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In vitro efficacy of eco-friendly and chemical approaches to manage *Fusarium oxysporum*, a dry root rot disease causing pathogen in mulberry

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Abstract
Root rot disease poses a significant threat to mulberry cultivation. The present study investigates the isolation and *in vitro* management of *Fusarium oxysporum*, the causal agent of dry root rot disease in mulberry. The pathogen was isolated from diseased root samples and rhizosphere soils, and its identity was confirmed based on characteristic cultural and microscopic features. On PDA, colonies were fast-growing with dense white to cream-pink mycelia and a pinkish-yellow reverse. Microscopic observations showed hyaline, septate hyphae, short conidiophores, and slightly sickle-shaped, 3 to 4 septate macroconidia. The antagonistic efficacy of fungal biocontrol agents, fungicides, and herbal oils against *F. oxysporum* was evaluated. Among the biocontrol agents, *Trichoderma viride* exhibited the highest mycelial inhibition (77.21%), significantly outperforming *T. asperellum* (66.68%) and *T. harzianum* (63.00%). Fungicide evaluation across six concentrations showed Hexaconazole (82.35%), Propiconazole (81.88%), and Carbendazim (81.77%) as the most effective inhibitory ability compare to Tebuconazole, Thiophanate methyl, and Tricyclazole. Further, Neem oil (60.90%) and Eucalyptus oil (59.68%) recorded the highest inhibition of mycelial growth followed by Clove and Cinnamon oils. Basil and Pongamia oils were least effective. Overall, *T. viride*, triazole fungicides particularly Hexaconazole and Propiconazole, and selected herbal oils (Neem and Eucalyptus) demonstrated significant antagonistic potential against *F. oxysporum* and may serve as effective components in integrated management strategies for mulberry root rot disease.

Keywords: Biocontrol agents, Fungicides, Herbal oils, *in vitro* Antagonism, Mulberry, Root rot disease

1. Introduction
Mulberry (*Morus* spp.) holds significant economic and agricultural importance as a primary food source for silkworms in sericulture and for its nutritional and medicinal properties. As mulberry leaf is the basic raw material in sericulture, the quality of leaf is of paramount importance for the industry where damage due to diseases and pests constitute a major challenge. The perennial nature of mulberry may be one of the factors for it's prone to soil borne diseases. Root rot disease poses a severe threat to mulberry cultivation, causing wilting, stunted growth, and eventual plant death, leading to substantial economic losses. The root rot diseased mulberry plant shown up to 35% leaf yield loss, reduction in leaf size, deterioration of leaf quality, and plant mortality. Four forms root rot diseases are identified in mulberry. The fungal pathogens of *Fusarium solani* and *F. oxysporum* leads to dry root rot, *Lasiodiplodia theobromae* (syn. *Botryodiplodia theobromae*) causes black root rot, *Helicobasidium mompa* causes violet root rot, *Rosellinia necatrix* causes white root rot diseases in mulberry (Philip *et al.*, 1996; Sharma *et al*., 2003) [19, 3]. *Fusarium*, a widespread fungal pathogen, is known to induce root rot in various plant species, including mulberry. The dry root rot causing *F. solani* and *F. oxysporum* is considered to be one of the most devastating fungal pathogens, which causes root rot disease on more than 500 plant species worldwide (Srivastava *et al*., 2001) [25]. Muthusamy (2019) [16] identified an eleven diverse *Fusarium* species of fungi causing root rot disease in mulberry and *F. solani* shows the highest infection with a disease incidence of 90% compare to rest of the other species.
Fusarium induced root rot in mulberry has focused on pathogen identification, disease symptomatology, and preliminary management strategies. However, significant gaps remain in understanding the host-pathogen interactions, genetic basis of resistance, and effective

control measures. Addressing these problems is difficult to handle due to compounded nature of the fungi and mulberry plant interaction. Therefore, prevention and timely control measures need to be taken up to protect mulberry plant. Disease management strategy largely depends upon the stage/form of pathogen responsible for carrying over a particular disease from one season to another and role of various agencies such as soil, stubbles and weeds in its perpetuation, thus to evolve a suitable and efficient system for disease control. Effective Integrated Disease Management (IDM) with cultural practices, chemicals and bio-control agents seems to be most appropriate and suitable to interrupt this crucial synergistic soil borne disease cycle particularly root rot disease in mulberry. Hence, the present study aims to evaluate the efficacy of fungicides, biocontrol agents and commercially available herbal oils against the *F. oxysporum* fungus.

2. Methodology

2.1. Isolation of root rot disease causing pathogen and pathogenicity test

The rot diseased root samples and soil samples were collected from the mulberry gardens of Paduvanagere and Balechennavalase, Kanakapura Taluk, Ramanagara Dist. The fungus of *F. oxysporum* was isolated from the collected diseased mulberry roots and soil samples. It was confirmed by macroscopic and microscopic study. 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.

2.2. *in-vitro* antifungal activity test

The antifungal activity was studied by using a biocontrol fungus, fungicides and commercially available plant oils. Antagonistic study of biocontrol agents, *T. asperellum*, *T. harzianum* and *T. viride* were tested against root rot disease causing fungus i.e., *F. oxysporum* by *in vitro*. Sterilized and cooled Potato Dextrose Agar (PDA) 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 five times and incubated at room temperature. The mycelial growth in the treated plates was recorded when fungal growth reaches periphery in 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 growth media (PDA) reached about 40 °C, specific initial concentrations of fungicides and herbal oils were added 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 control plate and at the same time the colony diameter of test fungus was recorded in treatment plates. The rate of mycelial growth inhibition (GI%) was calculated by following the above-mentioned formula (Vincent, 1947).

2.2.1. *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, *Fusarium*.

2.2.2. *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 *F. oxysporum* was conducted.

The data were subjected to ANOVA for a completely randomized design (CRD) and statistical tools such as mean and percentage analysis were also employed to meet the study's objective.

3. Result

The fungal pathogen, *F. oxysporum* was isolated from the root rot diseased samples and rhizosphere samples of mulberry plants. The morphological features of *F. oxysporum* were observed macroscopically on culture media (PDA) and microscopically under a microscope. The fungal colony was typically rapid-growing and the mycelia was white and cottony (fluffy), often becoming denser and raised. The colony color was white, cream to pink. The bottom of the colony *i.e*., reverse side of the colony was pinkish to yellowish (Fig. 1). The microscopic fungal structure shows a septate hyphae and hyaline. The conidiophores are typically short and simple. The conidia were hyaline and slightly sickle shaped with 3-4 septate. The pathogenicity test was conducted by employing a 2 method of irrigation *i.e*., one is irrigating for every 10 days and irrigation is once in 25 days. But there was not much difference in the development of root rot disease. Antagonistic study of fungal biocontrol agent, fungicides and commercially available herbal oils were tested against *Fusarium*.

3.1. 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 fungal pathogen, *F. oxysporum*. 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. Statistical analysis revealed significant differences in the mean percentage inhibition among the biocontrol agents against all three pathogens. *T. viride* demonstrated the highest percentage of inhibition (77.21%) against *F.*

oxysporum mycelial growth and it is significantly greater than both *T. asperellum* (66.68%) and *T. harzianum* (63.00%). Though, *T. asperellum* showed slightly higher inhibition than *T. harzianum*, this difference was not statistically significant.

3.2. Antagonistic study of fungicides

The *in vitro* efficacy of seven different fungicides *i.e*., Carbendazim, Difenoconazole, Hexaconazole, Propiconazole, Tebuconazole, Thiophanate methyl, and Tricyclazole against the fungal pathogen, *F. oxysporum* of root rot disease in mulberry was evaluated by assessing the percentage inhibition over a control at 6 varying concentrations (0.10%, 0.20%, 0.40%, 0.60%, 0.80%, and 1.00%). There is a significant difference in the percentage of growth of fungal mycelial inhibition among the tested fungicides (Table-2). Hexaconazole exhibited the highest overall mean percentage inhibition (82.35%), which was statistically superior to Difenoconazole, Tebuconazole, Thiophanate methyl, and Tricyclazole. Propiconazole (81.88%) and Carbendazim (81.77%) also demonstrated highest efficacy compare to Hexaconazole, but significantly greater than Tebuconazole, Thiophanate methyl, and Tricyclazole. Difenoconazole (70.47%) showed intermediate efficacy, significantly greater than Tebuconazole, Thiophanate methyl, and Tricyclazole but less effective than the top three fungicides. Tebuconazole (53.74%) and Thiophanate methyl (54.07%) showed the lowest overall efficacy, with no significant difference between their means. Tricyclazole (57.28%) exhibited relatively lower efficacy, significantly less than the top three fungicides. The effect of concentration on percentage inhibition was also statistically significant. Hexaconazole and Propiconazole generally maintained high inhibition across the concentrations, Difenoconazole showed a more pronounced increase in efficacy at higher concentrations (0.80% and 1.00%). Similarly, the efficacy of Thiophanate methyl remained relatively low across all tested concentrations. Generally, higher concentrations tended to result in greater fungal inhibition. A significant interaction effect was observed between the type of fungicide and the concentration indicating that the effectiveness of each fungicide varied differently across the tested concentrations.

3.3. 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, *F. oxysporum* in mulberry. Among these treatments, Neem oil (60.90%) and Eucalyptus oil (59.68%) recorded the highest inhibition, compare to Basil oil (33.07%) and Pongamia oil (35.59%). Clove oil (57.78%) and Cinnamon oil (55.18%) also showed significantly higher inhibition compared to the least effective oils. A steady increase in inhibition was observed with increasing concentration. The highest inhibition (55.05%) was recorded at 5.00%, which was significantly greater than that at 1.00% (45.12%), suggesting an inhibition rate is a dose-dependent response. Further, different oils responded across their concentrations. For instance, Eucalyptus oil showed a sharp increase of inhibition from 48.89% at 1.00% to 68.89% at 5.00%, while Neem oil remained relatively stable across concentrations (58.52% to 62.22%). Remarkably, Neem, Eucalyptus, Clove, and Cinnamon oils are most potent inhibitors of *F. oxysporum* mycelial growth. The statistical analysis indicates that all type of herbal oil and its concentration influence the inhibition percentage significantly.

4. Discussion

The similar *in vitro* evaluation was worked by various researchers against *Fusarium* (Philip *et al.*, 1996; Bandyopadhyay *et al*., 2003; Sachendra Bohra *et al*., 2005; Seetha Ramulu *et al*., 2010). But, the holistic approach of plant extracts, biocontrol agents and fungicides impact on *Fusarium* is done by us. Pratheesh Kumar (2019) evaluated 10 fungicides, 11 chemicals and 10 plant materials against the *F. solani* and *F. oxysporum* root rot causing pathogens in mulberry. Among them, Carbendazim and Benomyl completely suppressed *F. solani* and *F. oxysporum*, while Mancozeb and Captan showed strong inhibitory effects. Trichloroisocyanuric acid (TCCA) and phosphorus acid were highly effective, fully suppressing *Fusarium* growth in solid media. while, *Allium sativum* and *Azadirachta indica* completely inhibited the growth of *F. solani* and *F. oxysporum* in solid media. Extracts from *Pongamia pinnata*, *Tamarindus indica*, and *Brassica nigra* also reduced the fungal growth significantly. Sita Ram Bana *et al*., (2017) conducted an *in vitro* and *in vivo* studies by using different fungicides and biocontrol agents against root rot disease causing fungus *F. oxysporum* in Fennel (*Foeniculum vulgare* Mill.) plant. The maximum inhibition (%) of fungal growth (*F. oxysporum*) was in Carbendazim followed by Mancozeb and Calcium chloride. The biocontrol agents *T. harzianum* shown a maximum antagonism compare to *T. viride* and *P. fluorescens*. They also shown that carbandazim showed least disease incidence followed by mancozeb, calcium chloride and *T. harzianum* under *in vivo* study. Naveen Chandra Reddy *et al*., (2024) [17] conducted *in vitro* evaluation seven fungal and five bacterial bio control agents and eleven botanical extracts against mulberry root rot pathogen *F. solani.* They found that *T. viride, T. harzianum* and *B. subtilis* had the best inhibition impact on mycelial growth of the pathogen. It was also found that neem had the good antagonistic effect against *F. solani*, whereas, the extracts of Pongamia and Tulsi shown a moderate inhibition ability against mulberry root rot causing fungus.

The fungicides used in the present study belongs to 3 Groups according to Fungicide Resistance Action Committee (FRAC) based on their mode of action. They are Group 1 (Carbendazim and Thiophanate methyl), Group 3 (Difenoconazole, Hexaconazole, Propiconazole and Tebuconazole) and Group 16 (Tricyclazole). Carbendazim and thiophanate methyl binds to fungal β-tubulin, preventing the assembly of the protein subunits into functional microtubules during mitosis in the fungal pathogen. This prevents the development of new fungal hyphae and inhibits spore germination (Zhou *et al*., 2016) [30]. Resistance to carbendazim fungicides is related to alterations in the binding sites on the β-tubulin protein and overexpression of the β-tubulin protein may also contributing to increased tolerance (Chen *et al*., 2009) [6]. Difenoconazole, Hexaconazole, Propiconazole, and Tebuconazole are systemic fungicides belonging to the triazole group (a subclass of azole fungicides). They are

Demethylation Inhibitors (DMIs) because they inhibit sterol 14α-demethylase (CYP51), essential for the synthesis of ergosterol, a vital component of the fungal cell membrane. Blocking ergosterol synthesis disrupts the integrity and function of the fungal cell membrane, leading to growth inhibition and cell death. The affinity of azole group to the fungal CYP51 enzyme inhibits the fungal growth. The fungal CYP51 point mutations may leads to resistance against azole-based fungicides (Parker *et al*., 2014) [18]. Therefore, the inhibitory reciprocation by the *F. oxysporum* to Hexaconazole, Propiconazole and Tebuconazole in the present study is due to the binding ability of azole to sterol 14α-demethylase (CYP51) and point gene mutations of CYP51 or overexpression of CYP51. Tricyclazole inhibited the melanin biosynthesis in fungal appressoria and reduced the sporulation, spore size and number of septa in conidia. As a result, appressorium unable to penetrate the host plant's epidermis, thus blocking the infection process (Anirudha Chattopadhyay *et al*., 2013) [2]. The prolonged and excessive application of synthetic fungicides has caused pathogen resistance, environmental pollution, and detrimental effects on soil health and non-target organisms (Fisher *et al*., 2020) [8]. Consequently, sustainable disease management strategies, particularly biological control agents and plant-based extracts have emerged as vital, eco-friendly alternatives to mitigate these ecological risks (Lugtenberg and Kamilova, 2009) [14]. In the present study, it has been proved that *T. viride* demonstrated the highest percentage of inhibition against *F. oxysporum* mycelial growth. *Trichoderma* species represent the most extensively researched and commercially adopted biocontrol agents for managing soil-borne pathogens, particularly *F. oxysporum* in various plant crops. Prominent species such as *T. harzianum*, *T. viride*, *T. asperellum*, and *T. atroviride* exert their antagonistic effects through the secretion of lytic extracellular enzymes specifically chitinases, glucanases, and proteases, which degrade fungal cell walls (Benítez *et al*., 2004). Furthermore, this enzymatic activity is complemented by the production of potent secondary metabolites like peptaibols, gliotoxins, and harzianopyridones (Vinale *et al*., 2008) [27], which actively suppress pathogen growth, inhibit sporulation, and disrupt virulence mechanisms. From the present study, the herbal oils of eucalyptus, neem, clove and cinnamon oils shown a promising inhibition capability against the *F. oxysporum*. Gaumann (1958) [10] opined that the pathogenicity relies heavily on the secretion of fusaric Acid, a non-host-specific toxin that induces vascular wilt. Geraldo (2010) [11] found that neem oil decreases and even inhibited the production of fusaric acid. Eugenyl acetate, eugenol, and β-caryophyllene are the most significant phytochemicals in clove oil (Batiha, 2020) [4]. The microscopic observations made by Zhou (2023) [29] revealed that eugenol disrupted the hyphal morphology of *F. oxysporum* by *d*estroying the cell membrane integrity of the pathogenic fungi, resulting in the leakage of intercellular contents, including electrolytes, soluble proteins, nucleic acids, and malonydialdehyde. The antifungal activity of cinnamon oil against a fungal pathogen, *F. verticillioides* was studied by the Fuguo Xing (2014) [9]. They found an irreversible deleterious morphological and ultrastructural alterations, such as lack of cytoplasmic contents, loss of integrity and rigidity of the cell wall, plasma membrane disruption, mitochondrial destruction, folding of the cell. 1,8-Cineole (Eucalyptol) is the major active constituent compound of Eucalyptus oil. Kajal Singh *et al*., (2014) demonstrated that 1,8-cineole significantly downregulates genes responsible for ergosterol biosynthesis (e.g., *ERG* gene family). This depletion of ergosterol prevents the proper formation of the fungal cell membrane.

Table 1: *In vitro* study of antagonistic effect of fungal biocontrol agents against *F. oxysporum.*

Sl. No.	Fungal biocontrol agent	% inhibition over control
1	*T. asperellum*	66.68
2	*T. harzianum*	63.00
3	*T. viride*	77.21
SEm±		1.062
CD @ 5 %		3.273

Table 2: *In vitro* evaluation of fungicides against root rot disease causing fungal pathogen, *F. oxysporum.*

Sl. No.	Fungicides	% inhibition over control						
		0.10 %	0.20 %	0.40 %	0.60 %	0.80 %	1.00 %	Mean
1	Carbendazim	76.30	80.74	83.70	83.93	83.70	82.22	81.77
2	Difenoconazole	59.93	65.26	64.81	73.70	76.96	82.15	70.47
3	Hexaconazole	79.26	83.70	80.74	82.96	85.19	82.22	82.35
4	Propiconazole	74.81	81.85	82.59	80.96	82.96	88.11	81.88
5	Tebuconazole	53.33	52.59	53.70	52.96	57.26	52.59	53.74
6	Thiophanate methyl	52.59	51.48	54.15	54.81	55.11	56.30	54.07
7	Tricyclazole	55.56	57.78	62.22	58.52	55.56	54.07	57.28
Mean		64.54	67.63	68.85	69.69	70.96	71.10	68.79
		Fungicide		Concentration		Fungicide X Concentration		
SEm±		0.855		0.791		2.093		
CD @ 1 %		2.409		2.230		5.900		

Table 3: *In vitro* evaluation of herbal oils against root rot disease causing fungal pathogen, *F. oxysporum.*

Sl. No.	Herbal Oils	% inhibition over control						
		1.00 %	1.50 %	2.00 %	3.00 %	4.00 %	5.00 %	Mean
1	Basil Oil	28.52	30.00	32.52	34.07	35.19	38.15	33.07
2	Cinnamon Oil	48.15	52.59	56.30	57.78	57.04	59.26	55.18
3	Clove Oil	55.56	56.30	58.52	59.26	54.08	62.96	57.78
4	Eucalyptus oil	48.89	53.33	57.04	64.44	65.48	68.89	59.68
5	Neem oil	58.52	61.48	61.48	62.22	59.92	61.78	60.90
6	Pongamia oil	31.11	33.19	36.30	35.56	38.15	39.26	35.59
Mean		45.12	47.82	50.36	52.22	51.64	55.05	50.37
		Herbal Oils		Concentration		Herbal Oils X Concentration		
SEm±		1.103		1.103		2.701		
CD @ 1 %		3.115		3.115		N/A		

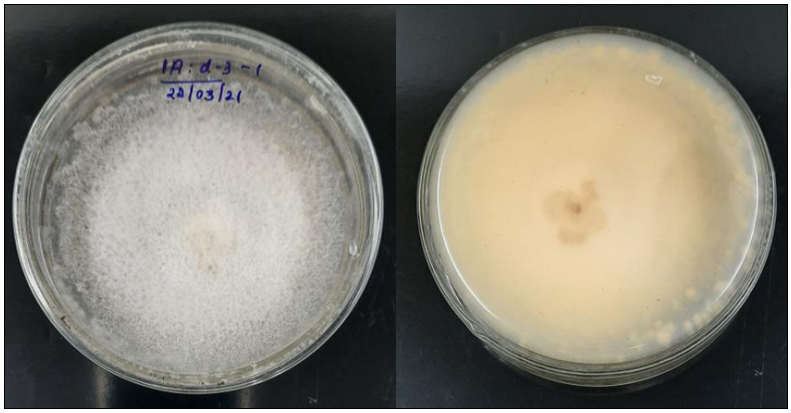

Fig 1: Macroscopic feature of *F. oxysporum* on PDA after 7 days at 25°C (upper and reverse side).

5. Conclusion

An adoption of IDM is proposing to manage soil root rot disease in mulberry, which includes cultural practices, chemicals and bio-control agents. The combined practices lead to synergistic effects in disease suppression. The present study result indicates that fungicides (Hexaconazole, Carbendazim, Propiconazole) are generally more effective in inhibiting the growth of mycelia of *F. oxysporum* than the fungal biocontrol agents (*Trichoderma* sp.) and herbal oils tested. Among the biocontrol agents and the herbal oils, *T. viride* and Neem oil respectively showed the most promising complementary management strategies for *F. oxysporum*, a root rot causing pathogen in mulberry.

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