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Emerging threat of a leaf spot disease in *Cattleya* by *Lasiodiplodia theobromae*: Symptoms, Etiology and Management

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Abstract
In an orchid nursery of Cattleya in Thiruvananthapuram district of Kerala, a leaf spot disease was noticed prominent. The pathogen isolated was identified as *Lasiodiplodia theobromae* based on cultural, morphological and molecular characterisation. To the best of our knowledge, this is the first report of *L. theobromae* causing leaf spot disease in Cattleya from India. The efficacy of eight fungicides was evaluated against *L. theobromae*, of which the fungicides *viz*., cymoxanil 8% + mancozeb 64%, carbendazim 12% + mancozeb 63%, propineb and 1% Bordeaux mixture completely inhibited the mycelial growth, while azoxystrobin was found to be least effective against the pathogen. Among the biocontrol agents tested, plant growth promoting microorganisms (PGPM) showed 100% inhibition, followed by plant growth promoting rhizobacteria-II (PGPM-II) (50.41%) and *Trichoderma asperellum* (20.41%), while *Pseudomonas fluorescens* did not show any inhibition.

Keywords: Cattleya, leaf spot, *Lasiodiplodia theobromae*, *in vitro* evaluation

1. Introduction
Orchids have always gained attention among the floriculture crops owing to their demand for cut flowers and ornamental plants. Among different orchid genera, Cattleya and its intergeneric hybrids are gaining popularity in cut flower trade. However, the cultivation of Cattleya is often hindered by fungal, bacterial and viral diseases. Many fungal diseases such as anthracnose (*Colletotrichum karstii*), leaf spot diseases (*Cercospora* spp., *Lasiodiplodia theobromae*, *Neoscytalidium orchidacearum*), wilt (*Sclerotium rolfsii*), black rot (*Phytophthora cactorum*, *Phytophthora palmivora and Pythium ultimum*) and root rot (*Rhizoctonia solani*) were reported worldwide (Kevorkian 1940 [9]; Bag 2003 [2]; Cating et al. 2010 [6]; Cabrera and Cúndom 2013 [5]; Suwannarach et al. 2018 [16]; Silva et al. 2021 [15]; Hsieh et al. 2023 [8]). The present study was carried out to find out the etiology of leaf spot disease in Cattleya observed in Thiruvananthapuram district of Kerala, and to evaluate the efficacy of fungicides and biocontrol agents against the pathogen under *in vitro* condition.

2. Materials and Methods
The present investigation was carried out in Department of Plant Pathology, College of Agriculture, Vellanikkara, during the month of May, 2022.

2.1 Sample collection, isolation of pathogen and pathogenicity studies
Diseased samples of Cattleya showing leaf spot symptom were collected from College of Agriculture, Vellayani, located in Thiruvananthapuram district of Kerala. The symptoms of the disease under field conditions were studied. The pathogen was isolated by tissue segmentation method (Rangaswamy and Mahadevan, 1999) [13]. The pathogenicity of fungal isolate was tested, and Koch' postulates were proved by mycelial bit inoculation method (Rocha et al. 1998). Healthy leaves of Cattleya were collected, wiped with 70% alcohol and small pin prick injuries were made on leaves. Mycelial disc of 8 mm diameter of the four-day-old fungal culture was placed on the pin pricked area, and kept under humid chamber. The leaves without inoculated pathogen served as control. The inoculated leaves were observed daily for symptom development. The fungus was reisolated from symptomatic leaves and compared with the original isolate.

2.2 Characterisation of the pathogen

The cultural characters of the pathogen such as colour, texture, growth rate, growth pattern, sporulation, pigmentation, colour on the reverse side of Petri plate, and the presence of fruiting bodies were observed. The morphological characters of the pathogen were studied using compound microscope. Species level identification was done by sending the culture to Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram for LSU-rRNA (large subunit ribosomal ribonucleic acid) gene sequencing. Sequences were analysed through the BLASTn programme of NCBI http://ncbi.nlm.nhm.gov/blast.

2.3 *In vitro* evaluation of fungicides

The efficacy of different fungicides against the pathogen, *Lasiodiplodia theobromae* was tested by poisoned food technique (Zentmeyer 1955) [19]. Seven fungicides at three doses (lower, recommended and higher doses) and 1% Bordeaux mixture were evaluated against the pathogen. The required quantity of fungicide was mixed with potato dextrose agar (PDA) medium to get the desired concentration and poured into sterile Petri plates @ 20 ml per plate. Mycelial bits of 8 mm diameter were cut from actively growing culture of the fungal pathogen and placed at the centre of each Petri dish containing poisoned medium. Three replications were maintained for each treatment. Media without fungicides served as control. The inoculated Petri dishes were incubated at 27±2 °C. The diameter of the mycelial growth of the pathogen was recorded when it attained full growth in the control plates. The details of fungicides used for *in vitro* evaluation are given in Table 1.

The per cent inhibition of growth over control was calculated by the formula given by Vincent (1927) [18].

$$\text{Per cent inhibition of pathogen} = \frac{C - T}{C} \times 100$$

C - Radial growth of pathogen in control (cm)
T - Radial growth of pathogen in treatment (cm)

2.4 *In vitro* evaluation of biocontrol agents

The efficacy of biocontrol agents *viz.*, *Trichoderma asperellum* (KAU reference culture) and *Pseudomonas fluorescens* (KAU reference culture) was tested *in vitro* by dual culture technique (Morton and Stroube 1955) [11], whereas that of PGPR-II and PGPM obtained from Kerala Agricultural University was evaluated by poisoned food technique (Zentmeyer 1955) [19].

Table 1: Fungicides used for *in vitro* evaluation along with their doses

Treatment No.	Treatments	Concentration (%)
T_1	Copper hydroxide	0.1, 0.2, 0.3
T_2	Hexaconazole	0.05, 0.1, 0.15
T_3	Propineb	0.1, 0.2, 0.3
T_4	Difenoconazole	0.05, 0.1, 0.15
T_5	Carbendazim 12% + mancozeb 63%	0.1, 0.2, 0.3
T_6	Cymoxanil 8% + mancozeb 64%	0.1, 0.25, 0.3
T_7	Azoxystrobin	0.05, 0.1, 0.15
T_8	Bordeaux mixture	1.0
T_9	Control	

For evaluating the efficacy of *T. asperellum*, 8 mm mycelial disc from actively growing culture of the pathogen was placed at a distance of 2 cm from the periphery of a sterilized Petri plate containing PDA medium. The inoculated plates were kept for incubation for 48 h. A mycelial disc of 8 mm diameter was later cut from a five-day-old culture of *Trichoderma asperellum*, and placed 2 cm away from the periphery of the Petri dish opposite to the pathogen. The observations were taken until the pathogen attained full growth in control plates.

The antagonistic activity of *P. fluorescens* was evaluated by dual culture technique (Morton and Stroube 1955) [11]. Mycelial disc (8 mm) of pathogen was inoculated at the centre of Petri plate and bacterial antagonist, *P. fluorescens* was streaked on either side of the pathogen, 2 cm away from the periphery. Three replications were maintained for each treatment, and the pathogen without antagonist served as the control. PGPR- II (2%) and PGPM (2%) were evaluated against the pathogen by poisoned food technique (Zentmeyer 1955) [19] and observations were taken until the pathogen attained full growth in control plates.

Per cent inhibition of the pathogen by bio control agents was calculated using the following formula (Vincent 1927) [18].

$$\text{Per cent inhibition of pathogen} = \frac{C - T}{C} \times 100$$

C - Radial growth of pathogen in control (cm)
T - Radial growth of pathogen in treatment (cm)

2.5 Statistical analysis

The data obtained from *in vitro* studies were analysed using General R-shiny based Analysis Platform Empowered by Statistics (GRAPES).

3. Results and Discussion

3.1 Sample collection, isolation of pathogen and pathogenicity studies

The symptom of leaf spot disease observed in Cattleya was characterised by greyish white, irregular sunken spot with thick brown margin. Lesions enlarged and coalesced to form blight symptom (Fig. 1). The pathogen produced similar symptoms upon artificial inoculation (Fig. 2). The pathogen could be reisolated and Koch' postulates were proved. The symptoms match with the infection of *Lasiodiplodia theobromae* in *Cattleya* sp. reported in Argentina (Cabrera and Cúndom 2013) [5].

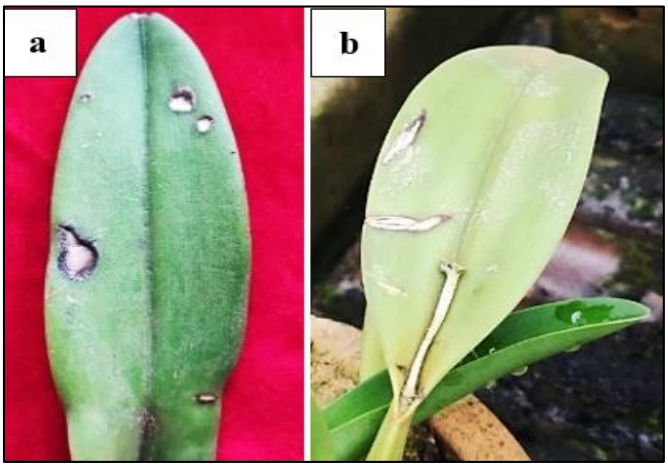


Fig 1: a) Greyish white, irregular sunken leaf spot on Cattleya with thick brown margin. b) Lesions enlarge and coalesce to form blight symptom

Fig 2: Symptom produced under artificial inoculation

3.2 Characterisation of the pathogen

3.2.1 Cultural and morphological characterisation

The fungal pathogen cultured on PDA attained full growth four days after inoculation forming dull white, abundant, fluffy, aerial mycelia, and turned olive-grey or grey over time (Fig. 3a). The reverse side of the Petri plate became greyish to black (Fig. 3b). Pycnidia were observed after two weeks of incubation. The immature conidia of the fungal isolate were unicellular, hyaline, ellipsoid-ovoid with broadly rounded apex and truncate or round base. Conidia on maturation turned brown in colour with transverse septa and longitudinal striations. The average size of the conidia was 20.0 x 8.6 µm under 400X magnification (Fig. 3c). Based on cultural and morphological characters, fungal pathogen was tentatively identified as *Lasiodiplodia theobromae*

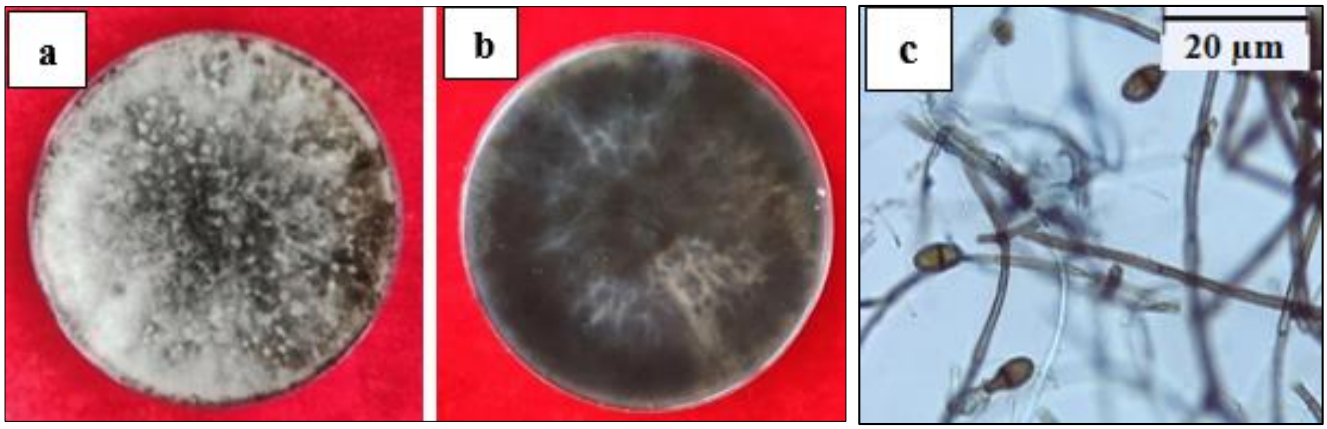


Fig 3: a) Culture of *Lasiodiplodia theobromae* b) Reverse side of the Petri plate c) Conidia of *Lasiodiplodia theobromae*

3.2.2 Molecular characterisation

The pathogen was identified by amplification and sequencing of large subunit ribosomal ribonucleic acid (LSU rRNA) using primers LROR and LR7. The BLASTn analysis showed 97.13 per cent similarity with *Lasiodiplodia theobromae* isolate VTCA having accession number KC442316.1 and 94% query coverage (Fig. 4). The culture was deposited in NCBI GenBank with an accession number of OQ348267. The infection of *Lasiodiplodia theobromae* in *Cattleya* sp. was reported earlier by Cabrera and Cúndom (2013) [5] in Argentina. The pathogen was also reported to cause leaf blight in *Catasetum fimbriatum* (Lopes et al. 2009).

3.3 *In vitro* evaluation of chemicals and biocontrol agents

When the pathogen, *Lasiodiplodia theobromae* was treated with different fungicides, complete inhibition of mycelial growth was observed in all three concentrations of carbendazim 12% + mancozeb 63%, cymoxanil 8% + mancozeb 64%, propineb and 1 per cent Bordeaux mixture (Fig. 5). The findings are in conformity with Amrutha (2020) [1] who reported cent per cent inhibition of the pathogen with carbendazim 12% + mancozeb 63% and cymoxanil 8% + mancozeb 64% at all three concentrations and 81.11 per cent inhibition by Bordeaux mixture. Hegde et al. (2013) also reported complete inhibition of the pathogen by carbendazim 12% + mancozeb 63%. Hexaconazole at its higher concentration showed maximum inhibition percentage (100 per cent), whereas higher concentration of difenoconazole showed an inhibition of 74.58 per cent. This is in agreement with the observations of Amrutha (2020) [1], who recorded 76.67 per cent inhibition of mycelial growth at 0.15 per cent concentration. Copper hydroxide at 0.1, 0.2 and 0.3 per cent showed an inhibition percentage of 58.33, 73.75 and 82.5 per cent respectively (Table 2). Azoxystrobin at 0.05, 0.1 and 0.15 per cent showed inhibition ranging from 37.08 to 50.0 per cent and was found to be least effective against the pathogen. The results are in line with the findings of Rafi (2021) [12], who reported low efficacy of who reported low efficacy of copper hydroxide and azoxystrobin against *Lasiodiplodia theobromae* under *in vitro* condition.

Table 2: *In vitro* efficacy of fungicides against *Lasiodiplodia theobromae*

Sl. No.	Fungicide	Concentration (%)	*Per cent inhibition (%)
1	Copper hydroxide	0.1	58.33 (7.62)^{f}
		0.2	73.75 (8.58)^{cd}
		0.3	82.50 (9.08)^{b}
2	Hexaconazole	0.05	69.58 (8.34)^{d}
		0.1	70.0 (8.36)^{d}
		0.15	100.0 (10)^{a}
3	Propineb	0.1	100.0 (10)^{a}
		0.2	100.0 (10)^{a}

		0.3	100.0 (10)[a]
4	Difenoconazole	0.05	63.75 (7.98)[e]
		0.1	70.41 (8.39)[cd]
		0.15	74.58 (8.63)[c]
5	Carbendazim 12% + Mancozeb 63%	0.1	100.0 (10)[a]
		0.2	100.0 (10)[a]
		0.3	100.0 (10)[a]
6	Cymoxanil 8% + Mancozeb 64%	0.1	100.0 (10)[a]
		0.25	100.0 (10)[a]
		0.3	100.0 (10)[a]
7	Azoxystrobin	0.05	37.08 (6.09)[i]
		0.1	45.0 (6.71)[h]
		0.15	50.0 (7.07)[g]
8	Bordeaux mixture	1.0	100.0 (10)[a]
	Coefficient of Variance (CV)		1.814
	Critical Difference (CD) (0.05%)		0.267

***** Mean of three replications
Values in parentheses are √ (x + 0.5) transformed

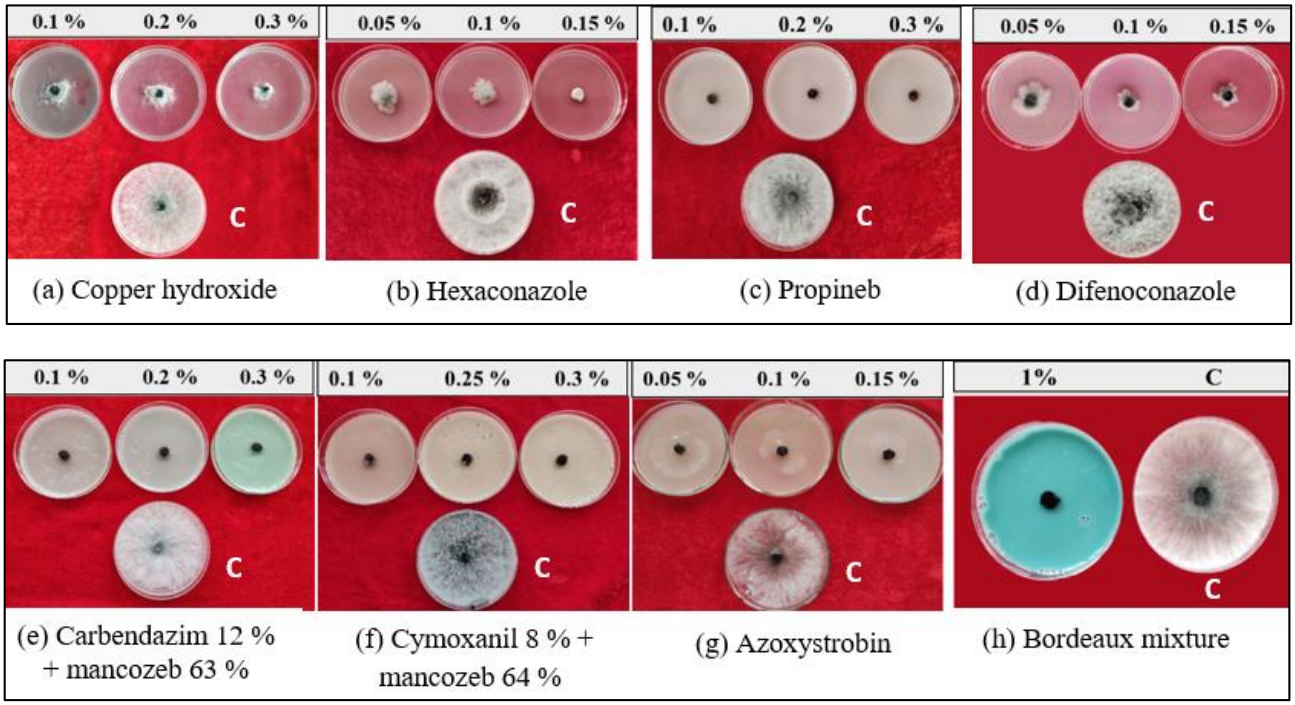


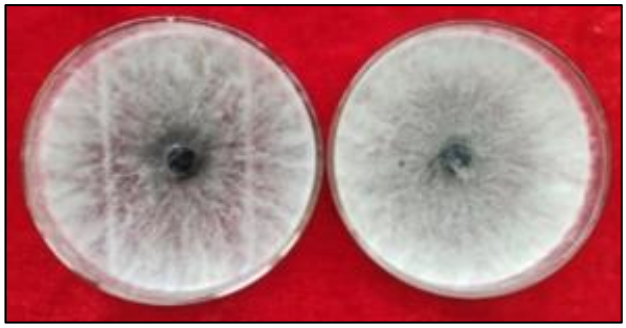
Fig 5: *In vitro* efficacy of fungicides against *Lasiodiplodia theobromae*

Table 3: *In vitro* efficacy of biocontrol against *Lasiodiplodia theobromae*

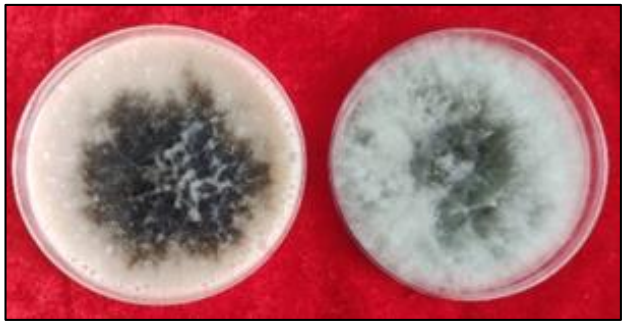
Biocontrol agent	Per cent inhibition of pathogen (%)
Trichoderma asperellum	20.41
Pseudomonas fluorescens	0
PGPR-II	50.41
PGPM	100.00

Among the biocontrol agents tested, PGPM showed 100% inhibition (Fig. 6), followed by PGPM-II (50.41%) and *Trichoderma asperellum* (20.41%), while *Pseudomonas fluorescens* did not show any inhibition (Table 3). The results are in conformity with Rafi (2021) [12], who recorded complete inhibition of the pathogen when treated with PGPM. The studies conducted by Bhadra et al. (2014) [4] revealed low inhibition of *L. theobromae* by volatile metabolites from *Trichoderma viride* (33.3 per cent). The results are also in line with the observations of Amrutha et al. (2020) [1], who found zero inhibition of *L. theobromae* by *Pseudomonas fluorescens.* According to the previous studies, PGPR served as effective antagonists of plant pathogens by different mechanisms such as competition, lysis, antibiosis, parasitism, and by inducing host plant resistance (Beneduzi et al. 2012; Tariq et al. 2017) [3, 17]. Therefore, the present investigation has thrown light upon etiology of emerging threat of leaf spot disease in Cattleya along with its *in vitro* management. To the best of our knowledge, this disease has not been reported so far in India

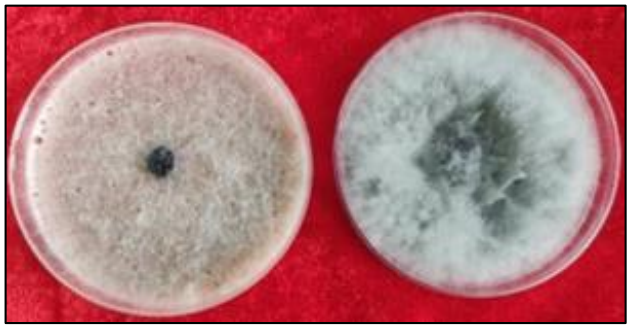
(a) *Trichoderma asperellum*



(b) *Pseudomonas fluorescens*



(c) PGPR II



(d) PGPM

Fig 6: *In vitro* efficacy of bio control against *Lasiodiplodia theobromae*

Conclusion

Fungal diseases are a major threat to orchid production worldwide. The present study identified *L. theobromae* as the causal organism of leaf spot disease in Cattleya based on cultural, morphological and molecular characterization. To the best of our knowledge, this is the first report of *L. theobromae* causing leaf spot disease in Cattleya from India. The efficacy of eight fungicides was tested against *L. theobromae*, of which the fungicides *viz*., cymoxanil 8% + mancozeb 64%, carbendazim 12% + mancozeb 63%, propineb and 1% Bordeaux mixture completely inhibited the growth of the pathogen. Among the biocontrol agents tested, plant growth promoting microorganisms (PGPM) showed complete inhibition, followed by plant growth promoting rhizobacteria-II (PGPM-II) (50.41%) and *Trichoderma asperellum* (20.41%), while *Pseudomonas fluorescens* was found to be least effective in inhibiting mycelial growth of fungus.

4. Conflicts of interests

On behalf of all authors, the corresponding author states that there is no conflict of interest.

5. References

1. Amrutha P, Vijayaraghavan R. Evaluation of fungicides for the management of Lasiodiplodia crown rot of strawberry (*Fragaria× ananassa* Duch.) in Kerala. Chinese Chem. Lett. 2020;9:425-431.
2. Bag TK. Orchid wilt incited by *Sclerotium rolfsii* on some Indian orchids. Indian J of Hill Farming. 2003;16(1/2):97-98.
3. Beneduzi A, Ambrosini A, Passaglia LM. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. Genet and mol bio. 2012;35:1044-1051. https://doi.org/10.1590/S1415-47572012000600020
4. Bhadra M, Khair A, Hossain MA, Sikder MM. Efficacy of *Trichoderma* spp. and fungicides against *Lasiodiplodia theobromae*. Bangladesh J Sci Ind Res. 2014;49(2):125-130. https://doi.org/10.3329/bjsir.v49i2.22008
5. Cabrera MG, Agueda Cúndom M. Occurrence of *Lasiodiplodia theobromae* in *Cattleya* spp in Corrientes, Argentina. Summa Phytopathologica. 2013;39:143-143. https://doi.org/10.1590/S0100-54052013000200014
6. Cating RA, Palmateer AJ, Stiles CM, Rayside PA. Black rot of orchids caused by *Phytophthora cactorum* and *Phytophthora palmivora* in Florida. Plant Health Progress. 2010;11(1):39. https://doi.org/10.1094/PHP-2010-0614-01-DG
7. Hegde YR, Hiremani NS, Keshgond RS, Chavhan TL. Evaluation of fungicides against *Botryodiplodia theobromae* causing collar rot in *Jatropha curcas*. Int J Plant Prot. 2013;6(1):45-47
8. Hsieh YC, Chang CW, Wang CJ. First report of *Rhizoctonia solani* AG-4 HG-I causing leaf blight disease on Cattleya× hybrid in Taiwan. J of Gen Plant Pathol. 2023;89(2):132-135. https://doi.org/10.1007/s10327-022-01108-y
9. Kevorkian A. Orchid diseases. Revista de Agricultura de Puerto Rico. 1940;32:3.
10. Lopes UP, Zambolim L, Pereira OL. First report of *Lasiodiplodia theobromae* causing leaf blight on the orchid *Catasetum fimbriatum* in Brazil. Aust Plant Dis Notes. 2009;4(1):64-65.
11. Morton DT, Stroube NH. Antagonistic and stimulatory effects of microorganism upon *Sclerotium rolfsii*. Phytopathol. 1955;45(8):419-420.
12. Rafi N. Etiology and characterisation of diseases of Anthurium (*Anthurium andraeanum* L.) in Kerala. M.Sc. (Ag) thesis, Kerala Agricultural University, Thrissur; c2021. p. 269.
13. Rangaswamy G, Mahadevan A. Diseases of crop plants in India. (4th edition) Prentice Hall of India Pvt Ltd New Delhi; c1999. p. 607.
14. Rocha JDRDS, Oliveira NTD, Menezes M. Comparison of inoculation methods efficiency for evaluation of *Colletotrichum gloeosporioides* isolates pathogenicity on passion fruits (*Passiflora edulis*). Brazilian Arch of Biol and Technol. 1998;41:140-148.
15. Silva AL, Salcedo-Sarmiento S, Mansur PSC, Barreto R.W *Colletotrichum karstii* causes anthracnose on the orchid *Cattleya walkeriana* in Brazil. Aust Plant Dis Notes. 2021;16(1):1-4. https://doi.org/10.1007/s13314-021-00431-1
16. Suwannarach N, Kumla J, Lumyong S. Leaf spot on Cattleya orchid caused by *Neoscytalidium orchidacearum*

in Thailand. Can J Plant Pathol. 2018;40(1):109-114. https://doi.org/10.1080/07060661.2017.1414882
17. Tariq M, Noman M, Ahmed T, Hameed A, Manzoor N, Zafar M. Antagonistic features displayed by plant growth promoting rhizobacteria (PGPR): A review J Plant Sci Phytopathol. 2017;1(1):38-43.
18. Vincent JM. Distortion of fungal hyphae in the presence of certain inhibitors. Nature. 1927;159:850.
19. Zentmeyer GA. A laboratory method for testing soil fungicides with *Phytophthora cinnamomi* as test organism. Phytopathol. 1955;45:398-404.