Management of a major fungal disease in black pepper (*Piper nigrum* L.) nursery

by

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(2019-11-221)

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

PADANNAKKAD, KASARAGOD - 671314

KERALA, INDIA

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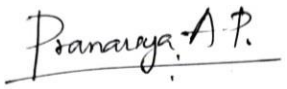
KERALA, INDIA

2022

DECLARATION

I, hereby declare that the thesis entitled **"Management of a major fungal disease in black pepper (*Piper nigrum* L.) nursery**" is a bonafide record of research work done by me during the course of research and that this thesis has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other university or society.



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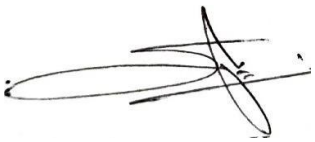
CERTIFICATE

Certified that this thesis entitled **"Management of a major fungal disease in black pepper (*Piper nigrum* L.) nursery"** is a bonafide record of research work done independently by Ms. Pranavya. A. P. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.



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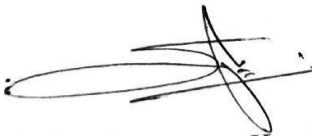

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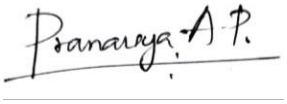


Pranavya. A. P.

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS USED

et al.	And other co workers
%	Per cent
^{0}C	Degree celsius
cm	centimeter
mm	millimeter
m	meter
ha	hectares
dia	diameter
s	second
min	minutes
h	hours
mg	milligram
g	gram
kg	kilogram
t	tonne
mt	metrictonne
µl	microlitre
ml	millilitre
l	litre
DAI	Days After Inoculation
CD	Critical Difference
SE	Standard Error
sp. or spp.	Species (singular and plural)
viz	Namely
Fig.	Figure
No.	Number

LIST OF APPENDICES

INTRODUCTION

1. Introduction

Black pepper (*Piper nigrum* L.) is one of the major spice crops in the world. Black pepper originated from the Western Ghats of southern India and belongs to the family Piperaceae. It is one of the plantation crops that is mainly grown as an intercrop with coconut and arecanut based cropping system. India is the largest producer, consumer, and exporter of black pepper in the world. In Kerala districts such as Kannur, Kasargod, Kozhikode, Wayanad and Idukki are the major black pepper growing regions for commercial purposes (Regeena, 2016). The fruits berries are valuable products from the crop. The dried or white berries have high market values due to their pungency, odour, and seasoning properties (Ravindran, 2000). In the year 2016-17, the area of cultivation was 132 ha and total production was 72 mt, with a productivity of 546 Kg/ha. But in the year 2018-19, the area of cultivation was increased to 139ha but the total production and productivity were decreased to 62 mt and 449 Kg/ha respectively (GOI, 2020).

In Kerala, pre-monsoon showers are preferable for flowering and fruit settings. Two-three noded cuttings are selected for planting in nursery and field conditions. For large production of pepper cuttings serpentine method was adopted. (KAU, 2016). Nowadays, nurseries are affected by many biotic stresses, resulting in the production of unhealthy seedlings. The biotic stresses are insect infestation and diseases (fungi, bacteria, virus, and nematodes) in which the fungal diseases are mostly affecting both the nursery as well as field conditions.

A major reason for the reduction in productivity of black pepper was the incidence of various diseases (Ratishmon and Scaria, 2019). Major fungal diseases observed in pepper seedlings that cause high economic losses are *Phytophthora* foot rot, anthracnose, *Pythium* root rot, *Sclerotium* / *Rhizoctonia* stem rot, Fusarium basal wilt (Anandaraj and Sarma, 1995). Prabhakaran (1997) estimated that there was nine percent yield loss in black pepper due to foot rot caused by *Phytophthora capsici* and 13 percent loss due to fungal pollu caused by *Colletotrichum gloeosporiodes.*

The major fungal diseases observed in the black pepper nursery are seedling blight caused by *Phytophthora capsici*, anthracnose disease caused by *Colletotrichum gloeosporiodes,* or *Colletotrichum capsici*. Collar rot caused by

Sclerotium rolfsii, basal wilt caused by *Rhizoctonia solani*, seedling blight caused by *Pythium* sp, *etc* (Anandaraj and Sarma, 1995). The diseases occurred due to climatic change with unpredictable rainfall. The nursery level management of this disease may have controlled the loss to some extent. The main management strategies including biocontrol agents, chemical fungicides, and organic preparations (Thangaselvabal *et al*., 2008).

The most prevalent disease of black pepper *i.e*, the foot rot infections were effectively managed by a combination of fungicide Metalaxyl (8%) + Mancozeb (64%) 72 WP (0.2 %) (Kumar *et al*., 2018). The anthracnose disease caused by *Colletotrichum gloeosporioides* was significantly lower in vines treated with a combination of fungicide containing carbendazim + mancozeb (0.1%) followed by carbendazim (0.1%) (Kurian *et al*., 2008). The endophytic isolates of *Trichoderma viride* and *T. pseudokoningii* from black pepper caused 64.4 and 65.6 percent inhibition of mycelial growth of *Phytophthora capsici in vitro* (Mathew *et al*., 2011). The organic extracts *i.e*, the dried garlic extract treatment was very effective in reducing the disease severity by *P. capsici* (39.8 %) (Demirci and Dolar, 2006).

The integrated disease management strategies are the need of the hour for the sustainable agriculture. So the combined effect of bio-agents, organic preparations and chemicals such as drenching of *Trichoderma harzianum* 50 g per vine with 1Kg of neem cake to the root zone application with potassium phosphonate (0.3%) as spraying (2 l/vine) was giving significant results in managing *Phytophthora* foot rot with respect to reduction in foliar yellowing and defoliation, less infection of vines, and highest green berry yield (Sivakumar, 2012).

The prophylactic application of biocontrol agents, chemical fungicides and organic preparations may reduce the loss due to the incidence of diseases. The nursery level management is one of the effective strategies to reduce the disease in field condition as well as to produce the quality planting materials.

With this background to reduce the loss from nursery fields, the present study was proposed to characterize the major fungal disease and pathogen-associated with black

pepper nursery and evolve an effective management strategy including biocontrol agents, chemical fungicides, and organic preparations.

The objective of the study was:

- Identify and characterize the major fungal pathogen associated with black pepper nursery.
- Formulate a disease management strategy against the pathogen.

REVIEW OF LITERATURE

2. Review of literature

Black pepper (*Piper nigrum* L.) is known as the 'King of spice'. In India, Kerala is the leading producer, with around 50 percent country's production from the state itself (Kumar and Swarupa, 2017). In general, rooted cuttings are used for planting in the main field and these cuttings are raised from nurseries through serpentine methods (KAU, 2016). In Kerala, black pepper cuttings raised in nursery conditions affected by many diseases lead to huge economic loss and the unavailability of disease-free planting materials.

Diseases are caused by various microorganisms like fungi, bacteria, and nematodes, and were found to be aggressive in the nursery condition. Among them phytophthora infection during the rainy season is the major concern as the condition is uniform and congenial throughout the seedling production period (Anandaraj and Sarma, 1995). Basal wilt/rot caused by *Sclerotium rolfsii*, leaf rot, and blights caused by *Rhizoctonia solani, Pythium sp., Colletotrichum gloeosporioides* are the other major fungal diseases reported in black pepper through different studies.

2.1. COLLECTION OF SAMPLES

Anandaraj and Sarma (1995) included the leaf spot disease that comes under the group of nursery diseases. The symptoms were observed as a yellow halo surrounded by necrotic spots. According to Kurian *et al*. (2008) anthracnose disease in black pepper was caused damages in the nursery and field conditions. In nursery conditions,it was mostly showing the foliar symptom.

Huq (2011) was conducted a survey to analyze PDI of leaf rot (*P. parasitica*) and leaf spot (*C. capsici*) disease in betel vine growing in India from June to May. The highest PDI was observed for leaf rot in August (77.90%) and leaf spot in May (63.30%).

Sahoo *et al*. (2012) surveyed the betel vine garden at Balasore district in Odisha. The PDI of leaf spot disease caused by *C. capsici,* highest PDI observed in Chandrakona (5.4%) and the lowest is in Sanchi (1.9%).

Biju *et al*. (2013) conducted a study on the epidemiology of anthracnose in black pepper and it was found that disease incidence was maximum (39.1%) in September and minimum (7.5-14.7%) at February to May which showed that on the monsoon season disease was severe compared with the winter season.

Samples of foot rot in black pepper were obtained from the commercial and backyard of Sarawak by following detection and delimitating surveys. The severely affected samples were collected and stored for further studies (Farhana *et al*., 2013).

George *et al.* (2015) studied the incidence of foot rot or quick wilt in black pepper in Kodagu region of Karnataka. Roving surveys were conducted in three taluks and found that 16.8 percent disease incidence in Virajpet, 12.88 percent disease incidence in Madikeri and least in Somwarpet taluk and it is about 9.92 per cent.

Jahan *et al*. (2016) collected the samples of sclerotium foot and root rot infected betel vine samples, the samples were put in polythene bags and kept at 4^0C for the isolation of the pathogen.

Parvin *et al*. (2016) collected the sclerotium foot rot affected stems of betel vine from different locations of Pabna districts. The samples were preserved at 4^0C in the refrigerator for isolation.

Thomas and Naik (2017) reported that foot rot caused by *P. capsici* is the most important and serious disease which causes the highest incidence of 65 percent in Mathodu village and 50 percent incidence recorded in Kabilaseathve village in Karnataka State.

Nguyen (2017) concluded that there is a two percent yearly reduction in total pepper area due to the outbreak of quick wilt in black pepper due to the biological characteristics, climatic conditions, and cultivation practices

Kifelew and Adugna (2018) conducted a survey by using a stratified random sampling method specifically using ’W’ path sampling. The quick wilt-infected black pepper samples like root, collar, leaves, and vines were collected for the isolation of the pathogen.

The phytophthora leaf spot of betel vine sample were collected from fields of Anjangaon Surji taluk and placed in sterile plastic bags for isolation of the pathogen (Hedawoo and Makode, 2019).

Garain *et al*. (2020) surveyed to find out the major diseases of Mitha Pata variety of betel vine in West Bengal and concluded that the disease severity of phytophthora leaf rot (14.40±0.46%) and sclerotium root rot disease incidence (17.67± 3.18 %).

Rahman *et al*. (2020) conducted a survey on the foot and root rot disease of betel vine under the different climatic conditions in Bangladesh. In which major betel vine growing areas were observed maximum incidence of disease at soil pH of 5.4, the temperature of 32^0C, relative humidity of 82 percent and light intensity at 53 x100 lux.

The collar rot infected stem portion of the betel vine was collected from the orchards, put in polyethylene bags, and was preserved at 4 degree celsius (Parvin *et al*., 2020).

The betel vine was infected with leaf rot, leaf spot and foot and root rot diseases in major betel vine growing locations in Bangladesh. The samples were collected from each district such as Barisal, Jhinaidha, Kustia, Rajshahi and Chittagong and placed at 4^0C for isolating*P. parasitica, C. piperis,*and *S. rolfsii* (Masud *et al*., 2020).

Betel vine anthracnose disease incidence and severity were analyzed after the survey conducted at Thiruvananthapuram (Kalliyoor, Vellayani and Kattakada), Kollam (Kareepra) and Alappuzha (Cherthala). The highest disease incidence and severity were observed at Cherthala about 80 and 20 per cent (Nisha and Heera, 2021)

2.2. ISOLATION OF THE PATHOGEN

Singh *et al*. (2003) collected the collar rot infected betel vine and sclerotia from the collar region were washed and sterilized with mercuric chloride ($HgCl_2$) at 0.2% for 2 min then washed with milli-q-water it placed on PDA containing Petri plates, incubated at room temperature for 3-4 days.

The diseased samples of leaves were cut into bits and sterilized with sodium hypochlorite (NaOCl) (1%) solution for 1 min and three consecutive washing with

distilled water. The surface disinfected samples were placed on the malt extract agar (MEA) and potato dextrose agar (PDA) plates and incubated at 25^0C for 8 days (Javaid *et al*., 2007).

The collar rot infected stem portions were washed and cut into pieces, then surface sterilized with 0.05% sodium hypochlorite solution for two minutes and washed with sterile water twice. After drying with tissue paper and placing them on PDA for one week at 28°C (Doley and Jite, 2012).

S. rolfsii infected tissues along with healthy tissues were cut into small bits and surface sterilized with 1% sodium hypochlorite for 3 min. The bits were rinsed with three changes of water and dried with blotting paper. The bits were plated on a 2% water agar medium containing 200 ppm of streptomycin. The plates were incubated at 26°C for 7 days (Bhuiyan *et al*., 2012).

Farhana *et al*. (2013) were collected the roots of the infected black pepper and washed to remove the pebbles and disinfection was done with 10% sodium hypochlorite solution in 30sec, then rinsed with water and placed on the rose bengal agar for incubation 28^0C for 3days.

Sclerotium rot infected betel vine stems, and washed to remove the specks of dirt. The samples were cut into small bits and washed with sterilized distilled water further rinsed with sodium hypochlorite (0.1%) solution. After drying with blotting paper, placed on water agar and incubated at room temperature (Sarker *et al*., 2013).

Akram *et al*. (2015) collected the black pepper sample with sclerotial bodies and collar infection, surface disinfected with 1% sodium hypochlorite solution for 1 minute, washed thrice in distilled water and plated it on PDA medium. Incubated at 25^0C for 7 days and purified to grow in PDA slants and kept at 4^0C for further study.

Jahan *et al*. (2016) isolated the sclerotium from infected betel vine samples through the moist blotter method and agar plate method. The moist blotter method in which the samples are placed on filter paper and moistened regularly and observe the growth of the fungus. In another method, the diseased parts were cut into bits washed

with sodium hypochlorite (NaOCl) 1% and then three times with sterile water, then was placed on the PDA containing Petri plates.

Dasgupta *et al*. (2016) collected betel vine samples with typical foot and leaf rot symptoms the progressive symptoms observing portion along with healthy portion cut into small sections. Then surface sterilized with 0.1 percent mercuric chloride ($HgCl_2$) for 1 min then by washing three times with water. Then they were placed on V8 juice agar media, under incubation for three days at 28^0C.

Ann and Mercer (2017) isolated *C. gloeosporioides* from leaves and berries obtained from infected plant parts. The specimens surface sterilised with sodium hypochlorite (5%) for five min then washed with sterile water. Later the bits were placed on PDA media for incubation.

C. gloeosporiodes was isolated from the infected thippali samples using potato dextrose agar media followed by standard procedure (Kendre *et al*., 2017).

V 8 juice media amended with antibiotics such as pimaricin (100 µg/ml), penicillin (50 µg/ml), polymyxin B (50 µg/ml) and rifampicin (10 µg/ml) in quantity to avoid bacterial growth in the culture plate were used for the direct tissue isolation technique. Collar part with lesions washed properly and sheared into bits, surface disinfected with 0.1 percent sodium hypochlorite solution for 60s. washed in three changes of water and plated on media, incubated for one week for 27^0C (Prasad *et al*., 2017).

The part of the collar or stem region showing typical symptoms of the disease was cut into small pieces. These pieces were surface sterilized with 0.1% mercuric chloride solution for 30 seconds. Such pieces were washed thoroughly in sterile distilled water thrice to remove traces of mercuric chloride, if any and then aseptically transferred to sterilized potato dextrose agar (PDA) plates. They were incubated at 27±1°C for three days for the growth of the fungus. Later, a loop full of fungal growth was transferred to PDA slants (Pandi and Naik, 2017).

Thomas and Naik (2017) isolated the foot rot in black pepper causing pathogen from infected plant parts and soil. In infected plant parts following the standard

isolation technique. By using cornmeal agar medium incorporated with ampicillin and streptocycline and incubated at 20-25^{0}C for 5 days then it purified by using PDA media. The isolation of pathogens from the soil was done by baiting techniques. For that unripe apple used for baiting from soil kept 3-4 days incubation at room temperature.

The causative organism of sclerotium foot and root rot disease of betel vine were isolated from basal stem portions. Stem cut into small bits disinfected with sodium hypochlorite (NaOCl) (1%) for 5 minutes and washed with 3 changes of water. After drying placed on the PDA medium, and incubated at room temperature for 4 days (Rahman *et al*., 2019).

The betel vine phytophthora leaf spot causing pathogen was isolated from the samples using direct isolation method and was plated on PDA media (Hedawoo and Makode, 2019).

Behera *et al*. (2019) isolated *C. gloeosporioides* from infected black pepper leaves. The leaves were cut into bits washed to remove clods then surface sterilised with mercuric chloride. Then placed on PDA media poured Petri plates after the three consecutive washing with sterile water.

Biju *et al*. (2020) isolated the *C. gloeosporioides* from black pepper by cutting the infected portion along with the healthy region. The cut bit was washed with sodium hypochlorite (NaOCl) for 2 min and subsequent washing with sterile water 3 times. The bits were transferred to PDA plates and kept for 2-3 days at room temperature.

Rahman *et al*. (2020) were isolated the pathogen from the basal stem of the betel vine 1% sodium hypochlorite (NaOCl) used for surface sterilisation, washed with water 3 times, plated on PDA media incubated at 27^{0}C for five days. Observe the mycelial growth on the plate.

The betel vine samples were infected with leaf rot, leaf spot and foot and root rot were cut into small bits and disinfected with alcohol (70%) and sodium hypochlorite (1 %) (NaOCl), washed twice in distilled water and plated on PDA media. The plates

were incubated for 10 days at room temperature and observed mycelial growth (Masud *et a*l., 2020).

Sun *et al*. (2020) collected betel vine samples with the sclerotial bodies that were selected and surface disinfected. After the sterilization dried the sclerotia and placed in the PDA containing Petri plates and incubated at 25^0C.

Seedling blight infected black pepper leaves with black coloured lesions were cut into pieces and washed 4-5 times in sterile water and plated in PDA media containing sporidex as an antibiotic. Plates were kept for incubation at 28^0 C for 3 days. The mycelial bits were transferred to PDA plates to get the pure culture of the pathogen and to slants long-time storage (Kollakodan *et al*., 2021).

Nisha and Heera (2021) were incised colletotrichum leaf spot samples and washed to remove soil particles. The incised bits were disinfected using 0.1% mercuric chloride for 1 min and three consecutive items of washing in distilled water. The bits were dried with sterile filter paper, plated on PDA media for 7 days at 27^0C.

Jibat and Alo (2021) were collected the infected plant root and incised it into small bits. The cut bits were disinfected with 10% sodium hypochlorite for 30-60 s. Then washed with tap water, then transferred to rose bengal agar and incubated at 28^0C for 3 days. Observed the mycelial growth and then transferred into potato dextrose agar media.

2.3. PATHOGENICITY TEST

Troung *et al*. (2005) tested the pathogenicity of *P. capsici* was used to inoculate on the pepper cuttings. The inoculum grew on millet seeds and mixed to the soil and observed the symptom development.

According to Shashidhara (2007) the pathogenicity of the isolate was studied on healthy seedlings. The test was done by inoculating 10^5 sporangia per ml for leaves and 2 ml, containing 10^5 sporangia per ml for roots.

Troung *et al*. (2008) tested the pathogenicity of *P. capsici* by selecting 10 seedlings the root and leaf were inoculated and maintained at 25-30^0C under humid

conditions. A control plant with sterile water inoculated was also kept. The pots were flooded and drained at daily intervals. The symptom development was observed daily.

Sclerotium isolate from foot rot samples of betel vine was inoculated to the healthy stem of the betel vine. Small injuries were made on the stem and inoculated with fresh cultures of the isolate and covered with moistened cotton and placed on moisture chamber for maintaining adequate climatic conditions (Sarker *et al*., 2013).

Dasgupta *et al*. (2016) selected five betel vine stems for the study, the stems were made with wounds, sprayed with sporangial suspension ($5x10^5$ sporangia/mL). The water sprayed on the control. The stems were kept at room temperature for 3-4 days for symptoms development. Reisolation has done after the symptoms occur in the stem.

Masud *et al*. (2020) were analyzed the virulence of the isolates from the leaf rot, leaf spot and foot and root rot samples, the healthy plants with leaves and stem of betel vine was chosen for it. The parts were surface sterilized with ethanol at 70 per cent, small wounds were created with sterilized needles and inoculated with the pathogen a (mycelial disc) and covered with moist cotton.

Biju *et al*. (2020) selected a pepper variety to study the infection capacity of the isolate by producing conidial suspension in 10 ml of sterile water. The conidial suspension contains 3 x 10^6 ml of spores and is poured into the leaf surface after creating wounds. Moistened cotton was placed on the spot of inoculation to maintain humidity and it was kept for 7 days for symptom development on the inoculated spot.

The virulence of the isolate was tested by the detached leaf inoculation method. The young leaves were collected and washed in tap water on the ventral side of the leaf made with pinprick wounds. On the wounds, mycelial discs were placed and covered with moistened cotton. The controls were inoculated with fresh media, the whole leaf was kept under incubation at 28^0C. The infection was observed after 2 days of inoculation (Kollakodan *et al*., 2021).

2.4. IDENTIFICATION OF PATHOGEN

2.4.1. Cultural characterization

Phytophthora isolates were grown on the oatmeal agar media and observed after one day with colony diameter up to full-grown on the Petri plates, cultural characteristics are observed (Dasgupta *et al*., 2016).

Pandi *et al*. (2017) was grown the sclerotium isolate on the PDA media and observed the cultural aspects by visual examination.

Quick wilt of black pepper pathogen allow to grow in different media Carrot agar, Cornmeal agar, Host leaf extract agar, malt extract agar, Oatmeal agar, Potato carrot agar, Potato dextrose agar and Rye-B agar at 27^0C for 7days. The growth characters were observed and recorded (Shivakumar and Somasekhara, 2018).

Kifelew and Adugna (2018) the colony characters were determined after one week of growth on potato dextrose agar (PDA), carrot agar (CA) and tomato agar (TA) at 25^0C in the dark. The plates were visually analyzed for colony texture and appearance.

The sclerotium isolates were grown on fresh PDA and observed the colony and sclerotial characters. Sclerotia formation, size, and shape of sclerotia and arrangement of sclerotia were recorded after two weeks of growth (Rahman *et al*., 2019).

Biju *et al*. (2020) studied the cultural growth characters by growing the colletotrichum isolate in PDA at 25^0C temperature.

The cultural characters of *C. gloeosporioides* such as radial growth, growth pattern and colony colour were observed (Nisha and Heera, 2021).

2.4.2. Morphological characterization

Malt extract agar (MEA) and Richard's V8 medium (RV8) were used to study the colony characteristics of the pathogen. Cultures were incubated at 20, 25 and 30°C in the dark. For a week, colony diameters were recorded. The cultures were grown in

PDA used for the study of the size and shape of the conidia after one-week growth at 28^0C (Photita *et al.,* 2005).

The seedling blight isolate was grown in PDA and observe the mycelia to study the specific morphological features and obtained data analyzed with the web-based "IDphy" lucid key which is used for identification of *Phytophthora spp*. (Kollakodan *et al*., 2021).

Weir *et al*. (2012) observed the cultural character under PDA plates at 18°C with a 12 h photoperiod. Conidial structures were observed by staining with lactophenol, appressoria were observed with slide culture technique.

Farhana *et al*. (2013) studied the morphological features of *Phytophthora spp*.by which mycelia structures and type of sporangia were observed on compound microscopy.

Dasgupta *et al*. (2016) observed the morphological characters of foot rot isolate such as length, breadth, the shape of the sporangia and chlamydospores were taken by ocular and stage micrometers.

The pathogen grown in PDA were studied the morphological characters such as colony morphology, mycelial growth rate, sclerotial number, size and colour, were observed under the microscope (Magnification 45x × 10x) by using ocular and stage micrometers (Pandi *et al*., 2017).

Hedawoo and Makode (2019) identified phytophthora isolate from betel vine by observing morphological characters by mounted using stain lacto phenol cotton blue dye. With the help of a compound, microscope observes the stains hyphae, conidiophores, and conidia with different magnifications.

Sun *et al*. (2020) observed the hyphal characters of the mycelium produced by *C. truncatum* using stained with 0.03% safranin-O and 3% KOH and counted using an Olympus CX31 18 microscope (400× magnification).

The *C. gloeosporioides* isolated from betel vine were observed under Leica DM 750 at 400X magnification for studying the morphological characters like mycelia, the morphology of conidia and appressoria (Nisha and Heera, 2021).

2.4.3. Molecular characterization

Extraction of DNA was carried out with CTAB buffer and the primers ITS4 and ITS5 used for the PCR cycle. PTC-200 thermal cycler used for the PCR reaction, PCR products were separated by using 1.4% agarose gels, staining is done with ethidium bromide, and visualized with a UV transilluminator in electrophoresis. Concert Rapid Gel Extraction System, used for purifying the products, ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit used for the sequencing. After sequencing with ABI 377 automated DNA sequencer, deposited on Genbank (Photita *et al.*, 2005).

Farhana *et al*. (2013) harvested the mycelia from the phytophthora isolate grown in PDB on a microcentrifuge tube and the agarose gel electrophoresis study was done at isolated fungal DNA and stored in-20^0C. The DNA was amplified with PC-1 and PC-2(specific primers).

Sun *et al*. (2020) used the CTAB buffer method for the extraction of genomic DNA, PCR detection was done using three isolate-specific primer pairs SCR-F/R, SRI TSF/R and SRLSUF/R and sequencing was done with universal primers ITS1/ITS4 in ITS region.

Molecular identification of isolate was performed by 18S rDNA sequencing, NucleoSpinVR Plant II Kit used for the DNA extraction. The PCR reaction was done in GeneAmp PCR System 9700, BigDye Terminator v3.1 Cycle sequencing kit used for the sequencing of the amplified products. Sequence Scanner Software v1 was used for checking the quality of the sequences and nucleotide BLAST analysis was used for the confirmation of the isolate (Kollakodan *et al*., 2021).

2.5. SYMPTOMATOLOGY OF DISEASE

Santhakumari and Aravind (2003) found out the occurrence of anthracnose-causing pathogens in bush black pepper. The symptoms were observed as small black

spots, later blighting was observed in leaves and on fruits with sunken areas and mummification.

Shashidhara (2007) observed as in nursery leaves with typical sunken lesions and wilting from collar to tip, resulting in yellowing which observed in quick wilt in black pepper.

Vijayakumar and Arumugam (2012) reported that foot rot in betel vine, diseased parts produced that infection on roots, stem and leaves, wilting of tender shoots. Leaf spot of betel vine caused by *Colletotrichum capsici* characterized with small brown spots on the leaves with a yellow halo. The spots become lesions with grey centers and enlarge to form a blighted appearance (Sahoo *et al*., 2012). Sclerotium foot rot of betel vine was observed as infection on the collar region with white mycelial growth (Sarker *et al*., 2013).

Jahan *et al*. (2016) studied the symptom development on the leaves and shoots of root and root rot affected betel vine which shows yellowing, withering and drying of the plant. Rotting of the root stem region with white mycelial growth as the disease progresses the entire plant gets affected.

Billah *et al*. (2017) reviewed that sclerotium rot infected betel vine causing yellowing and wilting of the affected portion. White mycelial growth with small, light to deep brown sclerotial bodies is observed on stems in contact with soil.

Kifelew and Adugna (2018) observed the symptoms on leaves as light and dark lesions with water-soaked margins with yellowing, wilting and defoliation. The symptoms of sclerotium root rot of betel vine as lesion development on the stem near the soil level which was resulting in wilting. Mycelial growth was produced from the infected region which spread on the soil surface (Rahman *et al*., 2019).

Nisha and Heera (2021) observed the symptom in leaf spot infected betel vine as necrotic spots with a yellow halo, leaf blight symptoms, drying of the vines and complete damage of the plants.

2.6. *IN VITRO* EVALUATION OF BIOCONTROL AGENTS, CHEMICAL FUNGICIDES AND ORGANIC PREPARATIONS AGAINST THE PATHOGEN

2.6.1. Biocontrol agents

Anandraj and Sarma (1995) found that the management of quick wilt in black pepper in the main field and nursery can be done by *T. viride* and *T. harzianum*.

Rajan *et al*. (2002) tested the effect of *Trichoderma spp* against foot rot disease of black pepper. Out of the five isolates were tested against the fungus, it was found that *T. harzianum* -26 and *Trichoderma virens* -12 were effective against *P. capsici* also *T. harzianum* -26 was the most rhizospheric adaptive one.

The antagonistic activity of *P. fluorescens* against the pathogen causing foot rot of black pepper was up to 72 percent. The percent inhibition varied from 89 – 98 percent in sporangium germination and 90 percent inhibition in germination of the pathogen (Paul and Sarma, 2006).

Hussain *et al*. (2008) observed the influence of the biocontrol agents *Trichoderma viride, T. harzianum* and *T. hamatum* against the growth of the *C. gloeosporioides* and found that *T. hamatum* was showing maximum inhibition (50.40%) in the growth of pathogen than other isolates

Patil *et al*. (2009) studied the effect of different biocontrol agents such as *T. viride*, *T. harzianum* and *P. fluorescence* against the colletotrichum blight of betel vine. The maximum inhibition (66.29%) in the growth of the pathogen was recorded in case of *T. viride* followed by *T. harzianum* (58.06%) and *P. fluorescens* (15.01%).

Lokesh *et al*. (2011) observed that application of systemic fungicides along with soil application of antagonistic organism *T. harzianum* @50 g cfu 10^{7} and 1 Kg of neem cake reduced the foot rot in black pepper.

Dasgupta *et al*. (2011) analyzed the biological control of foot rot disease in betel vine caused by *P. parasitica* and observed that *Trichoderma harzianum* inoculated with oil cake (500 Kg) was showing better control of the disease in betel vine than *P. fluorescens*.

Bhuiyan *et al*. (2012) collected around twenty isolates of *T. harzianum* were collected from the soil and studied against the *S. rolfsii*, each of the isolates were showing the antagonistic activity ranges from 65.01 to 83.06 percent against the pathogen. The study revealed that showed the highest inhibition (83.06%) of radial growth of *S. rolfsii* was observed in case of isolate TH-18 of *T. harzianum* compared with other isolates.

Dasgupta *et al*. (2015) studied the application of biocontrol agents such as *Trichoderma* spp that grew on organic amendments such as wheat grain, rice husk, sawdust, cow dung, wheat husk and mustard oil cake, etc for the antagonistic activity against the *Phytophthora* spp that cause rot in betel vine. The *Trichoderma* spp applied pre monsoon, monsoon and post monsoon period and found that the lesser disease incidence was recorded in biocontrol agent inoculated with wheat grain as compared with other combinations of formulations.

Parmar *et al*. (2015) studied the antagonistic activity of biocontrol agents such as; *T. harzianum*, *T. hamantum*, *T. virens*, *T. viride*, *T. koningii*, and *T. pseudo koningii* against the *Sclerotium rolfsii*. The result revealed that maximium inhibition (61%) in the growth of the pathogen was observed in case *Trichoderma viride* followed by *T. harzianum* (55%) as compared with other biocontrol agents

Parvin *et al.* (2016) studied the efficacy of biocontrol agents *T. harzianum* and *P. fluorescens* against foot and root rot disease of betel vine in Bangladesh. They found that after four days of inoculation, maximium inhibition (42.77%) in the mycelial growth of the pathogen was observed in the case of *Trichoderma harzianum* followed by *P. fluorescens* (27.66%).

Thomas and Naik (2017) studied the antagonistic activity of biocontrol agents against the *P. capsici* and in which *P. fluorescens* was most effective in inhibiting the growth of the fungus by 56.39 per cent.

Bharathi and Benagi (2018) the biocontrol agent isolates from Dharwad include *T. harzianum*, *P. fluorescens* and *B. subtilis*, biocontrol agent isolates from Kakol include *T. harzianum*, *P. fluorescens* and *T. viride* were selected for evaluating the efficacy against sclerotium isolate causing which disease in betel vine under *in vitro*

conditions. *T. harzianum* from Dharwad was showing maximum inhibition (71.33%) followed by *T. harzianum* from Kakol (70.37%).

The antagonistic study of three Trichoderma species; *T. afro-harzianum* (T8A4), *T. reesei* (T9i12) and *T. guizhouensis* (T4) were tested against the *Phytophthora capsici*. The percentage inhibition in growth of the pathogen was recorded highest (84.7 %) for *T. afro-harzianum* (T8A4) confronted to the pathogen (Mokhtari *et al*., 2018).

Behera *et al*. (2019) evaluated the antagonistic activity of biocontrol agents like *P. fluorescens* and *T. viride* against *C. gloeosporioides* causing anthracnose disease in black pepper. The maximum inhibition (88.97%) was observed by *T. viride* in the growth of the fungus followed by *P. fluorescens*(67.84%).

Das *et al*. (2019) tested biocontrol agents like *T. harzianum* strain from Chittor, *T. brevicompactum* strain from Marayur, *T. asperellum* strain from Palakkad and *T. asperellum* strain from Marayur against the *P. capsici* and the inhibition of the growth of the pathogen was observed as high (65.3%) in *T. harzianum* strain from Chittor treated plates as compared with other biocontrol agents.

Rahman *et al*. (2019) evaluated the biocontrol agents against the root and foot rot of betel vine and showed 63.64 percent reduction in the disease incidence was observed in case of *T. harzianum.*

Nandeesha and Ravindra (2020) studied the effect of biocontrol agents such as *T. harzianum* 1, *T. harzianum* 2 and *B. subtilis* against *Sclerotium rolfsii* causing root rot in the betel vine. The maximum antagonistic activity (62.64%) was recorded in case of *T. harzianum*1 against the fungi

Parvin *et al*. (2020) observed the number of sclerotial bodies production in culture media inoculated with *T. harzianum* and *P. fluorescens*. As compared to control the percent reduction in the number of sclerotial production was observed 74.99 percent in *T. harzianum* treated plate as compared to *P. fluorescens* treated plate (56.09%).

2.6.2. Chemical fungicides

Gawande *et al*. (2006) tested ten fungicides against the colletotrichum blight incidence in thippali. Out of these, maximum inhibition (90.70%) on the mycelial growth of the pathogen was observed in one percent of bordeaux mixture and followed by carbendazim (0.1%) and mancozeb (0.25%) as 85.70 percent and 76.5 percent respectively.

Javaid *et al*. (2007) studied the sensitivity of chemical fungicides acrobat MZ 75/667WP dithane m-45 WP applied recommended (R), 0.50R and 0.15R, aliette 80% WP (w/w) and ridomil gold 72%WP(w/w) against colletotrichum isolate with different concentrations included recommended (R), 0.50R and 0.25R. The dithane and ridomil gold at treated doses recorded a gradual reduction in the growth of the pathogen. the aliette and acrobat mz were found to be ineffective against the pathogen infection.

Kurian *et al*. (2008) evaluated the systemic fungicides and contact fungicides for the management of *C. gloeosporioides* on black pepper at the Idukki district of Kerala. They found that the severely infected vines treated with carbendazim + mancozeb (0.1%) followed by carbendazim (0.1%) significantly reduced the disease incidence

Hussain *et al*. (2008) observed that *C. gloeosporiodes* were managed by following chemical fungicides like captan (100 mg/l and 300 mg/l), mancozeb (100 mg/l and 300 mg/l) and results that mancozeb 300 mg/L inhibit the growth by 100%.

Patil *et al*. (2009) evaluated the efficacy of different fungicides like mancozeb (0.25%), chlorothalonil (0.1%), copper oxychloride (0.3%), carbendazim (0.1%), propiconazole (0.1%), tridemorph (0.1%), mancozeb + carbendazim (0.2%), mancozeb + tricyclozole (0.2%), zineb + hexaconazole (0.2%) against the leaf blight in betel vine. The cent per cent inhibition in the growth of the pathogen was observed in case of propiconazole (0.1%) and mancozeb + carbendazim (0.2%) and least inhibition was observed in mancozeb (0.25%).

Lokesh *et al*. (2011) reported that black pepper vines were treated with ridomil gold @ 2.5g/vine showed reduced infection by *P. capsici.*

Bhuiyan *et al*. (2012) screened the efficacy of six fungicides namely provax-200, bavistin, ridomil, dithane m-45, rovral 50 wp and tilt at a concentration of 100, 200 and 400 ppm against the mycelial growth of *S. rolfsii*. The complete inhibition was observed in the case of provax-200 and tilt at all the selected concentrations against the pathogen. The radial growth of the fungus was inhibited up to 93.88% by rovral 50WP, it was higher than the effect of dithane M-45. at the highest concentration. The lower inhibition was observed in the case of bavistin and ridomil.

Ahmed *et al*. (2014) observed that the complete inhibition of the anthracnose of betel vine was found in the application of propiconazole (25%) at 0.2 percent, tebuconazole (25%) at 0.3 percent and tricyclazole (75%) at 0.2 per cent.

Parvin *et al*. (2016) analyzed the efficacy of different fungicides such as bavistin 50 WP, topgan, tilt 250 EC, ridomil gold 68 WP, rovral-50 WP and dithane M-45 against the *S. rolfsii* which causing foot and root rot in betel vine. There was higher inhibition of growth of the pathogen observed in the case of Bavistin 50 WP (70%) and lowest in the case of dithane M-45 (14.44%).

According to Vinitha *et al*. (2016) the isolates of *P. capsici* from IISR around 82 were evaluated the virulence against metalaxyl-mancozeb, around 19 isolates were sensitive to metalaxyl-mancozeb and the combination of fungicides can effectively use in the field for the management of the *P. capsici*.

Ann and Mercer (2017) tested the efficacy of trifloxystrobin and tebuconazole against *C. gloeosporioides* infestation in black pepper. The concentration was selected are 0.4g/l, 0.2g/l and 0.1g/l. Higher inhibition in mycelial growth observed in 0.4g/l about 96.30% followed by 0.2g/l (88.48%) and 0.1g/l (31.55%).

Thomas and Naik (2017) evaluated the efficacy of systemic fungicides and combination fungicides against the mycelial growth of the *P. capsici* by poisoned food technique. In which higher inhibition was recorded in azoxystrobin 23% SC.

Kumar *et al*. (2018) studied about the effect of epidemiological factors and control of anthracnose leaf rot in betel vine with fungicides like; copper sulfate+ Calcium oxide+ water- (Bordeaux mixture)-(0.5%) and (1%), mefenoxam + mancozeb

(WP) - (0.2%) and (0.4%), locally available plant botanical based fungicide - (0.3%) and (0.6%) carbendazim 12% + mancozeb 63% WP (0.2%) and (0.4 %), metalaxyl 8% + mancozeb 64% WP (0.2%) and (0.4%), fenamidone 10% + mancozeb 50% WG (0.2%) and (0.4%), tebuconazole 25 EC (0.2%) and (0.4%). Whereas, carbendazim 12% + mancozeb 63% WP at 0.2 per cet was reduced the disease by 73.0 percent and followed by tebuconazole 25 EC (2%) which reduced the disease by 65.1 per cent.

Rahman *et al*. (2019) evaluated the efficacy of fungicides against the root and foot rot of betel vine and provax 20 was highly effective compared with score 250EC.

Behera *et al*. (2019) conducted a study to find the effect of fungicides against *C. gloeosporioides* causing anthracnose in black pepper. Carbendazim (0.2%), mancozeb (0.2%) and mancozeb + carbendazim (0.1%) were treated against the fungus and 91.24% inhibition in mycelial growth observed in the mancozeb + carbendazim 0.1% treatment.

Islam *et al*. (2020) analysed the effect of different fungicides like Carbendazim, fenamidone + mancozeb, mancozeb, trifloxystrobin + tebuconazole (0.1%) and propiconazole (0.05%) against the leaf spot causing pathogen in betel vine. The maximum inhibition (81.95%) in the growth of the pathogen was observed in trifloxystrobin + tebuconazole and the least inhibition (70.88%)was recorded in mancozeb.

Rahman *et al*. (2020) assessed the efficacy of different fungicides against sclerotium rot *viz.* antracol 70 WP, ridomil gold MZ 68 WP, secure 600 WG, bavistin DF and dithane M-45 (T5) against the growth of the pathogen in lab conditions. The fungicides were. The complete inhibition was observed in the dithane M-45 treated plates and the least inhibition was recorded in secure 600 WG (37.33%) at 6 days after inoculation.

2.6.3. Organic preparations

Demirci and Dolar (2006) selected plant parts of cabbage, garlic, peppermint, alfalfa, onion, radish and lentil plant. *etc*. were dried and taken extracts and

incorporated into the media. The same media was grown up with the pathogen and observed that severity of disease was reduced by the application of garlic (39.8%).

Khan and Zhihui (2010) analysed the garlic root extract at four concentrations (0.02%, 0.04%, 0.06% and 0.08% at w/v) against the pathogen. The root exudates inhibited the growth of the pathogen up to a certain extent, as concentration increases the inhibition was also increased. The maximum inhibition was observed in the case of 0.08 percent concentration and minimum inhibition (25%) was observed in the case of 0.02 per cent concentration.

Amin *et al*. (2013) used different sources of plant origin compounds *viz*. ginger rhizome, turmeric rhizome, neem leaf, tobacco leaf in water for the management of foot and root rot in betel vine. No growth of the pathogen was observed in tobacco leaf in cow's urine, lowest inhibition was observed of all concentrations of neem extract.

Parvin *et al*. (2016) selected the botanicals garlic, onion, ginger, neem, and allamanda for the study of analysis of antifungal activity against *S. rolfsii* which causes foot and root rot in betel vine. The higher inhibition (25.56%) in the mycelial growth of the pathogen was observed in case of garlic at four days after inoculation.

Sneha *et al*. (2016) studied the efficacy and antifungal activity of extracts from the garlic, ginger, nutmeg, black pepper, ajowan, myrobalan and fenugreek (3, 4 and 5 percent concentration against the pathogen under *in vitro* condition. The inhibition in the mycelial growth of the pathogen was observed in the case with aqueous extracts of garlic, ginger, nutmeg, black pepper, myrobalan and ajowan at 3, 4 and 5 % while the negative effect on mycelial growth was observed in the case of fenugreek.

Mohsan *et al*. (2017) studied the effect of plant extracts of white top weed, neem, sweet basil, giant milkweed, cassia and yellow oleander against the pathogen, in which cent percent inhibition in the growth of the mycelia was recorded in case of top weed.

Bharathi and Benagi (2018) reported the *in vitro* efficacy of different plant extracts such as leaves of black tulsi, neem, bulbs of garlic and rhizome of turmeric

against sclerotium wilt in betel vine at 5, 10 and 15 percent. The highest mean inhibition in the growth of the pathogen was observed in neem leaves treated plates (42.28%).

Abarna *et al*. (2019) studied the effect of plant extracts from bulbs of onion, garlic and leaves of papaya and beach pine at the concentration of 5,10,15 and 20 percent against the *P. capsici* using the paper disc method or well method. Neem was recorded 47.65 percent inhibition at 20 percent concentration under *in vitro* conditions against the pathogen.

Rahman *et al*. (2019) evaluated water extracts of leaves of neem, mahogany, mehandi and tulsi, rhizome extract of turmeric and ginger rhizomes; and clove extract of garlic against *C. capsici* at 5, 10 and 15 percent concentration. Among seven aqueous extracts used for the study they were most effective (72.66%) was observed in 15 percent aqueous ginger against anthracnose followed by turmeric (61.25 %).

Rahman *et al*. (2019) evaluated the plant extracts against the root and foot rot of betel vine and observed that garlic clove extracts were highly effective than allamanda extract in managing disease.

Nandeesha and Ravindra (2020) evaluated the efficacy of plant extracts viz., Garlic, turmeric, noni, nilgiris, mehandi, tulasi, neem, marigold at 5, 10, 15 percent concentration against the incidence of wilt caused by *S. rolfsii*. The higher inhibition (62.19%) in the growth of the fungi was recorded in tulsi.

Parvin *et al*. (2020) observed the efficacy of extracts from garlic, onion, ginger, neem and allamonda for reducing the sclerotial formation in media by the pathogen *Sclerotium rolfsii*. The maximum reduction in sclerotial formation (60.49%) was observed in garlic extract followed by neem extract (59.55%).

2.7. *IN VIVO* EVALUATION OF BIOCONTROL AGENTS, ORGANIC PREPARATIONS AND CHEMICAL FUNGICIDES

Demirci and Dolar (2006) studied the effect of plant extracts against *P. capsici* causing infection in black pepper and the plant materials such as dried garlic, peppermint, cabbage, alfalfa, onion, radish and garden cross were used for this study. The colony diameter of *P. capsici* reduced by the action of extracts from alfalfa, garlic,

cabbage and peppermint but it was increased by the onion, garden cross, lentil and radish. The severity of phytophthora blight in pepper by garlic was reduced to 39.8 percent in pot trials and in field conditions severity of the disease was reduced by 40 per cent.

Fungal pollu disease in black pepper was managed by the application of *B. subtilis, P. fluorescens, T. harzianum* and *T. harzianum + P. fluorescens + B. subtilis* at 10^9cfu/ml concentration in field experiment. Highest disease control was observed in the treatment of *T. harzianum + P. fluorescens + B. subtilis* as 66.96 percent and the lowest was *T. harzianum* (29.24%) (Vijayan *et al*., 2014).

Kendre *et al*. (2017) analyzed the effect of chemical fungicides such as carbendazim + mancozeb @1Kg/ha, biocontrol agents like *T. viride* @10kg/ha, and botanicals include neem cake @ 500 Kg/ha in colletotrichum blight causing isolate in *Piper longum.* The results revealed that the lowest disease incidence was observed in neem cake + *Trichoderma viride* + (carbendazim + mancozeb) applied plants as 11.34 percent and followed by carbendazim + mancozeb applied plants as 13.00 per cent.

Kumar *et al*. (2018) studied about the control of leaf rot in betel vine with help of fungicides such as; carbendazim (12%) + mancozeb 63% WP (0.2%) and (0.4 %), metalaxyl 8% + mancozeb 64% WP (0.2%) and (0.4%), fenamidone 10% + mancozeb (50% WG) (0.2%) and (0.4%), tebuconazole 25 EC (0.2%) and (0.4%), copper sulfate + calcium oxide-water- (bordeaux mixture)-(0.5%) and (1%), mefenoxam + mancozeb (WP) - (0.2%) and (0.4%), locally available plant botanical based fungicide - (0.3%) and (0.6%).The combination of fungicide metalaxyl 8% + mancozeb (64% WP) at 0.2% was found to be effective and reduced the disease severity by 76.2 percent followed by bordeaux mixture (0.5%) with 49.1 per cent.

Prasad *et al*. (2018) formulated an integrated disease management strategy for the foot rot complex disease in betel vine at Karnataka district. The biocontrol agent *Trichoderma harzianum* enriched with FYM at the rate of 2 Kg/vine along with *Pseudomonas fluorescens* 10 g/vine and organic amendments neem cake 1kg/vine and the chemical fungicides metalaxyl + mancozeb (78WP) (3 g/L) recorded the highest reduction in the disease incidence and maximum yield.

Rahman *et al*. (2019) selected provax 200, tilt 250EC, score 250 EC, pencozeb 80WP fungicides, Garlic clove extract and allamanda extract and *T. harzianum* in the field for the management of sclerotium foot and root rot of betel vine causing pathogen. The maximum reduction (90.91%) in disease incidence was recorded in Provax 200 in comparison with other treatments.

MATERIALS AND METHODS

3. Materials and methods

The study, entitled 'Management of a major fungal disease in black pepper (*Piper nigrum* L.) nursery' was conducted in the Department of Plant Pathology, College of Agriculture, Padannakkad during the period of 2019-2021. The detailed descriptions of materials and methods performed for the experiments are given below

3.1. COLLECTION OF SAMPLES

Infected samples of black pepper nursery plants were collected from various nurseries of Kasaragod and Kannur district and studied symptoms. The collected samples were taken to the laboratory for isolation of the pathogen and its further studies.

3.1.1. Assessment of percent disease incidence and percent disease index

Percent disease incidence and disease index of different fields were recorded during the sample collection by using score charts. Score charts for seedling rot disease (Shashidhara, 2007) (Table1), and anthracnose disease (Kurian *et al*., 2008) (Table 2) were used for assessing the percent disease index. Percent disease incidence was calculated based on the equation given below (Wheeler, 1969).

$$\text{Percent disease incidence} = \frac{\text{No. of plants infected}}{\text{Total no. of plants observed}} \times 100$$

The Percent disease index (PDI) was calculated using the equation (Mc Kinney, 1923).

$$\text{Percent disease index} = \frac{\text{Sum of grades of each leaf}}{\text{No. of leaves assessed} \times \text{Maximum disease grade used}} \times 100$$

Table 1. Score chart of seedling blight disease of black pepper

Sl. No	Scale	Percent of leaf area affected
1	0	Healthy plant
2	1	Up to 5% leaf infection
3	2	6-15% leaf infection
4	3	16-30% leaf infection
5	4	31-50% leaf infection
6	5	>50% leaf infection

Table 2. Score chart of anthracnose disease of black pepper

Sl. No	Scale	Percent of leaf area affected
1	0	No visible symptom
2	1	<1% leaf area infected
3	2	1-10% leaf area infected
4	3	11-25% leaf area infected
5	4	26-50% leaf area infected
6	5	>50% leaf area infected

3.2. ISOLATION OF THE PATHOGEN

Infected black pepper plant samples collected from different nurseries of Kannur and Kasaragod were isolated separately under *in vitro* conditions. Plant parts expressing the typical symptoms of seedling blight disease, anthracnose disease, and basal stem rot were washed thoroughly and then cut into small pieces having both healthy and diseased portions. The isolation procedure was performed under aseptic conditions for each specimen. Surface sterilization of the plant tissues was done using mercuric chloride ($HgCl_2$) (0.1%) followed by three consecutive washings with sterile distilled water. Washed pieces were allowed to dry on sterilized filter paper and then placed into the sterile Petri plates containing a 15 ml solidified sterile potato dextrose agar (PDA) medium (Appendix I) and Petri dishes were sealed. The Petri dishes were kept for incubation at 28 ± 2^0C for 3 days. The fungus was purified by the hyphal tip method (Rangaswami, 1972) and the different isolates were refrigerated at 15^0C for further studies.

3.3. PATHOGENICITY TEST

To prove Koch's postulates, the isolates from different locations were inoculated to the black pepper seedlings. Black pepper cuttings grown in polythene bags. The isolate which caused anthracnose and seedling blight/ foot rot was grown on the PDA media and one-week growth of the plate was selected for the study. Small agar bits of 8mm diameter cut using cork borer placed on the leaves for anthracnose and seedling blight symptom development. The pathogen which cause basal stem rot was multiplied in paddy grains as well as in fresh PDA culture plates. After one week of growth, these were used as inoculum for the soil inoculation for basal stem rot symptom development. Always maintained the congenial conditions for symptom development. Then the observations such as the number of days of mycelium development and the number of days symptom development in the plant were recorded based on the growth of the culture in the media and the occurrence of the symptoms on artificiallyinoculated plant parts respectively. Followed by this, reisolation of the pathogen was performed from the symptom observed plant parts and compared with the previously

isolated pathogen to prove Koch's postulates. Most virulent pathogen selected for further studies.

3.3.1. Culture of fungal inoculum

The isolates from seedling blight, anthracnose and basal stem rot disease were inoculated to the PDA media. The isolates were inoculated at the center of Petri plates with PDA media, sealed properly and kept for incubation at 25^0C for one week. The mycelial growth observed was purified by the hyphal tip method in PDA slants and stored at 15 degree celsius.

3.3.2. Multiplication of fungal inoculum

The isolates which showed seedling blight and anthracnose diseases were inoculated in PDA media and incubated for one week at room temperature. The isolates were subcultured at regular intervals.

The basal stem rot isolates were multiplied with paddy grains, 100 g disease-free paddy grains were soaked in water for overnight and washed properly to remove the unwanted materials and stones. Grains were boiled in water for 20 min and filled at one-third portion of the test tube, covered properly and autoclave at 121^0C and 1.05 kg/cm^2 for one hour. Sterilized grains were allowed to cool at room temperature and aseptically inoculated with the 3 mycelial disc of culture from the PDA medium. Inoculated test tubes were then incubated at room temperature for 15 days for sclerotia formation. Infected grains with mycelial growth were selected for the inoculation (Chaurasia *et al*., 2014).

3.3.3. Inoculation of the pathogen on the leaf

Artificial inoculation of seedling blight and anthracnose isolate was done on the healthy leaf of black pepper seedlings. Three leaves were selected and wiped with 70 % alcohol, with the help of a sterilized needle pinpricks were made on the leaf. The fully grown culture of the isolates was selected and an 8 mm sized culture disc of the pathogen was placed on the pinpricked area. The inoculated region is covered with a

thin layer of cotton moistened with sterilized water. The leaves which are inoculated with plain agar disc were considered as control. Inoculated plant parts were then covered by polythene cover to provide adequate climatic conditions for symptom development. The plants were observed regularly.

3.3.4. Inoculation of the pathogen in the soil

The black pepper seedlings were selected and from the test tube, five to ten paddy grains with sufficient fungal mycelium and sclerotia were incorporated in the soil near the collar region of the plant (Chaurasia *et al*., 2014). Black pepper seedlings with uninoculated soil were kept as control. To provide suitable moisture conditions the plants were covered with polythene bags. The plants were observed regularly for symptom development.

3.3.5. Virulence rating of pathogen

Based on the number of days required to complete the growth in the media by the fungus in the Petri dish, the number of days required for disease development during pathogenicity test and extent of loss of plants in the nursery under congenital condition, virulence rating of the different fungal isolates was carried out. The selected isolates from the pathogenicity test were inoculated into different black pepper varieties collected from the High Tech black pepper nursery of the College of Agriculture, Padannakkad. The seedling blight and anthracnose isolates were inoculated on the leaves and basal stem rot isolate was inoculated at the collar region and observed the disease incidence, percent disease index and lesion size in the inoculated region. Based on the disease incidence and disease index the virulent isolate were selected and further studies were performed with the most virulent pathogen. The black pepper variety with higher disease incidence was selected for further studies.

3.3.6. Maintenance of culture

The most virulent fungal isolate selected through virulence was subcultured in PDA medium and carrot agar medium, actively growing region of the mycelium was cut aseptically and placed in PDA slants. After obtaining sufficient fungal growth in the slants, these were kept in the refrigerator at 15^0C. Virulence of the pathogen was

maintained by inoculating the fungus and reisolating the same from the host plant in three month intervals.

3.4. IDENTIFICATION OF THE PATHOGEN

The most virulent pathogen was identified based on cultural, morphological and molecular characterization. Cultural, morphological and molecular characterization were done for the most virulent fungal isolate along with cultural and morphological variability studies among the isolates (Ristaino, 2016).

3.4.1. Cultural characterization

Cultural and morphological characteristics of most virulent fungal isolate was recorded by owererving its growth in PDA medium. A mycelial disc of 8mm size from three-day old culture was inoculated aseptically at the center of solidified PDA medium in Petri dish. Three replications were maintained for each isolate. After the incubation at room temperature 28^0C, the following observations were recorded daily till 7 days after inoculation.

- Rate of growth of mycelium
- Colour of mycelium
- Pattern of mycelia growth

3.4.2. Morphological characterization

Microscopic characteristics of fungal hyphae such as septation, branching pattern, sporangiospores, sporangium and cellular arrangement of the isolate were studied. To observe the spore characters and hyphal characters of the anthracnose isolates, a small portion from the one-week-old culture plate was stained with lactophenol cotton blue. The sporangial characters of seedling blight isolate, observed by collecting the mycelial growth from the mycelial disc which was placed in sterile water overnight for sporangium formation. The lactophenol cotton blue was used for staining (Appendix II). To observe basal stem rot causing isolate, sclerotial characters were analyzed by cutting the sclerotia into two equal halves. A thin layer of the cross-section was made ensuring that both rind and medullary portions were included in the section. This section was stained with lacto phenol observed under Carl Zeiss binocular microscope 400 X magnification.

3.4.2. Molecular characterization

Most virulent isolate from the virulence testing was selected for molecular characterization and the sample was carried out at UniBiosys Biotech Research Labs at South Kalamassery, Cochin. Identification of microbial culture was done by using D1/D2 region of LSU (Large Sub Unit: 16S rDNA) based molecular technique.

3.4.3.1. DNA isolation

0.01 g of mycelium were ground with mini grinder using 75µl of STE extraction buffer (320 mM Sucrose, 10mM Tris-Cl, 20mM EDTA, 75mM NaCl and 2.5mlof 20% SDS) along with 5mg of Polyvinyl pyrrolidone and 0.1g of silica powder, incubated at 65°C for 10 minutes. Centrifuged the sample at 13,000 rpm for 10 minutes. To the supernatant, an equal volume of chloroform: isoamyl alcohol was added and repeated the centrifugation. To the aqueous layer, added 2/3 volume of isopropanol and centrifuged at 13,000 rpm for 10 min. The pellet was washedwith 70% ethanol by centrifuging and the pellet was dried, dissolved in 50µl TE buffer.

3.4.3.2. Analysis of DNA purity and quality

The DNA stock samples was quantified using UV spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. The Ultra violet (UV) absorbance was checked at 260 and 280nm for determination of DNA concentration and purity. Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. The DNA having ratio between1.8 to 2.0 was considered to be of good purity. Concentration of DNA was estimated using the formula.

Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor

Quality of DNA was again checked by agarose gel electrophoresis.

The 0.8 % agarose was prepared (0.8 g agarose power / 100ml 1 X TBE), and was melted. 30 ml agarose was poured into the casting tray. The gel was allowed to solidify and the comb and tape were removed. 1 X TBE (Tris-Borate-EDTA; electrophoresis buffer) was added to the chamber until the buffer just covers the

top of the gel. The samples were loaded with Bromophenol blue loading dye, taking care not to puncture the well bottoms. The power pack was turned on andrun at 100V. The gel was viewed on a UV transilluminator after electrophoresis. The DNA was used further for PCR.

3.4.3.3. Polymerase Chain Reaction

ITS1 and ITS4 region was amplified by PCR from fungal genomic DNA using PCR universal primers:

Details of primers used for PCR

ITS1 - 5' -GGTGAACCTGCGGATGGA -3'

ITS4 - 5'- TCCTCCGCTTATTGATATGC-3'

PCR was carried out in a final reaction volume of 25 µl in a 200 µl capacity thin- wall PCR tube. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred toa thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given below **(**Table 3). The PCR reactions are included the Table 4.

3.4.3.4. Analysis of DNA amplification by AGE

a. Standard DNA Markers.

A commercially available 100bp ladder was used as standard molecular weight DNA (ThermoScientific USA).

b. PCR-Product Electrophoresis

Loaded 3µl of PCR product with 4µl bromophenol blue (Loading Dye) in 1.5% agarose gel. Ran the gel at a constant voltage of 100V and current of 45A for a period of 30 min till the bromophenol blue has traveled 6 cms from the wells. Viewed the gels on a UV transilluminator and a photograph of the gel was taken using a gel documentation system (Alphaimager, USA).

Table 3. Preparation of Reaction mix for PCR

Components	Vol. per reaction
Deionized water	18.7 µl
Taq buffer with $MgCl_2$ (10 X)	2.5 µl
Forward Primer (10 pM/µl)	0.5 µl
Reverse Primer (10 pM/µl)	0.5 µl
dNTPs mix (10 mM each)	0.5 µl
Taq DNA Polymerase (5U/µl)	0.3 µl
Template DNA (25 ng/µl)	2.0 µl
Final Volume	25.0 µl

Table 4. PCR conditions

Step	Process	Temperature	Time
1	Initial denaturation	95^0C	5m
2	Denaturation	94^0C	30s
3	Annealing	62^0C	30s
4	Extension	72^0C	45s
Go to step 2 -4 for 29 times			
5	Final Elongation	72^0C	10m
End			

3.4.3.5. Purification of DNA samples

Amplified PCR product was purified using column purification as per manufacturers guidelines (Thermo Scientific USA), and further used for the sequencing reaction.

3.4.3.6. Sequencing of purified 16SrDNA gene segment

The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI3730xl Genetic Analyzer (Applied Biosystems, USA).

Sequence analysis of ITS1 & ITS4 region

- Each nucleic acid sequence was edited manually to correct falsely identified bases and trimmed to remove unreadable sequences at the 3 ’and 5’ ends (considering peak and Quality Values for each base) using the sequence analysis tools.
- The edited sequences were then used for similarity searches using the BLAST (Basic Local Alignment Search Tool) program in the NCBI GenBank (www.ncbi.nlm.nih.gov) DNA database for identifying the fungal strains.

3.5. SYMPTOMATOLOGY OF DISEASE

Symptomatology of major diseases was studied under natural and artificial conditions. In natural conditions, it was studied by using the leaf inoculation method by placing an 8 mm disc of pathogen grown in PDA medium to the tender leaves of selected pepper plants. The seedlings with 2-3 leaf stages were selected for this study. The leaves were washed to remove the contaminants, and further surface sterilized with alcohol and cotton. After that, the wounds were created using sterilised needles. A disc of the pathogen was placed on the wounds and covered with moist cotton. The entire seedlings were covered with polythene cover to maintain humidity which is essential for symptom development.

3.6. *IN VITRO* EVALUATION OF BIOCONTROL AGENTS, CHEMICAL FUNGICIDES AND ORGANIC PREPARATIONS AGAINST THE PATHOGEN

In vitro study was undertaken with an aim to evaluate the efficacy of biocontrol agents, chemical fungicides and organic preparations against the major fungal disease-causing pathogen.

3.6.1. *In vitro* evaluation of biocontrol agents

The efficiency of the following KAU-released biocontrol agents such as *Trichoderma spp.*, *Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated against the pathogen by dual culture technique (Skidmore and Dickinson, 1976). The experimental design selected for statistical analysis was completely randomized design (CRD) with four treatments and four replications (Design: CRD, Treatment: 4, Replication: 4) (Table 5).

3.6.1.1. In vitro evaluation of fungal antagonist against the pathogen

Antagonism of *T. harzianum* against the pathogen was tested by dual culture technique. 8 mm culture disc of pathogen and antagonist were cut out from the actively growing margin of their fourth day old culture in the Petri dishes. These were placed on the PDA medium at opposite edges of the Petri plates (2 cm away from the edge) having a 9 cm diameter. Each treatment was replicated four times. Control plates were kept by inoculating pathogen at the center of the Petri dish without antagonist fungi. Both dual culture and control plates were incubated at room temperature 28^0C. Observations were taken at the interval of 24 h. Radial growth of the pathogen was recorded when complete growth was observed in control plates. Percent inhibition of the pathogen by antagonist fungi was calculated by using the following formula given by Vincent, (1927).

$$\text{Percent Inhibition \% (I)} = \frac{C-T}{C} \times 100$$

Where

I: Percent inhibition

C: Mycelial growth in control (cm)

T: Mycelial growth in treatment (cm)

3.6.1.2. In vitro evaluation of bacterial antagonist against the pathogen

KAU released bacterial biocontrol agents such as *Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated against isolated fungal pathogen by dual culture method. It was performed by inoculating the pathogen at the center of the sterile Petri plate with a potato dextrose agar (PDA) medium. Bacterial cultures were streaked as parallel lines at both sides of the pathogen keeping a 2 cm distance from the periphery of the plate. Pathogen inoculated at the center in potato dextrose agar (PDA) media without bacterial antagonists were kept as control. Four replications were maintained for each treatment. All the inoculated plates were incubated at room temperature 28^0C. Measurements from treatment plates were taken when the pathogen completed its growth in the control plate. Percent inhibition of mycelial growth of pathogen was calculated based on the equation.

$$\text{Percent Inhibition \% (I)} = \frac{C-T}{C} \text{ X } 100$$

Where

I: Percent inhibition

C: Mycelial growth in control (mm)

T: Mycelial growth in treatment (mm)

3.6.2. *In vitro* evaluation of chemical fungicides

The relative efficacy of contact and systemic fungicides will be tested against most virulent isolate by poisoned food technique (Nene and Thapliyal, 1993) (Design: CRD, Treatment: 28, Replication: 3) (Table 6). For the poisoned food technique double strength potato dextrose agar (DPDA) medium was used. Initially, 50 ml double strength potato dextrose agar (DPDA) medium was prepared in a 250 ml conical flask. 50 ml distilled water, DPDA and Petri dishes (9.0 cm diameter) were kept for sterilization in an autoclave at 121^0C and 1.05 Kg/cm^2 for 20 min. After sterilization, the required concentration (gm/ µl) of the fungicide was added to 50 ml sterilized water

and shaken well for uniform mixing. Well, mixed fungicidal suspension then transferred to 50 ml molten DPDA ensuring that required concentration was obtained. 15 ml poisoned media were poured into the Petri dishes. After solidification 8 mm mycelial disc of four days old culture of the pathogen was inoculated at the center of the Petri dish. Each treatment was repeated three times. Control plates were maintained by inoculating pathogens in the poison-less single-strength PDA medium. All procedures mentioned above were strictly followed aseptic conditions. Treatment plates were kept for incubation at room temperature 28^0C. Growth of pathogen in the poisoned media was recorded and compared with control plate showed full growth of the fungus. Percentage inhibition was estimated by using the equation.

$$\text{Percent Inhibition \% (I)} = \frac{C-T}{C} \times 100$$

Where

I: Percent inhibition

C: Mycelial growth in control (mm)

T: Mycelial growth in treatment (mm)

3.6.3. *In vitro* evaluation of organic preparations

The relative efficacy of organic preparations will be tested against the most virulent isolate by poisoned food technique (Gupta and Banerjee,1970) (Table 7).

Design: CRD, Treatment: 10, Replication: 3

To begin with filter, the organic preparations through a clean muslin cloth. The filtrate again passes through the Whatman No.1 filter paper. The collected filtrate passes through a bacteria filter inside a laminar airflow chamber (Dixit *et al*., 1982). Take 10ml of the filtrate and mix with 90 ml of molten potato dextrose agar media, pour 15 ml media into the sterile Petri plates. After the solidification of media inoculates 8mm culture disc at the center of the Petri plate. Culture without amendment is kept as

control. Treatment plates were kept for incubation at room temperature 28^0C. Measurements were taken after the full growth of the pathogen in the control plate. Percentage inhibition was estimated by using the equation.

$$\text{Percent Inhibition \% (I)} = \frac{C-T}{C} X100$$

Where

I: Percent inhibition

C: Mycelial growth in control (mm)

T: Mycelial growth in treatment (mm)

Table 5. Biocontrol agents selected for *in vitro* evaluation against pathogen

Sl. No.	Treatment	Biocontrol agents
1	T1	*Trichoderma* spp.
2	T2	*Pseudomonas fluorescens*
3	T3	*Bacillus subtilis*
4	T4	Control

Table 6. Chemical fungicides selected for *in vitro* evaluation against pathogen

Sl. No.	Treatment	Chemical fungicides	Concentration
1	T1	Mancozeb (75 WP)	0.1%
2	T2	Mancozeb (75 WP)	0.2%
3	T3	Mancozeb (75 WP)	0.3%
4	T4	Copper oxychloride(50WP)	0.1%
5	T5	Copper oxychloride(50WP)	0.2%
6	T6	Copper oxychloride(50WP)	0.3%
7	T7	Metalaxyl (35WS)	0.05%
8	T8	Metalaxyl (35WS)	0.1%
9	T9	Metalaxyl (35WS)	0.2%
10	T10	Carbendazim(50WP)	0.05%
11	T11	Carbendazim(50WP)	0.1%
12	T12	Carbendazim(50WP)	0.2%
13	T13	Propiconazole(25EC)	0.05%
14	T14	Propiconazole(25EC)	0.1%
15	T15	Propiconazole(25EC)	0.2%
16	T16	Azoxystrobin(23SC)	0.05%
17	T17	Azoxystrobin(23SC)	0.1%
18	T18	Azoxystrobin(23SC)	0.2%
19	T19	Carbendazim 12% + Mancozeb 63% (75WP)	0.05%
20	T20	Carbendazim 12% + Mancozeb 63% (75WP)	0.1%
21	T21	Carbendazim 12% + Mancozeb 63% (75WP)	0.2%
22	T22	Tebuconazole 50%+ Trifloxystrobin 25% (75 WG)	0.02%
23	T23	Tebuconazole 50%+ Trifloxystrobin 25% (75 WG)	0.04%
24	T24	Tebuconazole 50%+ Trifloxystrobin 25% (75 WG)	0.06%
25	T25	Metalaxyl 8%+Mancozeb 64% (72 WP)	0.2%
26	T26	Metalaxyl 8%+ Mancozeb 64% (72 WP)	0.4%
27	T27	Metalaxyl 8%+ Mancozeb 64% (72 WP)	0.6%
28	T28	Control	

Table 7. Organic preparations selected for *in vitro* evaluation against pathogen

Sl.No.	Treatment	Concentration
T1	Neemazal	0.1%
T2	Neemazal	0.2%
T3	Neemazal	0.3%
T4	Garlic extract	0.1%
T5	Garlic extract	0.2%
T6	Garlic extract	0.3%
T7	Ready to Use Neem Oil Garlic Soap	1%
T8	Ready to Use Neem Oil Garlic Soap	2%
T9	Ready to Use Neem Oil Garlic Soap	3%
T10	Control	

3.7. *IN VIVO* EVALUATION OF BIOCONTROL AGENTS, ORGANIC PREPARATIONS AND CHEMICAL FUNGICIDES

Most effective fungicides, biocontrol agents and organic preparations were shortlisted based on their efficacy under *in vitro* evaluation. The effectiveness of selected treatments against the disease was assessed by a pot culture experiment.

Design: CRD, Treatments: 7, Replication: 3

T1-T2: Effective treatments from 3.6.1

T3-T4: Effective treatments from 3.6.2

T5-T6: Effective treatments from 3.6.3

T7: Control

Crop: Black pepper

Variety: Selected from the virulence testing 3.1.1

3.7.1 Fumigation (Godfrey and Young, 1943)

A potting mixture containing sand, soil, and cow dung (in equal proportions) is required to fill earthen pots of the same size and 21 in numbers. Small pits were made mixture at a rate of 4 pits per m^2 with 12 inches depth. Formaldehyde solution (40 %) was poured into each hole at 25 ml/m^2. By filling sand to cover the pits for aeration. After it was moistened with water, polythene sheets of 150- 200gauge thickness were used to cover the entire bed. The soil was used to seal the sides of the bed and kept as such for 60 days. The sheet was removed, the soil was raked and moistened with water for five days.

3.7.2. Planting of pepper cuttings

The black pepper variety used for the pot culture experiment was selected from virulence testing 3.1.1. One plant per pot was maintained for the study. The plants were sustained for proper growth of the plant (KAU, 2016).

3.7.3. Inoculation of the pathogen

Most virulent pathogen isolates were selected for leaf inoculation mentioned in 3.4.1. to induce seedling blight foliar symptoms. The Petri plates with complete growth of the

mycelium were cut with a sterilized cork borer and mycelial discs were placed on the underside of the tender leaves.

3.7.4. Application of treatments

The treatments and method of application were determined based on the pathogen (Table 8).

3.7.5. Main items of observations

Disease incidence, lesion size, the occurrence of other pests, and diseases of the plant were examined in all the treatments and control plants. Data obtained by the above observations were subjected to statistical analysis.

3.8. STATISTICAL ANALYSIS

Data obtained from *in vitro* and *in vivo* studies were undergone analysis of variance (ANOVA) by using GRAPES software after appropriate transformations wherever needed. Critical difference (CD) values were calculated for each observation in significant treatments using t' values at a 5 percent level of significance and the significance of the treatments was compared with CD values.

Table 8. *In vivo* application of biocontrol agents, chemical fungicides and organic preparations

Sl. No	Treatment (Concentration in percentage)	Method of application
T1	Effective treatment from 3.6.1	Based on virulent pathogen after virulence testing 3.1.1
T2	Effective treatment from 3.6.1	
T3	Effective treatment from 3.6.2	
T4	Effective treatment from 3.6.2	
T5	Effective treatment from 3.6.3	
T6	Effective treatment from 3.6.3	
T7	Control	

RESULTS

4. Results

Black pepper (*Piper nigrum* L.) is one of the important spice and plantation crops native to the Western Ghats of India, well grown in Kerala condition. Seedling blight, anthracnose, and basal stem rot are the major fungal diseases more prevalent in black pepper in nurseries as well as field conditions before and after the rainy season. Hence, a study on "Management of a major fungal disease in black pepper (*Piper nigrum* L.) nursery" was conducted during 2019-2021 to identify and characterize a major fungal disease in black pepper followed by evaluation of various biocontrol agents, chemical fungicides and organic preparations against the pathogen. The results of experiments carried out under *in vitro* and *in vivo* conditions are described below.

4.1. COLLECTION OF SAMPLES

Plants showing symptoms of stem rot, anthracnose, basal rot and quick wilt in black pepper were collected from nurseries in different locations of Kannur and Kasargod district such as Panniyur, Padannakkad (College of Agriculture, Padannakkad), Kanakappalli, Balal and Periya during the year 2019-2020. Seedling blight was found in Padannakkad and Kanakappalli region, anthracnose infection was found in Panniyur, Padannakkad, Balal and Periya and basal stem rot were more prevalent in the nurseries of Periya, Panniyur and Padannakkad. Symptoms were observed in different seedlings in the nurseries. Seedling blight symptoms were observed during the June-July months and observed as black lesions on leaves and stems which become water-soaked lesions and dried off the affected parts. Anthracnose was observed as brown irregular spots on leaves smaller in size later enlarged and grey center with a yellow margin found in all the seasons. Yellowing and wilting of aerial parts, stem with water-soaked lesions on the collar region, formation of white mycelial strands of a pathogen, mustard-like brown sclerotial bodies, and rotting of collar region were the characteristic symptoms noticed at the basal region of stem portion in the case of stem rot infection (Plate 1). Seedling blight and anthracnose symptoms were distinguished by the first one producing the circular-shaped lesion which becomes rot in humid conditions, the later one with spots on the leaves which irregular in shape with the yellow outer layer. Details regarding sample collected, location of collection and the part infected are included below (Table 9).

Table 9. Disease samples collected from different nurseries in Kannur and Kasargod district

Sl.no.	Location	Latitude & longitude	Symptom
Seedling blight			
1	Padannakkad	12°15'12.8" N 75°7'5.8" E	Black water-soaked lesions on leaves
2	Kanakapalli	12°36'93.66" N 75°26'12.82" E	Stem with lesions, coalesce to form dried region
Anthracnose			
1	Padannakkad	12°15'12.8" N 75°7'5.8" E	Lesions on leaves which are round with greyish center and yellow halo
2	Periya	12°21'19.8" N 75°5'41.7" E	Leaves with black spots
3	Balal	12°20.6'15" N 75°24'23.2" E	Brown spots on the leaves form necrotic area
4	Panniyur	12°4'50.7" N 75°24'0.8" E	Leaves with brown spots and yellow halo
Basal stem rot			
1	Padannakkad	12°15'12.8" N 75°7'5.8" E	White mycelial growth on the stem region
2	Periya	12°21'19.8" N 75°5'41.7" E	Large spots on leaves with mycelial growth
3	Panniyur	12°4'50.7" N 75°24'0.8" E	Leaves with necrotic lesions

Panniyur

Pandannakkad

Balal

Periya

Kanakapalli

Plate 1. Disease symptoms observed in different black pepper nursery

4.1.1. Assessment of percent disease incidence and percent disease index

As per 3.1.1. disease incidence and disease index in infected fields were assessed. A score chart was prepared based on the area of a leaf affected by the disease and the diseases index was determined. The disease index was recorded on a scale of 0-5 rating. Percent disease incidence (PDI) was calculated for all plants with seedling rot, anthracnose and basal stem rot symptoms, while percent disease index (PDI) was noted only for plants with seedling blight, anthracnose (Table 2). Among the different isolates, seedling blight disease which has percent disease incidence and percent disease index showed a range of 44 to 74 percent and 17 to 32 percent respectively in case of seedling blight disease, 10 to 30 percent 2 to 25 percent respectively in case of anthracnose disease and 10 to 20 percent and 8 to 28 percent respectively in case of stem rot disease. The highest seedling blight disease was noticed at Padannakkad (74 %) followed by Kanakapalli (44 %) whereas the highest disease index was recorded at Padannakkad (32 %) followed by Kanakapalli (17%). The highest disease incidence and disease index of anthracnose was observed in Panniyur (30% and 25% respectively) followed by Periya (20% and 10% respectively). The highest incidence of basal stem rot disease was observed in Padannakkad (20%) followed by Periya (16%) andPanniyur (10%). Details regarding disease incidence and disease index are given (Table10).

4.2. ISOLATION OF THE PATHOGEN

Pathogen associated with seedling blight, anthracnose and basal stem rot diseases were isolated from infected samples collected from different nurseries in Kannur and Kasargod districts. Isolation of pathogen from infected samples were carried out with standard procedures depicted under 3.2. White mycelial growth of fungus in PDA was observed from 1-4 days of incubation at room temperature. Hyphal disc cut from the actively growing tip of the fungal colony was transferred to another PDA plate and then to the PDA slant for pure culturing. Nine different isolates were obtained from the survey locations. Seedling rot showing isolates were named with different prefixes such as ‘Pc’ such as Pc1 and Pc2, anthracnose showing isolates were named with different prefixes such as ‘Cg’ such as Cg1, Cg2, Cg3 and Cg4 and basal stem rot showing isolates were named with different prefixes such as ‘Sr’ such as Sr1,

Sr2 and Sr3. Slants of each isolate were maintained in the refrigerator at 15^0C and used for further studies.

Comparing the number of days taken for the mycelial growth of isolates Pc1 and Pc2, Pc1 completed the growth in 7 days after inoculation (DAI) and Pc2 in 9DAI. Comparing the anthracnose showing isolates Cg 1, Cg 2, Cg 3 and Cg 4, Cg 1 completed the growth in 7 DAI, Cg2, Cg3 and Cg4 in 9DAI. Basal stem rot isolates Sr1, Sr2 and Sr3, in which the Sr1 completed the growth in 5 DAI, Sr2 in 7 days and Sr3 in 8 days (Table 11).

4.3. PATHOGENICITY TEST

Evaluation of pathogenicity of the isolates was done by following Koch's postulates. All the isolates were selected for the pathogenicity test. Soil and leaf inoculation methods were used for pathogenicity testing on black pepper grown in polythene bags. Virulence testing was carried out to find the most virulent isolate and symptom development on the plant from the different isolates.

4.3.1. Radial growth of the mycelium

The seedling blight showing isolates were Pc1 and Pc2, anthracnose showing isolates were Cg1, Cg2, Cg3 and Cg4 and stem rot showing isolates were Sr1, Sr2 and Sr3 were inoculated to the potato dextrose agar media and observed the radial growth of the mycelium up to 9DAI and observations were taken 5DAI (Table 12).

Isolate Pc1 grows 7.9 cm as compared to Pc2 (4.8cm) in 5 DAI. in PDA media. Cg1(8.12 cm), Cg2 (7 cm), Cg3 (5.75 cm) and Cg4 (5.42 cm) growth in PDA media after 5 days of inoculation and Sr1 (8.96 cm), Sr2 (7.16 cm) and Sr3 (5.66 cm) growth were observed in 5 DAI.

Table 10. Disease incidence and disease index of diseases in black pepper at different locations

Sl.No	Location	Percent disease incidence	Percent disease index
Seedling blight disease			
1	Padannakkad	74	32
2	Kanakapalli	44	17
Anthracnose disease			
1	Padannakkad	30	25
2	Periya	20	10
3	Balal	14	12.6
4	Panniyur	10	2.0
Basal stem rot disease			
1	Padannakkad	20	*
2	Periya	16	*
3	Panniyur	10	*
* Disease index was indicted as the death of the plant and not as calculated			

Table 11. Number of days for the mycelium development on PDA media

Sl. No.	Isolate code	Initiation of mycelial growth (DAI)	Complete growth of the mycelia (DAI)
Seedling blight			
1	Pc1	1	7
2	Pc2	1	9
Anthracnose			
1	Cg1	1	7
2	Cg2	1	9
3	Cg3	1	9
4	Cg4	1	9
Basal stem rot			
1	Sr1	1	6
2	Sr2	1	9
3	Sr3	1	10

Table 12. Radial growth of isolates in PDA

Sl. No.	Isolate code	Colony diameter of isolate in PDA in petridish (cm)				
		1DAI	3DAI	5DAI*	7DAI	9DAI
1	Pc1	1.5	4.8	7.9	9.0	9.0
2	Pc2	1.0	3.6	4.8	7.0	8.0
3	Cg1	3.0	5.4	8.12	9.0	9.0
4	Cg2	3.0	5.0	7.0	9.0	9.0
5	Cg3	1.5	4.0	5.75	8.0	9.0
6	Cg4	1.8	3.5	5.42	7.2	9.0
7	Sr1	2.7	5.8	8.96	9.0	9.0
8	Sr2	1.8	4.7	7.16	8.0	9.0
9	Sr3	1.2	3.9	5.66	6.8	8.4
* Observations were taken in 5 DAI						

4.3.2. Multiplication of fungal inoculum

The isolates Pc1 and Pc2 (Plate 2) and isolates Cg1, Cg2, Cg3 and Cg4 (Plate 3) were grown in PDA media. The isolates Sr1, Sr2 and Sr3 were multiplied in paddy grains and used for soil inoculation (Plate 4). White fungal mycelia covered entire paddy grains in the test tube within three days of inoculation and sclerotial production started after five days of incubation.

4.3.3. Inoculation of the pathogen on the leaf

The different isolates such as Pc1 and Pc2, Cg1, Cg2, Cg 3 and Cg4 were inoculated to the underside of the leaves and observed the number of days for symptom development on black pepper seedlings and lesion size was calculated 7 DAI. Black water-soaked lesions on leaves produced by Pc1 and Pc2 isolates and black spots were produced on the leaves by Cg1, Cg2, Cg3 and Cg4.

The isolate Pc1 produced symptoms within 2 days and lesion size was 58.10 cm^2 in 7 DAI, Pc2 produced symptoms within 4 days and lesion size was 34.23 cm^2 in 7 DAI (Table13).

Among these isolates Cg1, Cg2, Cg3 and Cg4, Cg1showing the symptoms in 5 days and lesion size was 7.06 cm^2 in 7 DAI, Cg2 requires 6 days for symptom production and 3.14 cm^2 in lesion size, Cg3 symptom developed in 6 days and lesion size 2 cm^2 and Cg4 symptom developed in 7 days with lesion size 0.50 cm^2 in 7 DAI (Table 14).

4.3.4. Inoculation of the pathogen in the collar region

The isolates of pathogen showed the difference in number of days taken for infection and lesion size (Table13, Plate 4). A white thread-like mycelial mat was formed on the soil near the collar region within two to four days of inoculation and produced mustard-like sclerotial bodies. The Sr1 isolate takes 2 days for symptom development and lesion size was 17.90 cm^2 Sr2 require days for symptom development and lesion size 11.69 cm^2 and Sr3 require 4 days for symptom development and lesion size 6.72 cm^2 in 7 DAI.

Table 13. Number of days of symptom development and lesion size in seedling blight isolates inoculated to black pepper

Seedling blight			
Sl.no.	Isolate	Foliar symptom development	
		Number of days for symptom development	Lesion size on leaf (cm^2) *
1	Pc1	2	58.10^a
2	Pc2	4	34.23^b
		CD (0.05)	3.51
		SE(m)	1.07

* mean of four replications

Values in parenthesis are not transformed

Observations were taken after 7 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

Table 14. Number of days of symptom development and lesion size in anthracnose isolates inoculated to black pepper

Anthracnose			
Sl.no	Isolate	Foliar symptom development	
		Number of days for symptom development	Lesion size on leaf (cm^2) *
1	Cg1	5	7.06[a]
2	Cg2	6	3.14[b]
3	Cg3	6	2.00[c]
4	Cg4	7	0.50[d]
		CD (0.05)	0.19
		SE(m)	0.02

* mean of four replications

Values in parenthesis are not transformed

Observations were taken after 7 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

Table 15. Number of days of symptom development and lesion size in basal stem rot isolates inoculated to black pepper

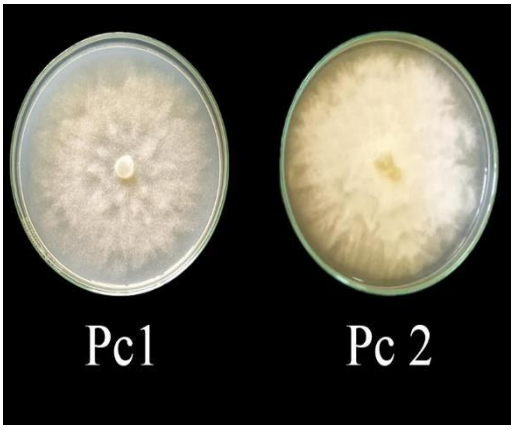
Basal stem rot			
Sl. No.	Isolate	Collar symptom development	
		Number of days for symptom development	Lesion size on leaf (cm^2) *
1	Sr1	2	17.90^a
2	Sr2	3	11.69^b
3	Sr3	4	6.72^c
		CD (0.05)	0.02
		SE(m)	0.08

* mean of four replications

Values in parenthesis are not transformed

Observations were taken after 7 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05



Pathogen grown in PDA media **Pathogen inoculated in plants**

Plate 2. Pathogenicity test of Pc1 and Pc2 isolate in black pepper

Pathogen grown in PDA media **Pathogen inoculated in plants**

Plate 3. Pathogenicity test of Cg1, Cg2, Cg3 and Cg4 isolate in black pepper

Pathogen mass multiplied in paddy grains **Pathogen inoculated at the collar portion of the plant**

Plate 4. Pathogenicity test of isolates Sr1, Sr2 and Sr3 in black pepper

4.3.5. Virulence testing of different isolates of the pathogen

Virulence testing was carried out with the fast-growing and earlier symptom-producing isolates such as Pc1, Cg1 and Sr1. The test was conducted at shaded conditions with different black pepper varieties including Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4, Panniyur 5, Panniyur 6, Panniyur 8, Panniyur 9, Vijay and Karimunda. *etc*.

4.3.5.1. Virulence testing of Pc1 isolate in different black pepper varieties

For the virulence testing, the isolate Pc1 was inoculated to the plant by leaf inoculation method and observed DI, PDI and lesion size after 5 days of inoculation. The radial growth of the isolate was observed at 5 DAI in the pathogenicity test chosen for virulence testing (Table 16).

The symptomatic plants show black lesions on the inoculated leaves (Plate 5). The DI ranges from 0-92%, PDI ranges from 0- 62.4% and lesion size 0- 91.76 cm^2. In which higher DI has observed at Panniyur 2 variety (92%) with PDI of 62.4 percent and lesion size of 91.76 cm^2 as compared with other varieties. In Panniyur 6, Vijay and Karimunda symptom development was not observed.

4.3.5.2. Virulence testing of Cg 1 isolate in different black pepper varieties

For the virulence testing, isolate Cg1 was inoculated to the plant by leaf inoculation method and observe DI, PDI and lesion size after 5 days of inoculation (Table17). The Cg1 isolates produced black spots on leaves without yellow halo with fewer symptoms in the inoculated plants (Plate 6), in which higher DI was observed in Panniyur 2 with 8 percent. The PDI ranges from 0-8.2 percent and lesion size ranges from 0 - 1.39 cm^2. The isolate did not produce symptoms in Vijay and Karimunda.

4.3.5.2. Virulence testing of Sr1 isolate in different black pepper varieties

For the virulence testing, the isolate Sr1 was mass multiplied in paddy grains and was inoculated to the collar region of the plants (Table 18). The DI, PDI and lesion size after 5 days of inoculation were observed and in all varieties, it produced the symptoms of black lesions on the leaves with mycelial growth on the collar region

(Plate 7). The DI (13-52 %) and lesion size (9- 54 cm^2) were observed in the varieties. In Panniyur 2 the DI (52 %) and lesion size (54.35 cm^2) were observed after 5 DAI.

From the virulence testing study, it was concluded that the isolate Sr1 and Cg1 were fast-growing in the media, but in the inoculated plants they produced less incidence and symptoms. Based on the virulence testing isolate Pc1 was identified as the most virulent pathogen. The higher disease incidence observed was in Panniyur 2 as is considered as the most susceptible variety. So the isolate Pc1 was selected for further studies.

Table 16. Virulence testing of Pc1 isolate in black pepper varieties

5 DAI				
Sl. No.	Black pepper variety	Pc1		
		DI* (%)	PDI (%)	Lesion size on the leaf (cm^2)**
1	Panniyur 1	68(55.82)^{cd}	33.6	36.86^{b}
2	Panniyur 2	92(79.37)^{a}	62.4	91.76^{a}
3	Panniyur 3	68(55.82)^{cd}	48.0	41.39^{b}
4	Panniyur 4	80(63.43)^{b}	48.0	41.07^{b}
5	Panniyur 5	60(50.76)^{d}	24.0	25.11^{c}
6	Panniyur 6	0(1.28)^{e}	0	0^{e}
7	Panniyur 8	76(60.89)^{bc}	43.2	17.78^{d}
8	Panniyur 9	60(50.76)^{d}	24.0	15.89^{d}
9	Vijay	0(1.28)^{e}	0	0^{e}
10	Karimunda	0(1.28)^{e}	0	0^{e}
	CD (0.05)	7.45		7.01
	SE(m)	2.60		2.45

* mean of five replications

Values in parenthesis are arc sin transformed

Observations were taken after 5 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

* *mean of five replications

Values in parenthesis are not transformed

Observations were taken after 5 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

Panniyur 1 Panniyur 2 Panniyur 3 Panniyur 4

Panniyur 5 Panniyur 6 Panniyur 8 Panniyur 9

Vijay Karimunda

Plate 5. Virulence testing of isolate Pc1 inoculated in different black pepper varieties

Table 17. Virulence testing of Cg1 isolate in black pepper varieties

5 DAI				
Sl. No.	Black pepper variety	Cg 1		
		DI* (%)	PDI (%)	Lesion size on the leaf (cm^2)**
1	Panniyur 1	6(14.17) [c]	2.4	0.38[b]
2	Panniyur 2	8(16.42) [a]	8.2	1.39[a]
3	Panniyur 3	5(12.92) [d]	4.8	0.66[b]
4	Panniyur 4	4(11.53) [e]	2.0	0.25[b]
5	Panniyur 5	2(8.13) [g]	2.4	0.15[b]
6	Panniyur 6	7(15.34) [b]	8.0	0.31[b]
7	Panniyur 8	3(9.90) [f]	2.4	0.30[b]
8	Panniyur 9	4(11.53) [e]	4.8	0.55[b]
9	Vijay	0(1.28) [h]	0	0[b]
10	Karimunda	0(1.28) [h]	0	0[b]
	CD (0.05)	0.004		0.72
	SE(m)	0.002		0.25

* mean of five replications

Values in parenthesis are arcsin transformed

Observations were taken after 5 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

* *mean of five replications

Values in parenthesis are not transformed

Observations were taken after 5 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

Panniyur 1 **Panniyur 2** **Panniyur 3** **Panniyur 4**

Panniyur 5 **Panniyur 6** **Panniyur 8** **Panniyur 9**

Vijay **Karimunda**

Plate 6. Virulence testing of isolate Cg1 inoculated in different black pepper varieties

Table 18. Virulence testing of Sr1 isolate in black pepper varieties

5 DAI			
Sl. No.	Black pepper variety	Sr1	
		DI* (%)	Lesion size on the leaf (cm^2)**
1	Panniyur 1	36(36.82)^{b}	29.40^{e}
2	Panniyur 2	52(46.15)^{a}	54.35^{a}
3	Panniyur 3	16(23.30)^{de}	53.31^{a}
4	Panniyur 4	22(27.58)^{cd}	39.79^{d}
5	Panniyur 5	14(25.96)^{cde}	43.96^{c}
6	Panniyur 6	35(36.23)^{b}	40.69^{d}
7	Panniyur 8	17(24.29)^{cde}	47.75^{b}
8	Panniyur 9	24(29.22)^{c}	19.62^{f}
9	Vijay	16(23.30)^{de}	10.17^{g}
10	Karimunda	13(20.60)^{e}	9.07^{g}
	CD (0.05)	3.698	1.89
	SE(m)	1.801	0.66

* mean of five replications

Values in parenthesis are arcsin transformed

Observations were taken after 5 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

* *mean of five replications

Values in parenthesis are not transformed

Observations were taken after 5 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

Panniyur1 Panniyur 2 Panniyur 3 Panniyur 4

Panniyur 5 Panniyur 6 Panniyur 8 Panniyur 9

Vijay Karimunda

Plate 7. Virulence testing of isolate Sr1 inoculated in different black pepper varieties

4.4. IDENTIFICATION OF THE PATHOGEN

Most virulent isolate Pc1 was identified based on cultural, morphological and molecular characteristics.

4.4.1. Cultural characteristics

Colony characters of Pc1 such as rate of growth of mycelium, colour of mycelium, the pattern of mycelial growth, days have taken to complete the growth were studied in PDA (Plate 8). The pathogen was showing slow growth and covered Petri dish of 90 mm diameter within 7 days after inoculation. It produced a white colony with petaloid or rosaceous zonations in PDA. White loosely intertwined large branches of coenocytic fungal hypha which are hyaline (Table 19).

4.4.1. Morphological characteristics

Morphological characteristics of fungal hyphae and sporangium were examined through Carl Zeiss binocular microscope under 400X magnification (Plate 9). The hyphae were coenocytic and lemon-shaped or oblong or ovoid sporangia were observed under the microscope and the length, breadth of the sporangium were measured (Table 20).

4.4.3. Molecular characteristics

Molecular characterization of the most virulent isolate was carried out for the identification of fungus at the molecular level. Identification of microbial culture was done by using the D1/D2 region of LSU (Large Sub Unit: 16 SrDNA) based molecular technique. Consensus Sequence were given as follows (420bp) (Appendix III).

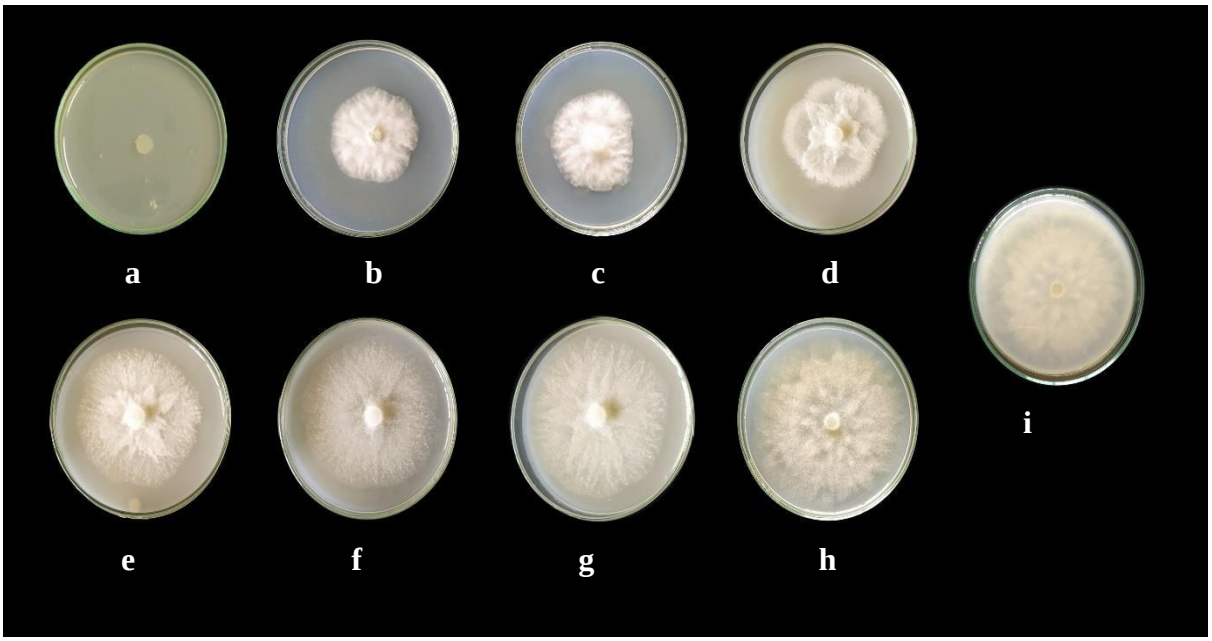
Based on the D1/D2 Region- PCR analysis, the fungal culture Pc1 showed 100 % similarity with *Phytophthora capsici* strain Bt3a (Accession No: AH015113.2) (Table 21). Based on the cultural, morphological and molecular characterization, isolate Pc1 responsible for seedling blight disease was confirmed as *Phytophthora capsici*.

Table 19. Cultural characters of isolate Pc1

Parameters	Observations
Culture colour	White
Reverse plate colour	Dull white
Culture margin	Irregular
Topography	Undulating with mycelia
Zonation	Petaloid
Colony diameter (cm)	Completes Petri plate (9 cm) on 7th day of inoculation
Mycelia	Coenocytic
Colour of mycelia	White

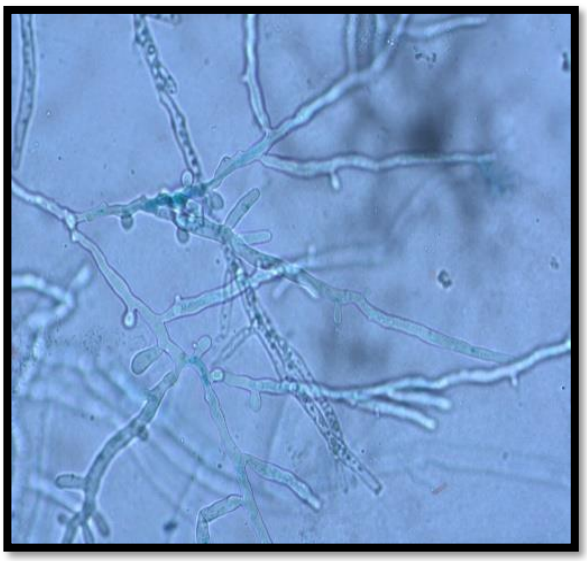
Table 20. Morphological characters of isolate Pc1

Parameters/sporangial characters	Observations
Length of sporangium	40.3µm
Breadth of sporangium	37µm
Shape of sporangium	Ovoid/oblong/lemon shaped
Papillate/ Nonpapillate	Papillate
Hyphae	Coenocytic



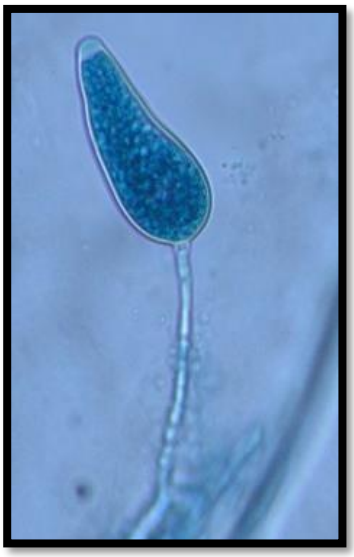
a) Pc1 isolate inoculated in PDA media b) 1DAI c) 2DAI d) 3DAI
e) 4DAI f) 5DAI g) 6DAI h) 7DAI i) Reverse side view

Plate 8. Cultural characters of Pc1 isolate

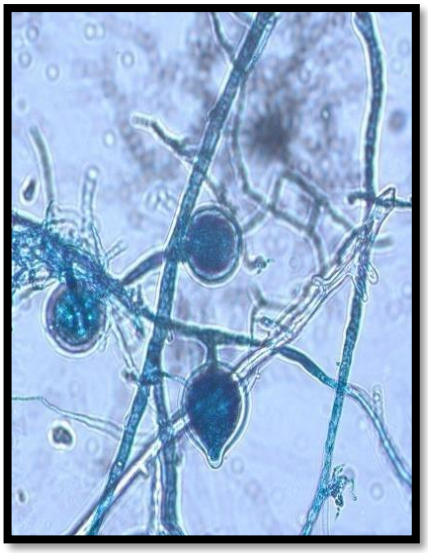


a

b



c



d

a) Mycelium b) Ovoid shaped sporangium c) Oblong shaped sporangium d) Lemon shaped sporangium

Plate 9. Morphological characters of Pc1 isolate

Table 21. Nucleotide identity of Pc1 isolate with reported isolates of *Phytophthora capsici*

Sl. No.	Description	Max. score	Query Coverage	E value	Identity (%)	Accession number
1	*Phytophthora capsici* strain Bt3a	776	100	0.0	100	AH015113.2
2	*P. mexicana* strain CPHSTBL24	752	100	0.0	99.05	MG865540.1
3	*P. capsici* strain Chla	752	100	0.0	99.05	AH015114.2
4	*P. capsici* isolate 09-02	752	100	0.0	99.05	KC311831.1
5	*P. capsici* WPCP0253	752	100	0.0	99.05	LC595782.1
6	*P. capsici* strain WPC21	752	100	0.0	99.05	JQ071941.1
7	*P. capsici* culture collection WPC:0782B1548	752	100	0.0	99.05	GU259440.2
8	*P. capsici* culture collection WPC:7535C587	752	100	0.0	99.05	GU254939.2
9	*P. capsici* culture collection WPC:10830A1599	752	100	0.0	99.05	GU259394.1
10	*P. capsici* clone WPC3375A793	752	100	0.0	99.05	FJ801841.2

4.5. SYMPTOMATOLOGY OF DISEASE

Symptomatology of seedling blight disease in black pepper caused by *P. capsici* was studied under natural and artificial conditions (Plate 10 and Plate 11). It was observed that similar symptoms were noticed in both conditions.

In natural conditions, the infection was produced as black spots on the leaves near the soil. Later enlarged the spots to become water-soaked, brown sunken lesions at the margins of the leaves. Two-three spots were combined to form larger lesions. The lesions spread entire leaves and dried the leaves. If the spots occur in the collar regions, the spots become enlarged lesions and later wilted the plants and complete drying of the plants.

The artificial conditions, symptoms were first observed as a black spot on the leaves, then enlarged to form large lesions as leading to drying of the leaves. Then shedding of leaves from the plants occurred. Entire plants with wilting symptoms were not observed in the plants.

4.6. *IN VITRO* EVALUATION OF BIOCONTROL AGENTS, ORGANIC PREPARATIONS AND CHEMICAL FUNGICIDES AGAINST THE PATHOGEN

In vitro evaluation was done with a set of biocontrol agents, chemical fungicides and organic preparation against the most virulent pathogen, which was showing more disease severity in nursery seedlings of black pepper

4.6.1. *In vitro* evaluation of biocontrol agents against the pathogen

The efficacy of KAU released biocontrol agents such as *T. harzianum*, *P. fluorescens* and *B. subtilis* were tested against the virulent isolate Pc1 of *Phytophthora capsici* by dual culture technique (Plate 12).

Comparing all the biocontrol agents, showed a higher percentage of inhibition (55.56%) on the growth of the pathogen was observed in the case of *T. harzianum*. But among the bacterial biocontrol agents a higher percentage (53.33%) of inhibition on the growth of pathogen was observed in the case of *P. fluorescens* as compared to *B. subtilis*. (Table 22).

Plate 10. Seedling blight disease symptoms observed in different nurseries

Plate 11. Seedling blight disease symptoms observed in artificial conditions

Table 22. Effect of biocontrol agents against *P. capsici in vitro*

Sl.no.	Treatment	Radial growth of the pathogen (cm)	Percentage mycelial inhibition of *P. capsici* (%)*
1	*Trichoderma harzianum*	4.0	55.56(1.75)[a]
2	*Pseudomonas fluorescens*	4.2	53.33(1.73)[b]
3	*Bacillus subtilis*	4.4	49.43(1.69)[c]
4	Control	9.0	0.00(0.06)[e]
		CD (0.05)	0.006
		SE(m)	0.002

*mean of four replications

Values in parenthesis are logarithmic transformed

Observations were taken after 7 days of inoculation

Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05

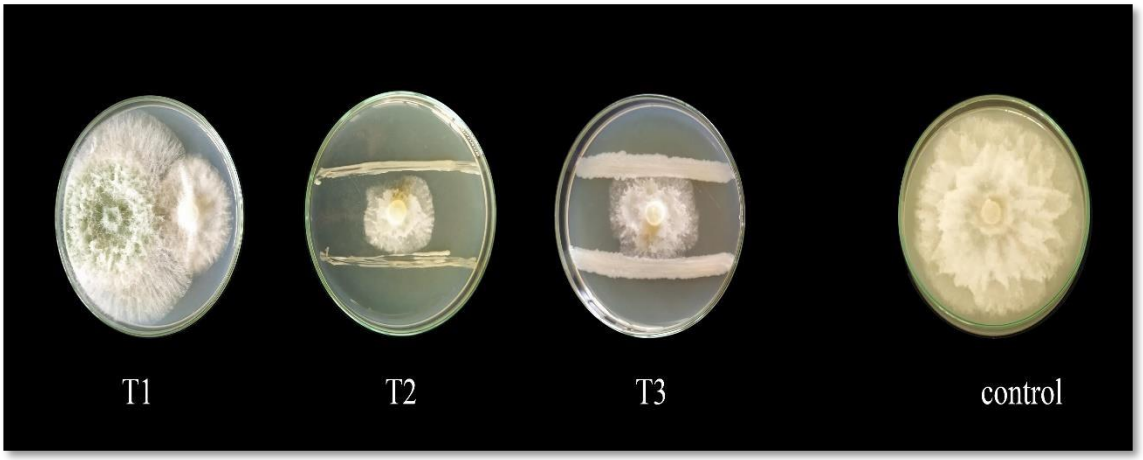


Plate 12. Effect of biocontrol agents against *P. capsici*

T1-*T. harzianum*

T2-*P. fluroscens*

T3- *B. subtilis*

T4- Control

4.6.2. *In vitro* evaluation of chemical fungicides against the pathogen

The relative efficacy of contact, systemic and combination fungicides tested against the virulent pathogen by poisoned food technique

In vitro evaluation fungicides was done at three concentrations against *P. capsici* by poisoned food technique in double strength PDA. All fungicides were tested at three different concentrations including recommended dosage as well as its lower and higher dosage (Table 23 and Plate 13).

The maximum (100 %) inhibition of the mycelial growth of the pathogen was observed in case of copper oxychloride(50WP) at 0.3 per cent and metalaxyl 8%+ mancozeb 64% (72 WP) 0.6 percent followed by copper oxychloride (50WP) at 0.2 and 0.1 percent and mancozeb at 0.1 per cent. The least inhibition in the mycelial growth of the pathogen was observed in the case of carbendazim (50WP) at its all concentrations, which was on par with that of the control followed by azoxystrobin (23SC) at 0.2 per cent.

4.6.3. *In vitro* evaluation of organic preparations against the pathogen

The relative efficacy of organic preparations tested against pathogen by poisoned food technique in potato dextrose agar media. The organic preparations such as neemazal, garlic extract and ready to use neem oil garlic soap were tested at three different concentrations including recommended dose and its lower and higher dose (Table 24).

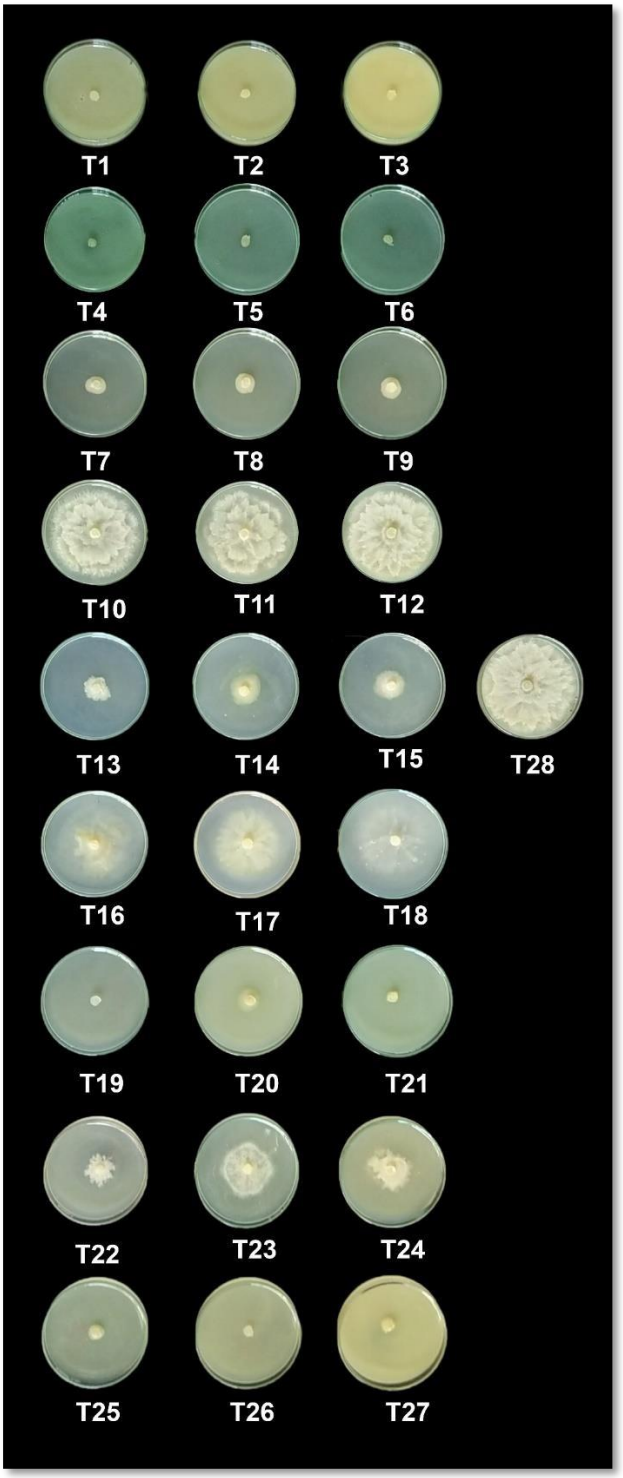
The maximum inhibition in the growth of the pathogen (31.11%) was recorded in the case of Ready to use neem oil garlic soap at 3 percent followed by garlic extract (28.88%) at 0.3 percent. the least inhibition (8.88%) in the growth of the pathogen was observed in Neemazal at 0.1 percent. as compared with other treatments (Plate 14). The is no significant difference between the effect of garlic extract at 0.2 percent and ready to use neem oil garlic soap at 0.2 per cent.

Table 23. *In vitro* evaluation of chemical fungicides against *P. capsici*

Sl.no.	Treatment concentration	Radial growth of the pathogen (cm)	Percentage mycelial inhibition of *P. capsici*(%)*
T1	Mancozeb (75 WP) 0.1%	0.92	89.85(1.958)^{g}
T2	Mancozeb (75 WP) 0.2%	0.90	89.92(1.958)^{g}
T3	Mancozeb (75 WP) 0.3%	0.80	91.03(1.96)^{def}
T4	Copper oxychloride(50WP)0.1%	0.46	94.81(1.98)^{c}
T5	Copper oxychloride(50WP)0.2%	0.26	97.03(1.99)^{b}
T6	Copper oxychloride(50WP)0.3%	0	100(2.004)^{a}
T7	Metalaxyl (35WS) 0.05%	0.9	90.07(1.959)^{fg}
T8	Metalaxyl (35WS) 0.1%	0.88	90.22(1.960)^{efg}
T9	Metalaxyl (35WS) 0.2%	0.78	91.33(1.965)^{d}
T10	Carbendazim(50WP) 0.05%	9.0	0.00(0.000)^{q}
T11	Carbendazim(50WP) 0.1%	9.0	0.00(0.000)^{q}
T12	Carbendazim(50WP) 0.2%	9.0	0.00(0.000)^{q}
T13	Propiconazole(25EC) 0.05%	2.68	69.62(1.84)^{h}
T14	Propiconazole(25EC) 0.1%	2.92	67.40(1.83)^{I}
T15	Propiconazole(25EC) 0.2%	3.12	65.18(1.82)^{j}
T16	Azoxystrobin(23SC) 0.05%	7.72	14.07(1.17)^{j}
T17	Azoxystrobin(23SC) 0.1%	7.92	11.92(1.11)^{o}
T18	Azoxystrobin(23SC) 0.2%	8.04	10.51(1.06)^{p}
T19	Carbendazim 12% + Mancozeb 63% (75WP) 0.05%	0.85	90.51(1.961)^{defg}
T20	Carbendazim 12% + Mancozeb 63% (75WP) 0.1%	0.84	90.66(1.962)^{defg}
T21	Carbendazim 12% + Mancozeb 63% (75WP) 0.2%	0.78	91.11(1.96)^{de}

T22	Tebuconazole 50%+ Trifloxystrobin 25% (75 WG) 0.02%	4.8	46.51(1.67)^{h}
T23	Tebuconazole 50%+ Trifloxystrobin 25% (75 WG) 0.04%	4.68	47.84(1.68)^{l}
T24	Tebuconazole 50%+ Trifloxystrobin 25% (75 WG) 0.06%	4.44	50.51(1.71)^{k}
T25	Metalaxyl 8%+ Mancozeb 64% (72 WP) 0.2%	0.84	90.58(1.96)^{defg}
T26	Metalaxyl 8%+ Mancozeb 64% (72 WP) 0.4%	0.82	90.80(1.96)^{defg}
T27	Metalaxyl 8%+ Mancozeb 64% (72 WP) 0.6%	0	100(2.00)^{a}
T28	Control	9.0	0.00(0.00)^{q}
		CD (0.05)	0.012
		SE(m)	0.004

*mean of three replications
Values in parenthesis are logarithmic transformed
Observations were taken after 7 days of inoculation
Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05



T1-Mancozeb (75 WP) 0.1%

T2-Mancozeb (75 WP) 0.2%

T3-Mancozeb (75 WP) 0.3%

T4-Copper oxychloride(50WP)0.1%

T5-Copper oxychloride(50WP)0.2%

T6-Copper oxychloride(50WP)0.3%

T7-Metalaxyl (35WS) 0.05%

T8-Metalaxyl (35WS) 0.1%

T9-Metalaxyl (35WS) 0.5%

T10-Carbendazim(50WP) 0.05%

T11-Carbendazim(50WP) 0.1%

T12-Carbendazim(50WP) 0.2%

T13-Propiconazole(25EC) 0.05%

T14-Propiconazole(25EC) 0.1%

T15-Propiconazole(25EC) 0.2%

T16-Azoxystrobin(23SC) 0.05%

T17-Azoxystrobin(23SC) 0.1%

T18-Azoxystrobin(23SC) 0.2%

T19-Carbendazim 12% + Mancozeb 63% (75WP) 0.05%

T20-Carbendazim 12% + Mancozeb 63% (75WP) 0.1%

T21-Carbendazim 12% + Mancozeb 63% (75WP) 0.2%

T22-Tebuconazole 50%+ Trifloxystrobin 25% (75 WG) 0.02%

T23-Tebuconazole 50%+ Trifloxystrobin 25% (75 WG) 0.04%

T24-Tebuconazole 50%+ Trifloxystrobin 25% (75 WG) 0.06%

T25-Metalaxyl 8%+ Mancozeb 64% (72 WP) 0.2%

T26-Metalaxyl 8%+ Mancozeb 64% (72 WP) 0.4%

T27- Metalaxyl 8%+ Mancozeb 64% (72 WP) 0.6%

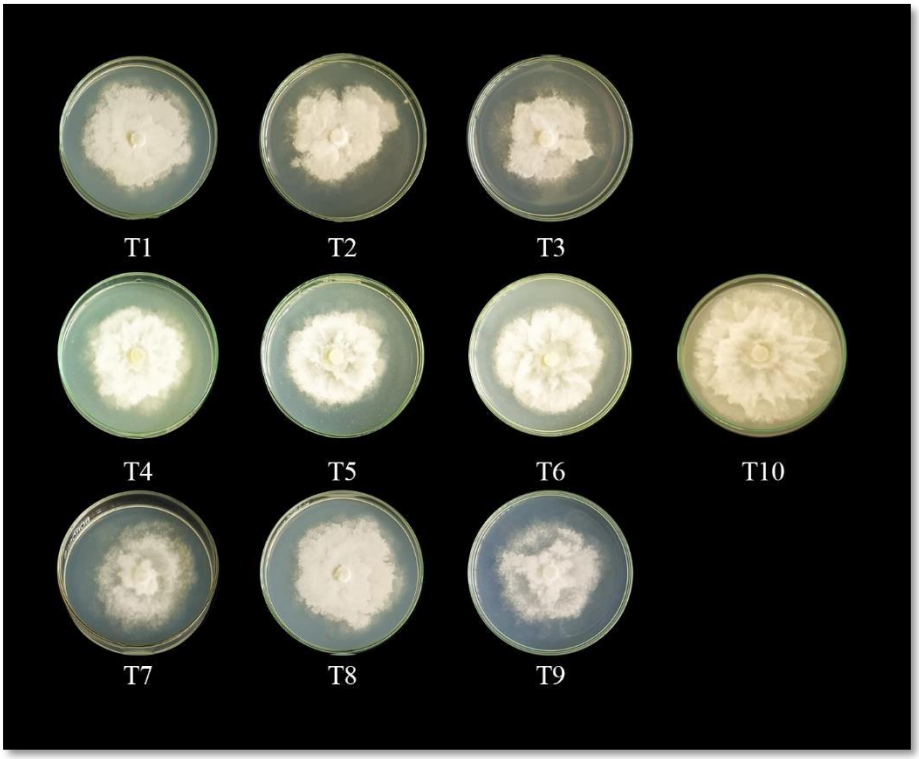
T28-Control

Plate 13. Effect of chemical fungicides on *P. capsici*

Table 24. *In vitro* efficacy of organic preparations against *P. capsici*

Sl. No.	Treatment- Concentration	Radial growth of pathogen (cm)	Percentage mycelial inhibition of *P.capsici*(%)
T1	Neemazal (0.1%)	8.2	8.88(0.98)^{h}
T2	Neemazal (0.2%)	7.8	13.33(1.15)^{g}
T3	Neemazal (0.3%)	7.6	15.55(1.21)^{f}
T4	Garlic extract (0.1%)	6.8	24.44(1.40)^{d}
T5	Garlic extract (0.2%)	6.6	26.66(1.44)^{c}
T6	Garlic extract (0.3%)	6.4	28.88(1.47)^{b}
T7	Ready to use neem oil garlic soap (1%)	7.0	22.22(1.36)^{e}
T8	Ready to use neem oil garlic soap (2%)	6.64	26.29(1.43)^{c}
T9	Ready to use neem oil garlic soap (3%)	6.2	31.11(1.50)^{a}
T10	Control	9.0	0.00(0.00)^{i}
		CD (0.05)	0.054
		SE(m)	0.018

*mean of three replications
Values in parenthesis are logarithmic transformed
Observations were taken after 7 days of inoculation
Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05



T1-Neemazal (0.1%)

T2-Neemazal (0.2%)

T3-Neemazal (0.3%)

T4-Garlic extract (0.1%)

T5-Garlic extract (0.2%)

T6-Garlic extract (0.3%)

T7-Ready to use neem oil garlic soap (1%)

T8-Ready to use neem oil garlic soap (2%)

T9-Ready to use neem oil garlic soap (3%)

T10-Control

Plate 14. Effect of organic preparations on *P. capsici*

4.7. *IN VIVO* EVALUATION OF BIOCONTROL AGENTS, ORGANIC PREPARATIONS AND CHEMICAL FUNGICIDES AGAINST THE PATHOGEN

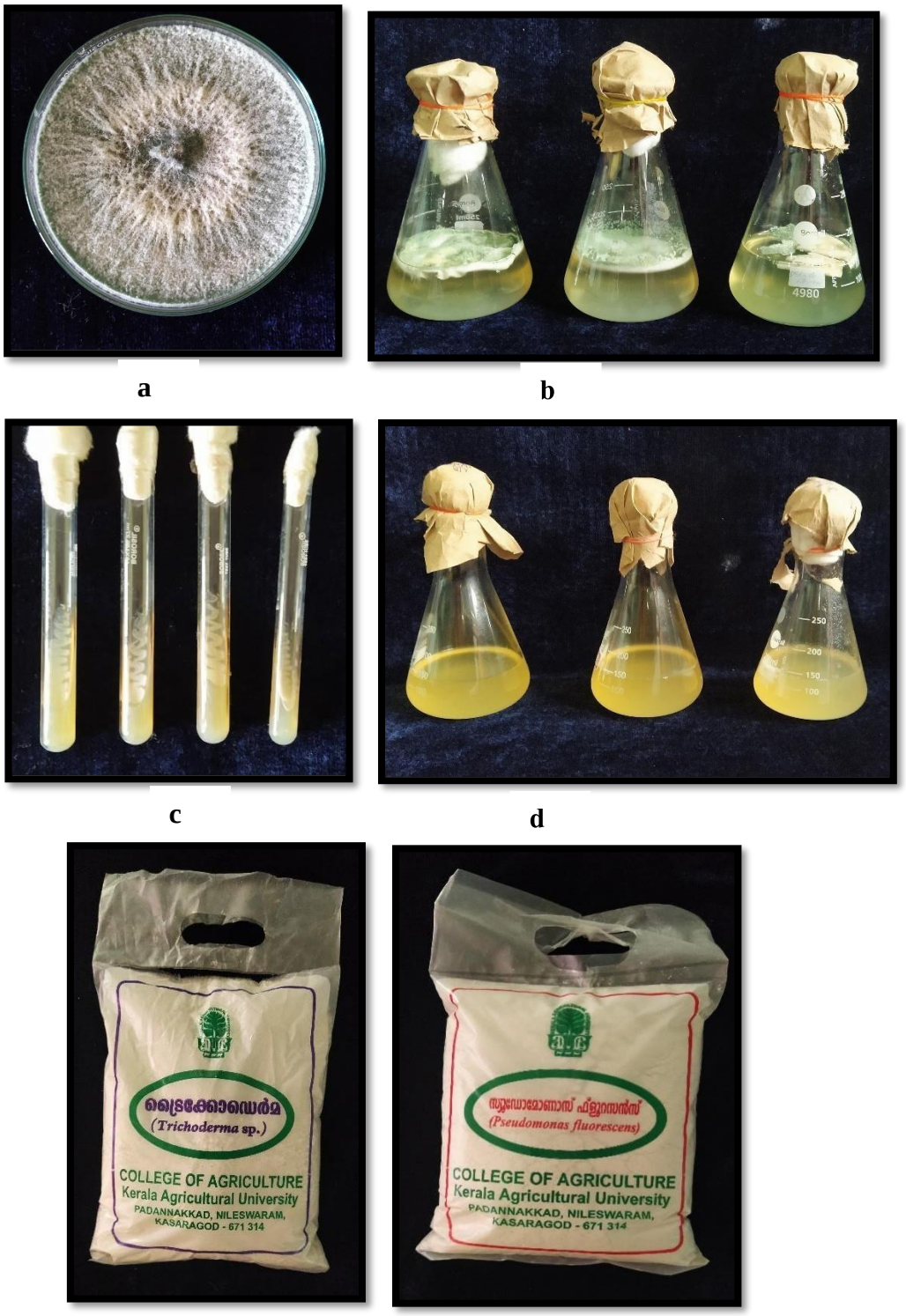
Best treatments from *in vitro* evaluation of biocontrol agents, chemical fungicides and organic preparations were selected for *in vivo* evaluation against the pathogen (Table 25).

The application of two biocontrol agents, one fungal biocontrol agent *T. harzianum* (2%) and one bacterial biocontrol agent *P. fluorescens* (2%) (Plate 15), two chemical fungicides at a higher concentration than recommended such as copper oxychloride (0.3%) and a combination of metalaxyl 8%+ mancozeb 64% (72 WP) (0.6%)and organic preparations such as garlic extract (0.3%) and ready to use neem oil garlic soap (3%) were applied to the 60 days old seedlings that grown in pots. The seven-day-old cultures of the pathogen were inoculated on the underside of the leaves and after 24 hrs of inoculation, the above-mentioned treatments were applied as a foliar spray. The symptom development observed on each day and disease incidence and severity observed after 7 days (Plate 16). The disease index was calculated after 7 days of growth, observing the lesion size and lesion area. Among these, no symptomatic expression was observed in the case of treatment metalaxyl 8% + mancozeb 64% (72 WP) (0.6%) followed by copper oxychloride (0.3%) The higher disease index (86.66%) was observed in the case of ready to use neem oil garlic soap (3%) as compared with other treatments. Lesion size and lesion area were observed after 7 days of inoculation, in which metalaxyl 8% + mancozeb 64% (72 WP) (0.6%) treated leaves with no further lesion development. A higher size of the lesion was observed in garlic extract (0.3%) treated plants was 3.9 cm and lesion area 47.84 cm^2 (Plate 17).

Table 25. Effect of biocontrol agents, chemical fungicides and organic preparations against the pathogen in the pot culture experiment

Sl.no.	Treatment	Method of application	PDI (%)	Lesion size (cm)	Lesion area on leaf surface (cm^2)
T1	*Trichoderma harzianum* (2%)	Foliar spray after 24 hrs of inoculation in leaves	80(63.43)^{c}	3.73	43.83
T2	*Pseudomonas fluorescens* (2%)		73(58.7)^{d}	3.63	41.47
T3	Copper oxychloride (0.3%)		64(53.14)^{e}	2.60	21.28
T4	Metalaxyl 8%+ Mancozeb 64% (72 WP) 0.6%		0.00(5.22)^{f}	0.00	0.00
T5	Garlic extract (0.3%)		74.33(59.63)^{cd}	3.90	47.84
T6	Ready to use neem oil garlic soap (3%)		86.66(68.85)^{b}	3.86	46.66
T7	Control		100(84.77)^{a}	3.98	49.98
		CD (0.05)	4.14		
		SE(m)	1.36		

*mean of three replications
Values in parenthesis are arcsin transformed
Observations were taken after 7 days of inoculation
Figures with the same letter do not have significant difference according to one way ANOVA at P=0.05



a) *T. harzianum* growth on PDA media b) Mass production of *T. harzianum*on PD Broth c) *P. fluorescens* growth in King's B slant d) Mass production of *P. fluorescens* on King's B Broth e) *T. harzianum* mixed with talc (CFU= $4x10^{8}$ g-1) f) *P. fluorescens* mixed with talc (CFU= $3x10^{8}$ g-1)

Plate 15. Mass production of biocontrol agents

a

b

c

d

e

a) Pots filled with potting mixture b) Fumigation of potting mixture for 45 days
c) Planting Panniyur 2 variety of black pepper d) Inoculation of pathogen
e) Symptom development on treated plants

Plate 16. *In vivo* evaluation of biocontrol agent, chemical fungicides and organic preparations against *P. capsici*

T. harzianum **Vs Control**

P. fluorescens **Vs Control**

Copper oxychloride Vs Control

Metalaxyl 4%+ Mancozeb 68% Vs Control

Garlic extract Vs Control

Ready to use neem oil garlic soap Vs control

Plate 17. Comparison with treated plant and control plant under *in vivo* experiment

DISCUSSION

5. Discussion

Black pepper (*Piper nigrum* L.) is one of the major spice crop and plantation crops in Kerala. The increased demand for berries in the world market day by day results in the focus on high production. The black pepper is mainly propagated by seedlings or cuttings. In nurseries, black pepper seedlings were infected with different diseases such as basal stem rot, seedling blight, and anthracnose. The study was conducted on "Management of a major fungal disease in black pepper (*Piper nigrum* L.) nursery" during 2019-2021 to identify and characterize a major fungal pathogen associated with nursery diseases in black pepper and develop a management strategy against those pathogen using various biocontrol agents, chemical fungicides and organic preparations.

5.1. COLLECTION OF SAMPLES

The major black pepper seedling-producing nurseries in Kannur and Kasargod districts were selected for the study. Government and nongovernment nurseries are there for the seedling production. Out of which, high tech pepper nursery in the instructional farm Padannakkad, State seed farm Periya, pepper research station, Panniyur, private nurseries in Kanakappalli, and Balal were visited for collecting the samples.

The seedling blight samples were collected from Padannakkad and Kanakappalli which showed black sunken lesions on the leaves. A similar type of survey was conducted using 'W' path sampling for collecting the infected samples for the isolation of the pathogen (Kifelew and Adugna, 2018; Shashidhara, 2007; Kollakodan *et al*., 2021). The disease incidence and percentage disease index were calculated, which was higher at Padannakkad as 74 per cent and 32 per cent respectively which was higher when compared with the maximum severity observed in the case of Phytophthora leaf rot in betel vine as14.40±z0.46% (Garain *et al*., 2020). Thomas and Naik (2007) reported as highest foot rot incidence in black pepper at Mathodu village of Shivamogga taluk as 65 percent and Kabilaseathve village of Chickamgaluru taluk as 50 percent, under nursery condition. From the study, it was concluded that seedling blight disease is prevalent in black pepper nurseries of Kasargod and Kannur districts.

The anthracnose symptoms such as circular to irregular spots with grey center and yellow halo showing samples were collected from Padannakkad, Periya, Balal, and Panniyur. Black spots with blighting symptoms were also collected from bush black pepper (Santhakumari and Aravind, 2003) and foliar blight from the black pepper nursery was collected by Kurian *et al*. (2008). The DI and PDI were higher at Padannakkad, 30 percent, and 25 percent respectively. The lower DI and PDI were observed at Panniyur as 10 and 2 percent respectively. Ahmed *et al*. (2014) was also conducted an alike study and concluded that the disease incidence in December was 10.87% and in July was 80.50%, the change in disease incidence may be due to the change in climatic conditions, especially the higher humidity during monsoon period.

Basal stem rot symptoms on the stem region were collected from the nurseries at Padannakkad, Periya, and Panniyur. A cognate type of infection along with yellowing and wilting symptoms betel vine samples were collected from different locations (Parvin *et al*., 2016; Jahan *et al*., 2016; Billah *et al*., 2017). The disease incidence was higher at Padannakkad (20%) and lower at Panniyur (10%). The higher disease incidence may be due to climatic parameters such as soil pH, temperature and relative humidity (Rahman *et al*., 2020). There was a corresponding observation reported in betel vine (Garain *et al*., 2020).

5.2. ISOLATION OF PATHOGEN

Following the standard procedures, three different groups of the pathogen were isolated from the collected samples and observed the number of days of mycelial development in media.

As comparing the mycelial growth of isolates Pc1 and Pc2, Pc1 completed the growth in 7 days after inoculation (DAI) and Pc 2 in 9DAI. Among the anthracnose showing isolates Cg1, Cg2, Cg3 and Cg4, Cg1 completed the growth in 7 DAI, Cg2, Cg3 and Cg4 in 9DAI. Basal stem rot isolates Sr1, Sr2 and Sr3, in which the Sr1 completed the growth in 5 DAI, Sr2 in 7 days and Sr3 in 8 days.

A comparable trend was observed in the case of *P. capsici* isolates (Kollakodan *et al*., 2021; Farhana *et al*., 2013; Rahman *et al*., 2020). *C. gloesporides* isolates (Behera

et al., 2019; Biju *et al*., 2020; Rahman *et al*., 2020; Masud *et a*l., 2020; Nisha and Heera, 2021), and *S. rolfsii* isolates (Sun *et al*., 2020).

5.3. PATHOGENICITY TEST

The pathogenicity of the Pc1 and Pc2 isolates was tested in black pepper varieties with leaf inoculation method and observed the symptom development. After two days of inoculation, the symptoms were observed as small round water-soaked lesions that enlarge and produce with yellow halo and later stages leaf showing the wilting symptoms. A resembling pattern of symptom development was observed in the case of a study conducted by the detached leaf inoculation method (Kollakodan *et al*., 2021) by plant inoculation methods (Shashidhara, 2007; Troung *et al*., 2008; Feng and Li, 2013).

The pathogenicity of the Cg1, Cg2, Cg3, and Cg4 isolates were tested in black pepper varieties by leaf inoculation method and observed the symptoms on the plants such as small round black spots with the yellow halo on the edges. The isolates Cg2, Cg3, and Cg4 produced the symptoms at a slow rate as compared with Cg1 isolate. The same pattern of symptomatic expression was observed in pepper on inoculation with *C. gloeosporoides* (Masud *et al*., 2020; Biju *et al*., 2020).

The pathogenicity of the Sr1, Sr2, and Sr3 isolates were tested in pepper varieties by stem inoculation method and observed the symptom like white mycelial growth on the collar region. The stem produces brown lesions with mycelia no wilting symptoms were observed in inoculated plants. Parallel type of observations was recorded by Yaqub and Shahzad (2005). Mustard-like sclerotial bodies structures that become brown or black on a mature stage (Sultana *et al*., 2012; Sneha *et al*., 2016; Eslami *et al*., 2015; Sarker *et al*., 2013). From the study, most virulent isolates were chosen as Pc1 and it was used for further studies.

5.4. IDENTIFICATION OF PATHOGEN

The most virulent isolate Pc1, selected for further studies was identified based on cultural, morphological, and molecular characters.

5.4.1. Cultural characters

The isolate Pc1 was grown in PDA media for 7 days and observed the colony characters, white culture color with dull white in reverse plate colour, irregular culture margin with undulating topography, and petaloid or rosaceous zonation on culture. The culture completes the growth on 7 DAI. Petaloid or rosaceous type of growth with dull white coloured colony was observed in Carrot agar, Cornmeal agar, Host leaf extract agar, malt extract agar, Oatmeal agar, Potato carrot agar, Potato dextrose agar, and Rye-B agar media (Shivakumar and Somasekhara, 2018). Dasgupta *et al*. (2016) observed the white mycelial growth with an undulating margin similar to the original observations. Resemblant colony characters were observed in potato dextrose agar (PDA), carrot agar (CA), and tomato agar (TA) media (Kifelew and Adugna, 2018).

5.4.2. Morphological characters

The isolate Pc 1 was identified based on morphological characters such as coenocytic mycelium with white colour. The sporangial characters were observed with oblong, ovoid, or lemon-shaped and papillate. The length of the sporangium was about 40.3µm and the breadth was 37µm The isolate with torulose hyphae, lemon-shaped sporangium with long pedicel was observed which was comparable to the Pc1 isolate (Farhana *et al*., 2013). Akter (2007) observed lemon-shaped sporangia with long pedicel which was also akin to the original observations. The length of the sporangium was observed as 48.9 µm and breadth was 45µm with lemon/ oblong shaped sporangia which was on par with the current characters (Dasgupta *et al*., 2016). The data obtained from the different studies showed that the isolate with lemon to ovoid oblong-shaped sporangia with pedicel and coenocytic hyaline hyphae is the characteristic feature of *P. capsici*, having a direct relation with the original results (Kifelew and Adugna, 2018; Photita *et al.,* 2005; Kollakodan *et al*., 2021; Weir *et al*., 2012; Hedawoo and Makode, 2019).

5.4.3. Molecular characters

Identification of microbial culture at the molecular level was done by using the D1/D2 region of LSU (Large Sub Unit: 16S rDNA) based molecular technique. Based

on the D1/D2 Region- PCR analysis, the fungal culture Pc1 showed100.00% similarity with *Phytophthora capsici* strain Bt3a (Accession No: AH015113.2).

rDNA ITS sequencing was used for the identification of *Phytophthora capsici* by Farhana *et al*. (2013). There was a 100% similarity with the small subunit ribosomal RNA gene sequence of *Phytophthora capsici* isolate CACAO by the PCR amplification of the 18S rDNA region (Kollakodan *et al*., 2021),

Based on the cultural, morphological and molecular characterization, isolate Pc1 responsible for seedling blight infection in black pepper was confirmed as *Phytophthora capsici*.

5.5. SYMPTOMATOLOGY OF THE DISEASE

Symptomatology of seedling blight disease in black pepper under natural conditions were compared with symptoms produced in artificially inoculated plants. The leaf infection was resembling in artificially inoculated plants as well as under natural conditions. The infection started as small water-soaked brown lesions at the base of the leaves which further expanded its area and completed destroy of the seedlings. It was observed from the study that symptoms in black pepper leaves as sunken lesions and wilting from collar to tip, resulting in yellowing and later stages it spread towards the collar region leads to complete drying of the plants (Shashidhara, 2007). An analogous pattern was observed in betel vine as black spots on the leaves, later become brown sunken lesions on the entire leaves and then dried off (Vijayakumar and Arumugam, 2012). The collar region with wilt, yellowing, and defoliation (Kifelew and Adugna, 2018) and black lesion on the leaves were observed during high humid conditions as the symptomatic expression in the case of pathogen *P. capsici* (Thomas and Naik, 2017). In this study, it was critically explained about the symptomatology of disease in the field as well artificial conditions.

5.6. *IN VITRO* EVALUATION OF BIOCONTROL AGENTS, CHEMICAL FUNGICIDES AND ORGANIC PREPARATIONS AGAINST THE PATHOGEN

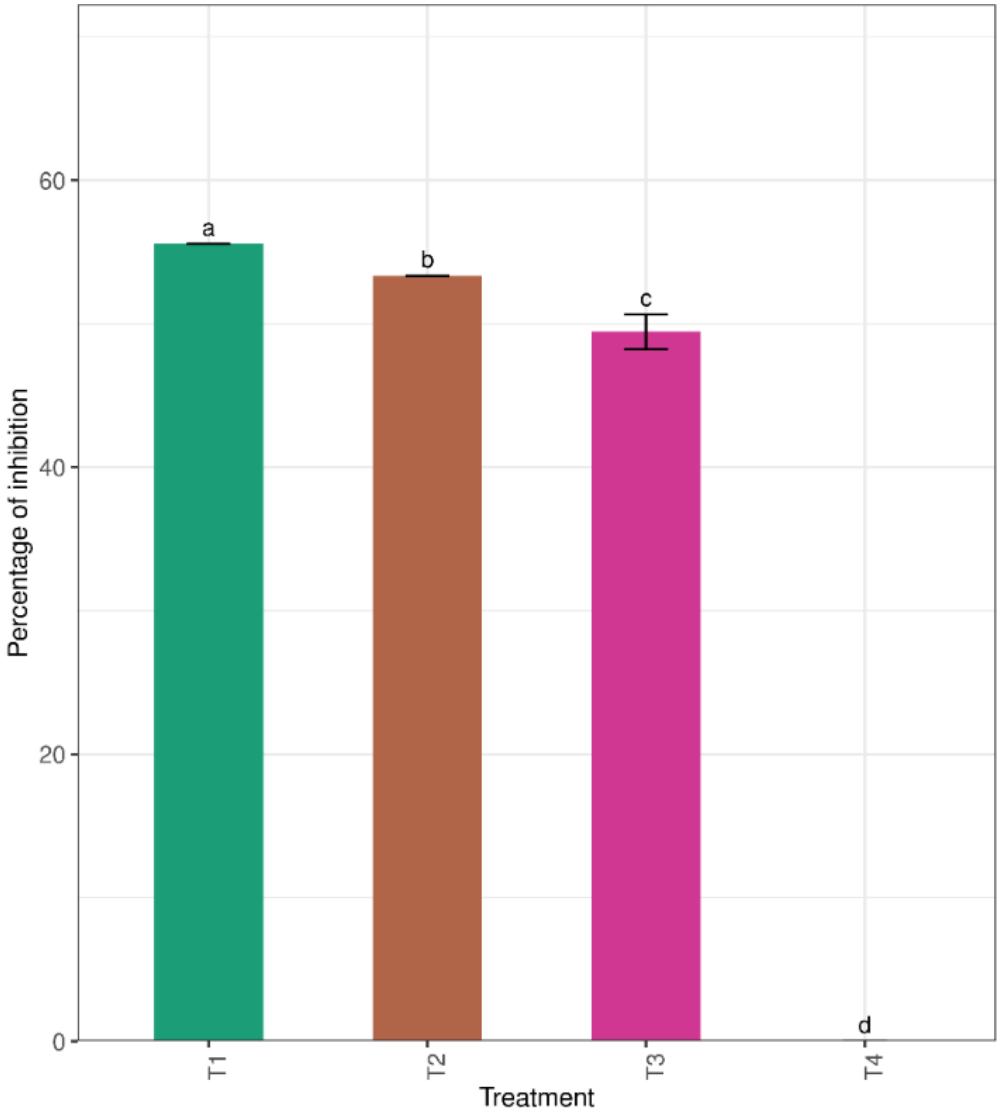
Two fungal biocontrol agents, two bacterial biocontrol agents released by KAU and systemic, contact and combination fungicides available in the market and organic

preparations available in the market and synthetic were evaluated against a most virulent isolate of *P. capsici* isolate Pc1 by dual culture and poisoned food technique respectively.

5.6.1. *In vitro* evaluation of biocontrol agents against the pathogen

KAU released biocontrol agents such as *T. harzianum, P. fluorescens,* and *B. subtilis* were evaluated to find out their potential against *P. capsici* by dual culture method. The highest percentage of inhibition on mycelial growth was observed in *T. harzianum* as 55.56 percent and *P. fluorescens* as 53.33 percent compared with other treatments (Fig. 1).

Das *et al*. (2019) observed that *T. harzianum* showed higher inhibition on the growth of the *P. capsici* by producing volatile metabolites which impart the antagonistic activity against the pathogen. The *Trichoderma* species were inhibiting the growth of the *P. capsici* by producing volatile and non-volatile compounds which show antagonistic activity against the pathogen (Osorio-Hernández *et al*., 2011). *Pseudomonas* was showing effective biocontrol activity against the pathogen by producing biosurfactant, indolic compounds, and siderophore production and reducing growth by inorganic phosphate solubilization, hydrogen cyanide activity, and detection of the phlD gene. The growth of the pathogen with a clear zone of inhibition was the result of phosphate solubilization of bacteria, extracellular enzymes like chitinase, protease, and cellulase produced by bacteria having lytic activities (Özyilmaz and Benlioglu, 2013). Effect of *Pseudomonas* spp. in the inhibition of growth of *P. capsici* by reducing the hyphal growth by the cell lysis with rupturing of zoospore formation results in complete inhibition (Zohara *et al*., 2016). Paul and Sarma (2006) observed as strains of *P. fluorescens* were antagonistic to the pathogen up to 72 percent, and the reasons were explained as the production of non-volatile metabolites, volatile-metabolites, hydrogen cyanide production, and formation of siderophores. Thomas and Naik (2017) studied the antagonistic activity of biocontrol agents against the *P. capsici* fungus and in which *P. fluorescens* has inhibited the growth of the fungus by 56.39 per cent.



T1- *Trichoderma harzianum*

T2- *Pseudomonas fluroscens*

T3- *Bacillus subtilis*

T4- Control

Fig 1. Effect of biocontrol agents against *P. capsici in vitro*

The mycolytic enzymes such as lipase, 1,4-glucanase, and 1,3-glucanase produced by *P. fluorescens* and *Trichoderma* result in the inhibition of growth of the pathogen (Diby *et al*., 2005). A matching type of inhibition was also observed under the current study.

5.6.2. *In vitro* evaluation of chemical fungicides against the pathogen

The contact, systemic and combination fungicides were tested at three different concentrations including recommended dose and its lower and higher dose against the pathogen by poisoned food technique. In which complete inhibition (100%) in the mycelial growth of the pathogen was observed in the case of copper oxychloride (50WP) (0.3%) and metalaxyl 8%+ mancozeb 64% (72 WP) (0.6%). The carbendazim does not affect the mycelial growth of the pathogen. The propiconazole and azoxystrobin the concentration of dose increases with a decrease in the inhibition of the growth of the pathogen (Fig. 2). The new generation fungicides were inhibiting the mitochondrial respiration by inhibiting the ETS cycle in the pathogen but in the case of providing high nutrient-rich media, especially the double strength media which provide an alternate source for the respiration of the pathogen which may be the reason for the growth in that condition (Matheron and Porchas, 2000).

Matheron and Porchas (2008) claimed that Ridomil gold is effective in the control of the *P. capsici* in the nursery conditions which was supporting the present results. Lokesh *et al*. (2008) observed that Ridomil gold @2.5g/vine was reducing *P. capsici* infection black pepper vines. The effect of Ridomil MZ against *P. capsici* was observed under various studies (Rather *et al*., 2012; Vinitha *et al*., 2016; Moosa *et al*., 2016).

5.6.3. *In vitro* evaluation of organic preparations against the pathogen

The maximum inhibition on the growth of the pathogen (31.11%) was recorded in the case of ready to use neem oil garlic soap at 3 percent followed by garlic extract (28.88%) at 0.3 percent. the least inhibition (8.88%) in the growth of the pathogen was observed in Neemazal at 0.1 per cent (Fig. 3).

The higher concentration of garlic root extract showed maximum inhibition may be due to the mycelial growth of pathogen specifically the hyphal morphology was affected by the extracts of garlic bulb. which inhibits the growth of the pathogen (Khan *et al*., 2011). The neem extract shows an inhibitory effect on the growth of the pathogen as similar to the action of ready to use neem oil garlic soap (Zakari *et al*., 2016; Mohsan *et al*., 2017). The inhibitory action of garlic may be due to the presence of allicin which is an important anti-microbial and antilipidemic agent. This agent exhibits anti-bacterial, anti-fungal, and anti-viral properties, helping to inhibit the growth of the pathogen (Khan and Cheng, 2010).

5.7. *IN VIVO EVALUATION* OF BIOCONTROL AGENTS, ORGANIC PREPARATIONS AND CHEMICAL FUNGICIDES AGAINST THE PATHOGEN

The disease index was calculated after 7 days of growth, observing the lesion size and lesion area. Among these, no symptomatic expression was observed in the case of treatment metalaxyl 8%+ mancozeb 64% (72 WP) (0.6%) followed by copper oxychloride (0.3%) The higher disease index (86.66%) was observed in the case of ready to use neem oil garlic soap (3%) as compared with other treatments (Fig. 4).

The same trend was observed in managing the Phytophthora leaf spot in the betel vine. There was a 76.2 percent reduction in disease severity by the application metalaxyl + mancozeb at 0.2 percent concentration. As the concentration increased the severity of diseases decreased and was very effective in managing the disease under field conditions (Kumar*et al*., 2018).

Under field conditions, the application of metalaxyl MZ 68 WP and potassium phosphonate showed effectiveness against the management of the foot rot disease also concluded that metalaxyl MZ 68 WP was much effective in managing the disease (Lokesh *et al*., 2011).

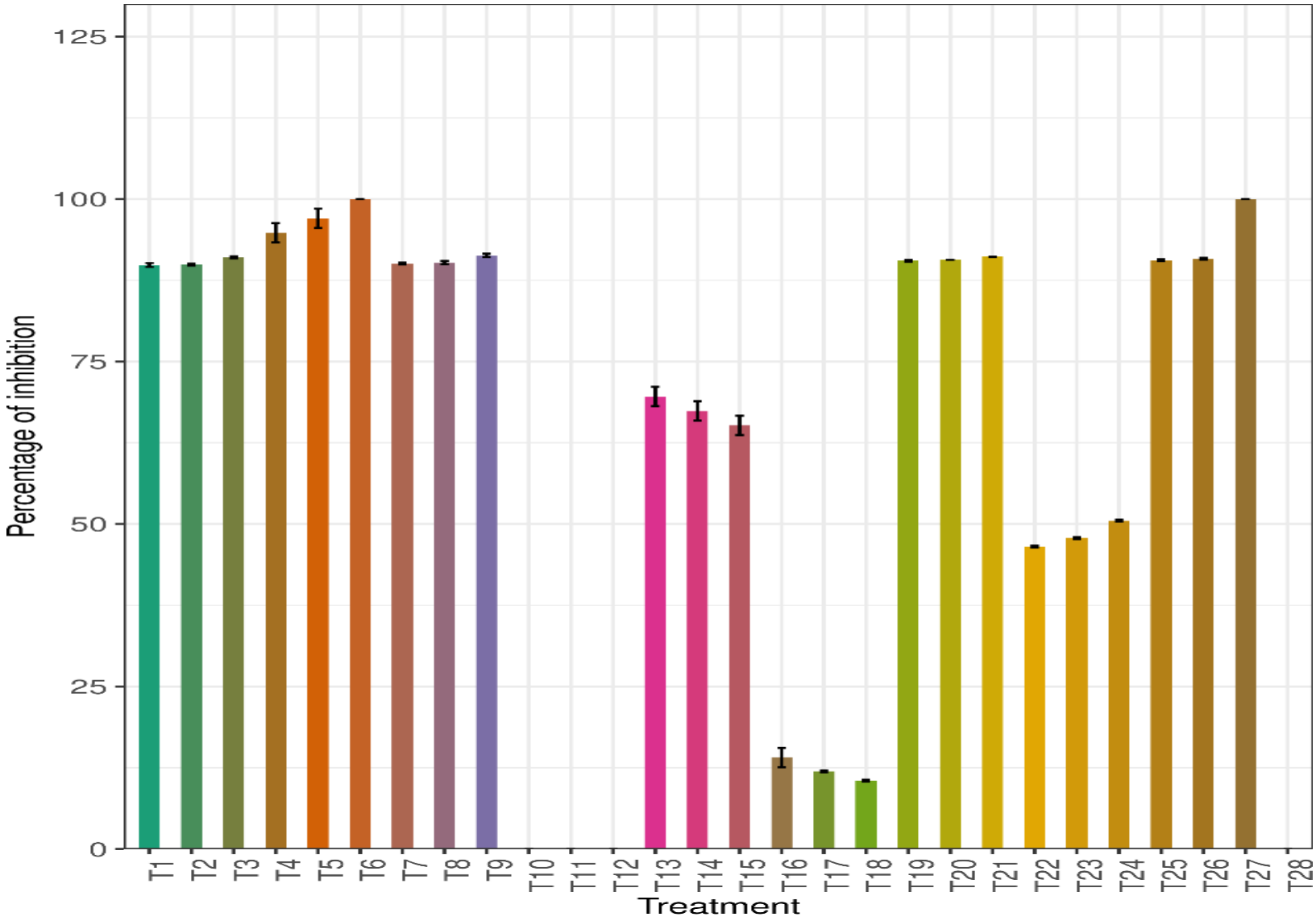
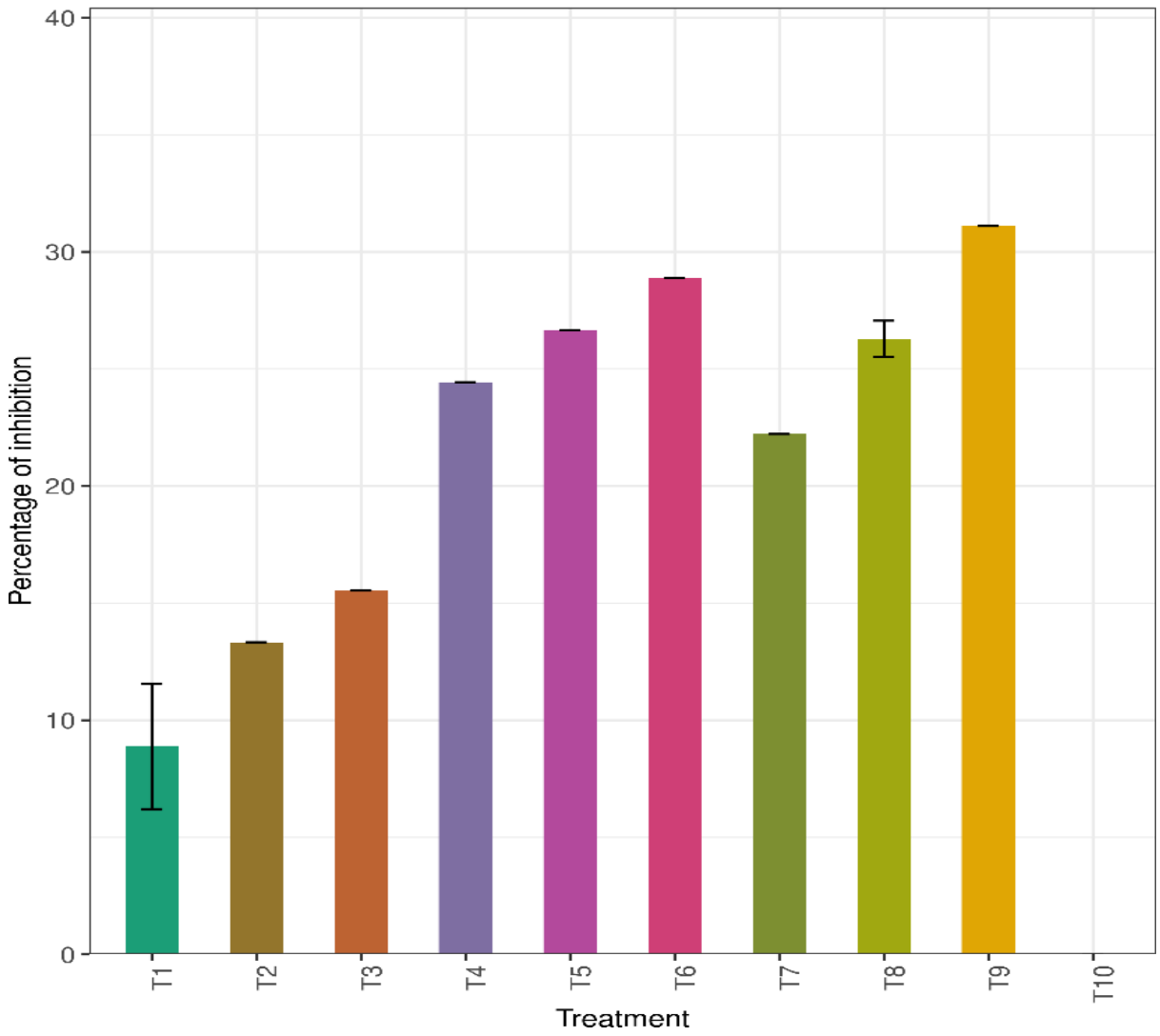


Fig 2. Effect of chemical fungicides on *P. capsici in vitro*



T1-Neemazal (0.1%)

T2-Neemazal (0.2%)

T3-Neemazal (0.3%)

T4-Garlic extract (0.1%)

T5-Garlic extract (0.2%)

T6-Garlic extract (0.3%)

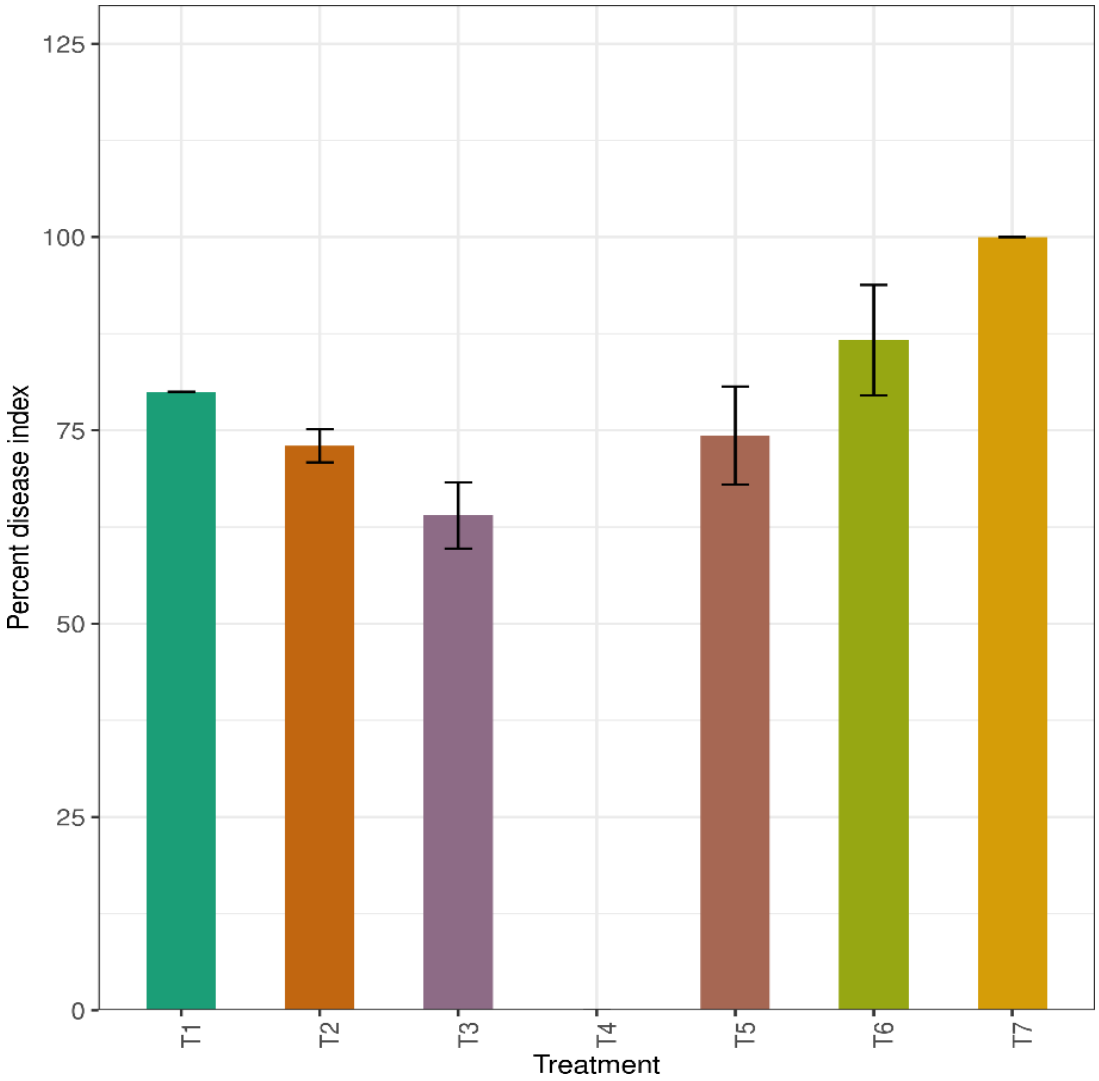
T7-Ready to use neem oil garlic soap (1%)

T8-Ready to use neem oil garlic soap (2%)

T9-Ready to use neem oil garlic soap (3%)

T10-Control

Fig 3. Effect of organic preparations on *P. capsici in vitro*



T1-*Trichoderma harzianum*

T2-*Pseudomonas fluroscens*

T3- Copper oxychloride (0.3%)

T4- Metalaxyl 8%+ Mancozeb 64% (72 WP) (0.6%)

T5-Garlic extract (0.3%)

T6-Ready to use neem oil garlic soap (3%)

T7-Control

Fig 4. Effect of different treatments on percent disease index in black pepper

SUMMARY

6. Summary

Black pepper (*Piper nigrum* L.) is one of the major spice crops and plantation crops are grown as the sole crop or intercrop in Kerala. In nurseries of black pepper major biotic stress is caused by bacteria, fungus, virus and nematode, out of this fungal infections are the leading ones. Among fungal infections, seedling blight, anthracnose and stem rot were found to be more severe in the nursery. With this background, the present study was conducted to identify a major fungal pathogen and formulate management strategies against those pathogens.

The different nurseries at Kannur and Kasargod were visited as part of the survey and collected the samples showing seedling blight symptoms were designated as Pc1 and Pc2 for the isolate from Padannakkad and Kanakappalli respectively. The samples showing anthracnose symptoms were collected from Padannkkad, Balal, Panniyur and Periya as designated as Cg1, Cg2, Cg3 and Cg4 respectively. The basal stem rot showing samples were designated as Sr 1, Sr 2 and Sr 3 for the isolate collected from Padannakkad, Periya and Panniyur respectively.

The disease incidence (DI) and percent disease index (PDI) were calculated and higher DI and PDI were observed in Pc1 as 74 percent and 32 percent respectively as compared with Pc2. In the case of anthracnose, higher DI and PDI were observed in Cg1 as 30 percent and 25 percent respectively as compared with Cg2, Cg3 and Cg4. In the case of basal stem rot higher DI was observed in Sr1 as 20 percent. All the isolates were tested for their pathogenicity, out of this Pc1 from seedling blight, Cg1 from anthracnose and Sr1 from stem rot were taken for its virulence test against the different black pepper varieties including Panniyur 1, Panniyur 2, Panniyur 3, Panniyur 4, Panniyur 5, Panniyur 6, Panniyur 8, Panniyur 9, Vijay and Karimunda. Pc 1 isolate was selected as the most virulent isolate and showed more severe damage as compared to other isolates in the most susceptible variety Panniyur 2 under favourable conditions. Through the cultural, morphological and molecular characterization, it was proved that the isolate Pc1 was *Phytophthora capsici* and symptomatology was studied under natural and artificial conditions.

In vitro evaluation of biocontrol agents, chemical fungicides and organic preparations were carried out to know the efficacy against *P. capsici*. Under *in vitro*

evaluation of biocontrol agents, *T. harzianum* was exhibited 55.56 percent inhibition followed by *P. fluorescens* (53.33%). Among the chemical fungicides, 100 percent inhibition was recorded in copper oxychloride at 0.3 percent and metalaxyl 8% + mancozeb 64% at 0.6 percent Among the organic preparations, the maximum inhibition (31.11%) was observed in ready to use neem oil garlic soap at 3 percent followed by garlic extract (28.88%) at 0.3 percent on the mycelial growth of the pathogen.

The effective treatments, from the *in vitro* evaluation were selected for the pot culture experiment by using the variety Panniyur 2. The *T. harzianum* and *P. fluorescens* (2%), copper oxychloride (0.3%) and metalaxyl 8% + mancozeb 64% (0.6%), garlic extract (0.3%) and ready to use neem oil garlic soap (3%) applied as foliar spray after 24 hours of inoculation. The PDI and lesion development were observed after 7 days of inoculation. The PDI was low in metalaxyl 8% + mancozeb 64% (72 WP) at 0.6 percent (0.00%) and it was higher (86.66%) in ready to use neem oil garlic soap at 3 per cent.

The results from the study put forward that the seedling blight was the major disease in black pepper nursery which was caused by *Phytophthora capsici* (Pc1 isolate). The metalaxyl 8% + mancozeb 64% (72 WP) at 0.6 percent was highly effective to manage the disease under nursery conditions compared with other treatments.

The future line of work should be focused on integrated disease management strategies including natural extracts, antagonistic agents, bioactive principles and newer molecules along with suitable disease forecasting methods. The periodical checking of the isolate for its resistance development help to reduce the incidence and better management of the disease at the field level.

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APPENDICES

APPENDIX - I

COMPOSITION OF MEDIA USED

1. Potato Dextrose Agar

Peeled and sliced potatoes -200 g

Dextrose - 20 g

Agar-Agar - 20 g

Distilled water - 1000 ml

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

2. Potato dextrose broth

Peeled and sliced potatoes -200 g

Dextrose - 20 g

Distilled water - 1000 ml

3. Double strength potato dextrose agar

Peeled and sliced potatoes -400 g

Dextrose - 40 g

Agar-Agar - 40 g

Distilled water - 1000 ml

4. Water agar

Agar - 2 g

Distilled water - 100 g

5. Nutrient agar

Peptone - 5 g

NaCl - 5 g

Beef extracts - 3 g

Agar - 20 g

Distilled water - 1000 ml

APPENDIX- II

COMPOSITION OF STAIN USED

1. Lacto phenol- cotton blue

Anhydrous lacto phenol - 67.0 ml

Distilled water - 20.0 ml

Cotton blue - 0.1 g

Anhydrous lacto phenol was prepared by dissolving 20 g phenol in 16 ml lactic acid and 3 ml glycerol.

APPENDIX- III

Molecular sequence of *P. capsici* isolate

C A T C A A A C T T G G C T T T C T T C C T T C C G T G T A G T
C G G T G G A G G A T G T G C C A G A T G T G A A G T G T C T
T G C G T G T T G T C C T T C G G G T C G G C T G C G A G T C C
T T T T A A A T G T A C T G A A C T G T A C T T C T C T T T G C
T C G A A A A G C G T G G T G T G G C T G G T T G T G G A G G
C T G C C T G C G T G G C C A G T C G G C G A C C G G T T T G T
C T G C T G C G G C G T T T A A A G G A G G A G T G T T C G A
T T C G C G G T A T G G T T G G C T T C G G C T G A A C A G G C
G C T T A T T G A A T G C T T T T C C T G C T G T G G C G T G A
T G G G C T G G T G A A C C G T A G C T G T G T T T G G C T T G
G C T T T T G A A T C G G C T T T G C T G T T G C G A A G T A G
G G T G C C G G C T T C G G C T G T C G A G G G T C G A T C C
A T T T T G G G A

ABSTRACT

Management of a major fungal disease in black pepper (*Piper nigrum* L.) nursery

by

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ABSTRACT OF THE THESIS

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ABSTRACT

Management of a major fungal disease in black pepper (*Piper nigrum* L.) nursery

Black pepper (*Piper nigrum* L.) is one of the major spice crops and plantation crops that grow as the sole crop or intercrop in Kerala. In nurseries of black pepper major biotic stress are caused by bacteria, fungus, virus and nematode, out of this fungal infection are the leading one. The seedling blight, anthracnose and collar rot were found to be more severe in the nursery. With this background, the present study was conducted to identify the major fungal pathogen associated with black pepper nursery and formulate disease management strategies against that pathogen.

A survey was carried out in black pepper growing nurseries to record the different diseases. Seedling blight, anthracnose and collar rot were observed. The fungal isolates associated with seedling blight in nurseries at Padannakkad and Kanakappalli were designated as Pc1 and Pc2 respectively. Anthracnose affected seedlings were collected from Padannkkad, Balal, Panniyur and Periya black pepper nurseries and the fungal isolates associated were designated as Cg1, Cg2, Cg3 and Cg4 respectively. The collar rot samples were collected from Padannakkad, Periya and Panniyur and isolates were designated as Sr1, Sr2 and Sr3.

In the case of seedling blight infection, highest disease incidence (DI) (74%) was recorded in Pc1. The highest percent disease index (PDI) in case seedling blight (32%) was recorded in Pc1 as compared to Pc2 isolate. In the case of anthracnose, higher DI (30%) and PDI (25%) were recorded in Cg1 in comparison with Cg2, Cg3 and Cg4. In case of collar rot, higher DI (20%) was recorded in case of Sr1 isolate.

All the isolates were tested for its pathogenicity; in case of seedling blight, the isolate Pc1 recorded higher radial growth (7.90 cm) and recorded lesser time (2days) for the symptom development as compared to Pc2 isolate. In case of anthracnose, isolate Cg1 recorded higher radial growth (8.0 cm) and took lesser time (5days) for the symptom development in comparison with Cg2, Cg3 and Cg4. In case of collar rot isolate Sr1 recorded higher radial growth (8.96 cm) and recorded lesser time (2days) for the symptom development as compared to Sr2 and Sr3.

A trial was laid out to identify the most susceptible variety of black pepper as well as the most virulent strain of the pathogen among different isolates on pepper varieties *viz*., Panniyur (P) 1-6, P8, P9, Vijay and Karimunda. Panniyur 2 was identified as most susceptible variety and Pc1 was the virulent isolate. Based on cultural and morphological characters and molecular characterization, Pc1 was identified as *Phytophthora capsici*.

In vitro evaluation of biocontrol agents, chemical fungicides and organic preparations were carried out to study the efficacy against *P. capsici*. Under *in vitro* conditions biocontrol agent *T. harzianum* (2%) exhibited maximum inhibition (55.56%) followed by *P. fluorescens* (53.33%). Among the chemical fungicides copper oxychloride (0.3%) and metalaxyl (8%) + mancozeb (64%) at 0.3 percent recorded 100 per cent inhibition. Among the organic preparations, ready to use neem oil garlic soap (3%) and garlic extract at 0.3 percent recorded maximum inhibition 31.11% percent and 28.88% percent respectively in the mycelial growth of the pathogen.

The best-performing treatments from the *in vitro* evaluation were selected for pot culture experiment on variety Panniyur 2. After 7 days of inoculation, the least PDI (0.00 %) as well as the least lesion development (0.00%) was recorded in case of metalaxyl (8%) + mancozeb (64%) at 0.6 per cent. The higher PDI (86.66%), as well as maximum lesion development (46.66%), was recorded in ready to use neem oil garlic soap (3%).

The study reveals that, the seedling blight caused by *Phytophthora capsici* can be effectively managed with the foliar application of metalaxyl (8%) + mancozeb (64%) at 0.6 per cent.

സംഗ്രഹം

കുരുമുളക് നഴ്സറിയിലെ പ്രധാന കുമിൾ രോഗ നിയന്ത്രണം

സുഗന്ധവിളയും വാണിജ്യവിളയുമായ കുരുമുളക് കേരളത്തിൽ പ്രധാനമായും കൃഷി ചെയ്യുന്നത് ഏകവിളയായോ ഇടവിളയായോ ആണ്. തൈചീയൽ, തണ്ടുചീയൽ, തണ്ടുണക്കം എന്നീ രോഗങ്ങളാണ് നഴ്സറിയിൽ സ്വാഭാവികമായും കണ്ടുവരുന്നത്. ഇതിൻ്റെ പശ്ചാത്തലത്തിലാണ്, കാർഷിക കോളേജ് പടന്നക്കാടിൽ ഈ വിഷയത്തെ ആസ്പദമാക്കി 2019-2021 കാലഘട്ടത്തിൽ പ്രസ്തുതപഠനം നടന്നത്.

ജില്ലയിലെ വിവിധ സ്ഥലങ്ങളിൽ സ്ഥിതി ചെയ്യുന്ന നഴ്സറികളിൽ സർവ്വേ നടത്തുകയും വിവിധ രോഗങ്ങളെക്കുറിച്ച് രേഖപ്പെടുത്തുകയും ചെയ്തു. തൈ ചീയൽ, തണ്ടുചീയൽ, തണ്ടുണക്കം എന്നിവയാണ് പ്രധാനമായും നിരീക്ഷിച്ചത്. തൈ ചീയലിനു കാരണമാകുന്ന രോഗകാരിയായ പിസി1, പിസി2 എന്നിങ്ങനെയും തണ്ടുണക്കം ബാധിച്ച തൈകളിൽ നിന്നും ലഭിച്ച കുമിളിനെ സിജി1, സിജി2, സിജി3, സിജി4 എന്നിങ്ങനെയും തണ്ടുചീയൽ ലക്ഷണം കാണിച്ച ചെടികളിൽ നിന്നും ലഭിച്ച കുമിളിനെ എസ്ആർ1, എസ്ആർ2, എസ്ആർ3 എന്നീങ്ങനെയും നാമകരണം ചെയ്തു. രോഗകാരികളായ എല്ലാ കുമിളുകളുടെയും രോഗവ്യാപനശേഷി ശാസ്ത്രീയമായി പരിശോധിച്ചതിൻ്റെ അടിസ്ഥാനത്തിൽ പിസി1 എന്നു നാമകരണം ചെയ്ത ഫൈറ്റോഫ്തോറ കാപ്സൈസി എന്ന കുമിളാണ് രോഗവ്യാപനശേഷി ഏറ്റവും കൂടിയതെന്ന് കണ്ടു പിടിച്ചു. കൂടാതെ രോഗവ്യാപനശേഷി കൂടുതലുള്ള ഇനങ്ങളെ കണ്ടെത്തുന്നതിനായുള്ള പരീക്ഷണത്തിൽ പന്നിയൂർ - 2 എന്നയിനം രോഗം. വരാൻ സാധ്യത കൂടുതലുള്ള ഇനമാണെന്ന് കണ്ടെത്തുകയും ചെയ്തു.

തൈചീയലിനു കാരണമായ ഫൈറ്റോഫ്തോറ കാപ്സൈസി എന്ന കുമിളിനെ പ്രതിരോധിക്കുന്നതിനായി ജൈവ നിയന്ത്രണമാർഗ്ഗങ്ങൾ, രാസകുമിൾനാശിനികൾ, ജൈവിക ഉല്പന്നങ്ങൾ എന്നിവയുടെ കാര്യക്ഷമത പരിശോധിച്ചു. ജൈവ നിയന്ത്രണ മാർഗ്ഗങ്ങളിൽ ട്രൈക്കോഡെർമ ഹാർസിയാനം എന്ന മിത്ര കുമിളും സുഡോമോണാസ് ഫ്ളൂറസൻസ് എന്ന മിത്ര ബാക്ടീരിയയും രാസ കുമിൾനാശിനികളിൽ കോപ്പർ ഓക്സിക്ലോറൈഡും മെറ്റാലാക്സൈൽ - മാൻകോസെബ് മിശ്രിതവും ജൈവിക ഉത്പന്നങ്ങളിൽ റെഡി ടു യൂസ് വേപ്പെണ്ണ വെളുത്തുള്ളി സോപ്പും വെളുത്തുള്ളി സത്തും രോഗകാരിയായ കുമിളിൻ്റെ വളർച്ചയെ പരിമിതപ്പെടുത്തുന്നതായി കണ്ടെത്തി. മികച്ച രോഗം നിയന്ത്രണ മാർഗ്ഗങ്ങളെ തിരഞ്ഞെടുത്ത് പന്നിയൂർ 2 കുരുമുളക് ഇനത്തിൽ പരീക്ഷണം നടത്തിയതിൻ്റെ ഭാഗമായി മെറ്റാലാക്സൈൽ -മാൻകോസെബ് മിശ്രിതം തളിച്ച തൈകളിൽ രോഗ തീവ്രതയും രോഗവ്യാപനവും വളരെയധികം കുറഞ്ഞതായി കണ്ടെത്തി. കുരുമുളക് നഴ്സറിയിലെ വിവിധ രോഗങ്ങൾക്കെതിരെ നൂതന നിയന്ത്രണ മാർഗങ്ങളും സംയോജിത രോഗനിയന്ത്രണ മാർഗങ്ങളും വികസിപ്പിച്ചെടുക്കുന്നതിന് ഇനിയും കൂടുതൽ പഠനം ആവശ്യമുണ്ടെന്ന് പ്രസ്തുത പഠനം തെളിയിച്ചു.