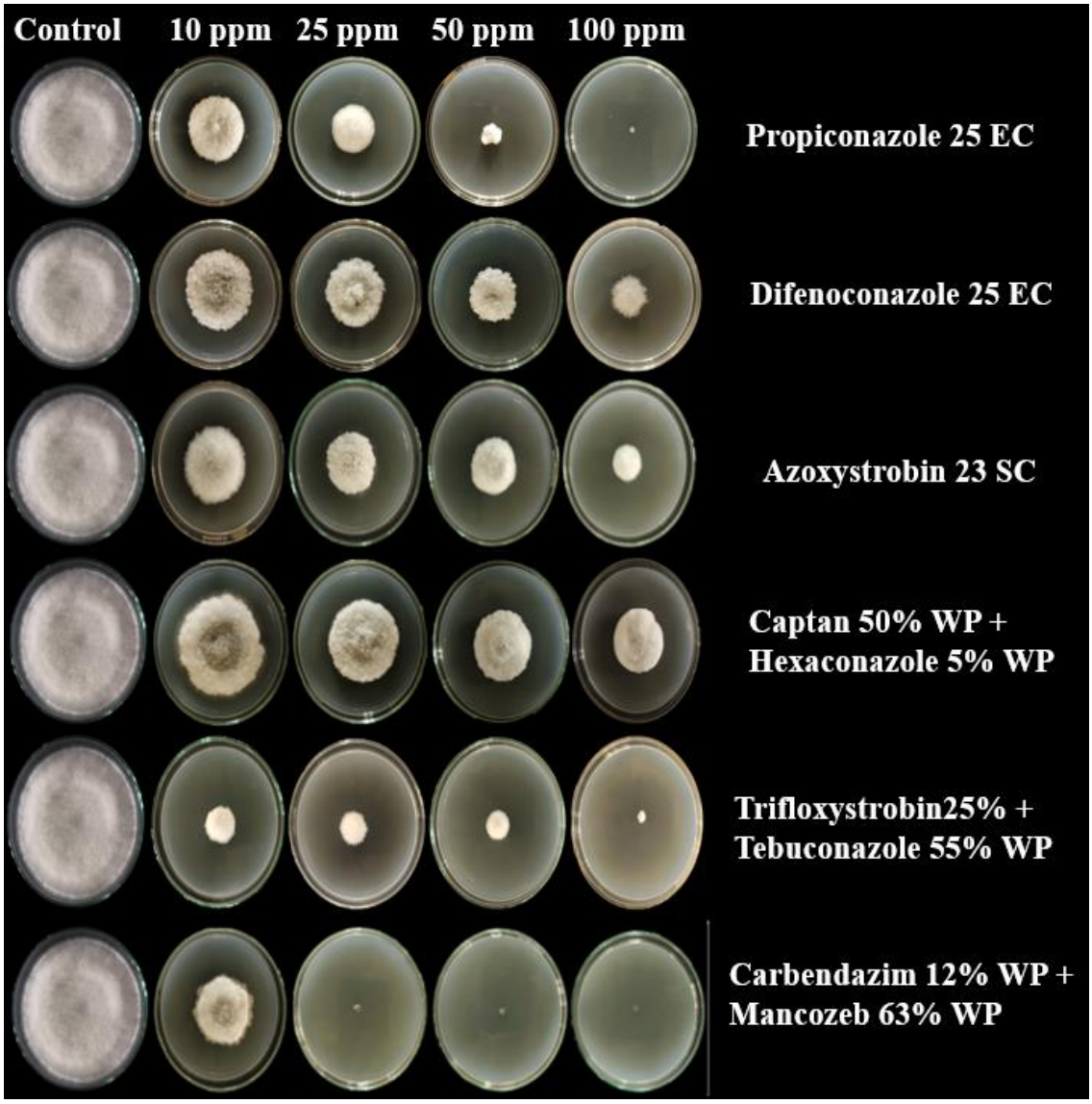


Plate 15. Per cent growth inhibition of *C. gloeosporioides* at different concentration of fungicides on PDA media at 7 DAI

Table 14. Nature of mycelial growth of *C. gloeosporioides* (C4) in fungicide amended PDA medium (Poisoned food technique)

Treatments	Dosage	Nature of mycelial growth	
		Front view	Rear view
T1-T4: Propiconazole 25 EC	10 ppm	White fluffy growth with regular margin	Orange colour
	25 ppm	Whitish fluffy growth with regular margin	Orange with grey radial zones
	50 ppm	Fluffy growth	Orange colour
	100 ppm	No growth	No growth
T5- T8: Difenoconazole 25 EC	10 ppm	White cottony growth	Orange colour with dark centre
	25 ppm	White cottony growth	Grey centre and white margin
	50 ppm	White cottony growth	Grey centre and white margin
	100 ppm	White cottony growth	Grey centre and white margin
T9-T12: Azoxystrobin 23 SC	10 ppm	White cottony growth	White with orange centre
	25ppm	White cottony growth	White with orange centre
	50 ppm	White cottony growth	White with orange centre
	100 ppm	White cottony growth	White with orange centre

Contd…

Table 15. Nature of mycelial growth of *C. gloeosporioides* (Poisoned food technique)

Treatments	Dosage	Nature of mycelial growth	
		Front view	Rear view
T13– T16: Captan 50% WP + Hexaconazole 5% WP	10 ppm	White cottony growth with grey centre	Off white with orange centre
	25 ppm	Grey cottony growth	Off white with orange centre
	50 ppm	White cottony growth	Off white with orange centre
	100 ppm	Off white cottony	White with grey centre
T17- T20: Trifloxystrobin 25% + Tebuconazole 55% WP	10 ppm	White fluffy growth	White with grey centre
	25 ppm	White fluffy growth	White with grey centre
	50 ppm	White fluffy growth	White with grey centre
	100 ppm	White fluffy growth	White
T21 -T24: Carbendazim 12% WP + Mancozeb 63% WP	10 ppm	White cottony growth	White with orange centre
	25 ppm	-	-
	50 ppm	-	-
	100 ppm	-	-
T25: Control		White fluffy mycelium	White with grey radiating zones

Table 16. Mycelial growth of *C. gloeosporioides* (C4) in fungicide amended PDA medium (Poisoned food technique)

Treatments	Dosage	Radial mycelial growth in cm (DAI)*			
		3rd	5th	7th	9th
T1-T4: Propiconazole 25 EC	10 ppm	0.84 (1.35)^{gh}	2.72 (1.92)^{cd}	2.72 (1.92)^{cd}	3.52 (2.12)^{efg}
	25ppm	0.00 (1.00)^{j}	1.02 (1.41)^{hij}	1.78 (1.66)^{hij}	1.78 (1.66)^{jklm}
	50 ppm	0.00 (1.00)^{j}	0.70 (1.30)^{j}	1.14 (1.43)^{kl}	1.30 (1.48)^{m}
	100ppm	0.00 (1.00)^{j}	0.00 (1.00)^{k}	0.00 (1.00)^{m}	0.00 (1.00)^{n}
T5-T8: Difenoconazole 25 EC	10 ppm	1.08 (1.43)^{efg}	2.72 (1.92)^{cd}	3.64 (2.15)^{cde}	4.20 (2.27)^{cde}
	25 ppm	1.00 (1.40) ^{fg}	2.40 (1.84)^{def}	3.52 (2.12)^{de}	3.94 (2.22)^{def}
	50 ppm	1.16 (1.46)^{def}	2.58 (1.89)^{cde}	3.32 (2.07)^{def}	3.52 (2.12)^{efg}
	100 ppm	0.00 (1.00)^{j}	0.80 (1.33)^{ij}	1.86 (1.68)^{hi}	2.76 (1.93)^{ghi}
T9- T12: Azoxystrobin 23 SC	10 ppm	1.540 (1.59)^{c}	2.38 (1.83)^{def}	3.24 (2.05)^{l}	3.86 (2.20)^{def}
	25ppm	1.96 (1.72)^{b}	2.06 (1.74)^{fg}	2.26 (1.80)^{gh}	4.08 (2.25)^{cde}
	50 ppm	1.32 (1.51)^{cde}	1.88 (1.69)^{g}	2.66 (1.91)^{fg}	3.16 (2.03)^{fgh}
	100 ppm	0.00 (0..00)^{j}	1.26 (1.50)^{h}	1.98 (1.72)^{hi}	2.40 (1.84)^{hij}

*Mean of five replication; DAI – Days after inoculation; Values in parenthesis are square root transformed values

Table 17: *In vitro* evaluation of new generation fungicides against *C. gloeosporioides* (Poisoned food technique) Contd…..

Treatments	Dosage	Radial mycelial growth in cm (DAI)*			
		3rd	5th	7th	9th
T13- T16: Captan 50% WP + Hexaconazole 5% WP	10 ppm	2.04 (1.74)^{b}	4.08 (2.25)^{b}	4.94 (2.43)^{b}	5.48 (2.54)^{b}
	25 ppm	1.40 (1.54)^{cd}	3.06 (2.01)^{c}	4.02 (2.24)^{cd}	4.66 (2.37)^{bcd}
	50 ppm	0.86 (1.36)^{gh}	2.12 (1.76)^{efg}	3.18 (2.04)^{ef}	3.82 (2.19)^{def}
	100 ppm	0.40 (1.17)^{I}	0.88 (1.34)^{j}	1.48 (1.51)^{jk}	1.88 (1.62)^{klm}
T17- T20: Trifloxystrobin25% + Tebuconazole 55% WP	10 ppm	0.74 (1.31)^{h}	1.38 (1.54)^{h}	1.72 (1.64)^{hij}	2.14 (1.77)^{ijk}
	25 ppm	0.00 (1.00)^{j}	1.12 (1.45)^{hi}	1.50 (1.58)^{ijk}	1.86 (1.69)^{jkl}
	50 ppm	0.00 (1.00)^{j}	0.22 (1.09)^{k}	0.62 (1.26)^{m}	1.44 (1.56)^{lm}
	100 ppm	0.00 (1.00)^{ja}	0.00 (1.00)^{k}	0.00 (1.00)^{m}	0.00 (1.00)^{n}
T21- T24: Carbendazim 12% WP + Mancozeb 63% WP	10 ppm	3.22 (1.92)^{a}	4.24 (2.28)^{ab}	4.40 (2.31)^{bc}	5.00 (2.44)^{bc}
	25 ppm	0.00 (0.70)^{j}	0.00 (1.00)^{k}	0.00 (1.00)^{m}	0.000 (1.00)^{n}
	50 ppm	0.00 (0.70)^{j}	0.00 (1.00)^{k}	0.00 (1.00)^{m}	0.000 (1.00)^{n}
	100 ppm	0.00 (0.70)^{j}	0.00 (1.00)^{k}	0.00 (1.00)^{m}	0.000 (1.00)^{n}
T25: Control		3.10 (1.89)^{a}	4.84 (2.41)^{a}	7.02 (2.83)^{a}	8.900 (3.14)^{n}
CD (0.05)		0.90	0.121	0.170	0.194
SEm		0.032	0.043	0.061	0.069

*Mean of five replication; DAI – days after inoculation; Values in parenthesis are square root transformed values

Table 18. Percentage mycelial inhibition of *C. gloeosporioides* by new generation fungicides

Treatments (Fungicides)		Per cent inhibition (%) at different concentrations* (7 DAI)			
		10 ppm	25 ppm	50 ppm	100 ppm
T1- T4	Propiconazole 25 EC	60.88 (51.28)[a]	80.22 (63.57)[b]	85.55 (70.11)[b]	100.00 (90.00)[a]
T4-T8	Difenoconazole 25 EC	53.33 (46.90)[b]	56.21 (48.57)[c]	60.88 (51.28)[c]	69.33 (56.36)[c]
T8-T12	Azoxystrobin 23 SC	57.10 (49.08)[b]	56.88 (48.94)[c]	64.88 (53.64)[c]	73.33 (58.89)[c]
T13-T16	Captan 50% WP + Hexaconazole 5% WP	39.10 (38.52)[c]	48.21 (43.95)[c]	57.55 (49.34)[c]	79.108 (68.29)[b]
T17-T20	Trifloxystrobin 25% + Tebuconazole 55% WP	76.21 (60.80)[a]	79.32 (62.95)[b]	83.99 (66.42)[b]	100.00 (90.00)[a]
T20-T24	Carbendazim 12% WP + Mancozeb 63% WP	44.44 (41.65)[b]	100.00 (90.00)[a]	100.00 (90.00)[a]	100.00 (90.00)[a]
CD (0.05)		5.927	2.602	6.892	10.685
SEm		2.019	0.886	2.347	3.639

*Mean of five replication; DAI – days after inoculation; Values in parenthesis are arc sine transformed values

Discussion

5. Discussion

Nutmeg (*Myristica fragtans* Houtt*)* is a highly remunerative spice crop and unique among all other tree spices as it yields two typical spices *viz.,* mace (aril on the kernel) and nutmeg (seed kernel). Nutmeg is universally accepted as a condiment and also possesses medicinal properties. However, the crop is affected by many diseases *viz.,* Die back (*Diplodia* sp), fruit rot (*Phytophthora* sp. and *Diplodia natalensis*), anthracnose or shot hole (*Colletotrichum gloeosporioides*), seedling wilt (*Cylindrocladium* sp., *Fusarium* sp., *Colletotrichum* sp., *Rhizoctonia bataticola* and *Phytophthora* sp.) and leaf spot (*Cladiosporium oxysporum)* (Thangaselvabai, 2011). Of the diseases, anthracnose and Phytophthora leaf fall were more prevalent in Kerala. The diseases are favoured by warm humid climate and affect the quality and quantity of the spice (Kumar *et al*., 2016). Presence of the diverse races of the fungus *C. gloeosporioides* makes their management troublesome. So, there is an urgent need to develop a new management strategy involving new generation fungicides with site specific action and higher efficacy at very low concentrations.

In this context the present study entitled "Host range studies and management of anthracnose of nutmeg caused by *Colletotrichum* spp." with the objectives to characterize the causal agent, study the host range of the pathogen and develop a new management strategy for the control of the disease by using new generation fungicides was conducted during 2018-2020 at Department of Plant Pathology, College of Agriculture, Vellayani.

5.1 PREVALENCE AND COLLECTION OF ANTHRACNOSE INFECTED SAMPLES

A survey for the collection of anthracnose infected nutmeg samples was conducted during August – September, 2019 in different districts under various agroecological zones of Kerala *viz.,* Thiruvananthapuram, Kottayam, Ernakulam and Idukki. Anthracnose disease was identified as the major disease and *C. gloeosporioides* as the causal agent in all locations. Anthracnose of nutmeg was first reported from Kerala in 1961 and was caused by an unknown species of *Colletotrichum*; later confirmed as *C. gloeosporioides* by Nair *et al.* (1978). The present study further confirmed C. *gloeosporioides* as the causal organism of anthracnose of nutmeg in Kerala.

Disease incidence and severity (PDI) during the survey period varied from location to location; even among the fields in the same location. Within the same agroecological zone

there was wide variation in the incidence and severity of the disease. The disease incidence in the surveyed locations varied from 20 - 90 per cent (Fig. 1). Ahmed *et al.* (2014) assessed disease incidence and severity of betelvine anthracnose and found disease incidence and severity was ranged between 10.87 % - 80.50% which was highest in July and lowest in December.

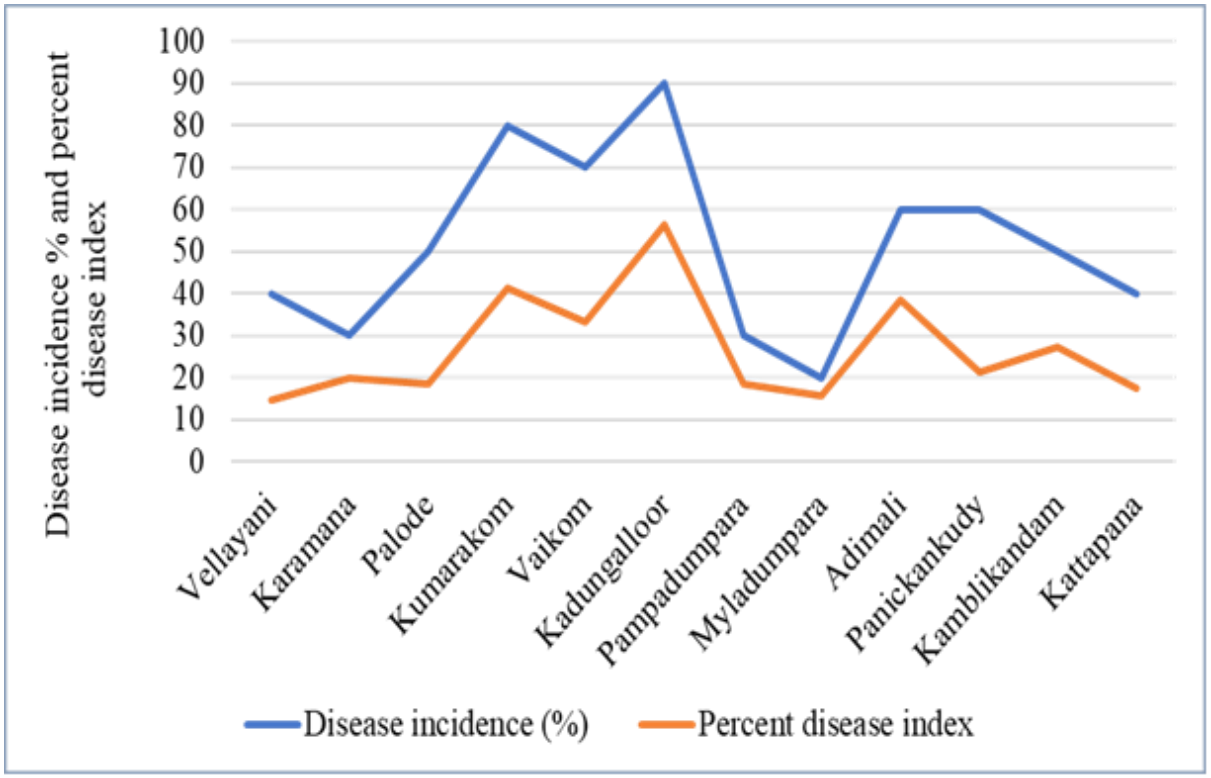


Fig. 1: Disease incidence and severity of nutmeg anthracnose in surveyed locations

5.2 SYMPTOMATOLOGY, ISOLATION OF PATHOGEN, PROVING PATHOGENICITY OF *Colleotrichum* spp. CAUSING ANTHRACNOSE OF NUTMEG

5.2.1. Symptomatology

Symptomatology of the anthracnose was studied in the surveyed locations. *C. gloeosporioides* infected nutmeg from seedlings to the mature plants and produced various symptoms *viz.,* necrotic leaf spot, necrotic spot surrounded by yellow halo, leaf blight, shot hole and fruit rot. The present investigation on the symptomatology revealed that the symptom initiates as leaf spot progressing to leaf blight, shot hole, twig blight and fruit infection ultimately leading to huge economic losses. Similar symptoms of leaf spot, shot hole and necrotic spots surrounded by a brown halo were observed by Chattopadhyay and Maiti (1990) in anthracnose infected betel vine. Black stem lesions were observed in anthracnose infected betel vines in addition to the necrotic spots with yellow halo and blight in the foliage (Shahzad and Zareen, 2000). Freire *et al*. (2002) also observed brown lesions, leaf blight and defoliation as the major symptoms of anthracnose in cashew. Gosh *et al*. (2003) reported the symptoms of brown spots, shot hole and fruit rot in anthracnose of mango.

5.2.2. Isolation of the pathogen

Leaves and fruits showing typical anthracnose symptoms were collected from different locations during the survey and were used for the isolation of the pathogen. The infected tissues along with the healthy portion were cut into small bits for isolation of the fungus. Eighteen isolates of *Colletotrichum* sp. were obtained and labelled as C1 – C18. Similar isolation techniques for anthracnose pathogen from nutmeg, clove and black pepper were reported by Kumar *et al*. (2016), Savani and Rajashekar (2016) and Behera *et al*. (2018) respectively.

5.2.3. Proving pathogenicity of *Colletotrichum* spp.

All the *Colletotrichum* isolates obtained from the different locations were separately/individually inoculated onto the healthy leaves of the nutmeg by pin prick method. The leaves inoculated with pathogen showed symptoms of brown lesions alone or brown lesion surrounded by the yellow halo and shot hole. Some isolates produced the acervuli and spore mass on the under surface of the leaves. From the eighteen isolates, seven isolates were selected and screened based on the days taken for symptom development and spread of the

disease (Fig. 2). Similar attempt to prove the pathogenicity of *C. gloeosporioides* of black pepper anthracnose was done by Sankar (2002) and Chandrakrant (2005).Pathogenicity of C. *gloeosporioides* in papaya was proved by using the spray of spore suspension at a concentration of 1.0 x 10^7 conidia ml^{-1} (Kumara and Rawal, 2004). Haralpatil (2006) inoculated both the spore suspension and mycelial bits for proving the pathogenicity of *C. gloeosporioides* on detached leaves of betel vine.

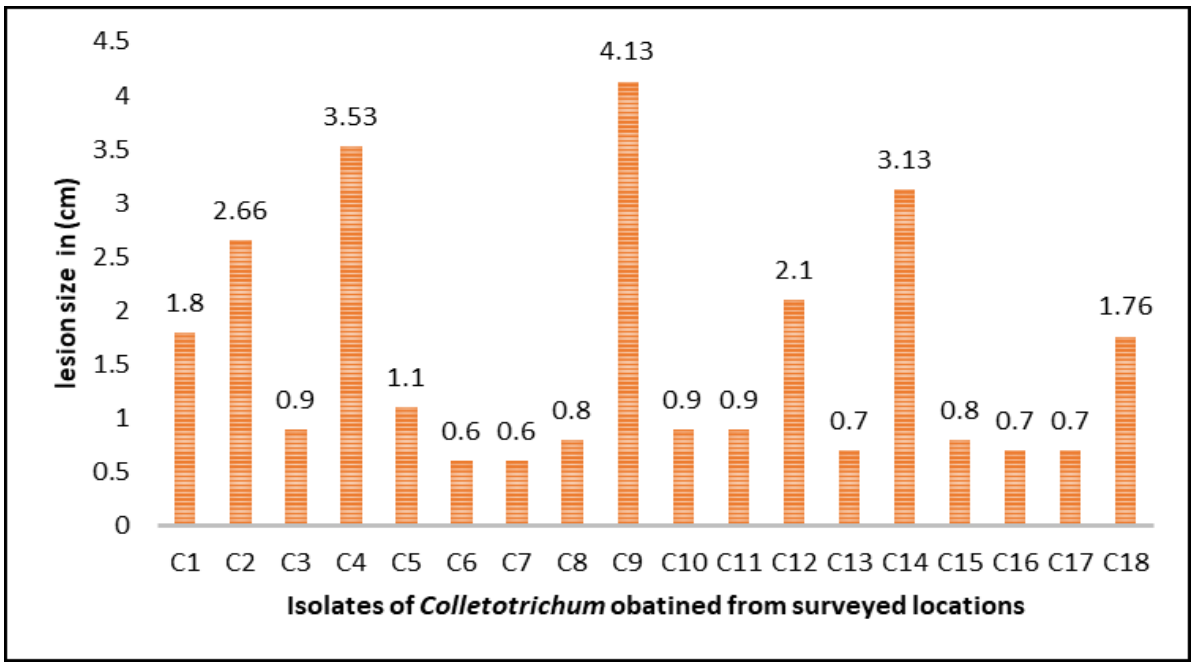


Fig. 2: Lesion size developed by different isolates of *Colletotrichum* causing anthracnose of nutmeg on artificial inoculation (Koch postulates)

5.3 MORPHOLOGICAL AND PATHOGENIC VARIABILITY OF *Colletotrichum* ISOLATES FOR SCREENING THE MOST VIRULENT ISOLATE

The morphological and culture characters of the seven isolates were studied in potato dextrose agar (PDA) medium.

5.3.1. Morphological characterisation of different isolates obtained from different localities

The mycelium was hyaline and septate; and width ranged from 0.46 µm to 2.48 µm. The isolate C4 had the maximum width of 2.48 µm followed by C3, C7, C6, C1, C2 and C5 with 2.34, 1.88, 1.59, 1.23, 1.14 and 0.46 µm respectively. The conidia were single celled with an oil globule at the centre and were oblong or dumbbell shaped. The size of the conidia

varied between 7.87 - 19.97 µm x 3.26 - 5.68 µm. Maximum conidial size of 19.97 µm. x 4.26 µm was exhibited by C7 isolate. The isolates were morphologically identified as *C. gloeosporioides.* Similar results were observed by Kumar *et al*. (2016) on conidia of *C. gloeosporioides* from nutmeg which were hyaline, single celled with round edges and the size ranged from 11.45 - 23.62 µm. x 3.58 - 5.72 µm. Sankar (2002) also reported the isolates of *C. gloeosporioides* from black pepper had a conidial size ranging from 13.10 -16.85 µm.x 3.28 - 5.43 µm indicating a positive correlation of the results with that of the pathogen from nutmeg. Mammooty (2003) observed mycelial width of the *C. gloeosporioides* ranging from 1.25 - 4.00 µm and conidial size 13.45 -16.45 µm x 3.86 - 5.36 µm.

The mycelia of *C. gloeosporioides* isolates obtained from papaya were brown to black and septate. The conidia were cylindrical with a size ranging 13.2 - 14.8 µm. x 4.6 - 5.1 µm (Bag, 2004). Similar observations on the conidial size and shape of *C. gloeosporioides* on *Cymbidium sinense* was made by Huang *et al*. (2012). Conidia were hyaline, single celled with round ends and size ranged from 11.45 to 23.62 µm in length and 3.58 to 5.72 µm in width (Savani and Rajashekar, 2016). Chavan *et al*. (2017) observed hyaline and cylindrical conidia in *C. gloeosporioides* isolates from different fruit crops; and the size of conidia were 7.00 -18.00 µm. x 3.32- 8.13 µm.

5.3.2. Cultural characterisation of different Colletotrichum isolates

The isolates of *Colletotrichum* sp. had whitish to greyish radial mycelial growth in the front view and whitish with grey concentric zonations, off white to pink colour in the rear. Days taken to complete growth in Petri dish varied between 7-10 days. The growth patterns of Colletotrichum isolates were either fluffy or sparse. The appearance of the mycelia was either white to grey with concentric or radiating zones and regular margin**.** Among the different isolates C4 and C2 were fast growing with the average growth rate of 1.32 cm day^{-1} and 1.31 cm day^{-1} and took 7 - 8 days for complete growth. The results obtained were in consonance with Sankar (2002) who reported mycelial growth of *C. gloeosporioides* isolates from black pepper were cottony with colour variation from white, light grey to dark grey. Similar observations were made in other spice crops. Naik and Hiremath (1986) isolated *C. gloeosporioides* from betel vine with grey colonies with an average growth rate of 1.04 - 1.28 cm day^{-1} and completed full growth in Petri dish within 7-9 days. Light to dark grey colony of *C. gloeosporioides* from black pepper was reported by Mammooty (2003). The colony appearance of *C. gloeosporioides* isolates of orchid exhibited greyish white, pinkish grey and orange colour in the front view whereas whitish, dark grey to orange in the rear (Chowdappa

et al., 2012; Huang *et al*., 2012). *C. gloeosporioides* isolates from cowpea were white to greyish colour in the front view and dark greyish to black colour in the rear view; and the isolates took 6 to 10 days for complete growth with an average growth rate of 0.80 – 1.38 cm day $^{-1}$ (Sreeja, 2014).

C. gloeosporioides from anthracnose infected nutmeg and clove took six days for complete growth in Petri dish (Kumar *et al*., 2016; Savani and Rajashekar, 2016). Sudha and Narendrappa (2016) reported the isolates of *C. gloeosporioides* from mango produced greyish to dark grey colonies in the front and rear view with orange to black pigmentation. The colonies of isolates exhibited fluffy to flat mycelial growth with smooth and regular to irregular margins. The cultures also produced orange coloured conidial mass and acervuli as black dots in the culture. Chavan *et al*. (2017) reported the isolates of *C. gloeosporioides* from different fruit crops *viz.*, pomegranate, papaya, banana, chickoo, mango, citrus, custard apple and guava with variation in the colony colour *viz.*, greyish pink to black, off white, cottony white, creamy white and dull white with smooth, raised to fluffy raised margins. Udhaykumar *et al*. (2019) observed variation in colony colour from pinkish-to-pinkish brown, normal white to light grey, greyish white, greyish brown, greenish grey in the isolates of *C. gloeosporioides* obtained from mango anthracnose. Morphological and cultural studies on the appearance, growth pattern, colony colour, rate of mycelia growth, mycelial size and conidial characteristics *viz.*, size and shape had considerable variation among the different isolates indicating the variability of *C. gloeosporioides*.

5.3.3 Pathogenic variability of *Colletotrichum* isolates for screening the most virulent isolate

The most virulent among the seven isolates of *C. gloeosporioides* was selected by assessing the virulence through artificial inoculation on to the detached nutmeg leaves. Virulence rating was based on the observations *viz.*, days taken for symptom expression, maximum lesion size and rate of development of lesion (Fig. 3). All the isolates produced symptoms within 2 - 4 days. The isolate C4 produced symptom within 2 DAI; while isolates C1, C2, C5 and C6 developed symptom in 3 days. The lesion size produced by the *Colletotrichum* isolates varied from 0.36 - 1.41 cm. The isolate C4 produced the maximum lesion size of 1.31 cm followed by C7 (1.09 cm). Seven days after inoculation, the isolate C4 showed a rapid increase in lesion size from 5.8 to 11.88 cm (Fig. 4). The isolate C4 had the maximum rate of lesion development (4.12) followed by C3 (3.86) and C2 (1.86 cm day^{-1})

The most virulent isolate C4 took just 2 days for symptom development; maximum lesion size of 11.8 cm on 9th DAI and higher rate of lesion development 4.12 cm day^{-1} compared to the other isolates. Similar observation was made by Sankar (2002) who conducted pathogenic variability studies with isolates of *C. gloeosporioides* from black pepper. The virulence rating was assessed as most virulent, moderate virulent and less virulent based on the lesion size at different days after inoculation. Virulence of the various *C. gloeosporioides* isolates obtained from cowpea was subjected to pathogenic variability study and the most virulent isolate was selected based on the days taken for symptom appearance (Sreeja, 2014). Martinez *et al*. (2009) reported only nineteen from thirty *C. gloeosporioides* isolates from mango were able to develop symptoms at 4 DAI indicating the virulent ones; whereas rest of the isolates failed to develop symptoms showing their avirulence. Figueiredo *et al*. (2012) did artificial inoculation of 18 isolates of *Colletotrichum* obtained from cashew leaves infected with anthracnose and the results revealed that 3-5 days were taken for the expression of the symptom. The variability of *C. gloeosporioides* isolates was characterised as most virulent, moderately virulent and less virulent based on the per cent disease index and they observed per cent disease index was varied and ranging 12.55 - 76.30 per cent (Devamma *et al*., 2012).

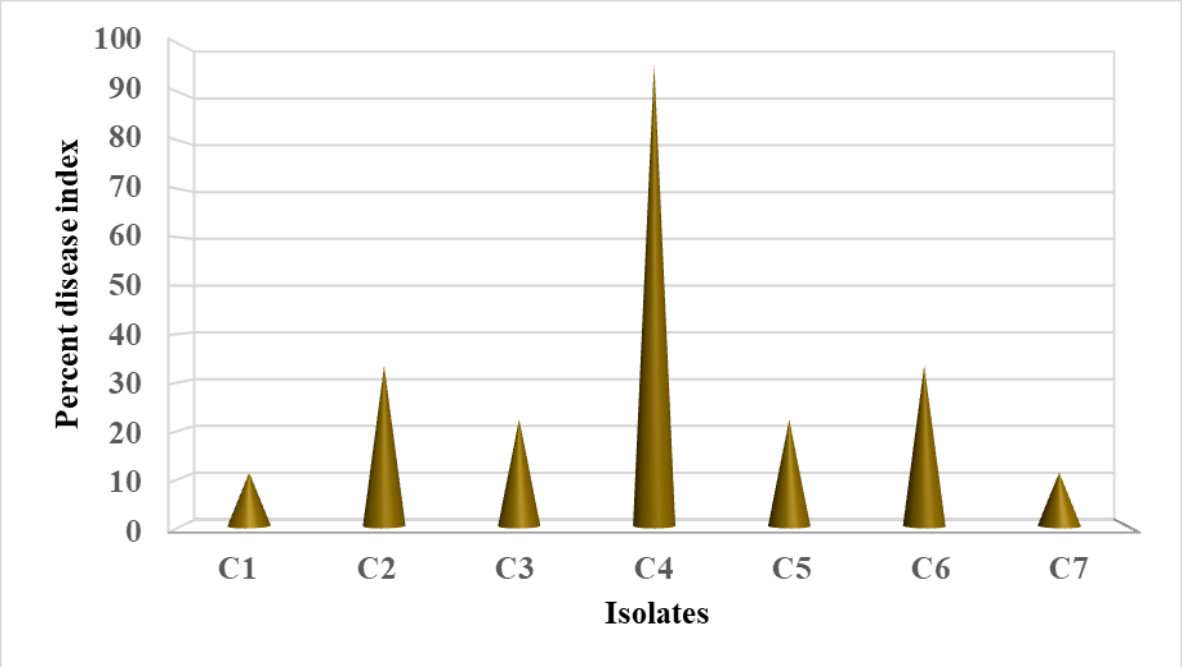


Fig. 3: Virulence rating of *C. gloeosporioides* isolates causing anthracnose in nutmeg (9 DAI)

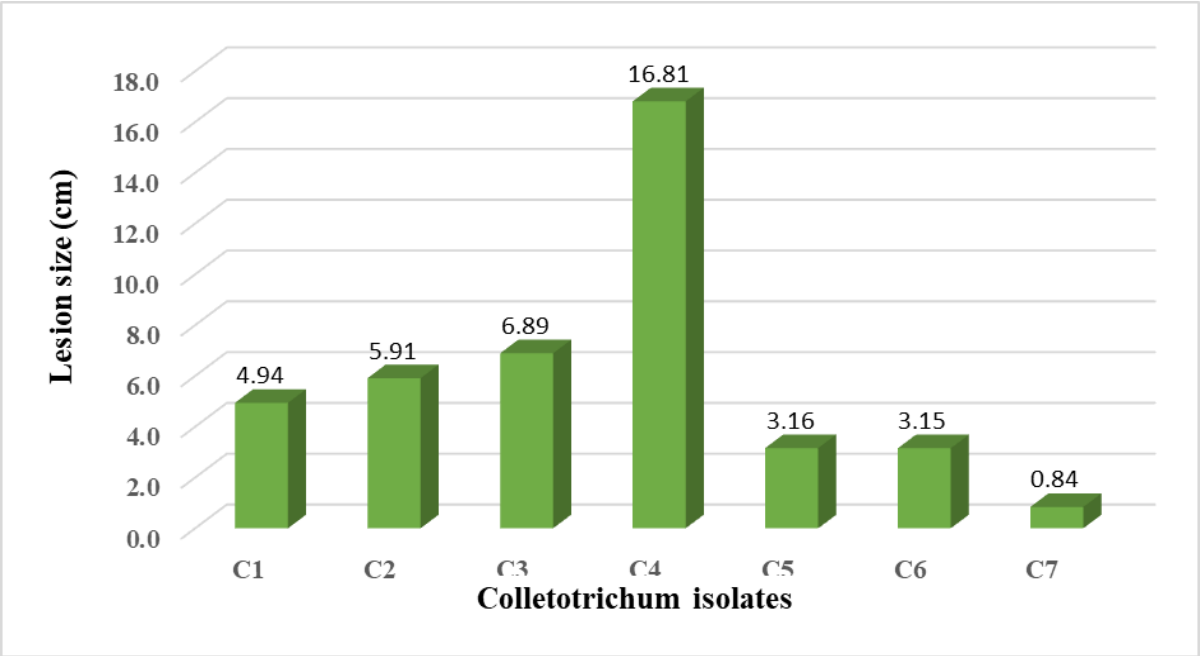


Fig. 4: Lesion size produced by seven isolates of *C. gloeosporioides* in nutmeg on artificial inoculation at 9 DAI

5.4. HOST RANGE OF THE MOST VIRULENT ISOLATE OF *Colletotrichum* spp.

Host range of the most virulent isolate of *C. gloeosporioides* from nutmeg was studied in other perennial tree spices and coconut. The selected perennial spices *viz.*, clove (*Syzygium aromaticum*) cinnamon (*Cinnamomum zeylanicum*), betelvine (*Piper betel*), allspice (*Pimenta dioica*), blackpepper (*Piper nigrum*) and coconut (*Cocos nucifera*) developed characteristic symptoms of brown necrotic lesions with a prominent yellow halo when inoculated with the nutmeg isolate of *C. gloeosporioides*; thus, indicating its cross infectivity on to other host plants. Lesion size on 3rd day after inoculation revealed a maximum lesion size of 0.66 cm and 0.63 cm in clove and betel vine followed by black pepper (0.52cm), coconut (0.47cm), cinnamon (0.41 cm) and allspice (0.37cm). The maximum lesion size of 5 cm was recorded in clove followed by 1.8 cm in betel vine and 1.1 cm in cinnamon at 9DAI. Minimum lesion size of 0.76 cm was observed in allspice (Fig. 5). The increased lesion size can be attributed to soft textured leaves of betel vine which might have facilitated rapid spread of the pathogen. The thickness of allspice leaves was comparatively higher than the other tree spices which might have hindered the further spread of the pathogen. Sanders and Korsten (2002) reported a single host plant can be affected by many *Colletotrichum* species strains and in the similar way one strain of *Colletotrichum* spp. can affect more than one host. *C. gloeosporioides*

obtained from avocado and mango showed bigger lesions on their original hosts and also produced lesions in other hosts *viz.,* strawberry, guava, papaya, and chilli; but failed to produce symptoms in citrus. Thus, the present study on the host range also revealed that single strain of *C. gloeosporioides* obtained from nutmeg was able to infect other tree spices *viz.,* cinnamon, clove, betelvine, allspice, black pepper and coconut. Similar observations were made by Kumara and Rawal (2004) who reported isolates of *C. gloeosporioides* from fruit crops *ie*., papaya, mango and banana were able to produce symptoms on other host while it could not infect grapes and pomegranate. Isolates of *C. gloeosporioides* obtained from custard apple developed symptoms of blackening when cross inoculated on various hosts *viz.,* curry leaves, chilli, papaya, mango, grape and guava (Gaikwad *et al*., 2005). Cross infection studies of *C. gloeosporioides* indicated its host range in 23 fruit crops *viz.,* ambarella, avocado, beli, cashew, citrus, syzygium, durian, guava, katu, mango, mangosteen papaya, passion fruit, pini, jambu, pomegranate, rambutan, rata, sapota, seeni, tomato,ugurassa, weralu and wood apple (Alahakoon and Brown, 1994). *C. gloeosporioides* isolates from papaya and mango produced bigger lesions in their original hosts while smaller lesions in other hosts on cross inoculation studies (Wijeratnam *et al*., 2008). Likewise, the current study also indicated the bigger lesions on the original host *i.e.,* nutmeg and comparatively lower lesion size in other hosts.

Lakshmi *et al.* (2011) reported that *C. gloeosporioides* obtained from mango showed anthracnose symptoms on other hosts *viz.*, papaya, guava, custard apple and pomegranate; but not in papaya. Bandgar *et al*. (2018) cross-inoculated fourteen isolates of *C. gloeosporioides* obtained from chilli, mango, papaya, turmeric, jasmine, onion, and garlic; and all the hosts developed symptoms within 7-10 days of inoculation indicating wide host range of *C. gloeosporioides*. Accordingly, the present study also revealed cross infection potential of *C. gloeosporioides* isolate of nutmeg in the other tree crops *viz.,* clove, betelvine, allspice, blackpepper and coconut indicating its wide host range.

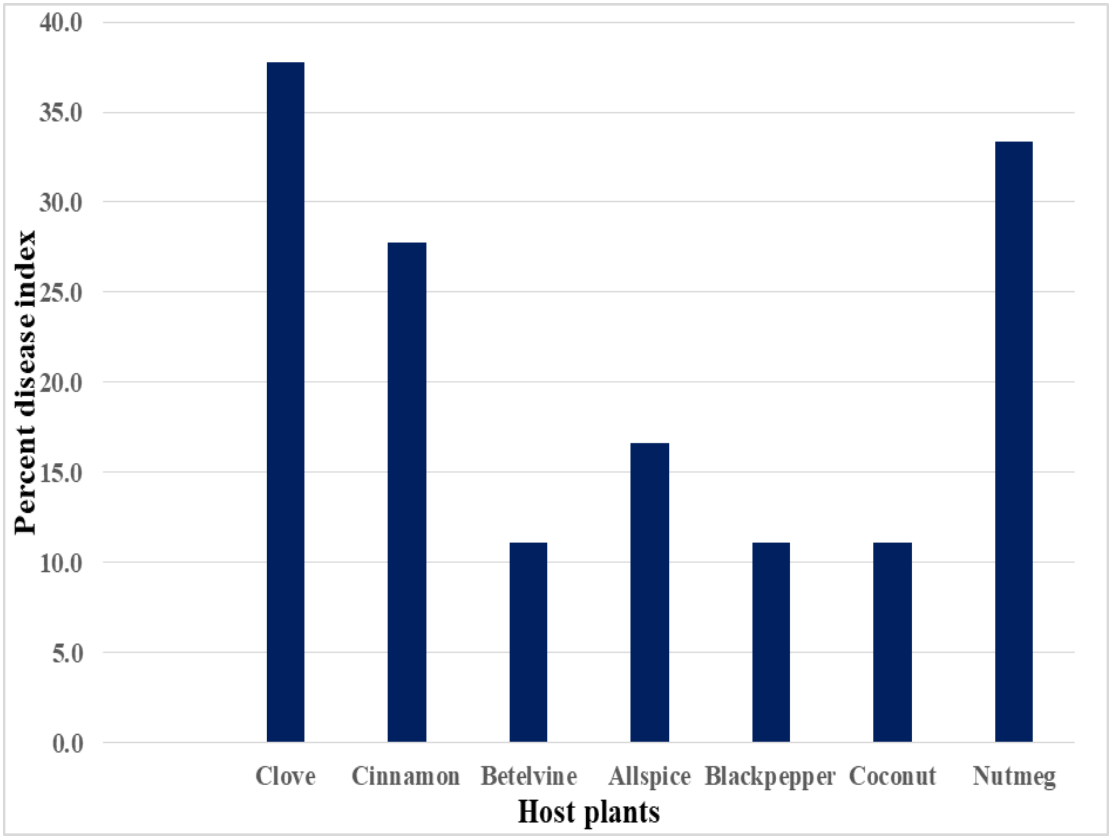


Fig. 5. Disease severity produced by the virulent isolate (C4) on different perennial hosts at 9 DAI

5.5 *IN VITRO* SCREENING OF FUNGICIDES AGAINST THE MOST VIRULENT ISOLATE OF *Colletotrichum* spp.

In vitro screening was carried out to find out the most effective fungicide against *C. gloeosporioides* by poisoned food technique. Different fungicides used in the experiment were Propiconazole 25 EC, Difenoconazole 25 EC, Azoxystrobin 23 SC, Captan 50% WP + Hexaconazole, 5% WP, Trifloxystrobin 25% + Tebuconazole 55% WP and Carbendazim 12% WP + Mancozeb 63% WP. These fungicides were evaluated at four different concentrations of 10, 25, 50 and 100 ppm. Present study of *in vitro* screening of new generation fungicides revealed that triazole fungicide Propiconazole 25EC at 100 ppm and combination fungicides, Carbendazim 12% + Mancozeb 63% WP at 25 ppm; and Trifloxystrobin 25% + Tebuconazole 55% WP at 100 ppm concentration resulted in cent per cent inhibition of the mycelial growth of the pathogen. Difenoconazole 25 EC, Azoxystrobin 23 SC and Captan 50% WP + Hexaconazole 5% WP showed mycelial inhibition of 69.33, 73.33 and 79.10 per cent respectively at 100 ppm (Fig. 6). Ashoka (2005) reported

Carbendazim + mancozeb and benomyl showed cent per cent mycelial inhibition of *C. gloeosporioides* @ 0.025%, 0.005% and 0.1% from vanilla. Chandrakrant (2005) reported the triazole fungicides, propiconazole, difenoconazole and hexaconazole at 0.05 per cent had completely inhibited mycelial growth of *C. gloeosporioides* from black pepper. Similar results were reported by Jadhav *et al*. (2008) where Carbendazim + mancozeb @ 0.25 per cent and propiconazole @ 0.1 per cent showed cent per cent inhibition of mycelial growth of *C. gloeosporioides*.

Positive correlation of result of the present study was reported from the *in vitro* studies conducted by Kurian *et al*. (2008). They reported effective control of black pepper anthracnose isolate of *C. gloeosporioides* using carbendazim + mancozeb @ 0.1 per cent. Prashanth *et al*. (2008) also reported pomegranate anthracnose caused by *C. gloeosporioides* was effectively inhibited by carbendazim + mancozeb @ 0.2 per cent (100 per cent inhibition). Carbendazim + mancozeb (89.23 %); and propiconazole and difenoconazole (90.78 %) were also effective at 0.1 per cent concentration. The triazole fungicides propiconazole, difenoconazole and hexaconazole (0.1 %) completely inhibited mycelial growth of *C. gloeosporioides* from sapota (Patil *et al*., 2010). Propiconazole and tebuconazole at 400 ppm effectively inhibited mycelial growth of *C. gloeosporioides* of mango (83.11 % and 80.33 % respectively) (Basalingappa, 2011). Azoxystrobin was totally ineffective in inhibiting the mycelial growth of *C. gloeosporioides* from nutmeg which was in contradiction to the observations made by Adhikary *et al*. (2013) who reported a mycelial inhibition of 99.69 per cent at 100 ppm concentration. Ahmed *et al.* (2014) reported the complete inhibition of *C. gloeosporioides* from betel vine even at 50 ppm of propiconazole, tricyclazole and tebuconazole.

In vitro evaluation of various fungicides carried out by Dev and Narendrappa (2016) revealed that trifloxystrobin + tebuconazole 75 WG (100 ppm, 250 ppm, 500 ppm and 1000 ppm) and triazole fungicide propiconazole (500ppm, 1000 ppm and 2000 ppm) gave 100 per cent mycelial inhibition. Difenoconazole 25 SC (1000 ppm) showed a mycelial inhibition of 85.85 per cent. Strobilurin fungicide, azoxystrobin 25 SC (1000 ppm) was found to show a minimum mycelial inhibition of 52.64 per cent. Parvathy and Girija (2016) reported fungicides *viz.,* propiconazole, tebuconazole, azoxystrobin and carbendazim + mancozeb, captan + hexaconazole each at 0.1 per cent completely inhibited the mycelial growth of *C. gloeosporiodies* causing black pepper anthracnose. Behera *et al. (*2019) evaluated the efficacy of carbendazim, mancozeb and its combination fungicide (carbendazim + mancozeb)

along with the biocontrol agents in inhibiting the *C. gloeosporioides* from black pepper. Carbendazim + mancozeb @ 0.1 per cent exhibited a maximum inhibition of 97.26 per cent under *in vitro* conditions. The results of the study were similar with respect to the above-mentioned references. Among the six fungicides, carbendazim 12% WP + mancozeb 63% WP was found to be effective in inhibiting the mycelial growth of the pathogen even at a lower concentration of 25 ppm. Propiconazole 25 EC was the most effective triazole fungicide against the anthracnose of nutmeg.

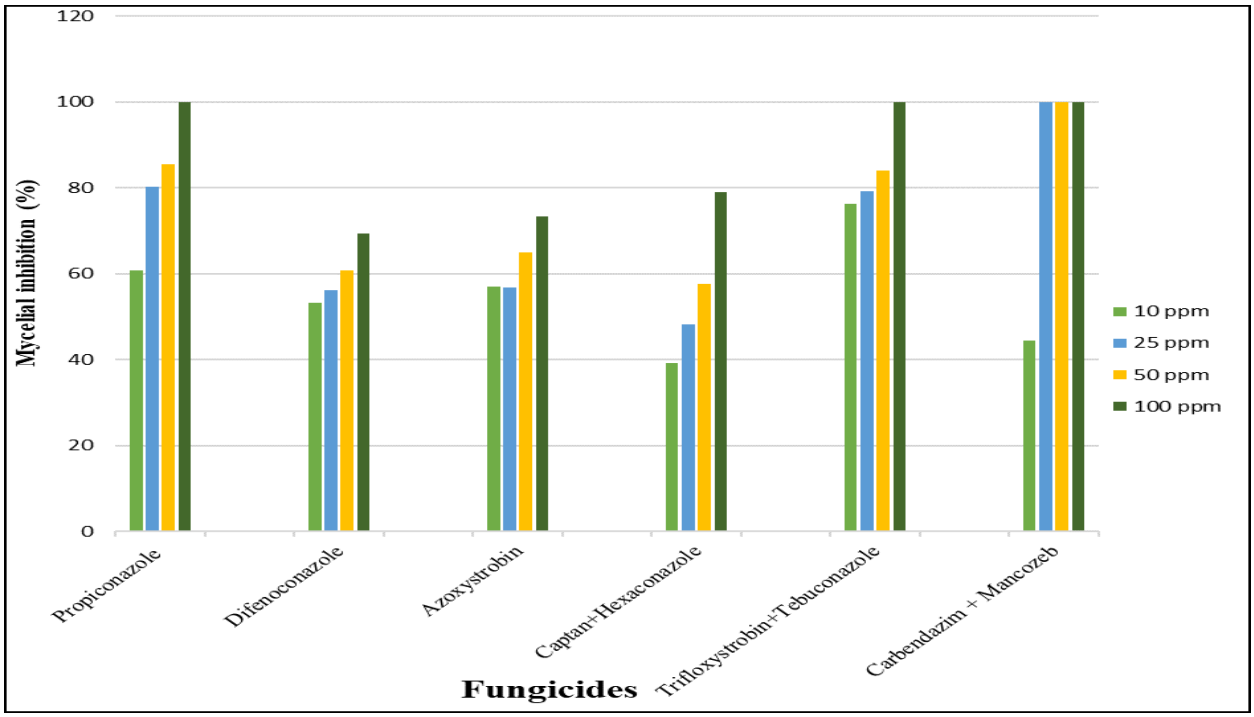


Fig. 6. Mycelial growth inhibition of C4 isolate at different concentration of fungicides on PDA medium at 7 DAI

Summary

6. Summary

Nutmeg, popularly known as a twin spice yields two products *viz.,* nutmeg from seed and mace from seed covering; both are used as spice due to its flavour, aroma, and fragrance. Being a remunerative crop, the production and productivity of the crop is affected by many diseases; among which the fungal disease "anthracnose" caused by *Colletotrichum* spp. is the major disease. It is a disease affecting the economical part which reduces the quality and quantity of spice. The wide host range of the pathogen and lack of information on practical application of new generation fungicides *i.e*, triazoles and strobilurins in the perennial tree spice crops make its unamenable to the common management strategy. In this context, a study entitled with "Host range studies and management of anthracnose of nutmeg caused by *Colletotrichum* spp." with the objectives to characterise the pathogen, study the host range of the pathogen and develop a management strategy using new generation fungicides was carried out at Department of Plant Pathology, College of Agriculture, Vellayani during 2018-2020.

A survey for the collection of anthracnose infected nutmeg was carried out during August – September, 2019 in four agro ecological zones of Kerala *viz.,* Vellayani, Karamana and Palode of Thiruvananthapuram district from southern zone; Kumarakom and Vaikom of Kottayam district from special problem zone; Kadungalloor of Ernakulam district from central zone; and Myladumpara, Pampadumpara, Adimali, Panickankudy, Kamblikandam and Kattappana of Idukki district from high range zone. The disease intensity and per cent disease index of anthracnose of nutmeg were also recorded. The disease was common during the rainy season in all the locations during the survey period. Maximum disease incidence was observed in Kadungallor of Ernakulam district with 90 per cent followed by Kumarakom and Vaikom of Kottayam district with 80 per cent and 70 per cent respectively; and the lowest disease incidence was recorded in Myladumpara of Idukki district with 20 per cent. Maximum per centage disease index (PDI) was recorded from Kadungalloor of Ernakulam district with 56.4. Minimum PDI was recorded at Vellayani, Thiruvananthapuram with 14.8 and Myladumpara of Idukki district with 15.53.

Different symptoms of the anthracnose were observed on the leaves as leaf spot, leaf blight, shot hole and fruit rot. The leaf spot developed as dark brown necrotic lesion surrounded by yellow halo; later leading to leaf blight. The leaves started drying up from the margin of the leaf and spreading to the entire lamina. In case of shot hole, centre part of the

necrotic lesion got torn off. On fruits initially water-soaked lesions developed; leading to the fruit rot.

Eighteen isolates obtained from different locations were studied and proved its pathogenicity. Seven isolates were screened from the eighteen isolates based on the days taken for symptom development and rate of spread of the disease from the point of inoculation. All the isolates were identified as *C. gloeosporioides.* Seven screened isolates (C1-C7) were further screened to determine the most virulent isolate. Artificial inoculation of the isolates produced typical symptoms as that of the original *viz.,* brown lesions alone or brown lesion typically surrounded by the yellow halo and shot hole symptom. Pathogenic variability studies revealed that, C4 isolate from Kumarakom was identified as the most virulent which produced maximum lesion size of 11.89 cm at 7 DAI, resulting complete blighting of leaf at 9 DAI and minimum period for symptom initiation.

Morphological and cultural characterisation revealed that the fungal isolates (C1- C7) produced either fluffy or sparse growth. Colony characters varied *viz.,* whitish to greyish discolouration in the front view and creamy white, dirty grey to pinkish discolouration in the rare view. Days taken to complete the full growth in Petri dish for the seven isolates ranged between 7 - 10 days. C4 isolate showed complete growth in petri plate in 7 days. The average growth rate of seven isolates ranged between 0.98 cm day^{-1} to 1.32 cm $day^{-1.}$ Per cent disease index on 9^{th} DAI ranged between 11.11 - 96.29. Mycelia of the different Colletotrichum isolates were hyaline, septate, branched and slender; and the size of the mycelium ranged between 0.46 µm - 2.48 µm. The maximum mycelial width of 2.48 µm was observed in C4 and the lowest in C5 (0.46 µm). The conidia of the seven isolates were either bullet shaped or oblong; and the size varied between 7.87 - 19.97 µm x 3.26 - 4.26 µm. Maximum conidial size of 19.97 x 4.26 µm was observed for C7 and the minimum conidial size of 7.87 x 3.26 µm for C2 isolate.

An experiment was conducted to study the host range of the pathogen by artificial inoculation on the leaves of detached nutmeg twigs by pin prick method. The study revealed that tree spices *viz.,* clove (*Syzygium aromaticum*) cinnamon (*Cinnamomum zeylanicum*), betelvine (*Piper betel*), allspice (*Pimenta dioica*), blackpepper (*Piper nigrum*) and coconut (*Cocos nucifera*) have developed symptoms related to the original host. The symptoms developed in other hosts appeared as brown lesions in clove and cinnamon, brown lesion with shot hole in betel vine, necrotic spot with yellow halo in allspice, black lesions in black

pepper, and necrotic lesion surrounded by prominent yellow halo in coconut. Days taken for symptom development varied between 2 - 4. Per cent disease index in the hosts ranged between 11.11 - 37.77.

An experiment to assess the efficacy of six new generation fungicides *viz.*, Propiconazole 25 EC, difenoconazole 25 EC, azoxystrobin 23 SC, captan 50% WP + hexaconazole, 5% WP, trifloxystrobin 25% + tebuconazole 55% WP and carbendazim 12% WP + mancozeb 63% WP at four different concentrations of 10, 25, 50 and 100 ppm was carried out against *C. gloeosporioides* by poisoned food technique. At 10 ppm, the maximum inhibition of 76.22 per cent was observed with the Trifloxystrobin 25% + Tebuconazole 55% WP; and the minimum of 39.10 in Captan 50% WP + Hexaconazole WP. At 25 ppm concentration, cent per cent inhibition was observed with Carbendazim 12% WP + Mancozeb 63% WP; and the least inhibition per centage of 48.21 with Captan 50% WP + Hexaconazole. At 50 ppm concentration, cent per cent inhibition was observed by Carbendazim 12% WP + Mancozeb 63% WP and minimum inhibition of 57.55 was observed with Captan 50% WP + Hexaconazole. At higher concentration of 100 ppm, cent per cent mycelial inhibition was observed in all the three fungicides *viz.*, Carbendazim 12% WP + Mancozeb 63% WP, Propiconazole 25 EC, Trifloxystrobin 25% + Tebuconazole 55% WP which was followed by Captan 50% WP + Hexaconazole with per cent inhibition of 79.11. The least inhibition per centage of 69.33 and 73.33 was observed in Difenoconazole 25 EC and Azoxystrobin 23 SC respectively.

Among the six new generation fungicides used, Carbendazim 12% WP + Mancozeb 63% WP was found to be most effective in inhibiting the growth of the mycelium even cent per cent at a lower concentration of 25 ppm and 50 ppm. This treatment was found to be significantly superior to all the other fungicides used for evaluation. Carbendazim 12% WP + Mancozeb 63% WP among combination fungicides and Propiconazole 25 EC among the triazole fungicides were found effective in managing the anthracnose disease of nutmeg.

In the current study on the "Host range studies and management of anthracnose of nutmeg caused by *Colletotrichum* spp." it was observed that the pathogen responsible for the disease was *C. gloeosporioides* with a wide host range capable of infecting other perennials tree spices and can be effectively managed by using the new generation fungicides *viz.*, triazole group fungicide Propiconazole 25 EC and combination fungicide Carbendazim 12% WP + Mancozeb 63% WP which would be a substitute for traditional copper fungicides.

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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

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

Abstract

HOST RANGE STUDIES AND MANAGEMENT OF ANTHRACNOSE OF NUTMEG CAUSED BY *COLLETOTRICHUM* SPP.

by

BOMMANA DIVYA

(2018-11-096)

ABSTRACT OF THE THESIS

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Abstract

The study entitled "Host range studies and management of anthracnose of nutmeg caused by C*olletotrichum* spp**."** was conducted at Department of Plant Pathology, College of Agriculture, Vellayani during 2018-2020 with the objectives to characterize the causal agent, study the host range of *Colletotrichum* spp. causing anthracnose and to develop effective management strategy to control the disease by using new generation fungicides.

As a part of the study, anthracnose infected samples were collected from four nutmeg growing districts of Kerala *viz.,* Thiruvananthapuram, Kottayam, Ernakulam and Idukki. For the infected sample collections, three locations from Thiruvananthapuram (Vellayani, Karamana and Palode), two locations from Kottayam (Kumarakom and Vaikom), six locations from Idukki (Myladumpara, Pampadumpara, Adimali, Kambilikandam, Panickankudy and Kattapana) and one location from Ernakulam (Kadungalloor) were surveyed. Disease incidence and severity were assessed from the surveyed locations. The highest disease incidence and severity were observed in Kadungalloor (DI - 90 % and PDI - 56.40 respectively) followed by Kumarakom (DI - 80 % and PDI - 41.33 respectively) and the lowest disease incidence and severity in Myladumpara (DI - 20 % and PDI - 15.53 respectively).

The symptoms of the anthracnose on nutmeg appeared as small necrotic spots with a prominent yellow halo on the leaf lamina. Several lesions coalesced together resulted in leaf blight, shot hole and defoliation. In Kambilikandam and Panickankudy. fruit rot was also observed along with leaf spot. The cultures of *Colletotrichum* spp. were isolated from the infected samples from different locations. Eighteen pure cultures of *Colletotrichum* sp. (C1 to C18) were obtained. Seven isolates of *Colletotrichum* sp. were selected for further studies based on the days taken for symptom development and rate of lesion development. The pathogenicity of the seven isolates of *Colletotrichum* sp. from different locations were proved by Koch postulates.

The morphological and culture characters of the seven different isolates were studied in potato dextrose agar (PDA) medium. The isolated cultures of *Colletotrichum* sp. produced whitish to greyish radiating mycelium; later turning to off white to pink coloured fluffy to sparse mycelium with regular margins. Days taken to grow the entire Petri dish ranged from 7 to 10. The mycelium of the fungus was hyaline and septate; and its width ranged from 0.46 µm to 2.48 µm. The conidia were single celled with an oil globule at the centre and were

oblong or dumbbell shaped. The conidial size varied from 7.87 to 19.97 µm x 3.26 to 5.68 µm. The isolates were morphologically identified as *C. gloeosporioides.*

The pathogenic variability of the seven isolates of *C. gloeosporioides* was assessed on detached nutmeg twigs by virulence rating. The isolate C4 was identified as the most virulent isolate which produced lesion size of 11.89 cm and 16.81cm at 7 DAI and 9 DAI respectively. The isolate C4 produced symptoms within two days after artificial inoculation and had a higher rate of lesion development of 4.12 cm day^{-1}. The other isolates took 3 to 4 days for symptom appearance on artificial inoculation of the pathogen.

Host range of the most virulent isolate of *C. gloeosporioides* (C4) obtained from nutmeg was studied in perennial tree spices *viz.*, clove, cinnamon, all spice, betel vine, black pepper and coconut. *C. gloeosporioides* isolate of nutmeg is capable of infecting the above-mentioned host plants. The isolate produced symptoms in all the hosts within 2 to 4 DAI and the symptoms developed varied from brown lesions, brown lesions with a shot hole to necrotic spots with prominent yellow halo. The maximum lesion size of 2.43 cm was observed in clove and minimum lesion size of 1.31 cm in all spice.

In vitro screening of new generation fungicides revealed that triazole group fungicide propiconazole 25EC at 100 ppm and combination fungicides, carbendazim 12% + mancozeb 63% at 25 ppm; and Trifloxystrobin 25% + Tebuconazole 55% WP at 100 ppm concentration were the most effective in completely inhibiting the mycelial growth of the pathogen.

The present study revealed the wide host range of the *C. gloeosporioides* isolate of nutmeg and also the effectiveness of new generation fungicides in managing the pathogen. The future line of work should include molecular variability between various isolates, cross infectivity among the isolates in other perennial hosts, and the efficacy of new generation fungicides under field condition.