Effectivity of *Invitro* Studies for the Management of Mulberry Root Rot Pathogenic Fungus, *Rhizopus* sp.

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A B S T R A C T

Mulberry, a perennial and economically important crop forming the foundation of the sericulture industry, is highly susceptible to soil-borne diseases, particularly root rot, which leads to severe plant mortality and reduced leaf yield. The present investigation clearly established *Rhizopus* sp. as a major root rot causing pathogen of mulberry, isolated from diseased roots and the rhizosphere. The comparative *in vitro* evaluation of biocontrol agents, fungicides, and herbal oils demonstrated significant variation in their antagonistic efficacy against the pathogen, highlighting the importance of a targeted and integrated disease management approach. Among the fungal biocontrol agents, all three *Trichoderma* species exhibited strong antagonistic activity against *Rhizopus* sp., with *T. viride* showing the highest mycelial inhibition, followed closely by *T. harzianum* and *T. asperellum*. The fungicide evaluation revealed marked differences in efficacy across fungicides and concentrations. Propiconazole and Carbendazim consistently recorded the highest inhibition of *Rhizopus* mycelial growth, followed by Hexaconazole and Difenoconazole, especially at higher concentrations. The assessment of herbal oils Eucalyptus oil emerged as the most effective herbal oil, followed by Cinnamon oil, while Clove, Pongamia, and Basil oils.

Introduction

Root-rot diseases are the most destructive soil-borne problems affecting woody perennial crops, frequently causing wilting, chlorosis, stunted growth, and plant death due to root system collapse and disruption of water and nutrient uptake. Because of their soil persistence, cryptic early symptoms, and complex etiology, root-rot pathogens present significant challenges to diagnosis and management in perennial agroecosystems including mulberry. Traditionally, mulberry root rot has been attributed to soil-borne fungi such as *Fusarium* spp., *Rhizoctonia solani*, *Macrophomina phaseolina*, *Botryodiplodia theobromae*, *Phytophthora*, *Pythium*, and *Armillaria* spp., as documented in surveys across Indian sericulture zones (Philip *et al.,* 1995; Sharma *et al.,* 2003; Pappachan *et al.,* 2020; Gnanesh *et al.,* 2022; Mahadeva *et al.,* 2025). Mulberry (*Morus* spp.) is an economically important perennial crop that forms the backbone of the sericulture industry by serving as the sole food source for

the silkworm *Bombyx mori* L. The productivity and nutritional quality of mulberry leaves directly influence silkworm growth, cocoon yield, and silk quality, thereby determining the sustainability and profitability of sericulture (Vijay *et al.,* 2021; Rafiqui & Ayoub, 2023). While its perennial nature ensures continuous leaf supply, it also predisposes plantations to prolonged exposure and accumulation of soil-borne pathogens, resulting in progressive yield decline and significant economic losses (Narayanan *et al.,* 2015; Belagihalli *et al.,* 2022). Ramya Harika *et al.,* (2024) reviewed and outlined common root-rot pathogens affecting mulberry (e.g., *Phytophthora, Pythium, Rhizoctonia, Fusarium, Armillaria*), predisposing factors (waterlogged soil, poor drainage, compaction), symptoms, and a broad set of control measures (cultural, biological, chemical). Root rot damages the root system, disrupts water and nutrient uptake, and leads to symptoms such as chlorosis, wilting, patchy drying, bark peeling with foul odour, and eventual plant death of mulberry (Sanoamuang & Saksirirat, 1984; Philip *et al.,* 1996; Anonymous, 2004). It spread through contaminated soil, irrigation water, and farm implements facilitates rapid transmission within plantations due to overlapping root systems. Loss of established perennial plant, mulberry further aggravates economic impact, as replanting requires substantial time and investment before normal productivity is restored.

However, accumulating evidence suggests that mulberry root rot is often a disease complex involving multiple pathogens and opportunistic fungi rather than a single causal agent. Such complexity complicates accurate diagnosis and effective management. Recent molecular-phylogenetic investigations have revealed the unexpected role of *Rhizopus oryzae* (family Mucoraceae), conventionally regarded as a saprophyte or post-harvest soft-rot fungus, as a primary root-rot pathogen of mulberry in Southern Indian states and its pathogenicity was confirmed by Gnanesh *et al.,* (2021) through controlled inoculation studies on susceptible genotypes. These findings challenge the traditional perception of *Rhizopus* spp. as secondary colonizers and highlight their epidemiological importance in mulberry.

It is well known fact that environmental and soil conditions play a decisive role in the onset and severity of mulberry root rot. Waterlogging, poor drainage, heavy or compacted soils, inadequate aeration, and prolonged high soil moisture conditions frequently encountered in intensively cultivated mulberry gardens, create a favourable niche for soil-borne fungi, including

Rhizopus, to colonize and proliferate in the rhizosphere (Saratha *et al.,* 2021). The emergence of *R. oryzae* as a root-rot pathogen necessitates refinement of diagnostic approaches. Reliance solely on morphological identification or diagnostics targeting a limited group of fungi may result in under estimation or misidentification of Mucoraceous pathogens. Molecular tools based on ITS and protein-coding genes (e.g., ACT, TEF) combined with multi-gene phylogenetic analysis have proven effective for accurate detection and differentiation of *Rhizopus* species from other soil borne fungi (Gnanesh *et al.,* 2022).

Disease management strategies must similarly evolve toward integrated and sustainable approaches. Although chemical fungicides are widely used against root rot, concerns regarding environmental safety, soil health, and adverse effects on silkworms necessitate alternative options. Biological control agents such as *Trichoderma*, *Bacillus*, and antagonistic actinobacteria have shown promising activity against mulberry root-rot pathogens, while cultural practices including improved drainage, soil management, and sanitation remain critical components of integrated disease management (Saratha *et al.,* 2022). It provides useful context for risk factors and management options. However, systematic evaluations of management strategies specifically targeting *Rhizopus* associated root rot under uniform conditions remain limited. In light of the emerging importance of *Rhizopus oryzae* and the complex etiology of mulberry root rot, the present study was undertaken to assess the efficacy of selected biological, chemical and commercially available herbal oils for management strategies for the suppression of *Rhizopus*, a root rot disease causing fungus in mulberry.

Materials and Methods

The rot diseased root samples and soil samples were collected from the mulberry gardens of Paduvanagere and Balechennavalase, Bangalore South District, Karnataka. The fungus was isolated from the collected diseased mulberry roots and soil samples. This fungus was mass cultured on rice, jowar and Potato Dextrose Broth. The same were used for the pathogenicity test *i.e.*, disease causing efficiency. 2 sets of experiment have been done in earthen pots. *i.e.*, one is irrigating for every 10 days and another one was once in 25 days (Mahadeva *et al.,* 2025). The pathogenicity was confirmed by the isolated fungus and it was confirmed as *Rhizopus* sp. by microscopic observation.

The antifungal activity was studied by using a biocontrol fungus, chemical fungicides and commercially available plant oils. Antagonistic effect of biocontrol agents, *T. asperellum, T. harzianum* and *T. viride* were tested against root rot disease causing fungus i.e., *Rhizopus* sp.*,* by *in vitro* study. Sterilized and cooled potato dextrose agar medium of 20 ml was poured into sterilized Petri plates. After solidification, the mycelial disc of 5 mm test fungus was inoculated at one end of petri plate and antagonistic fungus was placed opposite to it. A control plate was also maintained where in test fungal disc was placed and the center of medium without any biocontrol agents. Each treatment was replicated for three times and incubated at room temperature. The observation in treatment plates was recorded when fungal growth reaches periphery of petri plate in the control plate. The inhibition zone between test organism and antagonistic microorganism was measured and compared with control. The per cent inhibition growth of the pathogen was calculated by using formula as suggested by Vincent (1947). The rates of mycelial growth inhibition (GI %) was calculated as

GI % = dc-dt/dc*100

Where dc is mean colony diameter of control sets and dt is mean colony diameter of treatment sets.

The antifungal assay was carried out in Petri dishes (9 cm in diameter) containing Potato Dextrose Agar (PDA). When temperature of the medium (PDA) reached about 40 °C, specific initial concentrations of fungicides and herbal oils were added to PDA and mixed thoroughly and poured to sterilized Petri plates. The test fungal disc of the 5 mm was taken from actively grown culture and placed on center of petri plate. The control plate was maintained without any fungicides. Each treatment was replicated for three times. These plates were incubated to till the fungal growth reached periphery of Petri plates in case of control plate and at the same time the colony diameter of test fungus was recorded in treatment plates. The rates of mycelial growth inhibition (GI %) was calculated as mentioned above (Vincent, 1947).

i. *In-vitro* antifungal activity of fungicides against the root rot causing fungus: *in vitro* (Poisoned Food Technique, PFT) evaluation of 7 fungicides *i.e*., Carbendazim, Difenoconazole, Hexaconazole, Propiconazole, Tebuconazole, Thiophanate methyl and Tricyclazole of 6 different concentrations (0.10 %, 0.20 %, 0.40 %, 0.60 %, 0.80 % and 1.00 %) was conducted against root rot causing fungus, *Rhizopus* sp.

ii. *In-vitro* antifungal activity of herbal oils against the root rot causing fungus: *in vitro* (Poisoned Food Technique, PFT) evaluation of 6 commercially available herbal oils *i.e*., Neem oil, Eucalyptus oil, Clove oil, Cinnamon oil, Basil oil and Pongamia/Karanj oil of 6 different concentrations (1.00 %, 1.50 %, 2.00 %, 3.00 %, 4.00 % and 5.00 %) was conducted against *Rhizopus* sp. a root rot disease causing fungus was conducted. The data were subjected to ANOVA for a completely randomized design (CRD) for *in vitro* studies and statistical tools such as mean and percentage analysis were employed to meet the study's objective.

Results and Discussion

The fungal pathogen was isolated from the root rot diseased samples and rhizosphere samples of mulberry plants. The fungus was identified microscopically as *Rhizopus* sp. The microscopic character was resembled with as described by Yoshida *et al.,* (2001) (Fig. 1a-c). There was not much difference in the pathogenicity test with 2 forms of irrigating the mulberry plants. Antagonistic study of fungal biocontrol agent, fungicides and commercially available herbal oils were tested against *Rhizopus* sp.

Antagonistic study of biocontrol agents

The *in vitro* antagonistic potential of three fungal biocontrol agents (*T. asperellum*, *T. harzianum*, and *T. viride*) was evaluated against root rot disease causing three fungal pathogen, *Rhizopus* sp. by assessing the percentage inhibition over a control. The per cent inhibition of mycelial growth of fungus was calculated and results are presented in Table - 1. All three biocontrol agents exhibited high levels of antagonism to *Rhizopus* sp. fungal pathogen. *T. viride* showed the highest inhibition (74.67 %), followed by *T. harzianum* (72.63 %) and *T. asperellum* (68.65 %). The differences in the percentage inhibition among the three biocontrol agents against *Rhizopus* sp. were statistically significant. *T. viride* and *T. harzianum* were significantly better than *T. asperellum*. The statistically significant differences among the species indicate that antagonistic efficiency is species- and strain-dependent. Similar findings have been reported by Mokhtar and Dehimat (2012), who observed strong inhibition of *Rhizopus stolonifer* by *T. harzianum* both in vitro and in vivo, attributing the suppression to mycoparasitism and enzymatic degradation of pathogen hyphae. Likewise, Alka *et al.,* (2017) reported more than 70% inhibition of *Rhizopus* rot in tomato fruits using

Trichoderma spp. and their culture filtrates, supporting the high inhibition values recorded in the present study. The superior antagonistic activity of *T. viride* and *T. harzianum* observed here is consistent with earlier reports indicating that these species produce higher levels of antifungal metabolites and lytic enzymes such as chitinases, proteases, and β-1,3-glucanases. Haran *et al.,* (1996) demonstrated that these enzymes play a crucial role in degrading the fungal cell wall during mycoparasitism, resulting in hyphal lysis and growth inhibition. Microscopic studies by Elad (2000) further confirmed that *Trichoderma* spp. coil around pathogen hyphae, penetrate them, and cause structural disintegration. Although fewer studies have focused exclusively on *Rhizopus*, several authors have reported similar antagonistic behavior of *Trichoderma* spp. against other filamentous fungi such as *Rhizoctonia solani* and *Fusarium* spp. (Alka *et al.,* 2017; Redda *et al.,* 2018; Mousumi Das *et al.,* 2019). These pathogens share comparable cell wall compositions and growth characteristics with *Rhizopus*, suggesting that the mechanisms described are broadly applicable. The variation in inhibition among the *Trichoderma* species in the present study can therefore be attributed to differences in metabolite profiles, enzyme production, and competitive ability, as also emphasized by Benitez *et al.,* (2004). Overall, the results strongly support the potential of *T. viride* and *T. harzianum* as effective biocontrol agents for managing *Rhizopus*-induced root rot in mulberry.

Antagonistic study of fungicides

The *in vitro* study of percent inhibition of fungal mycelia over control of seven fungicides (Carbendazim, Difenoconazole, Hexaconazole, Propiconazole, Tebuconazole, Thiophanate methyl, and Tricyclazole) at six different concentrations (0.10 %, 0.20 %, 0.40 %, 0.60 %, 0.80 %, and 1.00 %) in controlling a *Rhizopus* shown statistical significance difference among the fungicides, the concentrations, and their interactions (Table-2). Propiconazole (84.81 %) and Carbendazim (83.46 %) exhibited the highest average inhibition for *Rhizopus* mycelial growth, followed closely by Hexaconazole (82.02 %) and Difenoconazole (77.09 %). Tricyclazole recorded the lowest mean inhibition (52.06 %), significantly lower than top-performing fungicides indicating significant variation among fungicides. Inhibition generally increased with higher concentrations, peaking around 0.80 % to 1.00 % for most fungicides. Interaction between fungicides and concentrations of

Propiconazole at 1.00 % (94.81 %) had the highest inhibition, followed by Carbendazim at 0.60 % (91.11 %) and Propiconazole at 0.60 % and 0.80 % (91.11 %). In contrast, Tricyclazole at 0.10 % (46.30 %) showed minimal efficacy. It is found that Propiconazole, Carbendazim and Hexaconazole proved most effective across concentrations. Further, Tebuconazole, Thiophanate methyl, and especially Tricyclazole were significantly less effective against the root rot disease causing *Rhizopus* fungus in mulberry.

The statistical inference confirms significant variation among fungicide, pathogen, concentration, and their interactions, reinforcing the importance of selecting the right combination. It implies that the efficacy of each fungicide varies not only by pathogen but also in combination with concentration and specific fungal species. Higher concentrations (0.60-1.00 %) generally yielded better inhibition, but the response was fungicide-specific. Propiconazole, Hexaconazole, and Carbendazim were the most effective fungicides across all three pathogens and concentrations. Further, Tricyclazole and Tebuconazole were consistently among the least effective, especially against *Rhizopus*. These findings highlight the necessity for pathogen-targeted and concentration-specific fungicide application strategies to achieve optimal fungal control. These results are in close agreement with previous studies reporting high sensitivity of *Rhizopus* spp. to systemic fungicides, particularly triazoles and benzimidazoles. Propiconazole and Hexaconazole belong to the demethylation inhibitor (DMI) group of fungicides, which disrupt ergosterol biosynthesis, leading to impaired cell membrane function and fungal growth arrest. Agrios (2005) highlighted the effectiveness of DMIs against a broad range of fungal pathogens, including fast-growing storage fungi such as *Rhizopus*. Carbendazim, a benzimidazole fungicide, interferes with mitotic spindle formation by binding to β-tubulin, thereby inhibiting cell division. Bautista-Baños, (2014) reported similar high inhibition of *Rhizopus* spp. by Carbendazim in postharvest disease management studies. The concentration dependent increase in inhibition observed in the present study corroborates the findings of Kumar *et al.,* (2018)**,** who reported enhanced fungicidal activity at higher concentrations due to increased uptake and prolonged persistence in the growth medium. However, the comparatively low efficacy of Tricyclazole and Tebuconazole against *Rhizopus* suggests that these fungicides may have limited target specificity or reduced interaction with the physiological pathways of this pathogen.

Table.1 *Invitro* study of antagonistic effect of fungal biocontrol agents against *Rhizopus* sp.

| Sl. No.	Fungal biocontrol agent	% inhibition over control
1	*T. asperellum*	68.65
2	*T. harzianum*	72.63
3	*T. viride*	74.67
	SEm±	0.460
	CD @ 5 %	1.419

Table.2 *Invitro* evaluation of fungicides against root rot disease causing fungal pathogen, *Rhizopus* sp.

Sl. No.	Fungicides	% inhibition over control						
		0.10 %	0.20 %	0.40 %	0.60 %	0.80 %	1.00 %	Mean
1	Carbendazim	72.59	79.26	88.15	91.11	83.70	85.93	83.46
2	Difenoconazole	59.26	78.52	74.07	76.30	87.96	86.44	77.09
3	Hexaconazole	77.78	80.00	82.22	85.93	88.41	77.78	82.02
4	Propiconazole	72.59	79.26	82.96	88.15	91.11	94.81	84.81
5	Tebuconazole	57.06	57.41	53.33	65.56	69.26	43.70	57.72
6	Thiophanate methyl	65.93	65.93	65.93	65.93	58.52	57.78	63.33
7	Tricyclazole	46.30	47.78	48.26	55.22	57.22	57.59	52.06
	Mean	64.50	69.74	70.70	75.46	76.60	72.01	71.50
		Fungicide		Concentration		Fungicide X Concentration		
	SEm±	0.796		0.737		1.950		
	CD @ 1 %	2.244		2.078		5.497		

Table.3 *In vitro* evaluation of herbal oils against root rot disease causing fungal pathogen, *Rhizopus* sp.

Sl. No.	Herbal Oils	% inhibition over control						
		1.00 %	1.50 %	2.00 %	3.00 %	4.00 %	5.00 %	Mean
1	Basil Oil	51.85	53.33	57.78	57.78	58.52	60.37	56.61
2	Cinnamon Oil	54.07	57.78	65.18	63.70	67.41	67.41	62.59
3	Clove Oil	54.82	54.07	59.26	57.04	57.78	68.15	58.52
4	Eucalyptus oil	50.37	55.56	72.59	71.85	74.07	75.19	66.61
5	Neem oil	60.00	41.48	48.89	50.37	62.22	37.04	50.00
6	Pongamia oil	53.33	55.56	57.78	58.52	60.00	63.70	58.15
	Mean	54.07	52.96	60.25	59.88	63.33	61.98	58.75
		Herbal Oils		Concentration		Herbal Oils X Concentration		
	SEm±	0.922		0.922		2.259		
	CD @ 1 %	2.606		2.606		6.383		

Fig.1 a) The growth of Rhizopus sp. on PDA plates (after 7 days at 25°C) showing cottony mycelia. b) bacside of the petriplate. (c) Hyphal structure of *Rhizopus* sp. with sporangiospores at the tip with dome structured zygospore at 40X.
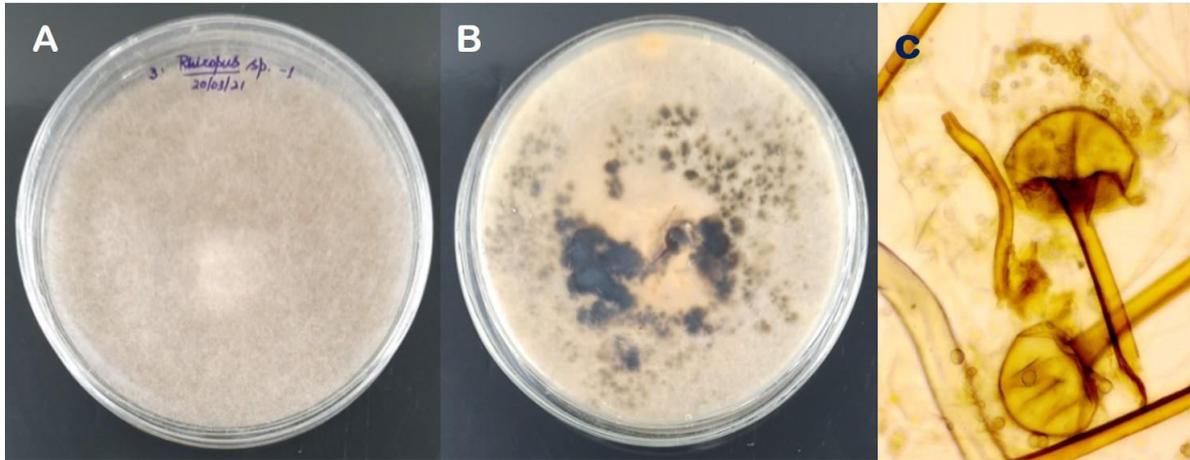
While chemical fungicides were more effective than biological and herbal oil treatments, their long-term use is associated with environmental hazards, pathogen resistance, and negative effects on beneficial microorganisms. These limitations reinforce the need for integrating chemical fungicides with eco-friendly alternatives.

Antagonistic study of herbal oils

The effectiveness of different commercially available 6 herbal oils (Basil Oil, Cinnamon Oil, Clove Oil, Eucalyptus oil, Neem oil and Pongamia oil) at 6 different concentrations (1.00 %, 1.50 %, 2.00 %, 3.00 %, 4.00 % and 5.00 %) was evaluated against the mycelial growth of root rot disease causing fungal pathogen of *Rhizopus* (Table-3). The percentage inhibition of *Rhizopus* fungal growth at different herbal oils at varying concentrations was evaluated. The mean percentage inhibition for each herbal oil across all tested concentrations was as follows: Basil Oil (56.61 %), Cinnamon Oil (62.59 %), Clove Oil (58.52 %), Eucalyptus Oil (66.61 %), Neem Oil (50.00 %), and Pongamia Oil (58.15 %). The mean percentage inhibition across all herbal oils at each concentration level was 1.00 % (54.07 %), 1.50 % (52.96 %), 2.00 % (60.25 %), 3.00 % (59.88 %), 4.00 % (63.33 %), and 5.00 % (61.98 %). A trend of increasing percentage inhibition with increasing concentration was observed for most of the tested herbal oils, although the relationship was not strictly linear for all. Among the tested oils, Eucalyptus oil exhibited the highest overall antifungal activity against *Rhizopus* mycelial growth with the inhibition rate of 66.61 %, reaching a peak inhibition of 75.19 % at 5.00 %. Cinnamon oil also demonstrated strong activity with a mean inhibition of 62.59 %, achieving its maximum inhibition of 67.41 % at both 4.00 % and 5.00 % concentrations. Clove oil and Pongamia oil showed moderate efficacy with mean inhibitions of 58.52 % and 58.15 %, respectively. Basil oil displayed a similar trend, with a slightly lower mean inhibition of 56.61 %. In contrast, Neem oil exhibited the least consistent activity across concentrations and recorded the lowest overall mean inhibition of 50.00 %, with notably reduced efficacy at higher concentrations (*e.g.*, 37.04 % at 5.00 %). The overall mean inhibition across all oils increased with concentration, peaking at 4.00 % (63.33 %) before slightly decreasing at 5.00 % (61.98 %), suggesting a plateau or potential decrease in efficacy at higher concentrations for some oils. The antifungal assay of herbal oils revealed that Eucalyptus oil and Cinnamon oil were the most effective against *Rhizopus* sp., while Neem oil showed the least inhibition. Similar observations were made by Bajpai *et al.,* (2008), who reported strong antifungal activity of cinnamon oil against storage fungi due to the presence of cinnamaldehyde, which disrupts membrane integrity and enzyme systems. Eucalyptus oil contains eucalyptol (1,8-cineole), a compound known to cause membrane destabilization and leakage of cellular contents. Prakash *et al.,* (2015) demonstrated that eucalyptus oil significantly inhibited *R. stolonifer* by altering

membrane permeability and inhibiting spore germination. Likewise, Zhang *et al.,* (2020) reported morphological deformities and cytoplasmic leakage in *Rhizopus* hyphae exposed to essential oils, supporting the present findings. The moderate efficacy of clove, pongamia, and basil oils observed in this study aligns with previous reports indicating that their antifungal activity depends on phytochemical composition and concentration. The inconsistent performance of neem oil against *Rhizopus* has also been reported by Dubey *et al.,* (2011), who suggested that some pathogens exhibit tolerance to azadirachtin-based compounds. The observed plateau or slight decline in inhibition at higher concentrations may be attributed to reduced diffusion of oil components or antagonistic interactions among volatile compounds, as suggested by Uzsakova *et al.,* (2024).

In the present investigation, *Rhizopus* sp. was successfully isolated and confirmed as the causal agent of mulberry root rot and assessed an integrated disease management strategy by using fungal biocontrol agents, chemical fungicides, and herbal oils under *in vitro* conditions. The results confirm that *Rhizopus* sp. can be effectively suppressed through a combination of potent biocontrol agents (*Trichoderma* spp.), highly effective fungicides (Propiconazole, Carbendazim, and Hexaconazole), and selected herbal oils, particularly Eucalyptus oil. These findings may be utilized for *in situ* study which strongly support an integrated, pathogen-specific management strategy that balances efficacy with sustainability for the long-term control of mulberry root rot.

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Author Contributions

Concept, design and Supervision: A Mahadeva. Acquisition, analysis, or interpretation of data: A. Mahadeva., Y.M. Suma., Ramya, M.N.., and Dholakiya Namratta. Drafting of the manuscript: A. Mahadeva., Ramya, M.N.., and Dholakiya Namratta. Critical review of the manuscript for important intellectual content: A. Mahadeva., and Ramya, M.N.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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