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Original Research Paper

First identification of *Curvularia xishuangbannaensis* Tibpromma & K.D. Hyde, sp. nov as a causal agent of leaf spot in oil palm nurseries and evaluation of fungicidal efficacy

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ABSTRACT

Leaf spot caused by *Curvularia* spp. represents a major constraint in oil palm nurseries, adversely affecting plant health and productivity. This study reports the first identification of *Curvularia xishuangbannaensis* as the causal agent of oil palm leaf spot, confirmed through colony and spore morphology, ITS-based molecular phylogenetic analysis, and pathogenicity testing using the disc placement method. The *in vitro* evaluation of fourteen fungicides using the poisoned food technique revealed complete inhibition of mycelial growth by eight fungicides across all tested concentrations, including azoxystrobin 8.3% + mancozeb 66.6% WDG, propiconazole 25% EC, hexaconazole 5% SC, difenconazole 25% EC, triflumizole, tebuconazole 25.9% EC, propiconazole 13.9% + difenconazole 13.9%, and flupyram 17.7% + tebuconazole 11.4% SC. To date, no reports exist on *Curvularia* species causing leaf spot in oil palm in India or systematic fungicide evaluations against this pathogen. These findings enhance understanding of the pathogenicity of *C. xishuangbannaensis* and support effective disease management strategies in oil palm nurseries.

Keywords: *Curvularia*, fungicides, leaf spot, management, oil palm

INTRODUCTION

Oil palm (*Elaeis guineensis*), often reckoned as the 'Golden palm', stands as cornerstone of the global edible oil industry due to its owing to its exceptional productivity of around 4 to 6 tonnes of vegetable oil per hectare per year (Vijay et al., 2016). The production and supply of disease-free seedling are critical for maintaining optimal plant population and ensuring productivity in oil palm plantation. Nursery environments, while designed to provide ideal conditions for seedling growth, inadvertently create a conducive atmosphere for the proliferation of pest and diseases. These infestations, if unmanaged, can compromise seedling health, potentially affecting the long-term yield and sustainability of the plantations.

Leaf spot disease is the most common and significant challenge to oil palm seedlings. These diseases can be rampant if neglected, often leading to defoliation and complete mortality. Under favorable conditions, this disease causes leaf necrosis and premature leaf senescence in young seedlings with 14% mortality reported over nine months (Brahima et al., 2023). The common fungi genera causing leaf spots are Cercospora, Curvularia, *Dreschelra, Neopestalotiopsis*, *Pestalotiopsis*, *Nigrospora, Oxydothis* and *Phyllosticta* (Kovachich, 1954; Sunpapao et al., 2014; Ismail et al., 2017; Zheng et al., 2017, Agustina et al., 2019; Nasehi et al., 2020). The genus *Curvularia* is frequently reported as major pathogen that cause 80% disease incidence in nurseries of Thailand and Indonesia with species such as *C. oryzae*, *C. eragrostidis* and *C. lunata* commonly implicated (Suwarnnarach et al., 2013; Martinez & Plata-Ruedo, 2013, Sunpapao et al., 2014; Shen et al., 2014; Azlan et al., 2018; Lekete et al., 2019, Augustina et al., 2019; Anauar et al., 2022). However, there is no published report on species of *Curvularia* causing leaf spots in oil palm in India.

Several fungicides, including mancozeb, propiconazole, and tebuconazole, have shown effective suppression of *Curvularia*-induced leaf spot diseases in different crops (Poole & Arnaudin, 2014). In oil palm nurseries, a combination of contact and systemic fungicides has been suggested for managing leaf spot diseases caused by related fungal pathogens

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(Brahima et al., 2023). However, there is a lack of comprehensive studies evaluating the efficacy of fungicides against *Curvularia* infecting oil palm in India. In light of these considerations, the present study aims to identify species delimitation and evaluate the efficacy of new-generation fungicides against *Curvularia*-induced leaf spots in oil palm.

MATERIALS AND METHODS

Pathogen isolation and purification

Five *Curvularia*-infected leaves were collected in fresh polythene bags from the oil palm nursery of ICAR-Indian Institute of Oil Palm Research, Pedavegi, Andhra Pradesh (8° 43′ 22.92º N, 77° 1′ 15.02º E) and brought to the laboratory. Leaf spot dimensions were measured using a Zeiss Stemi 305 stereo zoom microscope with ZEN 3.0 software. Infected tissues (1 cm) were surface sterilized with 70% ethanol (30 s), 4% sodium hypochlorite (1 min), and rinsed thrice in sterile distilled water before plating on PDA. Pure cultures were obtained using the hyphal tip technique (Brown, 1924). Cultural characteristics and spore morphology was examined using a Leica DM 5000 B microscope.

Molecular confirmation

Agar plugs (10 mm) from five-day-old *Curvularia* cultures were inoculated into potato dextrose broth, and actively growing mycelial mats (150 mg) were harvested on the seventh day and blot-dried. Genomic DNA was extracted using the HiPurA Fungal DNA Purification Kit (Himedia, India). DNA quality and quantity were assessed by NanoDrop spectrophotometry and 0.8% agarose gel electrophoresis. The nuclear ribosomal internal transcribed spacer (ITS) region was amplified using primers ITS4 and ITS5 (White et al., 1990). Each 15 µL PCR reaction contained PCR buffer, dNTPs, primers, Taq polymerase, and nuclease-free water. PCR cycling conditions were: initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 30 s, annealing at 51°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified products were electrophoresed on 1% agarose gel, stained with ethidium bromide, visualized under UV light, and compared with a molecular size marker. The PCR products were sequenced at Bionivid Technology Private Ltd., India.

Phylogenetic analysis

Identification was performed by comparing ITS, *RPB2*, and *tef1α* sequences with GenBank (NCBI) using BLAST. The top ten matching sequences were aligned using MUSCLE. Conserved, variable, parsimoniously informative sites, and singletons were determined for ITS. Phylogenetic relationships were inferred using the Neighbor–Joining method (Saitou & Nei, 1987) with 1000 bootstrap replications (Felsenstein, 1985). Evolutionary distances were calculated using the maximum composite likelihood method (Tamura et al., 2004), and analyses were conducted in MEGA X (Kumar et al., 2018).

Screening of fungicides for bioefficacy against *Curvularia*

The bioefficacy of fourteen fungicides against *Curvularia* was evaluated at seven concentrations (50–4000 ppm) using the poisoned food technique (Schmitz, 1930). Fungicide solutions were prepared based on active ingredient and incorporated into PDA (Lakshmi et al., 2024), with unamended PDA as control. Mycelial discs (5 mm) from seven-day-old cultures were placed centrally and incubated at 25±1°C. Radial growth was recorded after seven days, and percentage inhibition was calculated using Vincent's formula (Vincent, 1927) as follows. Per cent inhibition = [(C-T)/C]* 100, where C is the growth of the pathogen in the control plate (mm) and T is the mycelial growth of the pathogen in the treated plate (mm) (Vincent, 1927).

Pathogenicity assay

Planting material

Oil palm sprouts obtained from the Seed Processing Unit, ICAR- Indian Institute of Oil Palm Research, Pedavegi, India, were surface sterilised and planted in shallow pits (2–3 cm) in polyethylene bags (23 × 15 cm) filled with sieved, autoclaved soil. Soil was sterilised twice at 121°C for 20 min with a two-day interval. Plants were maintained in a net house under 16 h photoperiod, 28/25 °C (light/dark), >70% relative humidity, with regular watering, fertilization, and sanitation.

Inoculum preparation and Inoculation

The *Curvularia* isolate was cultured on PDA at 25°C for 14 days. Leaf inoculation was performed by placing 5 mm mycelial discs from seven-day-old

cultures on the abaxial leaf surface and securing with moistened cotton. Inoculated plants were covered with polythene bags to maintain humidity. Lesion development was monitored for 5–7 days in ten replicates, and disease severity was measured at seven days post-inoculation using a Zeiss Stemi 305 microscope with ZEN 3.0 software under 70% RH and 25±2°C conditions.

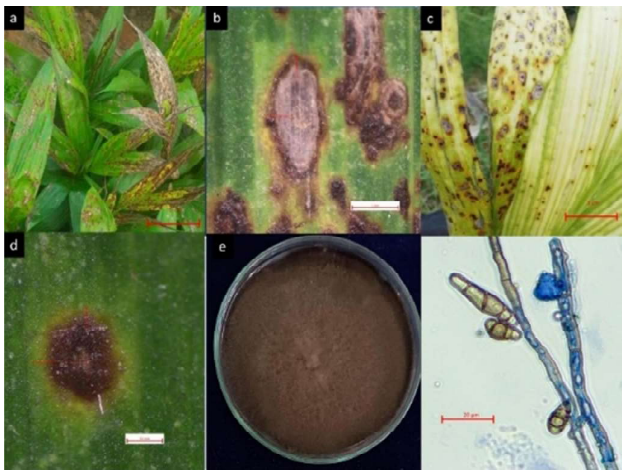
Statistical analysis

The data obtained from the fungicidal evaluation experiments were statistically analyzed using anlysis of variance (ANOVA), and the mean values were compared using LSD, performed using the Web Agri Stat Package (WASP 2.0 software, https://ccari.icar.gov.in/waspnew.html).

RESULTS AND DISCUSSION

Symptomatology

Curvularia leaf spot is characterised by yellow necrotic lesions that enlarge into round to irregular, dark brown to black spots surrounded by a yellow halo, with a lighter centre and occasionally a whitish core bordered by a raised oily brown margin. Severe infections result in coalescing lesions and leaf blight on mature leaves (Fig. 1a). Similar symptoms were also observed on young leaves, though lesions were comparatively smaller (Fig. 1c). Lesion length ranged from 0.25–0.68 mm in younger leaves and 0.558–2.810 mm in older leaves, as observed under a stereomicroscope



a. Infected mature plant leaves, b. Under a stereomicroscope of mature leaves, c. Infected young plant leaves, d. Lesions under a stereomicroscope, e. Colony morphology, f. Spore morphology under 100x magnification

Fig. 1 : Symptomatology, colony, and spore morphology of *Curvularia*

(Fig. 1b, 1d), consistent with earlier reports (Sunpapao et al., 2014; Priwiratama et al., 2023).

Morphological identification of *Curvularia*

All five *Curvularia* isolates exhibited similar colony morphology, characterised by dense, velvety mycelial growth with dark brown to black coloration, circular margins, and prominently raised colonies (Fig. 1e). Colonies initially appeared white with a dark grey centre and attained a radial growth of 9 cm within seven days. Conidia were smooth-walled, obclavate, melanised, 3–5 distoseptate with a dark basal hilum, borne on geniculate, septate conidiophores (Fig. 1f). Based on these traits, the isolate was identified as *Curvularia xishuangbannaensis*, consistent with published descriptions (Tibpromma, 2018).

Molecular identification of *Curvularia*

The ITS region has been useful for discriminating fungi at the species level and has been used extensively for identiûcation and phylogenetic analysis (Gardes & Bruns, 1993). For species identification, PCR-based molecular approach yielded 575 bp of internal transcribed spacer (ITS) region amplicon with sequence identity of 100% was obtained for ITS and compared to GenBank reference sequences (Fig. 2). The ITS consensus sequence generated in this study was deposited in GenBank under the accession number PP930726. The phylogenetic tree based on ITS sequences depicts relationships among *Curvularia* species, with *Curvularia xishuangbannaensis* isolate IIOPR1 clustering with *C. geniculata* and related taxa, while *Exserohilum rostratum* and *C. fallax* were used as outgroups to root the tree. This morpho-molecular approach enhances the accuracy and reliability of the identification process, laying a foundation for further studies on the pathogenicity and diversity of *Curvularia* isolates (Santos et al., 2018).

Pathogenecity

Lesions resembling those observed in the nursery appeared within 3 days of inoculation on all 10 plants inoculated with *Curvularia*, with lesion sizes ranging from 0.381 to 0.664 mm (Fig. 3 c&d). This rapid development of symptoms confirmed the pathogen's ability to infect under controlled conditions. In contrast, the non-inoculated control treatments, including plants treated with an empty PDA disc, sprayed with sterile water and untreated plants serving as absolute controls, remained symptomless

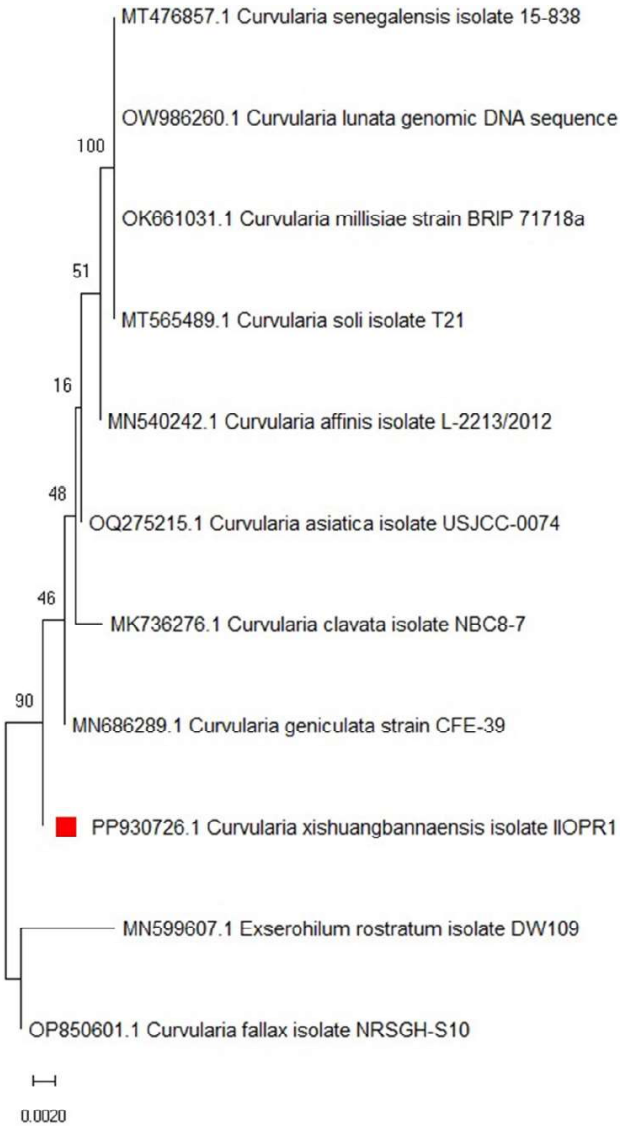
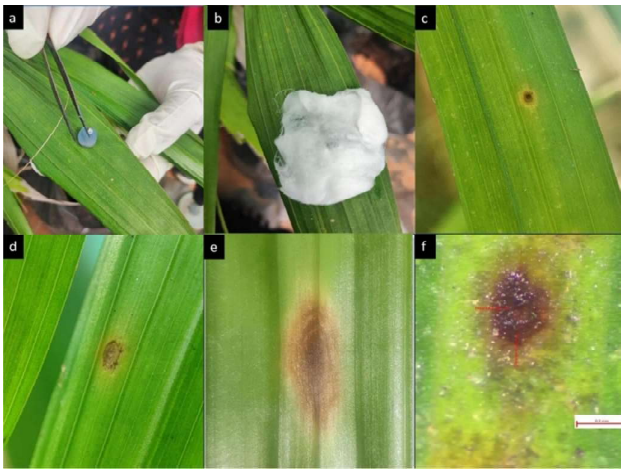


Fig. 2 : Phylogenetic Tree of *Curvularia* Species Based on Internal Transcribed Spacer (ITS) region sequences

The scale bar (0.0020) indicates the genetic distance, measured in substitutions per site

throughout the experiment. Furthermore, reisolation of the pathogen on PDA from the inoculated plants consistently yielded *Curvularia*, confirming Koch's postulates and establishing a direct link between the pathogen and the observed disease symptoms (Fig. 2f). These results demonstrate that the lesions were exclusively caused by the inoculation of *Curvularia*, thereby validating its pathogenicity and its role as the causal agent of the disease observed in the nursery. These findings agree with Garcia-Aroca et al. (2018), who confirmed *C. lunata* as the leaf spot pathogen on corn through conidial spray inoculation.



a&b. Disc placemenet method of inoculation, c,d&e. Disease progression at 3, 5 and 7 days post inoculation, f. Under a stereomicroscope at 5 days post inoculation

Fig. 3 : Proving of pathogenicity by Koch's postulates

Efficacy of fungicides against *Curvularia* using solid plate assay

The percentage inhibition of mycelial radial growth of *Curvularia* by different fungicides is presented in Table 1. Among the fourteen fungicides evaluated, eight fungicides *viz*., propiconazole 25% EC, hexaconazole 5% SC, difenconazole 25% EC, triflumizole 42.14% SC, tebuconazole 25.9% EC, and combination products including azoxystrobin 8.3% + mancozeb 66.6% WDG, propiconazole 13.9% + difenconazole 13.9%, and flupyram 17.7% + Tebuconazole 11.4% SC exhibited 100% inhibition of mycelial growth at all tested concentrations under *in vitro* conditions. These fungicides predominantly belong to the triazole group, which inhibits ergosterol biosynthesis and disrupts fungal cell membrane formation and strobilurins, which suppress mitochondrial respiration by inhibiting quinone oxidation (Hewitt, 1988). Similar efficacy of triazole fungicides against *C. ergarostidis* species has been reported in spider lily (Prajapati & Chakraborty, 2017) and pomegranate fruit spot caused by *C. geniculata* (Prithviraj et al., 2021).

Carbendazim 50% WP, a β-tubulin inhibitor (Hewitt, 1998), was the least effective systemic fungicide, showing only 33% inhibition at 50 ppm and a maximum of 88.9% inhibition even at 2000 ppm, indicating reduced sensitivity or resistance. Chlorothalonil 75% WP, a non-systemic contact fungicide, also exhibited low efficacy at lower concentrations, requiring 2000 ppm to achieve

Table 1 : *In vitro* evaluation of new generation fungicides against *Curvularia* spp.

Fungicide	Inhibition in mycelial growth (%)						
	50 ppm	100 ppm	250 ppm	500 ppm	1000 ppm	2000 ppm	4000 ppm
Copper sulphate 47.15% + Mancozeb 30% WDG	27.8±1.1^{e}	55.5±1.1^{e}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Carbendazim 50% WP	33±1.3^{d}	35.6±1.1^{g}	83.3±1.1^{b}	86.7±1.1^{b}	88.9±0^{b}	88.9±0	92.5 ±0
Azoxystrobin 8.3% + Mancozeb 66.7% WG	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Tebuconazole 50%+Trifloxystrobin 25% WG	91.1±0.6^{c}	91.1±0.6^{c}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Carbendazim 12% +Mancozeb 63% WP	33.3±0^{d}	57.8±2.2^{d}	64.4±1.1^{c}	68.5±0.6^{d}	77.8±1.1^{c}	100±0	100±0
Chlorothalonil 75% WP	33.3±1.1^{d}	44.1±1.3^{f}	55.2±1.2^{d}	75.6±1.1^{c}	88.5±1.6^{b}	100±0	100±0
Flupyram 17.7% +Tebuconazole 17.7% SC	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Propiconazole 13.9% + Difenconazole 13.9% EC	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Azoxystrobin 18.2% + Difenconazole 11.4% SC	93.3±1.1^{b}	94.4±0^{b}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Propiconazole 25% EC	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Hexaconazole 5% SC	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Triflumizole 42.14% SC	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Difenconazole 25% EC	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Tebuconazole 25.9 % EC	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0	100±0
Control	0±0^{f}	0±0^{h}	0±0^{e}	0±0^{e}	0±0^{d}	0±0	0±0
CD (0.05)	1.02	1.32	0.84	0.73	0.84	NS	NS
CV	0.82	1.01	0.6	0.45	0.5	-	-

Values represent the mean inhibition percentage (± standard deviation) of mycelial growth for each fungicide treatment

The letters (a, b, c, etc.) indicate significant differences between treatments at a 0.05 probability level, as determined by Duncan's Multiple Range Test (CD 0.05). The control treatment showed no inhibition, with 0% inhibition recorded at all concentrations

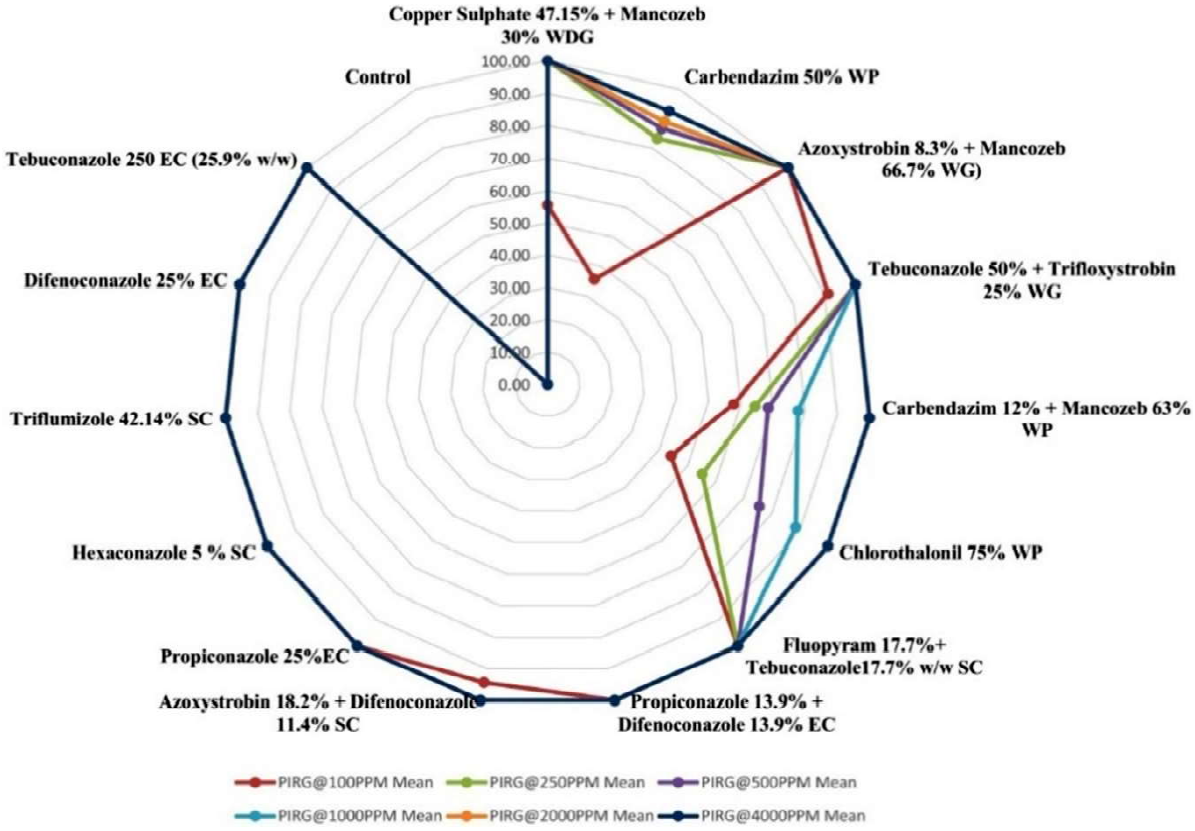


Fig. 4 : Graph representing the efficacy of new generation fungicides against *Curvularia* with respect percentage inhibition on mycelial growth

complete inhibition. Comparable responses have been reported in *Curvularia* leaf spot of rice and maize (Sumangala et al., 2008; Bisht et al., 2016). Combi-products containing systemic and contact fungicides demonstrated enhanced efficacy due to dual modes of action. Mancozeb, a multi-site contact fungicide that disrupts fungal enzymatic activity (Koli et al., 2019), showed maximum effectiveness when combined with systemic fungicides. Azoxystrobin + mancozeb was the most effective combination, achieving complete inhibition at 50 ppm, followed by copper sulphate + mancozeb at 250 ppm. Overall, systemic fungicides, particularly triazoles, were highly effective against *Curvularia*, while contact fungicides required higher concentrations, underscoring the importance of integrated fungicide strategies for sustainable disease management. The efficacy of new generation fungicides against Curvularia with respect percentage inhibition on mycelial growth is presented in Fig. 4.

CONCLUSION

The present study successfully identified *Curvularia xishuangbannaensis* as the causal agent of Curvularia leaf spot through a morpho-molecular approach, validating its pathogenicity *via* controlled disc placement inoculation and reisolation. Systemic fungicides, particularly triazoles demonstrated exceptional efficacy in suppressing fungal growth and managing the disease, outperforming non-systemic fungicides which required higher concentrations for similar effects. The integration of new-generation fungicides with advanced delivery systems offers a promising strategy for sustainable disease management by enhancing efficacy, reducing phytotoxicity and minimizing environmental impact. Future research should focus on *in vitro* and *in planta* evaluations at seedling and field levels to establish long-term effectiveness and refine application strategies for optimal disease control.

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Data availability: The ITS consensus of *Curvularia xishuangbannaensis* generated in this study have been deposited in NCBI GenBank under the accession numbers PP930726.

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