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Evaluation of fungicide sensitivity in *Colletotrichum gloeosporioides* causing arecanut leaf spot disease

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Abstract
Arecanut (*Areca catechu* L.) is an economically important plantation crop in India, but its productivity is increasingly threatened by leaf spot disease caused by *Colletotrichum gloeosporioides*. Effective disease management relies on identification of potent fungicides, particularly against virulent isolates. The present study evaluated the *in vitro* sensitivity of a highly pathogenic isolate (Shiralakoppa) to contact, systemic and combi-fungicides using poisoned food technique. Among the contact fungicides, Propineb (61.39% mean inhibition) was most effective, followed by Bordeaux mixture (49.44%), while other copper-based formulations recorded demonstrated minimal efficacy (Copper hydroxide 53.8% DF: 17.22%; Copper oxychloride 50% WP: 29.72%). Systemic fungicides exhibited significantly higher efficacy, with Tebuconazole (97.50%), Propiconazole (93.89%) and Carbendazim (89.17%) showing strong inhibition of mycelial growth. Strobilurins such as Azoxystrobin (70.83%) and Trifloxystrobin (84.17%) were moderately effective. Combi-fungicides performed best overall, with Mancozeb + Carbendazim (95.28%), Azoxystrobin + Tebuconazole (92.78%) and Zineb + Hexaconazole (89.72%) achieving near-complete inhibition at higher concentrations. The results highlight that triazoles, particularly Tebuconazole and Propiconazole and combi-products integrating systemic and contact fungicides, offer the most promising options for integrated management of *C. gloeosporioides* in arecanut and also demonstrate clear concentration-dependent responses for all fungicides, with combination products and systemic fungicides outperforming single-site contact fungicides. These findings provide a baseline for fungicide selection and resistance management strategies to control leaf spot disease under field conditions.

Keywords: *Colletotrichum*, fungicide sensitivity**,** arecanut, leaf spot

1. Introduction
Arecanut (*Areca catechu* L.), widely referred to as betel nut, is an important plantation crop cultivated across South and Southeast Asia. India is the world's largest producer, accounting for more than half of the global output, with Karnataka alone contributing nearly 70% of the national production. Beyond its socio-economic significance as a commercial crop, arecanut holds immense cultural and medicinal value, being an integral component of social and religious practices (Ahuja and Ahuja, 2011) [1]. However, its productivity is hampered by several biotic stresses, among which fungal diseases are the most destructive.

Leaf spot disease, caused predominantly by *Colletotrichum gloeosporioides*, has emerged as a major constraint in arecanut-growing regions. The pathogen infects leaves at various stages of crop growth, leading to characteristic necrotic lesions, premature leaf senescence, and reduced photosynthetic efficiency. Under conducive conditions, disease severity can escalate rapidly, resulting in substantial yield losses (Desai *et al*., 2019) [6]. Conventional disease management practices, including sanitation and cultural measures, often provide limited protection, thereby necessitating the use of fungicides as a primary control strategy.

Chemical control of *Colletotrichum* spp. typically involves both protectant and systemic fungicides. Contact fungicides such as copper formulations and dithiocarbamates provide a protective barrier but have limited curative action. In contrast, systemic fungicides including triazoles, benzimidazoles, and strobilurins interfere with fungal metabolism, offering higher efficacy (Gullino *et al*., 2010; Lamichhane *et al*., 2018) [14, 17]. Combi-fungicides that integrate systemic and contact components are increasingly favored for their broad-spectrum activity and potential to delay resistance development (Brent & Hollomon, 2007; FRAC,

2023) [4, 10]. However, continuous exposure to fungicides can lead to shifts in pathogen sensitivity, making periodic monitoring of fungicide efficacy essential for sustainable management.

In this context, the present study was undertaken to evaluate the *in vitro* sensitivity of a virulent *C. gloeosporioides* isolate against selected contact, systemic, and combi-fungicides. The objective was to identify effective molecules that can suppress mycelial growth and provide a scientific basis for field-level fungicide recommendations in integrated disease management programs.

2. Materials and Methods

2.1 Isolation and maintenance of the pathogen

Arecanut leaves showing typical leaf spot symptoms were collected from Shiralakoppa, Karnataka, India. Small segments of infected tissue were surface-sterilized with 1% sodium hypochlorite for 1 min, rinsed thrice in sterile distilled water, and placed on potato dextrose agar (PDA) plates. After incubation at 25±2 °C for 7 days, fungal colonies were purified using the single-spore isolation technique. The isolate was maintained on PDA slants at 4 °C for subsequent studies.

2.2 Morphological and Molecular Identification

The purified isolate was identified based on cultural and morphological characteristics including colony color, texture, growth pattern, and conidial morphology. For molecular identification, genomic DNA was extracted using the CTAB method. The ITS region was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR products were sequenced and compared with known sequences in the NCBI database using BLAST analysis.

2.3 Pathogenicity Test

Pathogenicity was confirmed using detached leaf assay on water agar. Healthy detached arecanut leaves were surface sterilized and artificially inoculated using the pinprick method with mycelial plugs (5 mm diameter) from 7-day-old cultures. Inoculated leaves were maintained in humid chambers at 25±1°C. Control leaves were inoculated with sterile PDA plugs. Disease development was monitored daily for 12 days and the pathogen was re-isolated from symptomatic tissues to fulfill Koch's postulates.

2.4 *in vitro* Fungicide Sensitivity Assay

The sensitivity of *C. gloeosporioides* to various fungicides was evaluated using the poisoned food technique. Three categories of fungicides were tested: (1) Contact fungicides: Propineb 70% WP, Bordeaux mixture, Mancozeb 75% WP, Zineb 75% WP, Copper oxychloride 50% WP, and Copper hydroxide 53.8% DF; (2) Systemic fungicides: Hexaconazole 5% EC, Tebuconazole 25.9% EC, Propiconazole 25% EC, Carbendazim 50% WP, Azoxystrobin 75% WP, and Trifloxystrobin 25% WG; (3) Combi-fungicides: Azoxystrobin 11% + Tebuconazole 18.3% SC, Tebuconazole 50% + Trifloxystrobin 25% WG, Mancozeb 63% + Carbendazim 12% WP, and Zineb 68% + Hexaconazole 4% WP.

Stock solutions of each fungicide were prepared in sterile distilled water or appropriate solvents and incorporated into PDA medium at four different concentrations (contact fungicides: 100, 200, 300, 400 ppm; systemic fungicides: 50, 100, 150, 200 ppm; combi-fungicides: 75, 150, 225, 300 ppm). A mycelial disc (5 mm diameter) from the margin of a 7-day-old culture was placed in the center of each plate. Control plates contained PDA without fungicide. Each treatment was replicated three times.

Radial growth (mm) of the fungus was measured after 7 days of incubation at 25±1 °C. Percentage inhibition of mycelial growth was calculated using the formula (Vincent, 1947) [27]

$$I = \frac{C - T}{C} X100$$

Where,

I = percent inhibition

C = Radial growth of pathogen in control plate

T = Radial growth pathogen in treatment (fungicide) plate

The isolates were designated as highly sensitive, sensitive, weakly sensitive. Moderately resistant, resistant, resistant and highly resistant based on the ED_{50} values as indicated below (Finney 1952) [9].

Sl. No.	Sensitive group	ED_{50} value (µg ml^{-1})
1	Highly sensitive	<150
2	Sensitive	150-250
3	Weakly resistant	250
4	Moderately resistant	250-350
5	Resistant	350-550
6	Highly resistant	>550

2.5 Statistical Analysis

The experiment was laid out in a completely randomized design (CRD). Percent data were arc sine transformed before analysis. Analysis of variance (ANOVA) was performed, and treatment means were compared at the 1% level of significance ($p \leq 0.01$).

3. Results and discussion

3.1 Morpho-Cultural Characterization of *Colletotrichum* spp.

Leaf spot symptoms in areca palm were initial appear on older leaves, where the severity of disease was higher. Early symptoms were in the form of small, round to oblong spots that ranged from light to dark brown colour, generally with brown to black borders. One concomitant *Colletotrichum* sp., was isolated from diseased tissue by the standard tissue isolation method. On Potato Dextrose Agar (PDA), the isolate produced greyish white colonies with a flat growth pattern and distinct concentric zonation, suggesting variance in melanin production and sporulation on PDA that were consistent with the observation of Weir *et al*. (2012). The fungus exhibited septate, hyaline mycelia and produced single-celled, hyaline, cylindrical conidia with rounded ends. Conidial dimensions averaged 11.50-15.80 µm in length and 4.20-4.90 µm in breadth, typically containing 1-2 oil globules. The results were in similarity with the findings of Desai *et al*. (2019) [6] presence of oblong or cylindrical, slightly dumbell, hyaline, aseptate with rounded ends containing 1-2 oil globules in *Colletotrichum* spp affecting areca palm.

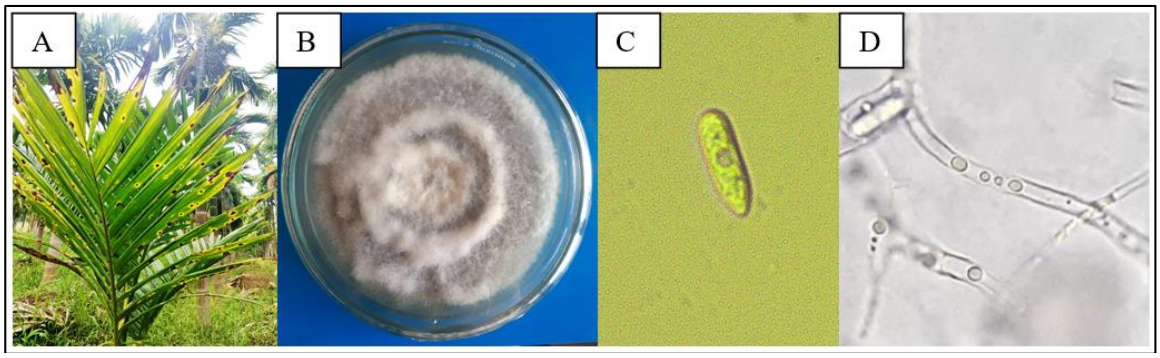

Fig 1: Symptoms of arecanut leaf spot disease and morphological characterization of the associated fungal isolate. (A) Early to mid-stage symptoms on a leaf, showing light to dark brown necrotic spots surrounded by a distinct yellow halo. (B) A 7-day-old pure culture growing on Potato Dextrose Agar (PDA) at 28±2°C. (C) Micrograph (40X) showing conidial morphology. (D) Micrograph (40X) highlighting septate mycelial structures.

Colletotrichum spp. demonstrated robust growth, achieving a mean colony diameter of 68.29 mm after 12 days of incubation (DAI), which was among the highest recorded and statistically on par with the top-performing isolates. Furthermore, *Colletotrichum* spp. exhibited excellent sporulation, producing more than 75 conidia per microscopic field at 40X magnification.

3.2 Pathogenicity and Molecular Identification

Pathogenicity was confirmed via a detached leaf assay on water agar. *Colletotrichum* sp. induced visible symptoms as early as 4 days post-inoculation, with lesions developing from small reddish-brown spots into greyish necrotic areas surrounded by a dark brown margin and a prominent yellow halo. The pathogen was successfully re-isolated from symptomatic tissue, fulfilling Koch's postulates. The result concur with previous research conducted by Cao *et al.* (2020) [5] and Pandian *et al.* (2024) [20], who also documented early symptom development and repeatable re-isolation in *Colletotrichum* pathogenicity tests.

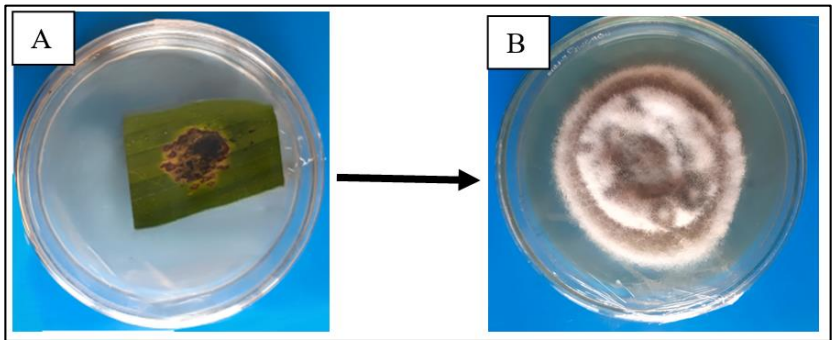

Fig. 02: Pathogenicity test of *Colletotrichum* sp. on arecanut leaf spot lesion. A) Inoculated diseased leaf tissue on water agar; B) subsequent mycelial growth of the pathogen after incubation.

Molecular characterization through sequencing of the ITS region confirmed the identity of pathogen as *Colletotrichum gloeosporioides* (GenBank Accession: PX247853). Specific PCR amplification with primers CgINT and ITS-4 yielded a 450 bp amplicon, further confirming its species designation. This result was similar to those reported by Serra *et al.* (2011) [25] and Zivkovic *et al.* (2017) [30] earlier.

3.3. *in vitro* Fungicide Sensitivity Assay

In vitro evaluation of fungicide efficacy against *Colletotrichum gloeosporioides* revealed significant differences in sensitivity across fungicide groups (Tables 1-3). Among contact fungicides, Propineb 70% WP demonstrated the highest mean mycelial growth inhibition (61.39%), in line with previous observations by Prashanth *et al.* (2008) [24] and Arunprasad (2022) [2]. Propineb is effective because it interferes in the metabolism of carbohydrates and proteins, in the cell membranes and disrupts integrity of membranes. Bordeaux mixture (49.44%) showed moderate inhibition, but other Copper-based fungicides showed minimal efficacy, with Copper hydroxide 53.8% DF and Copper oxychloride 50% WP inhibiting growth by only 15.19% and 17.22%, respectively, indicating likely adaptive resistance due to long-term field applications, as pointed out by Bhat *et al.* (1991) [3] and Padalkar *et al.* (1996) [19]. All contact fungicides exhibited concentration-dependent responses, with maximum inhibition observed at 400 ppm (Table.01).

Table 1: Sensitivity of *Colletotrichum gloeosporioides* to Contact Fungicides (% Inhibition)

Tr. No.	Fungicides	Mycelial growth inhibition (%) Concentration				Mean
		100 ppm	200 ppm	300 ppm	400 ppm	
T_1	Propineb 70% WP	51.11# (45.64)*	60.00 (50.77)	65.56 (54.07)	68.89 (56.10)	61.39 (51.64)
T_2	Bordeaux Mixture	38.89 (38.57)	50.00 (45.00)	52.22 (46.27)	56.67 (48.83)	49.44 (44.67)
T_3	Mancozeb 75% WP	36.67 (37.26)	40.00 (39.23)	43.33 (41.17)	50.00 (45.00)	42.50 (40.66)
T_4	Zineb 75% WP	35.56 (36.60)	37.78 (37.92)	40.00 (39.23)	41.11 (39.88)	38.61 (38.41)
T_5	Copper oxychloride 50% WP	5.56 (13.09)	11.11 (19.34)	23.33 (28.86)	28.89 (32.50)	17.22 (23.45)
T_6	Copper hydroxide 53.8% w/w DF	4.44 (11.22)	10.80 (18.93)	18.66 (25.57)	26.85 (31.21)	15.19 (21.73)
	Mean	28.70 (30.40)	34.95 (35.28)	40.52 (39.19)	45.40 (42.25)	
		S.Em±		CD ($p \leq 0.01$)		
	Fungicides (F)	0.60		1.70		
	Concentration (C)	0.49		1.39		
	F X C	1.19		3.40		

Mean of three replications
* Figures in the parenthesis are arcsine transformed values

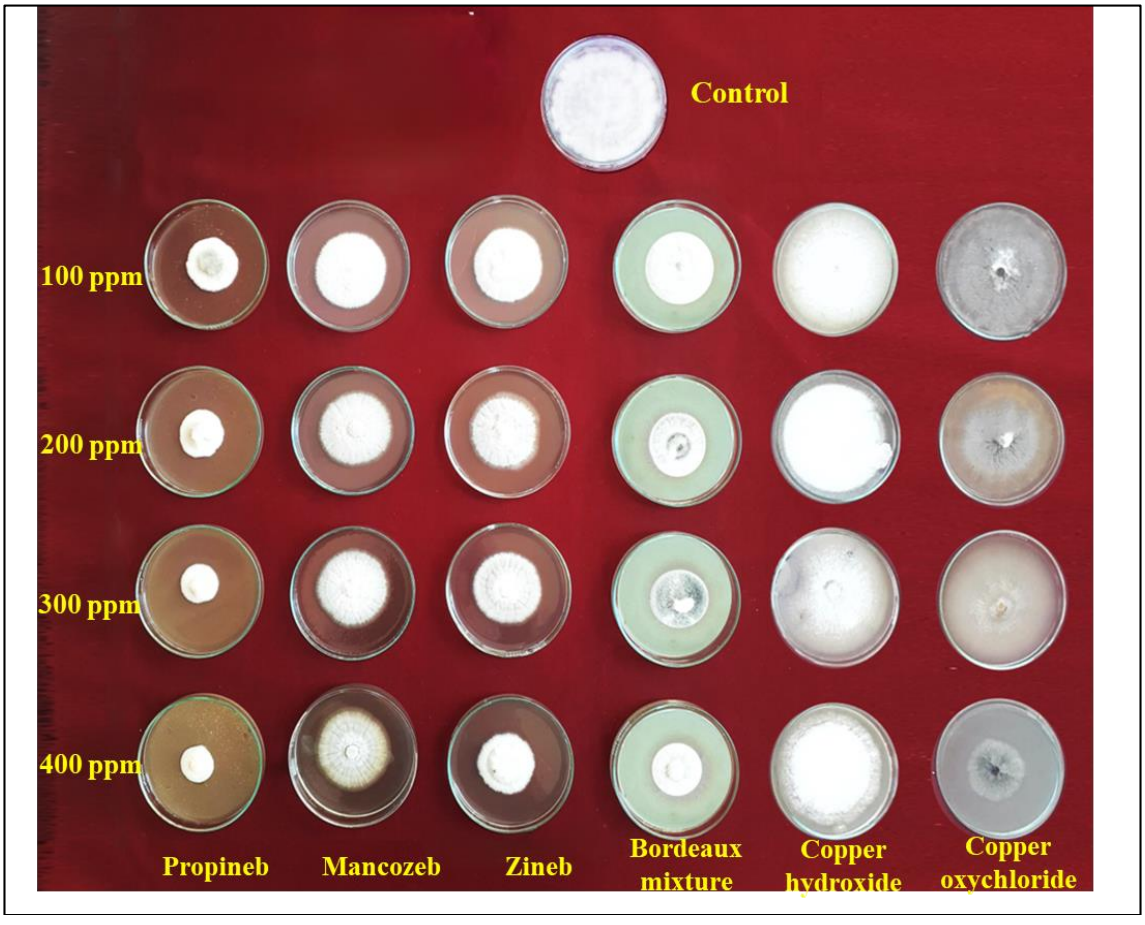

Fig 3: Sensitivity of *Colletotrichum gloeosporioides* isolate to contact Fungicides

Systemic fungicides demonstrated superior efficacy against *Colletotrichum gloeosporioides*. Tebuconazole 25.9% EC achieved the highest inhibition (97.50%), followed by Propiconazole 25% EC (93.89%) and Carbendazim 50% WP (89.17%). These triazole fungicides achieved nearly complete inhibition (100%) at concentrations of 150-200 ppm. Strobilurin fungicides showed moderate effectiveness, with Azoxystrobin 75% WP and Trifloxystrobin 25% WG inhibiting growth by 70.83% and 84.17%, respectively (Table. 02). These results were similar to the earlier reports made by Goswami *et al*. (1996) [12], Pradeep Kumar (2000) [23], Ekbote *et al*. (1997) [7], Gud and Raut (2008) [13], Patel (2009) [21] and Watve *et al*. (2009) [28], kulkarni (2009) [16], Pavitra *et al*. (2017) [22] that Carbendazim and Propiconazole have highest inhibition percentage on the growth of *C. gloeosporioides*. The effectiveness of Carbendazim in

growth inhibition of pathogen *C. gloeosporioides* is characterized by the inhibition of spindle formation during mitosis and there by killing the pathogen (Kalim *et al*., 2000) [15]. The effectiveness of the Triazole fungicides like propiconazole may be attributed to their interference with the biosynthesis of fungal sterols and inhibit the ergosterol biosynthesis. In many fungi, ergosterol is essential for the structure of cell wall and its absence cause irreparable damage to cell wall leading to death of fungal cell. A similar study was reported for the effectiveness of Triazoles, which inhibit the sterol biosynthesis pathway in fungi (Nene and Thapliyal, 1973) [18].

Table 2: Sensitivity of *Colletotrichum* to systemic Fungicides (% Inhibition)

Tr. No.	Fungicides	Mycelial growth inhibition (%) Concentration				Mean
		50 ppm	100 ppm	150 ppm	200 ppm	
T_1	Hexaconazole 5% EC	74.44# (59.64)*	76.67 (61.12)	78.89 (62.65)	82.22 (90.00)	78.06 (62.12)
T_2	Tebuconazole 25.9% EC	90.00 (71.57)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	97.50 (85.39)
T_3	Propiconazole 25% EC	85.56 (67.66)	90.00 (71.57)	100.00 (90.00)	100.00 (90.00)	93.89 (79.81)
T_4	Carbendazim 50% WP	75.56 (60.37)	81.11 (64.24)	100.00 (90.00)	100.00 (90.00)	89.17 (76.15)
T_5	Azoxystrobin 75% WP	64.44 (53.40)	68.89 (56.10)	73.33 (58.91	76.67 (61.12)	70.83 (57.38)
T_6	Trifloxystrobin 25% WG	76.67 (61.12)	83.33 (65.91)	86.67 (68.59)	90.00 (71.57)	84.17 (66.79)
	Mean	77.78 (62.29)	83.33 (68.16)	89.81 (76.69)	91.48 (77.96)	
		S.Em±		CD ($p \leq 0.01$)		
	Fungicides (F)	0.63		1.81		
	Concentration (C)	0.51		1.48		
	F X C	1.27		3.62		

Mean of three replications
* Figures in the parenthesis are arcsine transformed values

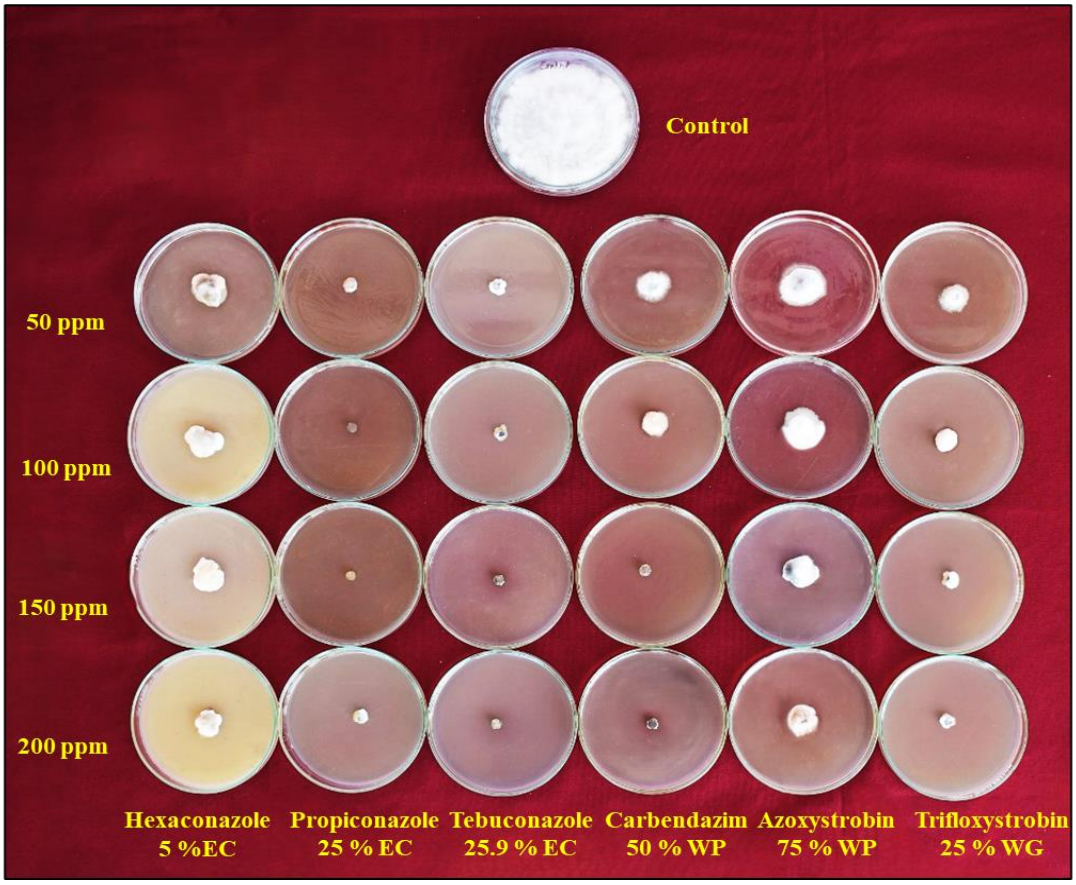

Fig 4: Sensitivity of *Colletotrichum gloeosporioides* isolate to Systemic Fungicides

The *C. gloeosporioides* isolate displayed resistance to single-site fungicides (e.g., strobilurins, benzimidazoles), likely due to target-site mutations such as G143A in the *cyt b* gene (Fernández-Ortuño *et al*., 2008). However, combi-fungicides showed strong synergy. Mancozeb + Carbendazim was highly effective (95.28% inhibition), as

the multi-site action of Mancozeb protects the single-site Carbendazim (Brent & Hollomon, 2007) [4]. Similarly, Azoxystrobin + Tebuconazole (92.78% inhibition) overcame strobilurin resistance via multi-site synergy, attacking both mitochondrial respiration and sterol biosynthesis (FRAC, 2023) [10]. These mixtures achieved 100% inhibition at 400 ppm, confirming their practical value.

Table 3: Sensitivity of *Colletotrichum gloeosporioides* to combi-fungicides (% Inhibition)

Tr. No.	Fungicides	Mycelial growth inhibition (%) Concentration (%)				Mean
		75 ppm	150 ppm	225 ppm	300 ppm	
T_1	Azoxystrobin 11% + Tebuconazole 18.3% SC	83.33# (65.91)*	87.78 (69.54)	100.00 (90.00)	100.00 (90.00)	92.78 (78.86)
T_2	Tebuconazole 50% + Trifloxystrobin 25%WG	74.44 (59.64)	85.56 (67.67)	87.78 (69.54)	100.00 (90.00)	86.94 (71.71)
T_3	Mancozeb 63% + Carbendazim 12% WP	81.11 (64.24)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	95.28 (83.56)
T_4	Zineb 68% + Hexaconazole 4% WP	82.22 (65.06)	86.67 (68.59)	90.00 (71.57)	100.00 (90.00)	89.72 (73.80)
	Mean	80.28 (63.71)	90.00 (73.95)	94.44 (80.28)	100.00 (90.00)	
		S.Em±		CD ($p \leq 0.01$)		
	Fungicides (F)	0.11		0.32		
	Concentration (C)	0.11		0.31		
	F X C	0.22		0.64		

Mean of three replications
* Figures in the parenthesis are arcsine transformed values

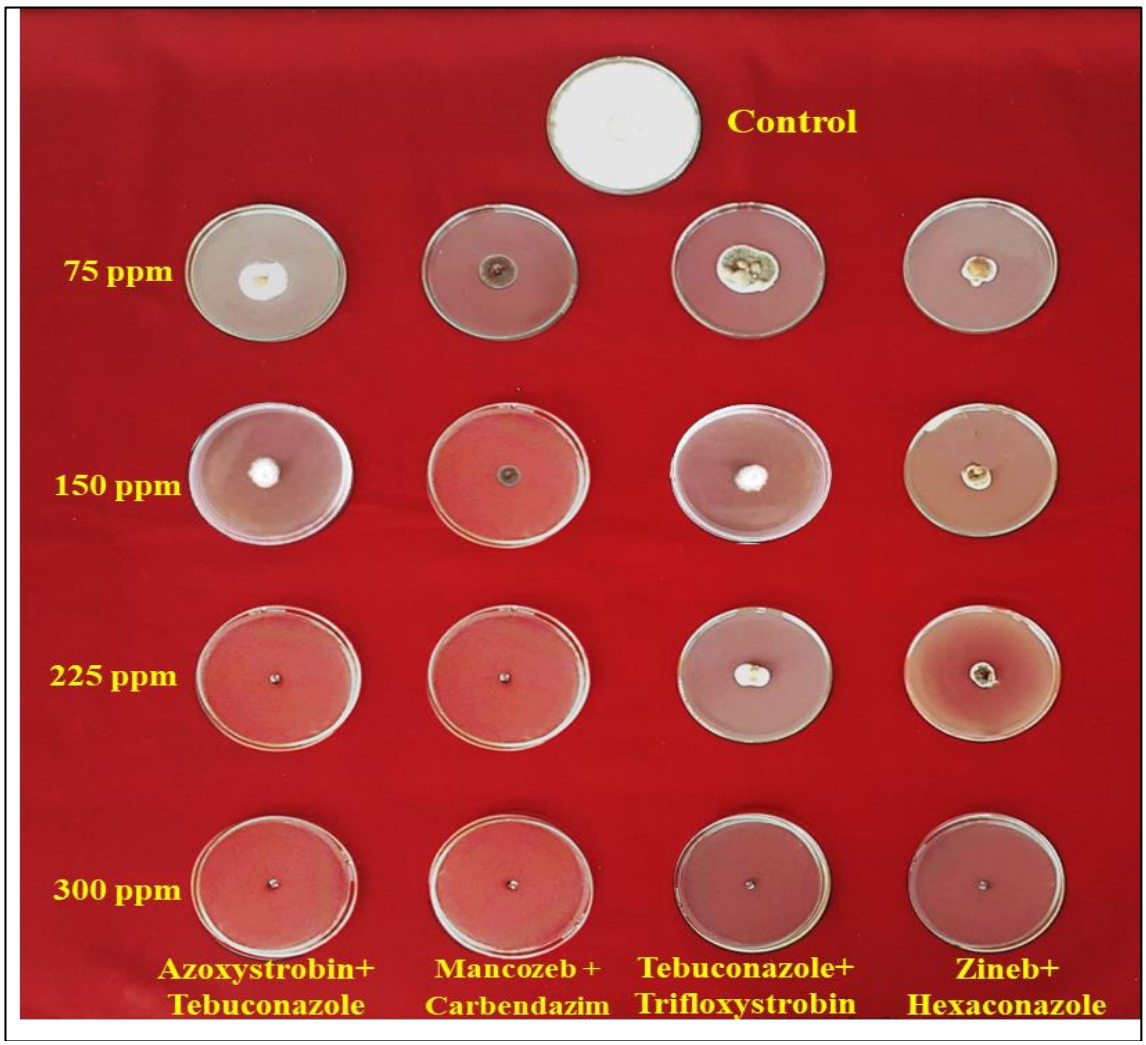

Fig 5: Sensitivity of *Colletotrichum gloeosporioides* to combi-fungicides

The effective dose (ED_{50}) values and sensitivity classification of the *Colletotrichum gloeosporioides* isolate to various fungicides are presented in Table 4. The results showed distinct patterns of sensitivity.

For the *Colletotrichum gloeosporioides* isolate, Propineb 70% WP was the most effective contact fungicide with the lowest ED_{50} value (56.62 µg/mL), classifying the isolate as highly sensitive. This is likely due to its efficient release of

zinc ions and its disruptive action on multiple enzymatic processes within the fungal cell (Gullino *et al*., 2010) [14]. Other contact fungicides like Bordeaux mixture (249.75 µg/mL) showed moderate effectiveness. Mancozeb 75% WP (423.32 µg/mL) showed resistant. the high resistance to Zineb, both belonging to the ethylenebisdithiocarbamate (EBDC) group, are particularly noteworthy. While these fungicides are also multi-site inhibitors, resistance mechanisms, though less common, can involve reduced uptake, enhanced efflux, or metabolic detoxification (Gisi and Sierotzki, 2015). The differential efficacy within dithiocarbamates, Propineb (effective) versus Zineb and Mancozeb (resistant), suggests that the specific metal ion (Zinc in Propineb, Zinc + Manganese in Mancozeb, Zinc only in Zineb) may influence bioavailability or the specific cellular targets for this isolate, warranting further investigation.

Table 4: Effective Dose (ED_{50}) and sensitivity classification of *Colletotrichum* isolate to various fungicides

Sl. No	Fungicide	*Colletotrichum gloeosporioides*	
		ED_{50} (µg/mL)	**Sensitivity Group**
1	Propineb 70% WP	56.62*	Highly sensitive
2	Bordeaux Mixture	249.75	Sensitive
3	Mancozeb 75% WP	423.32	Resistant
4	Zineb 75% WP	852.11	Highly resistant
5	Copper oxychloride 50% WP	648.78	Highly Resistant
6	Copper hydroxide 53.8% w/w DF	713.51	Highly Resistant

Copper-based fungicides (Copper oxychloride 50% WP and Copper hydroxide 53.8% DF) exhibited high resistance, with ED_{50} values exceeding 400 µg/mL. Copper ions are known to generate reactive oxygen species and cause non-specific protein denaturation (Lamichhane *et al*., 2018) [17]. Resistance to copper is often associated with over-expression of copper-binding proteins or efflux transporters that sequester or remove copper ions from the cell (Solel and Kimchi, 1997) [26]. The development of resistance to these traditional, multi-site fungicides is alarming because they are often considered baseline protectants and last-resort options in resistance management strategies.

The present study confirmed that *Colletotrichum gloeosporioides*, the causal agent of arecanut leaf spot, exhibits variable sensitivity to different fungicide groups. Among the contact fungicides, Propineb was most effective, whereas copper-based formulations showed poor efficacy, suggesting possible field-level resistance. Systemic fungicides, particularly Tebuconazole, Propiconazole, and Carbendazim, demonstrated strong inhibition of mycelial growth, while strobilurins were only moderately effective. Combi-fungicides, especially Mancozeb + Carbendazim and Azoxystrobin + Tebuconazole, provided the highest and most consistent suppression, underscoring their synergistic advantage in disease management. These findings provide a scientific basis for selecting fungicides with higher efficacy and reduced resistance risk for integrated management of arecanut leaf spot.

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