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Morpho-Molecular Characterization and *In vitro* Management of the *Pestalotiopsis palmarum* (Cooke) Steyaert Causing the Grey Blight of Coconut

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The solitary species of the genus Cocos is the coconut palm (*Cocos nucifera* L.). The coconut palm is affectionately known as "Kalpa Vriksha," which translates to "the tree of heaven. The causal organism of grey blight was identified based on the morphological characteristics *i.e.,* five-celled conidia had three middle cells that ranged in colour from light brown to dark brown further confirmed through PCR analysis as *Pestalotiopsis palmarum*. In cultural characteristics, maximum radial growth occurred in PDA, with V-8 juice agar showing the least growth (65.00 mm). The optimal pH

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for pathogen growth was 6 (338.11 mg). Fungicide evaluation revealed ziram as highly efficient among non-systemic fungicides, while carbendazim and thiophonate methyl were most effective among systemic ones. Trifloxystrobin 25% + tebuconazole 50% EC emerged as the most effective combi product, inhibiting pathogen growth by 88.02%. Among bio-agents, *T. viride*-2 displayed the highest mycelial inhibition (71.30%). Turmeric exhibited the highest botanical efficacy (48.03%), whereas lantana had the least impact (3.37%).

Keywords: Grey blight; Pestalotiopsis palamrum; acervuli; potato dextrose agar and fungicides.

1. INTRODUCTION

The solitary species of the genus Cocos is the coconut palm (*Cocos nucifera* L.), a member of the palm family *Arecaceae*. Coconut fruit is a drupe and not a real nut. A drupe is a fruit with a hard, rocky covering that encloses the seed (like a peach or an olive), and the term "drupe" originally meant an overripe olive. It has three layers: an exocarp, a mesocarp and an endocarp, just like other drupe fruits. The husk of the coconut is composed of the exocarp and mesocarp. The toughest section of the coconut, known as the mesocarp or "shell," is now visible. It is made of coir fibres. Once the husk is removed, the shell's three germination pores (stoma) or eyes are seen on its exterior. The tough, woody covering that covers the seed is known as the endocarp. It is a sizable palm that can reach heights of up to 30 meters (98 feet), is a perennial crop categorized as a fibrous one-seeded drupe and has fonds that are 4 to 6 metres (13–20 feet) long and pinnate leaves that are 60 to 90 cm long. Old leaves easily fall off, leaving the trunk smooth. The coconut palm is affectionately known as "Kalpa Vriksha," which translates to "the tree of heaven." Among many other things, it may be used to make food, fuel, cosmetics, traditional medicines, and building materials [1,2].

In India, there are 18 States and three Union Territories where coconut is grown. In India, four southern states—Kerala, Tamil Nadu, Karnataka, and Telangana— produce more than 90 per cent of the nation's coconuts. The crop, which was previously thought to only be produced in coastal regions, has now spread to unorthodox locations in the eastern and north-eastern regions of the nation. Even in traditional states, coconut farming has moved from coastal regions to interior regions. In addition to the 3975 crores in export revenue, coconut contributes more than Rs. 34100 crores to the GDP of the nation. More than 10 million people in India and 80 million people globally rely on the coconut industry for their livelihood [3].

The world's largest plantation crop, coconut, is cultivated on 11,906,000 hectares and yields 67128 million nuts. Karnataka is a large producer of coconut in India, with an area of 619.78 thousand hectares and a production of 4947.74 million nuts. In all, India produces coconuts on 21288.24 million hectares. In India, Kerala produces the most coconuts, followed by Tamil Nadu and Karnataka [1].

More than a dozen fungi have been reported from India to cause various diseases of coconut leaves. About 40 foliar disease-causing fungi have been reported in the world's largest coconut-producing countries. Of these, grey rot was the most common disease, reported in 28 countries (Joseph and Radha, 1979; Koshi, 2000 and Doraiswamy et al. 2003). A preliminary field survey revealed several leaf diseases with different symptoms in the main coconut-growing areas in Karnataka [4].

Since no specified findings, it became deemed important to conduct research on coconut grey blight in Karnataka. A survey on the grey leaf blight of coconut with inside the subject offers statistics approximately the volume of ailment prevalence and severity at the coconut palm leaves in different places and additionally the volume of harm at the yield of palm. Grey blight ailment is widespread, in particular on wet season/ excessive moisture conditions or even in potassium deficiency soils. Initially taken into consideration as a minor ailment, nowadays, turning into a chief ailment infecting the coconut palms; however, a systematic survey at the prevalence and severity within the southern element of Karnataka is lacking [5].

Studies on the morphological and cultural characteristics of the pathogen are very important to understand the nature and variability of the pathogen. Physiological studies help determine the optimal pH and temperature for a pathogen to grow and cause infection in the palm of your hand. The molecular characterization of

the pathogen is very crucial for confirmation of the pathogen.

There is little or no information on the control of grey blight of coconut. However, many chemicals are available on the market today and their bio-efficacy and suitability need to be verified by *in vitro* studies. Later it should be extended to the field condition.

2. MATERIALS AND METHODS

2.1 Collection, Isolation and Identification

Grey leaf blight infected leaves were collected from places *viz*., Bengaluru (GKVK) and Mandya district and used to isolate the fungus under *in vitro*. The fungus isolation was made by following a standard tissue isolation technique, as described by Petrini et al*.* [6]. Hyphal tip culture. As soon as the mycelial growth was observed in Petri plates, advancing hyphal tips growing out of tissue segments were cut off with sterilized inoculation needle and transferred to potato dextrose agar medium and allowed to grow for 20 days at 26 ± 1ºC and regularly observed for appearance of the conidia in the culture. The morphological characters of the fungus, such as mycelial and cultural characters, length and breadth of conidia, fruiting body were studied by using morphological traits following Maharachchikumbura et al*.* [2].

2.2 Proving Pathogenicity

Healthy leaflets were artificially inoculated by spraying the conidial suspension of *P. palmarum* (2.4×10^6 spore/ mL) on leaves. The plants without conidial suspension spray served as control. Observations were recorded every third day for two weeks. Re-isolation was made from the infected leaves and incubated at 26 ± 1 ^{o}C. Then that culture was compared with that of the original culture.

2.3 Cultural Characters of *P. palmarum* on Different Solid Media

The cultural characteristics of *P. palmarum* such as colony colour, colony diameter, nature of colony margin, zonation of the colony, pigmentation, colony texture and sporulation were studied on following different solid media *viz*., Potato dextrose Agar, Czapeck's Dox agar, Potato carrot agar V8 juice agar, Richard's agar, Oat meal agar, Corn meal agar and Sabouraud agar

2.4 Sporulation count

15 mL of each medium listed above was poured into petri plates of 90 mm diameter and allowed to solidify. Such plates were inoculated with 5 mm discs from 10 days old *P. palmarum* cultures which were cut by using a cork borer and a single disc was placed upside down at the centre of the plate. Each set was replicated thrice and the plates were incubated at 27±1°C. Observations on the colony diameter, rate of growth, colony colour, pigmentation, type of margin, colony texture and zonation on different solid media were recorded when the maximum growth was attained in any one of the media tested. Using a transparent plastic scale linear growth of the colony was measured in millimetre. In addition, the sporulation was observed from 10 days old culture of each isolate by making the spore suspension. A single block of 5 mm diameter was cut out from the fungal colony near the margin by sterilized cork borer. It was transferred to 5 ml sterile distilled water in a test tube, where it was mixed thoroughly to make a uniform spore suspension. One small drop of spore suspension was taken on a slide and the average spore count of three microscopic fields under low power (10X) objective of the microscope. The sporulation was graded as follows.

List 1. Spore count and gradation

Sl. No.	Sore	Grade	Conidia/ microscopic field (10X)
1	++++	Excellent	>75
2	+++	Good	51-75
3	++	Fair	26-50
4	+	Poor	1-25
5	-	No sporulation	--

2.5 Molecular Identification

2.5.1 DNA extraction of *P. palmarum* by C-TAB method

The mycelium of fungus collected from the potato dextrose broth after 7 days of incubation was filtered by using Whatsman No.40 filter paper. The mycelia were later dried by pressing in between folds of pre-autoclaved filter papers. The DNA extraction of fungus was carried out by following C-TAB method [7].

2.5.2 Sequencing of 18S rDNA region and in silico analysis

The 18S rDNA region was sequenced to confirm the identity of the organism. The PCR product was sequenced in both the directions using Sanger di-de-oxy method at Europhins, Bengaluru. The homology search was done using BLAST algorithm available at the VA3T.ncbi.nlm.nih.gov. Multiple alignments for homology search were performed using the Clustul W algorithm software and the phylogenetic tree was constructed.

2.6 Physiological Studies

2.6.1 Effect of temperature on growth of *P. palmarum*

The growth of the fungus was tested at 15, 20, 25, 30 and 35^{o} C. 15 ml of each medium was poured into each Petri plate and allowed to solidify. Such plates were inoculated with 5 mm discs of the pathogen cut from the periphery of the actively growing culture and incubated at 27 ±1 °C temperature. The experiment was conducted by using a Completely Randomized Design (CRD) and each treatment was replicated thrice. Observations were taken when the growth of any culture covers the entire Petri plate to know the optimum temperature for growth and development of test fungus.

2.6.2 Effect of hydrogen ion concentration on the growth of *P. palmarum*

The growth of the pathogen was tested at six different pH levels *viz*., 4, 5, 6, 7, 8, 9, respectively. The hydrogen ion (pH) concentration of the potato dextrose agar was determined by using pH meter. Adjustment of pH was done using 0.1 N alkali (Sodium hydroxide) and 0.1 N acid (Hydrochloric acid) and was sterilized in an autoclave at 121.6^{o} C for 15 minutes. 100 mL flasks containing 50mL of sterilized broth, 5 mm disc of the pathogen will be inoculated and incubated at 27 ±1 °C temperature. The experiment was conducted by using a Completely Randomized Design (CRD) and each treatment was replicated thrice. The ideal pH for growth of the fungus was determined by harvesting mycelial mat that will be filtered through Whatsman filter paper and dry mycelial weight observation was recorded at 9 days after incubation.

2.6.3 In vitro evaluation of fungicides against *P. palmarum*

Systemic, contact and combi product fungicides were evaluated at different concentrations under *in vitro* conditions. Nine systemic fungicides at the concentration of 100, 250 and 500 ppm, seven contact and five combi fungicides at the concentration of 250, 500 and 1000 ppm were evaluated against the pathogen under laboratory conditions by poisoned food technique using potato dextrose agar medium.

The poisoned medium was prepared by adding the required quantity of fungicides to the melted potato dextrose agar medium to obtain the desired concentration, 15 mL of poisoned medium was poured in each sterilized Petri dish and suitable checks were maintained without fungicides. Five mm of ten days old fungal disc taken from the periphery of the culture was placed in the centre of poisoned medium and incubated at 27 ± 1 °C. The experiment was conducted by using Completely Randomized Design (CRD) and each treatment was replicated thrice. The observations were recorded when the fungal growth was maximum in the untreated control. The per cent Inhibition of mycelial growth over the control was calculated using the formula [8].

$$\mathbf{I} = \frac{\mathbf{C} - \mathbf{T}}{\mathbf{C}} \times \mathbf{100}$$

Where,

I = Per cent growth inhibition of mycelium,
C = Growth of mycelium in control
T = Growth of mycelium in treatment

2.6.4 In vitro evaluation of bio-agents against *P. palmarum*

The antagonistic potential of bioagents *viz. Trichoderma viride, Trichoderma harzianum, Pseudomonas fluorescence* and *Bacillus subtilis* were tested by dual culture technique. The bacterial antagonists were streaked with a sterilized inoculating loop at one end of the PDA Petri plates. Just opposite to the bacterial streak 5 mm disc of the pathogen was placed with a sterilized cork borer. The inoculation of the pathogen alone on the centre in the plates serves as a control. The experiment was conducted by using CRD. Four replications of each treatment, including the control, were maintained. These plates were incubated at 27 ±

1 °C. The efficacy of antagonistic organisms will be recorded by measuring the colony diameter of the pathogen in each treatment and compared with the control. Per cent inhibition over control was calculated by using the formula given by Vincent [8].

2.6.5 In vitro evaluation of botanicals against *P. palmarum*

The efficacy of botanicals was tested against *P. palmarum* on PDA medium by using a poisoned food technique. For this, fresh plant parts of 100 g of each as mentioned below were collected and washed with tap water and crushed by adding sterile water of 100 ml. this solution gave 100 per cent and was used as stock solution. 5, 10, 15, and 20 ml of stock solution was mixed with 95, 90, 85 and 80 ml of PDA medium and then it was shaken for uniform mixing of plant extract. Later, the media was sterilized and allowed to cool. Twenty ml of medium was poured into sterilized Petri plates and then fungal disc of 5 mm was placed at the centre of the Petri plate and such plates were incubated at 27 ± 1 °C. the control plate was maintained on PDA medium without any plant extract. The radial growth of the fungus was recorded in treatment plates when colony growth reached the periphery in the control plate. The per cent inhibition of mycelial growth of test fungus over control was calculated using the formula [8].

3. RESULTS AND DISCUSSION

3.1 Isolation of Pathogen

Infected leaves with the typical symptoms were collected and the isolation was made using the standard isolation procedure under aseptic conditions (Fig. 2)

3.2 Identification of Pathogen

A white-coloured colony with a regular margin and cottony texture was observed in the isolated pathogen. However, several black-coloured fruiting bodies (acervuli) were visualized after fifteen days of isolation of the pathogen (Fig. 1).

The microscopic studies of the isolated fungus revealed that the conidia of the pathogen were five-celled, with three median cells that ranged in colour from brown to dark brown, while the apical and basal cells were hyaline. The basal appendages were hyaline, smooth, or even curled. There were e to three setulae, which are apical appendages (Fig. 2).

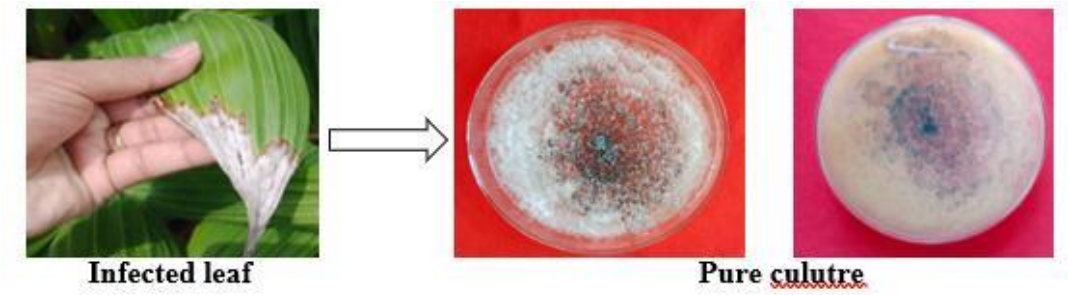
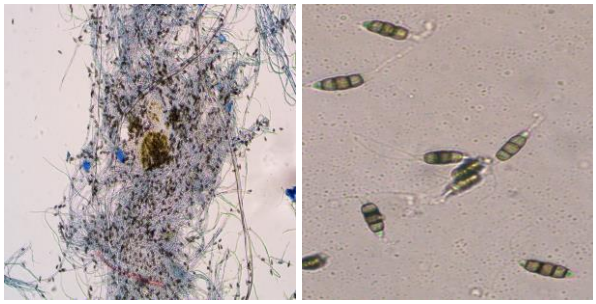


Fig. 1. Isolation of *Pestalotiopsis palmarum* from infected coconut leaves



a. Fruiting body Acervuli b. 5-celled conidia

Fig. 2. Morphological characteristics of *P. palmarum*

The present findings are supported by Fernandez *et al*. (2015) who reported that *Pestalotiopsis* on blueberry produced white cottony colonies on potato dextrose agar that grew to a diameter of three cm in just five days. There were several black, conidiomata with acervuli that exuded conidial masses. The conidia were straight, five-celled, and fusiform.

Similar findings were seen by Pruthviraj [9]. who identified *Pestalotiopsis microspora* on pomegranate with three to five-celled conidia, of which apical and basal cells were hyaline and three median cells were light brown with varying shades of olive-green colour. They also contain both apical and basal appendages

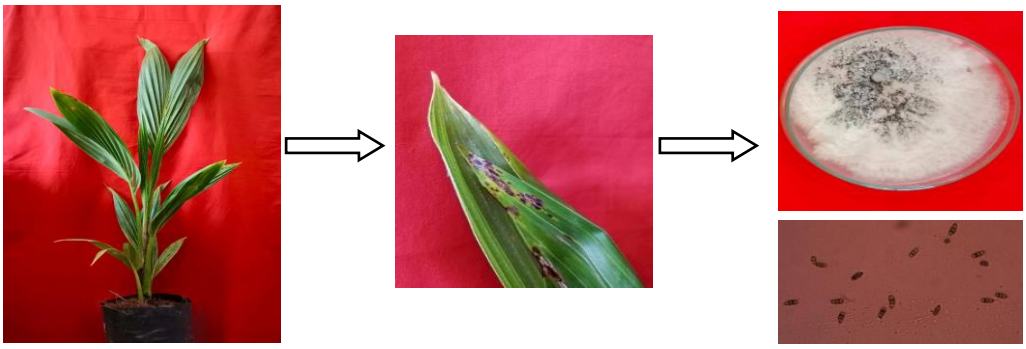
3.3 Proving the Pathogenicity

For the pathogenicity test, the pin-prick method was used on the leaf and observed for the development of the symptoms. Grey blight with brown to greyish centre surrounded by an irregular dark brown margin developed on inoculated leaves. Re-isolation of the pathogen from the symptom-developed area of leaves resulted in similar cultural and morphology to the earlier isolated pathogen (Fig. 3). The similar reports were recorded by Rokade [10].

3.4 Cultural Studies

P. palmarum growth on eight different solid media was tested in the present investigation. The differences in the colony characters among different solid media were observed and recorded *viz*., colour of colony, colour of margin, colony texture, pigmentation (Table 1 and Fig. 4).

Similar results were seen by Pruthviraj [9] who reported that maximum radial growth of *P. microspora* was observed on potato dextrose agar (90.00 mm), and oatmeal agar (90.00 mm). The results are in conformity with the findings of Kyada (2006) who observed that Richard's medium, corn meal agar and potato dextrose agar medium in solid-state are the most suitable media for the growth of *P. guepinii.*



Healthy plant **Development of symptoms** **Re-isolation of the pathogen**

Fig. 3. Pathogenicity of P. palmarum on coconut seedling

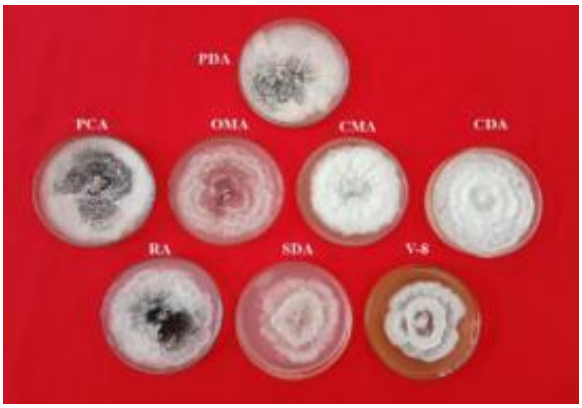


Fig 4. Effect of different solid media on growth of *Pestalotiopsis palmarum*

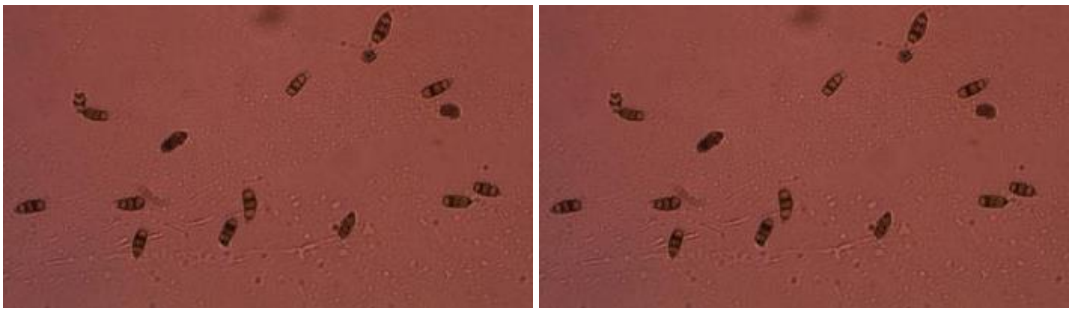


Fig. 5. Effect of different cultural media on the growth of *P. palmarum*

Table 1. Effect of different cultural media on the growth of *P. palmarum*

Sl. No.	Different media	Radial growth (mm)*	Colony colour	Margin of colony	Texture of colony	Growth nature	Pigmentation	Sporulation
1	Potato dextrose agar	90.00	white	regular	cotton	Aerial, raised	White	++++
2	Potato Carrot agar	89.00	white	Regular smooth	Slightly cottony	Surface growth	Black	++++
3	Oat meal agar	87.66	White	Irregular wavy	Cottony	Fluffy. Slightly raised	White	++
4	Corn meal agar	85.00	Dull white	Wavy	Sparsely cottony	Surface growth	Black	++++
5	Czapek's Dox agar	85.00	Dull white	Irregular	Sparsely cottony	Aerial, raised	Black	++++
6	Richard's agar	80.00	White	Regular smooth	Cottony	Fluffy	White	++
7	Sabouraud dextrose agar	70.00	Dull white	Irregular	Slightly cottony	Surface growth	Black	++
8	V8-juice agar	67.18	White	Irregular	Slightly cottony	Surface growth	White	+++
SEm ±		0.368						
CD @ 1%					1.106			

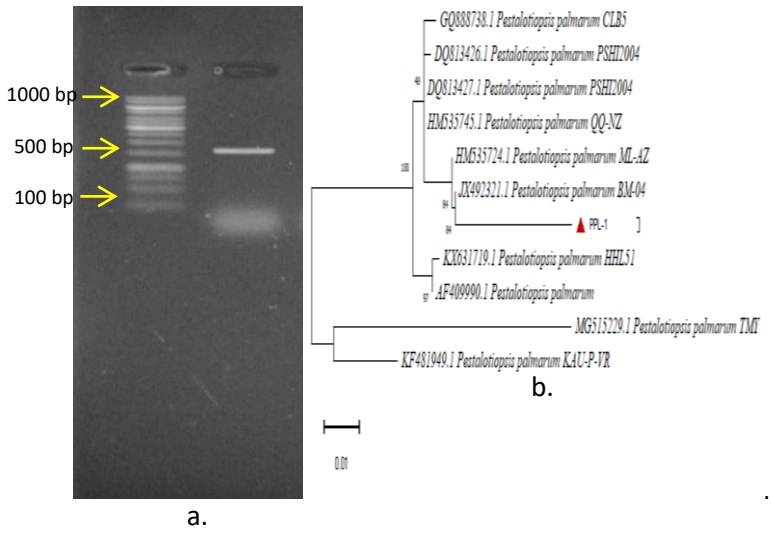
The growth on all solid media varied from a range of 65.00 mm to 90.00 mm. The radial growth of *P. palmarum* was significantly recorded highest on PDA (90 mm) which was followed by Potato Carrot agar (89.00 mm), Oatmeal agar (88.00 mm), Corn meal agar (85.00 mm), Czapek's Dox agar (85.00 mm), Richard's agar (80.00 mm), Sabouraud dextrose agar media (70.00 mm) whereas significantly least radial growth of *P. palmarum* was observed on V-8 juice agar (65.00 mm). Colony growth of the fungus was significantly different except in potato dextrose agar and potato carrot agar; Czepek's Dox agar and Corn meal aga.

The results in this study are confirmed by the findings of Rokade [10] who tested eight media, including semi-synthetic and synthetic in the solid and liquid state and found that potato dextrose in the semi-synthetic group, Czapeck's Dox and Richards' agar in the synthetic group proved significantly superior over the rest of the media for growth and sporulation of *P. palmarum.* Sporulation grading followed as mentioned in the material and methods.

3.5 Molecular Confirmation of the Pathogen *Pestalotiopsis palmarum*

The DNA from grey blight fungi was isolated from coconut and amplified through PCR using universal ITS primers *viz.,* ITS1 and ITS4. The amplified product was later subjected to gel electrophoresis in 1.5 per cent agarose gel. The PCR product was amplified with an amplicon size of 550 bp (Fig. 6).

The homology search was done by using the bioinformatics tool NCBI (National Centre for Bioinformatics) BLAST programme. The



a. b.

Fig. 6. a. PCR amplification of 18s rDNA fragment from the sample, b. Phylogenetic tree constructed using Mega X software

amplicon sequence showed 98.7 per cent similarity with the existing *P. palmarum* in NCBI Genebank through BLAST analysis. It was clearly confirmed that the grey blight of coconut is mainly caused by *Pestalotiopsis* spp. Phylogenetic tree was constructed with the aid of Mega X software. Similarly, the identification of fungal pathogens through phylogenetic relationships was successfully documented.

3.6 Physiological Studies

3.6.1 Effect of temperature on growth of *Pestalotiopsis palmarum* on liquid media

Among the different levels of temperature evaluated, the maximum dry mycelial weight of 312.66 mg was obtained at 25 °C which was significantly superior to other treatments, followed by the temperature of 30 °C with 259.00 mg growth.

Further, 231.56 mg of dry mycelial growth was found at 20 °C temperature, followed by a temperature of 35 °C with 201.7 mg and 15 °C with 160.43 mg of dry mycelial weight. And the minimum dry mycelial weight was obtained at the temperature of 5°C which was on par with the temperature of 10°C with 16.30 mg and 21.41 mg of dry mean mycelial weight (Table 2 and Fig. 6). Zahra Ibrahim El-Gali [11] evaluated the effects on the mycelial growth of three species of *Pestalotiopsis* (*P. fici*, *P. guepinii,* and *P. palmarum)* at different temperatures and pH.

Table 2. Effect of temperature on the growth of *Pestalotiopsis palmarum*

Sl. No.	Temperature (^{0} C)	Potato dextrose broth
		Dry mycelial weight (mg)
1	5	16.30*
2	10	21.41*
3	15	160.43
4	20	231.56
5	25	312.66
6	30	259.00
7	35	201.7
SEm ±		4.379
CD @ 1%		13.137

**Insignificant difference*

3.6.2 Effect of Hydrogen-ion concentration (pH) on the growth of *Pestalotiopsis palmarum* in liquid media

The hydrogen ion concentration influences the growth of fungi, this study was done to know the optimum pH required for the growth of *P. palmarum,* dry mycelial weight was noted at different pH levels *viz.,* 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0.

The growth of the pathogen was supported at all the different pH tests. The highest dry mycelial weight of *P. palmarum,* was recorded at the pH level 6.0 with a dry mycelial weight of

338.11 mg, followed by pH 5.00 (295.89 mg), pH 7.00(267.83 mg), pH 4.00 (201.42 mg), pH 8.00 (172.44 mg) and the lowest dry mycelial weight

was recorded at 9.00 pH with the dry mycelial weight of 134.19 mg (Table 3 and Fig. 7). Majumdar and Mandal [12] showed similar results.

Table 3. Effect of hydrogen ion concentration (pH) on growth of *Pestalotiopsis palmaru* in potato dextrose broth medium

Sl. No.	pH level	Dry mycelial weight (mg)
1	4.0	201.42
2	5.0	295.89
3	6.0	338.11
4	7.0	267.83
5	8.0	172.44
6	9.0	134.19
SEm ±		6.94
CD @ 1%		21.65

Fig. 7. Effect of temperature on the growth of P. palmarum on potato dextrose broth

3.6.3 *In vitro* evaluation of fungicides against *Pestalotiopsis palmarum*

Various fungicides have been evaluated under *in vitro* conditions, Six non-systemic fungicides, nine systemic fungicides and five combination product fungicides were evaluated against *P. palmarum* at four different concentrations in the laboratory by the poisoned food technique.

3.6.4 *In vitro* evaluation of non-systemic fungicides against *Pestalotiopsis palmarum*

The per cent inhibition of *Pestalotiopsis palmarum* mycelial growth at three different concentrations (500 ppm,750 ppm and 1000 ppm) of six non-systemic fungicides was observed (Table 4 and Fig. 8).

Among the different non-systemic fungicides which were evaluated, ziram and captan were found to be significantly superior and on par with mean mycelial inhibition of 93.58 and 93.52 per cent respectively. Ziram and captan at 1000 ppm concentration had cent per cent inhibition. The mean mycelial inhibition of 100, 93.70 and 87.04 per cent was observed at 1000 ppm, 750 ppm and 500 ppm in ziram respectively. The mean mycelial inhibition of 100, 92.04 and 88.52 per cent was observed at 1000 ppm, 750 ppm and 500 ppm in captan respectively. Mancozeb and chlorothalonil showed the mean mycelial inhibition of 76.48, 66.48, 60.93 and 73.15, 66.11, 57.96, at 1000 ppm, 500 ppm and 250 ppm respectively. Next to chlorothalonil, copper oxychloride had a mean mycelial inhibition of 62.78 per cent and the mean mycelial inhibition at 1000 ppm, 500 ppm, 250 ppm were 71.48, 69.63, 47.22 per cent. The least per cent mean mycelial inhibition was recorded in zineb (57.78) and the per cent mycelial inhibition at 1000 ppm, 500 ppm, 250 ppm was 83.70, 64.26 and 25.37 per cent respectively. The results are in support by the findings of Vishwas (2020) who showed that captan was found to be effective in inhibiting *P. microspora* growth of 89.99 per cent.

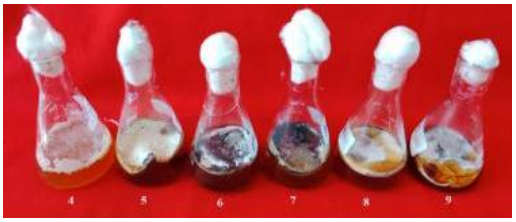


Fig. 8. Effect of Hydrogen ion concentration (pH) on the growth of P. palmarum on potato dextrose broth

3.6.5 *In vitro* evaluation of systemic fungicides against *Pestalotiopsis palmarum*

The per cent inhibition of *Pestalotiopsis palmarum* mycelial growth at three different concentrations (100, 250 and 500 ppm) of nine systemic fungicides were observed results are noted in the Table 5 and Fig. 9.

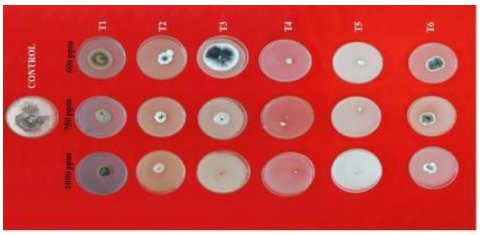


Fig. 9. *In vitro* evaluation of non-systemic fungicides against *P. palmarum*
T1:Copper oxy chloride, T2:Mancozeb, T3:Zineb, T4:Captan, T5:Ziram, T6:Chlorothalonil

Among the nine different systemic fungicides tested, cent per cent mycelial inhibition of *Pestalotiopsis palmarum* was recorded in carbendazim and thiophonate methyl in all three concentrations (100 ppm, 250 ppm and 500 ppm), mean of these two fungicides show statistical superior to all other fungicides. Further, propineb exhibited mean inhibition of 93.21 per cent and cent per cent mycelial inhibition at concentrations of 500 ppm, while 88.52 and 91.11 per cent inhibition was observed at 100 ppm and 250 ppm respectively. Tebuconazole and kresoxim methyl recorded mycelial inhibition of 78.15, 78.52, 81.30 and 66.3, 76.3, 87.59 per cent mycelial inhibition at 100 ppm, 250 ppm, 500 ppm respectively.

Propiconozole, difenconozole at 100 ppm, 250 ppm, 500 ppm recorded per cent mycelial inhibition of 61.48, 78.15, 78.52 and 44.63, 61.48, 78.15 respectively. Whereas the least mean mycelial inhibition was recorded in hexaconazole (48.15 %) which was on par with trifloxystrobin (49.81 %). The least mycelial inhibition was recorded in hexaconazole with 43.33, 44.63 and 61.48 per cent at 100 ppm, 250 ppm and 500 ppm respectively. The findings are in collaboration with the earlier findings of Surichandraselvan et al. [13].

Table 4. *In vitro* evaluation of non-systemic fungicides against *P. palmarum*

Sl. No.	Non-systemic fungicides	Per cent inhibition over control			
		Concentration			
		500 ppm	750 ppm	1000 ppm	Mean
1	Copper oxy chloride 50% WP	47.22 (43.39) *	69.63 (56.54) *	71.48 (57.70) *	62.78 (52.38) *
2	Mancozeb 75% WP	60.93 (51.29) *	66.48 (54.60) *	76.48 (60.97) *	67.96 (55.51) *
3	Zineb 75% WP	25.37 (30.23) *	64.26 (53.26) *	83.70 (66.16) *	57.78 (49.45) *
4	Captan 50% WP	88.52 (70.17) *	92.04 (73.58) *	100.00 (89.96) *	93.52 (75.22) *
5	Ziram 27% SC	87.04 (68.87) *	93.70 (75.44) *	100.00 (89.96) *	93.58 (75.29) *
6	Chlorothalonil 50% WP	57.96 (49.56) *	66.11 (54.38) *	73.15 (58.77) *	65.74 (54.15) *
Mean		61.17 (51.44) *	75.37 (60.22) *	84.14 (66.50) *	73.56 (59.03) *
		Fungicides (F)	Concentration (C)	Interaction (F×C)	
SEm ±		0.30	0.22	0.53	
CD @ 1%		1.17	0.83	2.03	

**Figures in the parenthesis are arc sine transformed value*

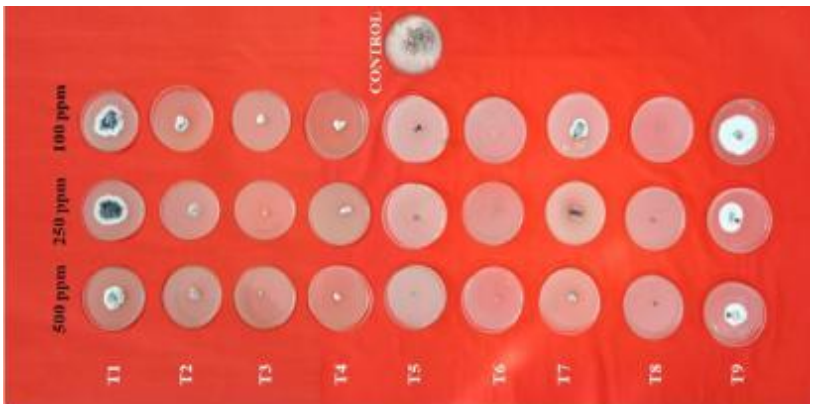


Fig. 10. *In vitro* evaluation of systemic fungicides against *P. palmarum*

T1:Trifloxystrobin, T2:Difenconazole, T3:Propiconazole, T4:Tebuconazole, T5:Propiconazole, T6:Thiophonate methyl, T7:Kresoxim methyl T8:Carbendazim, T9:Hexaconazole

Table 5. *In vitro* evaluation of systemic fungicides against *Pestalotiopsis palmarum*

Sl. No.	Systemic fungicides	Per cent inhibition over control Concentration			
		100 ppm	**250 ppm**	**500 ppm**	**Mean**
1	Trifloxystrobin 50% WG	43.33 (41.15) *	44.63 (41.90) *	61.48 (51.62) *	49.81 (44.88) *
2	Difenconazole 25% EC	44.63 (41.90) *	61.48 (51.62) *	78.15 (62.11) *	61.42 (51.58) *
3	Propiconazole 25% EC	61.48 (51.62) *	78.15 (62.11) *	78.52 (62.36) *	72.72 (58.49) *
4	Tebuconazole 25%EC	78.15 (62.11) *	78.52 (62.36) *	81.30 (64.35) *	79.32 (62.93) *
5	Propineb 70% WP	88.52 (70.17) *	91.11 (72.62) *	100.00 (89.96) *	93.21 (74.87) *
6	Thiophonate methyl 70%WP	100.00 (89.96) *	100.00 (89.96) *	100.00 (89.96) *	100.00 (89.96) *
7	Kresoxim methyl 44.3% SC	66.30 (54.49) *	76.30 (60.84) *	87.59 (69.35) *	76.73 (61.13) *
8	Carbendazim 50% WP	100.00 (89.96) *	100.00 (89.96) *	100.00 (89.96) *	100.00 (89.96) *
9	Hexaconazole 5% EC	33.15 (35.16) *	50.19 (45.09) *	61.11 (51.40) *	48.15 (43.92) *
	Mean	68.40 (59.61) *	75.60 (64.05) *	83.13 (70.12) *	75.71 (60.45) *
		Fungicides (F)	Concentration (C)	Interaction (F×C)	
	SEm ±	0.30	0.17	0.52	
	CD @ 1%	1.13	0.65	1.95	

**Figures in the parenthesis are arc sine transformed values*

3.6.6 *In vitro* evaluation of combi products against *Pestalotiopsis palmarum*

Five different combi-product fungicides were tested at three concentrations *viz*., 250 ppm, 500 ppm, 1000 ppm. The per cent inhibition of *Pestalotiopsis palmarum* mycelial growth in different combi product fungicides were observed and results are noted in the Table 6 and Fig.10. Out of five tested combi-product fungicides in this study, trifloxystrobin 25 per cent + tebuconazole 50 per cent EC was the most effective and superior over the other fungicides followed by iprovalicarb 5.5 per cent + propineb 61.5 per cent with mean mycelial inhibition of 84.75 per cent and per cent mycelial inhibition was 81.85, 85.93 and 86.48 at 250 ppm, 500 ppm and 1000 ppm respectively.

Captan 70 per cent + hexaconazole 4 per cent WP showed 71.30, 79.07 and 88.52 per cent mycelial inhibition at the concentrations of 250 ppm, 500 ppm, and 1000 ppm respectively. While tricyclazole 18 per cent + mancozeb 62 per cent WP speculated with 60.04, 76.85 and 84.26 per cent mycelial inhibition at concentration of 250 ppm, 500 ppm, and 1000 ppm respectively. The least mean mycelial inhibition of 57.35 per cent was recorded in zineb 68 per cent + hexaconazole 4 per cent.

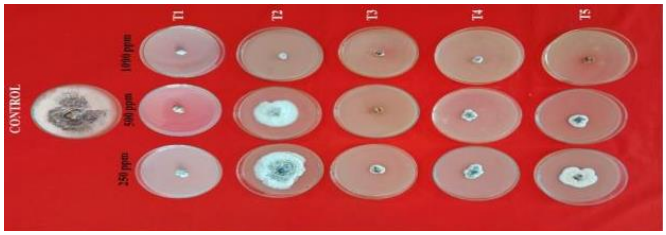


Fig. 11. *In vitro* evaluation of combi-product fungicides against *P. palmarum*

T1: Trifloxystrobin + Tebuconazole, T2: Zineb + Hexaconazole, T3: Iprovalicarb + Propineb, T4: Captan + Hexaconazole, T5: Tricyclazole + Mancozeb

Table 6. *In vitro* evaluation of combi products against *Pestalotiopsis palmarum*

Sl. No.	Combi product fungicides	Per cent inhibition over control			
		Concentration			
		250 ppm	500 ppm	1000 ppm	Mean
1	Trifloxystrobin 25% + Tebuconazole 50% EC (Nativo)	85.37 (67.49) *	89.07 (70.67) *	89.63 (71.19) *	88.02 (69.73) *
2	Zineb 68% + Hexaconazole 4% WP (Avatar)	35.37 (36.48) *	47.96 (43.81) *	88.70 (70.33) *	57.35 (49.20) *
3	Iprovalicarb 5.5% + Propineb 61.5% (Melody duo)	81.85 (64.78) *	85.93 (67.94) *	86.48 (68.40) *	84.75 (66.99) *
4	Captan 70% + Hexaconazole 4% WP (Taqat)	71.30 (57.78) *	79.07 (62.75) *	88.52 (70.17) *	79.63 (63.15) *
5	Tricyclazole 18% + Mancozeb 62% WP (Merger)	60.04 (51.94) *	76.85 (61.22) *	84.26 (66.60) *	74.38 (59.57) *
	Mean	67.19 (55.03) *	75.78 (60.49) *	87.52 (69.28) *	76.83 (61.20) *
		Fungicides (F)	Concentration(C)	Interaction (F×C)	
	SEm ±	0.31	0.24	0.54	
	CD @ 1%	1.21	0.93	2.09	

**Figures in the parenthesis are arc sine transformed value*

3.6.7 In vitro evaluation of botanicals against *Pestalotiopsis palmarum*

The study was carried out to know the antifungal activity nature of different plant extracts against *P. palmarum* by poison food technique. Based on the observation of radial growth of the fungus, the per cent inhibition was calculated. The effectiveness of different plant extracts in reducing the mycelial growth of *P. palmarum* is varied greatly.

The results presented in Table 7 revealed statistical difference between plant extracts per cent inhibition at four different concentrations with three replications. Turmeric (48.03 %) was found to be most effective and statistically on par with Ginger (44.13%), followed by Onion (36.66 %) which was on par with Garlic (32.87%), Simarouba (27.7 %), Pongamia (19.44%) which was found to be on par with subabul (19.42%), Neem (12.96 %) and Lemongrass (3.86 %). The least inhibition of mycelial growth was observed in Lantana (3.37 %) (Table 7 and Fig. 11). Among tested ten plant extracts, the highest mean inhibition was found in Turmeric (48.03 %) and the lowest in Lantana (3.37 %).

The fungal antagonistic microorganisms like *Trichoderma* were evaluated against *P.palmarum* by dual culture technique to know their antagonistic effect. The per cent inhibition of mycelial growth of fungus was calculated and results were noted

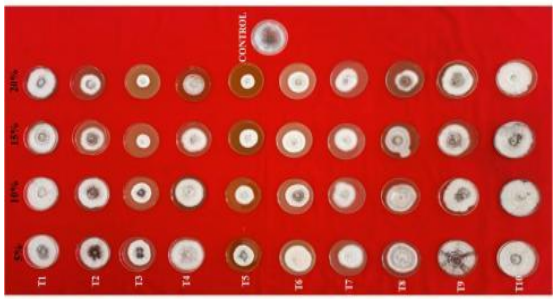


Fig. 12. *In vitro* evaluation of botanicals against *P. palmarum*

T1: Lemongrass, T2: Pongamia, T3: Ginger, T4: Subabul, T5; Turmeric, T6: Garlic, T7: Onion, T8: Simarouba, T9: Neem, T10: Lantana

Among the eight different fungal bio-agents tested, *T. viridae* (Tv-2) was found to be statistically superior to others which was on par with *T. harzianum*-B2 (Th-B2) with their mean mycelial inhibition of 71.30 and 70.56 per cent over the control respectively. *T. viridae* (Tv) which was on par with *T. harzianum*-55 showed 67.89 and 66.67 per cent mean mycelial inhibition over control. Next in order was *T. viridae*-1 (Tv-1) which had 65.44 per cent mycelial inhibition over control, followed by *T. viridae*-3 (Tv-3) with 61.11 per cent mycelial inhibition, and *T. harzianum*-56 (Th-56) with 59.17 per cent over control. The least mycelial inhibition was noticed in *T. harzianum*-44 (Th-44) which had 58.89 per cent mycelial inhibition (Table 8, Fig. 12).

Table 7. In vitro evaluation of botanicals against Pestalotiopsis palmarum

Sl. No.	Botanicals	Per cent inhibition over control				
		Concentration (%)				
		5 %	**10%**	**15%**	**20%**	**Mean**
1	Lemongrass	0.74 (4.94) *	2.04 (8.20) *	4.48 (12.22) *	8.19 (16.62) *	3.86 (10.49) *
2	Pongamia	5.56 (13.63) *	11.11 (19.46) *	27.78 (31.79) *	33.33 (35.25) *	19.44 (25.03) *
3	Ginger	25.56 (30.35) *	34.26 (35.81) *	55.59 (48.19) *	61.11 (51.40) *	44.13 (41.43) *
4	Subabul	3.33 (10.52) *	15.48 (23.16) *	24.44 (29.62) *	34.44 (35.92) *	19.42 (24.80) *
5	Turmeric	38.89 (38.56) *	45.26 (42.26) *	47.78 (43.71) *	60.19 (50.86) *	48.03 (43.84) *
6	Garlic	22.22 (28.11) *	34.81 (36.15) *	37.04 (37.47) *	37.41 (37.69) *	32.87 (34.85) *
7	Onion	32.22 (34.57) *	33.33 (35.25) *	36.67 (37.25) *	44.44 (41.79) *	36.66 (37.21) *
8	Simarouba	16.67 (24.09) *	22.22 (28.11) *	27.78 (31.79) *	44.44 (41.79) *	27.77 (31.44) *
9	Neem	0.74 (4.94) *	12.22 (20.45) *	16.67 (24.09) *	22.22 (28.11) *	12.96 (19.40) *
10	Lantana	1.11 (6.05) *	0.56 (4.27) *	0.70 (4.81) *	11.11 (19.46) *	3.37 (8.65) *
Mean		14.70 (19.57) *	21.12 (25.31) *	27.89 (30.09) *	35.68 (35.88) *	24.85 (27.7) *
		Botanicals (B)		Concentration(C)	Interaction(B×C)	
SEm ±		1.62		0.89	2.80	
CD @ 1%		6.09		3.33	10.54	

**Figures in the parenthesis are arc sine transformed values*

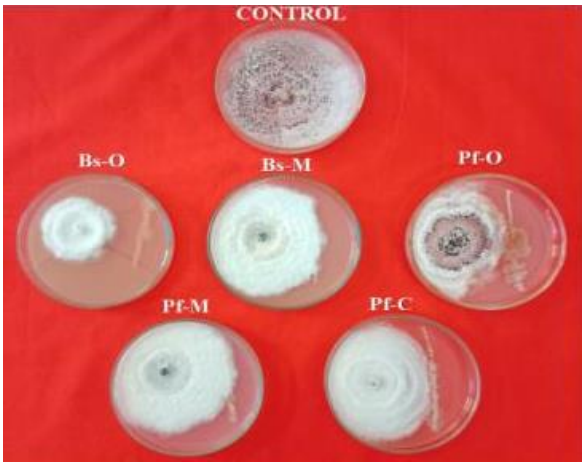


Fig. 13. In vitro evaluation of fungal bio-agents against P. palmarum

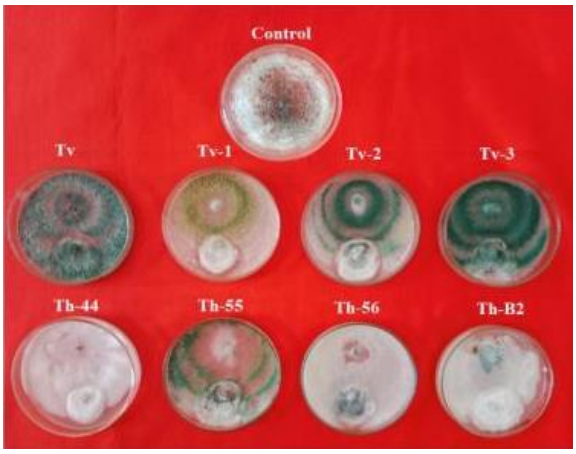


Fig. 14. In vitro evaluation of bacterial bio-agents against P. palmarum

3.6.8 In vitro evaluation of fungal bio-agents against *P. palmarum*

The findings are in confirmity to the earlier study by Saju et al. [14] who observed *T. viride* had significantly high inhibition (50.9 %) against *Pestalotiopsis* sp. in large cardamom.

3.6.9 In vitro evaluation of bacterial bio-agents against *P. palmarum*

The antagonistic action of five different bacterial bio-agents *viz*., *Bacillus subtilis, Pseudomonas fluorescence*, *Bacillus megatherium* isolates from different places were evaluated. Experiment was

Table 8. *In vitro* evaluation of fungal bio-agents against *P. palmarum*

Sl. No.	Fungal bio-agents	Per cent inhibition over control*
1	*Trichoderma viride*	67.89 (55.46)
2	*T. viride*- 1 (Tv-1)	65.44 (53.97)
3	*T. viride*-2 (Tv-2)	71.30 (57.58)
4	*T. viride-3* (Tv-3)	61.11 (51.40)
5	*T. harzianum-44* (Th-44)	58.89 (50.10)
6	*T. harzianum*-55 (Th-55)	66.67 (54.71)
7	*T. harzianum*-56 (Th-56)	59.17 (50.26)
8	*T. harzianum-B2*(Th-B2)	70.56 (57.11)
SEm ±		0.49
CD @ 1%		2.46

**Figures in the parenthesis are arc sine transformed values*

Table 9. *In vitro* evaluation of bacterial bio-agents against *P. palmarum*

Sl. No.	Bacterial bio-agents	Isolate	Per cent inhibition over control*
1	*Bacillus subtilis*	BS-O (Organic unit, GKVK)	52.22 (46.26)*
2	*Bacillus subtilis*	BS-M (Dept. of Agricultural microbiology, GKVK)	25.13 (30.07)
3	*Pseudomonas fluorescence*	PF-O (Organic unit, GKVK)	31.11 (33.89)
4	*Pseudomonas fluorescence*	PF-M (Dept. of Agricultural microbiology, GKVK)	22.22 (28.11)
5	*Pseudomonas fluorescence*	PF-C (Dept. of Plant pathology, Chintamani)	28.89 (32.50)
	SEm±	1.29	
	CD @ 1%	5.79	

**Figures in parenthesis are arc sine transformed values*

carried out by following dual plate culture method. There was statistically difference among the bacterial bio-agents evaluated with regarding to mycelial growth of *P. palmarum*.

Among different bacterial bio-agents evaluated, *Bacillus subtilis* (BS-O) isolate was found very effective in inhibiting the mycelial growth of *P. palmarum* and this was statistically superior over all other treatments with the mean mycelial inhibition of 52.22 per cent followed by *Pseudomonas fluorescence* (PF-O) which had mean mycelial inhibition of 31.11 per cent. Next in order was *Pseudomonas fluorescence* (PF-C) with mean mycelial inhibition of 28.89 per cent over the control. And the least mycelial inhibition was observed in *Bacillus subtilis* (BS-M) with 25.13 per cent mycelial inhibition (Table 9 and Fig. 13).

The maximum inhibition was recorded in *Bacillus subtilis* (BS-O) isolate (52.22 %). The minimum inhibition was recorded in *Pseudomonas fluorescence* (PF-M) (22.22%). The results are in confirmity with the earlier findings of Saju et al. [14] where *B. subtilis* had showed significantly high inhibition (62.6 %) against *Pestalotiopsis* sp. in large cardamom [15].

4. CONCLUSION

Grey blight infected sample used for isolation of the pathogen where white colony with regular margin and cottony texture was found. Black coloured fruiting bodies (acervuli) were speculated after fifteen days of isolation. Pathogen was initially identified based on the morphological characteristics observed under the microscope. Five-celled conidia had three middle cells that ranged in colour from light brown to dark brown, while the apical and basal cells were hyaline. Basal appendages were hyaline, smooth, or even curled. There were one to three setulae, which are apical appendages.

Pathogenicity test performed using pin prick method for confirmation of the isolated pathogen resulted typical symptoms appearance twelve days after artificial inoculation. The maximum radial growth of *P. palmarum* was observed in potato dextrose agar (90.00 mm), followed by potato carrot agar, oatmeal agar, and corn meal

agar, while the least radial growth was seen in V-8 juice agar (65.00 mm).

The PCR amplification by using ITS rDNA sequence analysis and homology search through BLAST programme, revealed that *P. palamrum* is considered as causal organism of grey blight of coconut. An investigation on the isolation with cultural, morphological, physiological and molecular characterisation of grey leaf blight in coconut revealed that isolated pathogen was *Pestalotiopsis palmarum*. The maximum radial growth of *P. palmarum* was observed in potato dextrose agar(90.00 mm), followed by potato carrot agar, oatmeal agar, and corn meal agar, while the least radial growth was seen in V-8 juice agar (65.00 mm).

The physiological experiments found that the maximum dry mycelial weight was at pH 6 (338.11 mg), and the least at pH 9 (134.19 mg). Among the various temperatures evaluated for this study, 25^{o}C (312.66 mg) produced the highest dry mycelial weight, while 5^{o}C (16.30 mg) produced the lowest dry mycelial weight. Ziram was found to be the most efficient among non-systemic fungicides in preventing *P. palmarum* development by 93.58 per cent, whereas zineb recorded the lowest fungal growth inhibition (57.78 per cent). Carbendazim and thiophonate methyl were found to be the most effective among the systemic fungicides tested, which inhibited pathogen growth by cent per cent, followed by propineb (93.21 per cent) and the least inhibition of fungal growth by hexaconazole (48.15 per cent). Among the combi products tested, trifloxystrobin 25 per cent + tebuoconazole 50 per cent EC was found to be the most effective, inhibiting pathogen growth by 88.02 per cent, followed by iprovalicarb + propineb (84.75 per cent).

Among the different fungal bio-agents tested, the per cent inhibition of mycelia of *P. palmarum*, was highest by 71.30 per cent in *T. viride*-2 (Tv-2) which was found to be on par with *T. harzianum* (Th-B2) by 70.56 per cent, whereas least inhibition was noticed in *T. harzianum* (Th-44) by 58.89 per cent. From the different bacterial bio-agents evaluated, *Bacillus subtilis* (Bs-O), isolate from organic farming unit, UAS, GKVK, had the highest inhibition of mycelia by 52.22 per cent and the least was recorded in *Pseudomonas fluorescence* (PF-M) by 22.22 per cent.

From the different botanical extracts that were tested, turmeric showed the highest per cent mycelial inhibition by 48.03 per cent and the least was observed in case of lantana by 3.37 per cent.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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