



Characterisation and *In-vitro* Fungicide Susceptibility of *Corynespora cassiicola* Associated with Leaf Spot Disease of *Aglaonema Ruby Garuda*

Sandra S. R. ^{a*}, Gleena Mary C. F. ^{a*},
Reshmy Vijayaraghavan ^a, Mini Sankar ^b,
Sible George Varghese ^a, Mufeeda K. T. ^c
and Shambhu Kumar ^c

^a Department of Plant Pathology, College of Agriculture, Vellanikkara, Kerala Agricultural University, Thrissur, 680656, India.

^b Department of Floriculture and Landscaping, Kerala Agricultural University, Vellanikkara, Thrissur, 680656, India.

^c Forest Pathology Department, Forest Health Division, KSCSTE-Kerala Forest Research Institute, Thrissur, 680653, India.

Authors' contributions

This work was carried out in collaboration among all authors. The authors GMCF, RV, MS and SGV contributed to the design and implementation of the research and the author SSR conceptualized, conducted the study and drafted the manuscript. The author GMCF provided valuable insights into the theoretical framework, and assisted with manuscript revision and other authors MKT and SK provided assistance and technical support for the completion of the study. All authors have read and approved the final manuscript.

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*Corresponding author: E-mail: sandrasr3012@gmail.com, gleena.m@kau.in;

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ABSTRACT

During 2020- 2022, a characteristic leaf spot symptom was recorded from the *Aglaonema* cultivar, Aglaonema Ruby Garuda Aglaonema Ruby Garuda from Ambunadu region, Ernakulam district of Kerala. The symptomatic plants formed dark brown, water-soaked, circular spots with concentric zonations and recorded 30 per cent disease severity. Pathogen associated with the symptom isolated using standard isolation methods and pathogenicity of the fungal isolates was established. Greyish brown mycelial growth was observed in culture plates with greyish black colour on the reverse side. Conidia of the fungus were sub hyaline to pale brown, solitary, straight to slightly curved, obclavate produced on brown septate mycelia with a dimension of 27.3-47.8 μm \times 4.2-12.8 μm . The amplicon sequences of internal transcribed spacer (ITS) and translational elongation factor 1- alpha (TEF1- α) regions of the pathogen were analysed with the nucleotide sequences in nBLAST database and identified as *Corynespora cassiicola*. Evolutionary relationship of the pathogen with related organisms were identified by constructing phylogenetic tree using ITS and TEF1- α sequences. *In vitro* efficacy of 11 chemical fungicides at different dosages and three biocontrol agents tested recorded, cent per cent inhibition with contact fungicides viz. mancozeb, propineb, Bordeaux mixture. Complete inhibition was observed with systemic fungicides, hexaconazole 5EC, tebuconazole 25.9EC, carbendazim 50WP at all test dosages. Among the combination fungicides, carbendazim 12%+ mancozeb 63% was superior with cent per cent mycelial inhibition. Among the biocontrol agents, the fungal antagonist, *Trichoderma asperellum* (KAU reference culture) recorded complete inhibition against *Corynespora cassiicola* by dual culture assay.

Keywords: *Aglaonema Ruby Garuda*; Leaf spot disease; *Corynespora cassiicola*; In-vitro evaluation; poisoned food technique; dual culture technique.

1. INTRODUCTION

Aglaonema spp. popular as Chinese evergreen are fleshy, evergreen perennial herb native to tropical and subtropical regions of southeast Asia and New Guinea [1,2]. The foliage plant, belonging to Araceae family consists of more than 50 species and characterised by thick, erect, unbranched stems with circular leaf scars [1]. The word “Aglaonema” derived from two Greek words “Aglaos” and “nema” which means shining stamen. According to Asian culture, these plants were believed to bring good luck to life. Aglaonema flourishes well under heavy shade (75–90%) with an air temperature of more than 95°F [3]. These plants were introduced to the western world in 1885 and further sparked the large scale development of *Aglaonema* cultivar [4]. They are highly valued for their attractive foliar variegations and high adaptability in low light conditions. These plants were believed to eliminate harmful toxins from the air and hence raised as an integral component in interior landscaping. Currently, there are different *Aglaonema* hybrids and cultivars which were recognised as popular choice in interior plant scaping including ‘Jubilee Petite’, ‘Peacock’, ‘White Rain’, ‘White Lance’, ‘Brilliant’, ‘Illumination’, ‘Black Lance’, ‘Emerald Stars’, ‘Jewel of India’, ‘Ruby Garuda’ etc [5]. Foliage plants are gaining due importance being an

unavoidable element in the indoor landscapes. The indoor plantscaping has a huge potential in the coming years and *Aglaonema* is one among the most important foliage used world wide. Even though the plants have huge marketing potential in domestic and international markets, its marketability is greatly hampered by several foliar pathogens. According to Uchida and Yhata [6] large-scale import of aroids during the 1970s was the major reason for the unintentional introduction of novel bacterial and fungal pathogens in Hawaii, leading to devastating foliar blight and crowns rot diseases in *Aglaonema* spp.

A perusal of review revealed *Gloeosporium graffi* [7], *Colletotrichum dematium* [8], *Colletotrichum gloeosporioides* [9,10], *Fusarium subglutinans* and *Phytophthora* spp. viz., *Phytophthora meadii* and *P. parasitica* [11] as the leaf blight and leaf spot pathogens of different *Aglaonema* spp. Among them, Anthracnose pathogen was the most prominent one causing greyish, irregular, extensive spots, with slightly elevated dark edges on the infected *Aglaonema* leaves [8,9]. *Fusarium subglutinans* induced collar rot and foliar blight with considerable crop losses in the area of commercial cultivation in Hawaii, United States. Pathogen produced a dark water-soaked lesion with a diffused yellow margin and on young leaves, symptoms are initiated as water-

soaked lesion that progress rapidly without any prominent yellow halo [6]. Co-infection of *Fusarium aglaonematis* and *F. elaeidis* enhanced disease severity in *Aglaonema modestum* compared to individual infection by each pathogen species Zhang et al. [12]. The current piece of work unveils *Corynespora cassiicola* as an emerging leaf spot pathogen of *Aglaonema* cultivar Ruby Garuda.

It has been observed that *Corynespora cassiicola* infects over 530 plant species from 380 different genera, encompassing ferns, monocots, dicots and cycads [13]. It exist as pathogen, saprophyte and endophyte based on the host characteristics [14,15]. Several economically important plants such as soyabean rubber, tomato, tobacco, cotton, blueberry and sweet potato were infected by the fungal pathogen [15-21]. It causes infection over the stem, root, leaves and fruits of a wide range of plants including crops, ornamental plants and weeds [22]. In addition to common host plants like rubber (*Hevea brasiliensis*), cotton (*Gossypium* sp.) and soybean (*Glycine max*) they cause infection on plants like cow pea (*Vigna unguiculata*), lettuce (*Lactuca sativa*), lantana (*Lantana camara*), *Hydrangea* spp. [16,23]. According to Fulmer et al. [24] *C. cassiicola* causes target-like symptoms and premature defoliation in cotton plants. Similar to the leaf spot symptoms on cotton the foliar infection on soybean was recorded with considerable yield reduction in Brazil [25]. According to Schulub et al [15] over the past 20 year the pathogen was emerged as a potential threat in the vegetable crop, tomato (*Solanum lycopersicum*) in Florida. Among the ornamental plants *corynespora cassiicola* were documented as potential leaf spot pathogen including different *Jasminum* spp. [26,27], *Codiaeum variegatum*

[28] *Hydrangea* sp. [29] and gerbera [30]. These reports underlines the emergence of this pathogen as a serious threat for new economically important hosts.

From India *Corynespora cassiicola* was reported pathogenic in cotton [31,32], pomegranate [33] castor bean [34] and tomato [35]. *Corynespora* leaf fall disease caused by *Corynespora cassiicola* was documented as an emerging threat in rubber plantations of Kerala with a yearly increase in the disease severity, Manju et al [36]. Rafi [37] reported the pathogenic nature of *Corynespora cassiicola* on Anthurium plants from Thrissur district of Kerala. From the existing piece of literatures, it was evident that *Corynespora cassiicola* has not been reported as pathogen on the ornamental foliage plant, *Aglaonema* in India. The present study reveals the *Corynespora* infection on *Aglaonema* and enlightens knowledge about the symptomatology, pathogenicity, morpho molecular characteristics of pathogen and *in vitro* susceptibility of commercial fungicides against the pathogen.

2. MATERIALS AND METHODS

2.1 Survey, Disease Assessment and Symptomatology Studies

An unusual leaf spot symptom was observed on the *Aglaonema* cultivar, *Aglaonema ruby garuda* from commercial nurseries of Ambunadu, Ernakulam districts of Kerala (10°07'13" N, 76°40'36" E) in April 2022. Symptoms associated with pathogen were documented under field and *in situ* conditions. Disease severity was assessed using the standard score chart of 0 - 6 scale developed by Mounika et. al [9] (Table 1) and per cent disease severity was calculated using the formula.

$$\text{Per cent disease severity (PDS)} = \frac{\text{Sum of all numerical}}{\text{Total no. of leaves observed} \times \text{maximum disease grade}} \times 100$$

Table 1. Disease score chart for per cent disease severity Mounika et al. [9]

Scale	Description
0	No infection
1	1-5% leaf area/ length covered by disease
2	6-10% leaf area/length covered by disease
3	11-25% leaf area/length covered by disease
4	26-50% leaf area/length covered by disease
5	51-75% leaf area/length covered by disease
6	76-100% leaf area/length covered by disease

2.2 Isolation, Purification and Pathogenicity Studies

The pathogen associated with symptom was isolated by tissue segmentation method [38]. Surface sterilised leaf bits of the infected sample (3 bits/plate) were placed in Petri plates with solidified PDA media and incubated at room temperature ($26 \pm 2^\circ\text{C}$). Single hyphal tip of the pathogen was transferred in to sterilised PDA medium for purification followed by long term storage at 4°C under refrigerated condition.

Pathogenicity of the fungal isolate was established by proving Koch's postulates by mycelial bit inoculation [39] on healthy detached leaves of *Aglaonema Ruby Garuda*. Inoculated leaves were incubated in polythene bags under room temperature ($26 \pm 2^\circ\text{C}$) for symptom development. The fungus reisolated was compared with original culture and further confirmed based on cultural, morphological and molecular characteristics.

2.3 Morpho- Molecular Characterisation and Phylogenetic Analysis

The fungal isolate was cultured on PDA medium for generic level identification of pathogen based on cultural and morphological features and compared with the descriptions of pathogenic fungi by Commonwealth Mycological Institute's [40]. The cultural characters like colony colour,

texture of mycelia, growth pattern, growth rate, pigmentations, colour. Morphological features like hyphal colour, branching pattern, hyphal septation, presence of sporulating structures, type, colour, spore characters and shape of spores, septation in spores and spore dimension were recorded and micrographs were captured.

Species level identification of pathogen was conducted by the amplified internal transcribed spacer (ITS) and translational elongation factor 1- alpha (TEF1- α) of the pathogen with the nucleotide sequences in NCBI BLASTn database. The genomic DNA of pathogen isolated from seven days old fungal culture by CTAB method [41] and PCR reaction was carried out by amplifying the ITS and TEF1- α nucleotide sequences of the pathogen using universal primer ITS1/ITS4 [42] and EF1-728F/EF1- 986R [43]. In silico analysis of the retrieved sequences was performed for the identification of pathogen.

A concatenated dataset of two genes (ITS, TEF1- α) was used for the phylogenetic analyses of *Corynespora* sp. using Maximum Likelihood method (ML). The consensus sequences of the isolate, *Corynespora* sp. (CCSR) and related species were assembled (Table 3). *Fusarium solani* was used as the outgroup. Evolutionary analyses conducted in MEGA X [44,45]. Kimura 2-parameter model and nearest neighbor-interchange search options with 1000 bootstrap replicates.

Table 2. ITS and Tef- 1 α sequences of *Corynespora* species along with their accession number

<i>Corynespora</i> sp.	Isolate/ strain	Gene bank accession numbers ^a	
		ITS	Tef-1 α
<i>C. cassiicola</i>	CCSR	OR412828	OR462816
<i>C. cassiicola</i>	ON3	AB539457	AB539235
	CYDB2	MK571286	MK589896
	CCC87	KP748287	KP748325
	LP 138	KU167046	KU167045
	ZM170454	MG132185	MG132189
	ZM170452	MG132183	MG132187
	ZM170455	MG132186	MG132190
	RLT-2	MN512632	MN512635
	XQ3-1	MH569606	MH572687
	BS1	KJ954117	KJ954135
	ACC18	KP748293	KP748331
	ACC39	KP748295	KP748333
<i>C. nabanheensis</i>	HJAUP C2048T	OQ060577	OQ067526
<i>C. pseudocassiicola</i>	CPC 31708	MH327794	MH327877
<i>C. smithii</i>	L120	KY984297	KY984435
<i>C. smithii</i>	L130	KY984298	KY984436
<i>C. thailandica</i>	CBS 145089	MK047455	MK047567
<i>Fusarium solani</i>	FSSC-C4383B	KC009602	KC161396

^a Sequences in bold are from present study

2.4 *In-vitro* Susceptibility Assay of Chemical Fungicides and Biocontrol Agents

The potential inhibitory effect of different commercial fungicides and biocontrol agents were assessed on the pathogen under *in vitro* conditions. Mycelial growth reduction of the pathogen on treatment with different chemical fungicides was conducted by poisoned food technique [46] and antagonistic activity of different biocontrol agents was performed by dual culture technique [47].

Different test dosages of 12 chemical fungicides at specified concentrations were mixed separately in 100 ml sterilized molten PDA and 8 mm mycelial plugs of the test pathogen was transferred to the fungicide amended media. Chemical fungicides and concentrations used for *in vitro* evaluation are detailed in Table 2.

The experiment was conducted in completely randomized design (CRD) with four replications for each treatment and a Petri plate with non-amended medium served as negative control. Growth rate of each pathogen in poisoned medium and control plates was recorded till the pathogen in control plate attained full growth under an incubation temperature of $26 \pm 2^\circ\text{C}$. Per cent inhibition of growth of the test pathogen was calculated using the formula suggested by Vincent et al. [48].

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

Where,

C - Growth of fungus in control plate (cms)

T - Growth of fungus in treatment (cms)

The efficacy of fungal biocontrol agent, *Trichoderma asperellum* (KAU reference culture) was evaluated by dual culture technique [47] and poisoned food technique was used for evaluating the efficacy of Plant Growth Promoting Microorganism, KAU (PGPM) and Plant Growth Promoting Rhizobacteria, KAU (PGPR II) against the pathogen. The inhibition of the growth of the pathogen by the antagonist was calculated as per Vincent et al. [48].

3. RESULTS AND DISCUSSION

Leaf spot sample of *Aglaonema Ruby Garuda* with 40 per cent disease severity was collected from Ambunadu, Ernakulam district of Kerala. Symptomatic plants initially formed circular to irregular, brown, water-soaked spots on the lamina which further matured into alternating light and dark brown bands on the affected area. The leaves containing multiple lesions subsequently enlarged and blighted (Fig. 1A, 1B). Similar symptom was observed on cotton plants grown in central India [49]. The pathogen associated with the symptom was isolated by tissue segmentation method [38] and pathogenicity of the fungal isolate established by mycelial bit inoculation on healthy detached leaves of *Aglaonema Ruby Garuda* with characteristic symptom appearance within four days of incubation and no symptom expression was observed on control (Fig. 1C, 1D). Cultural and morphological characteristics of the reisolated pathogen was compared with the original fungal culture and further confirmed as the same using ITS and TEF1- α genes.

Table 3. Fungicides and concentrations for *in vitro* evaluation studies

Sl. No	Fungicide	Concentration (Per cent)
1.	Mancozeb 75% WP	0.2, 0.25, 0.3
2.	Copper hydroxide 53.8% DF	0.1, 0.2, 0.3
3.	Propineb 70% WP	0.1, 0.2, 0.3
4.	Chlorothalonil 75% WP	0.1, 0.2, 0.3
5.	Hexaconazole 5% EC	0.1, 0.15, 0.2
6.	Tebuconazole 25.9% EC	0.05, 0.1, 0.15
7.	Difenoconazole 25.0% EC	0.1, 0.15, 0.2
8.	Carbendazim 50%WP	0.05, 0.1, 0.2
9.	Carbendazim 12% + Mancozeb 63% W	0.1, 0.2, 0.3
10.	Tebuconazole 50% + Trifloxystrobin 25% WG	0.03, 0.04, 0.05
11.	Azoxystrobin 18.2% + Difenoconazole 11.4% SC	0.05, 0.1, 0.2
12.	Bordeaux mixture	1

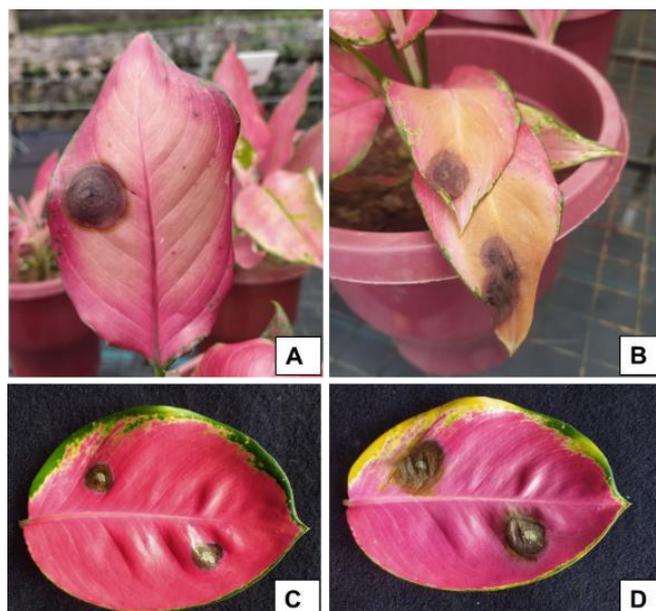


Fig. 1. Symptom under natural (A, B) and artificial conditions (C, D)

3.1 Characterisation of Pathogens

Greyish brown, regular, dense, aerial growth was observed on PDA medium with greyish black colour on reverse side (Fig 2). The fungal culture attained full growth in the Petri plate (9 cm) within 14 days of incubation at room temperature. Conidia sub hyaline to pale brown, solitary, straight to slightly curved, obclavate produced abundantly within the brown septate mycelia and $27.3\text{-}47.8\ \mu\text{m} \times 4.2\text{-}12.8\ \mu\text{m}$ [50].

Based on the cultural and morphological characteristics the pathogen was identified up to genus level as *Corynespora* sp. The morphological and cultural features of the pathogen were similar to that of target leaf spot pathogen of tomato and cucumber recorded by Kamei et al. [35] and Liu et al. [50].

The amplicon sequence of *Corynespora* isolate were analysed with the nucleotide sequences in nBLAST database. The ITS sequences showed 100 per cent similarity with *Corynespora cassiicola* with gene bank accession number KF577899.1. and TEF1- α sequences also recorded cent per cent similarity with *C. cassiicola* (MN887503.1). The sequences were deposited in NCBI database and accession numbers were obtained as OR412828 and OR462816 for ITS and TEF1- α regions. Concatenated sequences of ITS +TEF1- α

regions of different *Corynespora* spp. were used for phylogenetic analysis and observed that the pathogen shared a common clade of *Corynespora cassiicola* represented by the GenBank sequences in the phylogenetic tree (Fig.3.). This results reaffirms the isolated pathogen as *C. cassiicola* by morphological, cultural, and molecular sequence analysis.

3.2 In vitro Susceptibility Assay of Chemical Fungicides against *Corynespora cassiicola*

The fungicides viz., mancozeb 75WP, propineb 70WP, hexaconazole 5EC, tebuconazole 25.9EC, carbendazim 50WP, carbendazim 12%+ mancozeb 63%, and Bordeaux mixture completely inhibited the fungal pathogen (Table 3). The other fungicides, tebuconazole 50% + trifloxystrobin 25%, azoxystrobin 18.2% + difenoconazole 11.4%, copper hydroxide 53.8DF, chlorothalonil 75WP, and difenoconazole 25EC, also inhibited the pathogen with an inhibition ranging between 55.18-81.85 per cent (Fig. 4). Sowmya et al. [51] recorded cent per cent inhibition in the growth of *Corynespora cassiicola* with the contact fungicide, mancozeb at 0.05, 0.1, 0.15 and 0.2 per cent concentrations. The research findings of Manju et al. [52] is also agreeable with the current study and recorded cent per cent inhibition in the growth of *Corynespora cassiicola*, causing leaf fall disease in rubber with

the fungicides viz., mancozeb, hexaconazole, carbendazim and carbendazim + mancozeb at 250 ppm concentration. Similar results from

different studies reassures the susceptibility of the pathogen to different triazoles, carbamate and combination fungicides

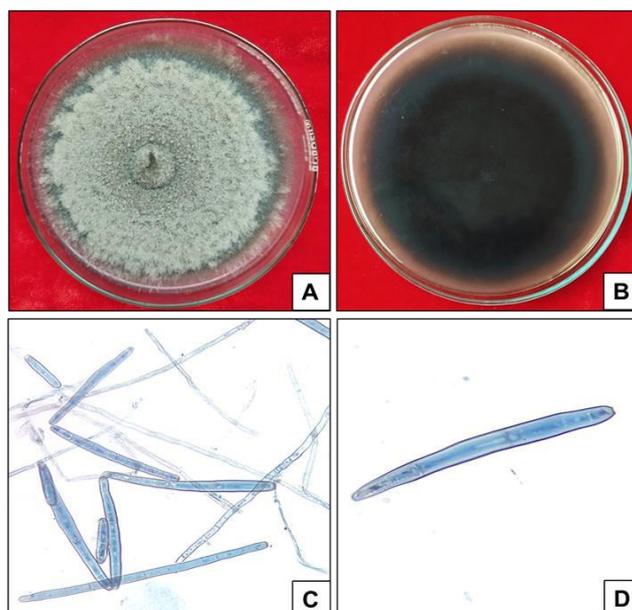


Fig. 2. Fungal colony on PDA medium and morphological features. Upper surface of *Corynespora* sp. colony on PDA (A), reverse side of the culture plate (B), sub hyaline to pale brown, solitary, straight to slightly curved, obclavate conidia with hyaline mycelium at x 40 (C) conidial morphology at x100 (D)

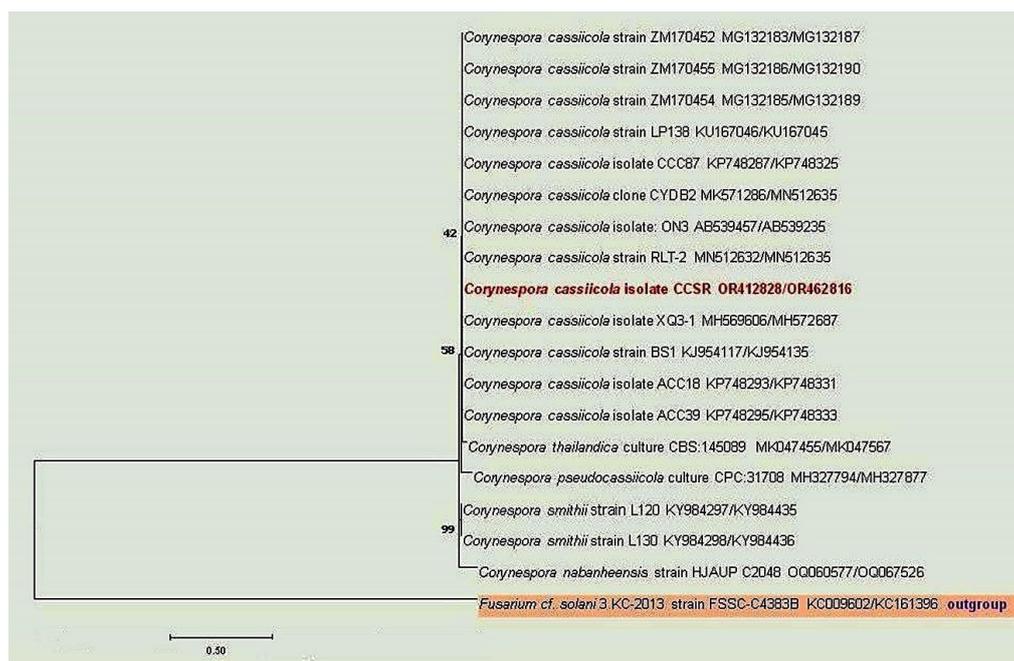


Fig. 3. Concatenated phylogenetic tree generated from Maximum Likelihood method (ML) analysis for the analyzed *Corynespora* sp. isolates using ITS-TEF1- α genes (Kimura 2-parameter model and nearest neighbour-interchange search options with 100 bootstrap replicates were used). The sequence from the present study is indicated in bold and in red. The scale bar represents the expected number of changes per site. The tree is rooted with *Fusarium solani* USM FSSC-C4383B

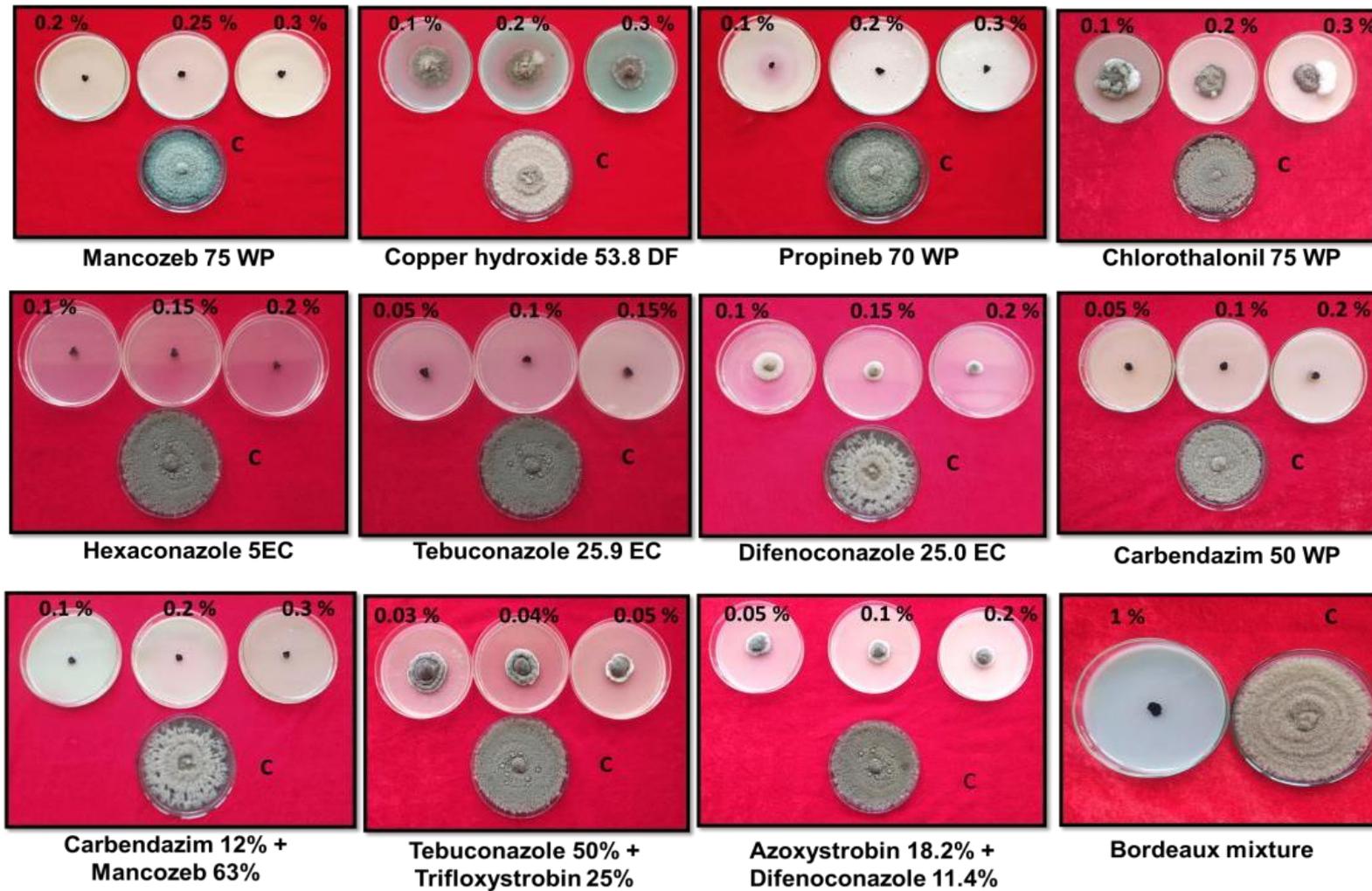


Fig. 4. *In vitro* evaluation of chemical fungicides against *Corynespora cassiicola*

Table 4. *In-vitro* evaluation of chemical fungicides against *C. cassicola*

Chemical fungicide	Concentration (%)	Inhibition (%)
Mancozeb 75WP	0.2	100 (10) ^a
	0.25	100 (10) ^a
	0.3	100 (10) ^a
Copper hydroxide 53.8 DF	0.1	62.96 (8.05) ^d
	0.2	64.07 (8.10) ^c
	0.3	64.44 (8.05) ^d
Propineb 70 WP	0.1	100 (10) ^a
	0.2	100 (10) ^a
	0.3	100 (10) ^a
Chlorothalonil 75 WP	0.1	55.18 (7.46) ^g
	0.2	57.78 (7.63) ^{fg}
	0.3	63.33 (7.98) ^e
Hexaconazole 5 EC	0.1	100 (10) ^a
	0.15	100 (10) ^a
	0.2	100 (10) ^a
Tebuconazole 25.9 EC	0.05	100 (10) ^a
	0.1	100 (10) ^a
	0.15	100 (10) ^a
Difenoconazole 25.0 EC	0.1	67.04 (8.21) ^d
	0.15	78.52 (8.88) ^b
	0.2	81.85 (9.07) ^b
Carbendazim 50 WP	0.05	100 (10) ^a
	0.1	100 (10) ^a
	0.2	100 (10) ^a
Carbendazim 12% + Mancozeb 63%	0.1	100 (10) ^a
	0.2	100 (10) ^a
	0.3	100 (10) ^a
Tebuconazole 50% + Trifloxystrobin 25%	0.03	59.16 (7.71) ^f
	0.04	60 (7.77) ^f
	0.05	65.41 (8.11) ^{de}
Azoxytrobin 18.2% + Difenoconazole 11.4%	0.05	71.25 (8.47) ^c
	0.1	73.75(8.61) ^c
	0.2	80.00 (8.97) ^b
Bordeaux mixture	1	100 (10) ^a

Square root transformed values are given in the parenthesis

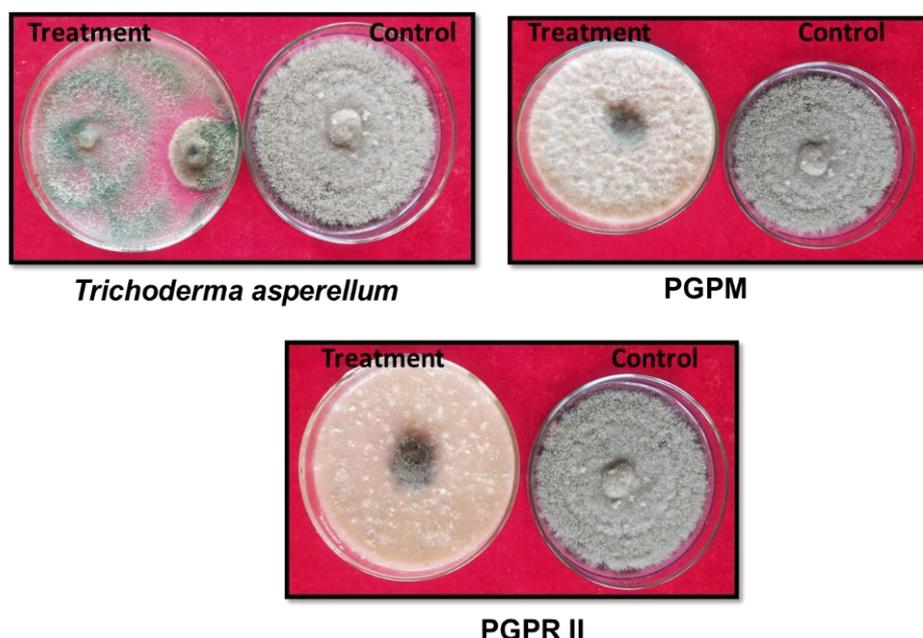


Fig. 5. *In vitro* evaluation of biocontrol agents against *C. cassiicola*

Table 5. *In vitro* efficacy assay of bioagents against *Corynespora cassiicola*

Biocontrol agents	Inhibition (%)
<i>Trichoderma asperellum</i>	100
plant growth promoting microorganism (PGPM)	75.55
plant growth promoting rhizobacteria (PGPR II)	68.88

3.3 *In vitro* Susceptibility Assay of Bioagents against *Corynespora cassiicola*

The sustainable management practices for plant pathogens reiterates the importance of biological control measures with potential microbes. The inhibitory activity of KAU reference cultures of *Trichoderma asperellum*, plant growth promoting microorganisms (PGPM) and plant growth promoting rhizobacteria (PGPR II) were assessed against the pathogen under laboratory conditions (Table4). Significant reduction in the growth of pathogen was recorded on treatment with fungal antagonist, *Trichoderma asperellum*. Radial mycelial growth of the pathogen was completely inhibited by the bioagents by the mechanism of overgrowth (Fig.5). Baiyee et al. [53] evaluated the efficacy of *T. asperellum* against *Corynespora cassiicola* causing infection on lettuce and recorded an inhibition per cent of 83.79.

The microbial consortium of plant growth promoting microorganisms also had a potential

impact in restricting the growth of pathogen. The PDA medium amended with PGPM showed 75.55 per cent inhibition in the mycelial growth of pathogen followed by the consortium of plant growth promoting rhizobacteria with 68.88 per cent inhibition. According to the studies, plant growth promoting rhizobacteria act as a potent inhibitor of plant pathogens by the mechanism of parasitism, hyper parasitism, competition, and by inducing resistance in plants [54,55,56].

4. CONCLUSION

The current piece of work record a new leaf spot disease of the *Aglaonema* cultivar, Ruby Garuda from Kerala. Symptomatology and pathogenicity studies revealed that the pathogen associated with the symptom as *Corynespora cassiicola* based on cultural and morphological characteristics and this was further confirmed using amplified ITS and TEF1- α sequences. This study also reveals the expansion of host range by the pathogen in the changing climatic scenario and can cause potential impact on the foliage industry. From the *in vitro* susceptibility

studies of 12 different chemical fungicides, the fungicides viz., mancozeb, propineb, Bordeaux mixture, hexaconazole 5EC, tebuconazole 25.9EC, carbendazim 50 WP and carbendazim 12%+ mancozeb 63% found effective with complete inhibition of pathogen at all tested dosages. Of the biocontrol agents, the fungal antagonist, *Trichoderma asperellum* (KAU reference culture) recorded complete inhibition against *Corynespora cassiicola* upon dual culture assay. Further research in the field is necessary for confirmation of the results attained during the laboratory studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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