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Etiology and management of shisham die-back disease in Pakistan forests

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The etiology of shisham (*Dalbergia sissoo*) die-back has been a subject of considerable scientific debate for hundred years. No single pathogen species is responsible for die-back outbreaks, making shisham die-back challenging to study. Symptoms of the disease include yellowing, the water-soaked appearance of leaves, thinning of leaves and crown, branch die-back (single or whole), bark splitting, gummosis, and lesion development. Symptomatic shisham were sampled from 117 sites across Pakistan. One hundred and ninety-two fungal isolations were made from 1170 shisham samples and were identified as *Ceratocystis* sp. and *Fusarium* sp. based on cultural morphology. Koch's postulates were completed using 23 isolates of *Ceratocystis* sp. and four isolates of *Fusarium* sp. on three shisham age groups a) 6-12-month-old nursery seedlings growing under greenhouse conditions, b) 1-3-year-old plants growing under natural field conditions, and c) 10-15-year-old tree growing under natural field conditions. Inoculation with *Ceratocystis* isolates resulted in typical shisham die-back symptoms. Inoculation with *Fusarium* sp. did not produce any symptoms alone or in combination with *Ceratocystis* sp. Four fungicides were evaluated for efficacy against *Ceratocystis* sp. using *in vitro* tests and field trials. In *in vitro* test, propiconazole resulted in the highest level of inhibition (88.88%) at day seven, followed by difenoconazole (84.75%), hexaconazole (81.11%), while benzimidazole (77.73%) was least effective against the tested fungus. The efficacy of these fungicides has declined throughout greenhouse trials conducted in this study. Additionally, these fungicides could not prevent tree loss in trees inoculated with *Ceratocystis* sp. in the field trials. Thus, shisham die-back continues to be of concern in shisham-growing areas in Indo-Pakistan. However, chemical management and continuing research on the epidemiology of this disease may provide a solution.
Keywords: Fungi; isolation techniques; characterization; *Ceratocystis*; disease control; chemical fungicides

INTRODUCTION

Shisham (*Dalbergia sissoo*) die-back is the most important and frequently identified disease impacting shisham trees in the Indo-Pak subcontinent. It has infected millions of mature trees in urban landscapes, fields, forests, and farms (Webb and Hossain, 2005; Rehman *et al*., 2012; Naqvi *et al*., 2019). Die-back of forest trees is found globally and is responsible for destroying numerous forest ecosystems (Ciesla and Donaubauer, 1994; Clatterbuck, 2006; Jump *et al*., 2006). A majority of published research on shisham die-back has a historical focus due to the considerable economic importance of the host.

Shisham is a high-demand commodity worldwide, specifically in the Indian sub-continent, for its value in forestry, agroforestry, and landscape horticulture (Ul Haq *et al*., 2023). Over the years, it has been extensively cultivated for its quality timber wood in South Asia. It has durable, fragrant, and decay-resistant wood and is highly valued for furniture and construction (Naqvi *et al*., 2019). Apart from its economic importance, it is cultivated as a decorative tree (Ijaz and ul Haq, 2021).

The general symptoms include yellowing of leaves, sparse foliage, branch death, crown dieback, the stag-headed appearance of branches, bark cracking due to the formation of fungal mats just beneath the bark, gum exudation, vascular staining and eventually tree death (Latif *et al*., 2021). Naturally infected trees showed three different types of symptoms 1) healthy-looking trees showed sudden wilting and death of the whole tree, 2) yellowing of green leaves and thinning of crown, and 3) partial wilting; wilting of one or a few branches at a time (Dayaram *et al*., 2003; Latif *et al*., 2021). The fungus grows into and eventually plugs up the xylem cells the tree uses to transport water. This causes the tree to wilt and die (Latif *et al*., 2021).

Reports of shisham die-back began over a hundred years ago, but recent observations of increasing mortality are becoming a significant concern in areas where the tree is cultivated. No

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single pathogen species is responsible for these outbreaks, making shisham die-back challenging to study. A complex of primarily fungal plant pathogens, including *Botryodiplodia theobromae* (Khan *et al*., 2004; Rehman *et al*., 2012), *Ceratocystis* spp. (Poussio *et al*., 2010; Al Adawi *et al*., 2013; Ul Haq *et al*., 2019). *Fusarium* spp. (Dayaram *et al*., 2003; Shakya and Lakhey, 2007; Rajput *et al*., 2008; Rehman *et al*., 2012). *Ganoderma lucidum* (Parker, 1915; Troup, 1921; Sharma *et al*., 2000) and *Phytophthora cinnamomi* (Gill *et al*., 2002; Rehman *et al*., 2012) have all been linked to die-back outbreaks and tree death. *Ceratocystis* and *Fusarium* spp. are major players in this disease complex.

Ceratocystis diseases have been observed to cause cankers, declines, die-backs and vascular wilts of trees (Kile, 1993; Marín Montoya and Wingfield, 2006; Roux and Wingfield, 2009). Many *Ceratocystis* species are wound-invading infectious pathogens of woody plants with broad host ranges (Kile, 1993). In recent years, there has been an alarming increase in newly emerging tree diseases caused by *Ceratocystis* spp (Tsopelas *et al*., 2017). The fungus has been reported on six continents with varying levels of virulence on several different hosts (Van Wyk *et al*., 2007).

Unfortunately, the management options for diseases caused by *Ceratocystis* spp. are limited. Preventing disease transmission entirely is the preferable method for control, but successful prevention relies on knowledge of the pathogen distribution and early and accurate detection. However, there are limited diagnostic tools that can detect *Ceratocystis* spp. before a transmission has occurred, in some cases, a combination of agronomic, cultural and pruning practices may be used to lower disease risk, but in many circumstances, these practices may harm the health of trees. To date, chemical sprays used alongside cultural management practices are the best option for controlling *Ceratocystis* infections once they have occurred (Agrios, 2005).

Studies attempting to elucidate the biology of shisham die-back are numerous. Identifying the etiology and overcoming the disease has proven challenging and the die-back is still a significant problem across the shisham growing regions of the world. This fact revealed that further research is needed to explore the pathological cause of the disease by employing classical and molecular pathological techniques to mitigate the disease. Our study seeks to apply modern scientific studies to the evidence-based historical narrative of this disease to understand its complex etiology. Our research foci include: First, what is the causal agent of shisham dieback disease in Pakistan? Second, are there effective chemical management strategies for those pathogens? Answering these questions may inform integrated pest management strategies for the disease and make the production of shisham in Pakistan more sustainable. We addressed these questions by estimating disease levels at 117 different sites across 23 sampling districts of Pakistan.

MATERIALS AND METHODS

***Sampling and fungal isolation*:** During 2017-18, shisham trees showing die-back symptoms were randomly selected for sampling from scattered shisham plantations along roadsides, farmer fields, canal banks and plantation forests from 117 locations across 23 sampling districts of the country (Table 1). Observed symptoms of the disease include thinning of leaves and crown and witling of individual branches, in some cases wilting of the entire crown, partial to complete die-back, stag-headed appearance, excessive gummosis on tree stem, bark splitting, epicormic growth, vascular discoloration, and vascular blockage of xylem vessels (Fig. 1).

Figure 1. A) Healthy shisham tree growing in natural conditions, B) die-back infected tree showing thinning of leaves and crown, C) shisham tree with partial die-back symptoms, D) stag-headed dead tree, E) excessive gummosis on tree stem, F) bark splitting, G) wilting throughout the crown of the tree, H) epicormic growth of die-back infected tree, I) one-sided wilting J) vascular discoloration (blue streaking along xylem vessels, typical of *C. fimbriata*) just beneath the bark, and K) vascular blockage of xylem vessels.

Table 1. Fungal isolates used for pathogenicity trials along with their FMB strain number and Genbank accession no (Ul Haq *et al*., 2019).

Sr. No.	FMB strain No.	District	Genbank accession No.		Fungi
			ITS	TEF1-α	
1	FMB 0146	Okara	MK816923	MK890310	*Ceratocystis* sp.
2	FMB 0147	Jhang	MK816925	MK890311	*Ceratocystis* sp.
3	FMB 0148	Lahore	MK816928	MK890312	*Ceratocystis* sp.
4	FMB 0149	Mandi Bahaudin	MK816929	MK890313	*Ceratocystis* sp.
5	FMB 0150	Gujranwala	MK816932	MK890314	*Ceratocystis* sp.
6	FMB 0160	Khushab	MK816933	MK890315	*Ceratocystis* sp.
7	FMB 0161	Rahim Yar Khan	MK816934	MK890316	*Ceratocystis* sp.
8	FMB 0162	Bahawalnagar	MK816957	MK890317	*Ceratocystis* sp.
9	FMB 0033	Muzaffargarh	MF767443	MK890321	*Ceratocystis* sp.
10	FMB 0163	Bahawalpur	MK811131	MK820654	*Ceratocystis* sp.
11	FMB 0164	Sahiwal	MK813838	MK890320	*Ceratocystis* sp.
12	FMB 0165	Sialkot	MK813839	MK890322	*Ceratocystis* sp.
13	FMB 0166	Khanewal	MK811133	MK820656	*Ceratocystis* sp.
14	FMB 0167	Faisalabad	MK811134	MK850855	*Ceratocystis* sp.
15	FMB 0168	Toba Tek Singh	MK811125	MK890319	*Ceratocystis* sp.
16	FMB 0169	Jaffarabad	MK811095	MK850853	*Ceratocystis* sp.
17	FMB 0170	Nasirabad	MK811097	MK820655	*Ceratocystis* sp.
18	FMB 0171	Thatta	MK811092	MK850852	*Ceratocystis* sp.
19	FMB 0172	Hyderabad	MK811093	MK850851	*Ceratocystis* sp.
20	FMB 0173	Naushahro Feroze	MK811094	MK850850	*Ceratocystis* sp.
21	FMB 0174	Kohat	MK811103	MK890318	*Ceratocystis* sp.
22	FMB 0175	Shangla	MK811102	MK910747	*Ceratocystis* sp.
23	FMB 0176	Peshawar	MK811101	MK850854	*Ceratocystis* sp.
24	FMB 0063	Bahawalpur	MG256017	-	*Fusarium* sp.
25	FMB 0064	Gujranwala	MG283134	-	*Fusarium* sp.
26	FMB 0065	Faisalabad	MG283133	-	*Fusarium* sp.
27	FMB 0067	Sialkot	MG283129	-	*Fusarium* sp.

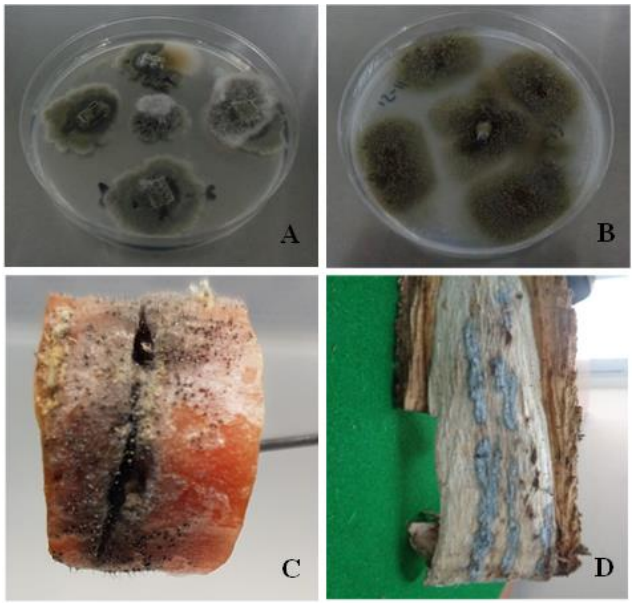

Figure 2. A) isolation of *Ceratocystis* sp. on PDA, B) sub-culturing of the fungus through single hyphal tipping, C) Perithecia embedded into the carrot baits, and D) Fungal colonies growing on wood chips incubated in a moist chamber.

Stem and root samples from symptomatic and asymptomatic tissues were collected by removing the bark at breast height. Wood samples were placed in plastic bags, labeled, and kept in an ice box until they could be brought to the Fungal Molecular Biology Laboratory (FMB) in Plant Pathology, University of Agriculture, Faisalabad, and stored at 4°C until ready for the user.

Samples from infected tissues were then tested for *Ceratocystis* spp., *Fusarium* spp., and *Botryodiplodia* spp. Infected wood samples were washed with tap water, cut into one-centimeter squares, surface sterilized using 1% sodium hypochlorite, washed with distilled water, and placed on paper towels to dry. Wood samples taken from stems and roots were plated directly onto various media types and placed on carrot baits and in moist chambers to induce sporulation (Figure 2), while root samples were processed on PDA amended with 1% Ampicillin only. An array of Agar media, including water agar (WA; 1L Water and15g/L Agar), Malt Extract Agar (MEA; 1L water, 30g/L Malt extract, 530g/L Mycological Peptone, and 15g/L Agar), and Potato Dextrose Agar (PDA; 1L water, 200g/L infusion from potato, 20g/L Dextrose and15g/L Agar) were used for isolation of fungal pathogens. Cultures were incubated at 25°C for 10-12 days. Carrot baiting was exclusively used to isolate *Ceratocystis*

spp., as described by (Moller and DeVay 1968). The carrot discs were incubated at 25°C for 4 to 8 days to induce fungal sporulation. Wood chips were placed in a moist chamber at room temperature (25°C) for 7–10 days to induce sporulation directly on infected tissue. Perithecia were removed from carrot slices (Figure 2C) and wood chips (Figure 2D) and transferred to MEA and PDA. The frequency of fungal isolates on synthetic media, carrot baits and the moist chamber was recorded. Various isolates from each plate were sub-cultured in duplicate on PDA and MEA using a single hyphal tip technique (Narayanasamy, 2001).

***Identification, Purification, and Preservation of fungal isolates*:** Fungal isolates were purified by a single spore method (Choi *et al*., 1999) and hyphal tip technique (Narayanasamy, 2001). Isolates chosen for morphological characterization were grown on PDA and incubated for 10-12 days at room temperature (25°C) with natural day/night light intervals. Morphological characterization was done by studying macroscopic (colony pigmentation and growth patterns) and microscopic characters (hyphae, macroconidia, microconidia, and perithecia). All the fungal isolates were categorized into 1) *Ceratocystis* sp. group or 2) *Fusarium* sp. The morphological characteristics of *Ceratocystis* sp. were compared with (Engelbrecht and Harrington, 2005; Latif *et al*., 2020). The morphological characteristics of the *Fusarium* sp. isolated from shisham trees were compared with Nelson, 1983. Samples were microscopically examined by mounting various fungal structures on glass slides. Observations of microscopic structures were made using the Nikon SMZ 18 stereo microscope and Nikon Eclipse Ni compound microscope with differential interference contrast (DIC) illumination. All the *Ceratocystis* sp. isolates were morphologically identical, so one representative was selected from each district for pathogenicity trials. Only four isolates of *Fusarium* were recovered from root samples. Therefore, 27 isolates were used in pathogenicity trials: 23 *Ceratocystis* and 4 *Fusarium* (Table 1). Fungal isolates were stored on PDA slants (Onions, 1971; Humber, 1997) and dry filter papers (Morales, 2008). All isolates were deposited in the Fungal Molecular Biology Culture Collection (FMB-CC-UAF) in the Department of Plant Pathology, UAF (Table 1).

***Description of plant material and fungal isolates*:** Three hundred and sixty-five seedlings rose from scion wood before bud break and were issued a pathogen challenge for pathogenicity trials. Scion wood was obtained from symptomless healthy shisham trees collected during the 2017-2018 survey described above. Four pathogenicity trials were conducted on three shisham age groups a) 6-12-month-old nursery seedlings growing under greenhouse conditions, b) 1-5-year-old plants growing under natural field conditions, and c) 10-15-year-old trees growing under natural field conditions. Koch's postulates were carried out to assess the symptomology and virulence of the 27 pathogen isolates on shisham. The first of four inoculation trials were designed to evaluate the pathogenicity of all fungal isolates on seedlings grown in greenhouse conditions. In the second trial, only *Ceratocystis* sp. were tested under local field conditions because *Fusarium* sp. isolates were not pathogenic in the first inoculation trial. The third trial was used to confirm the pathogenicity of *Ceratocystis* sp. on a naturally growing mature shisham tree. Finally, the last inoculation trial was to determine if *Ceratocystis* sp. had a synergistic disease effect when co-inoculated with *Fusarium* sp. in seedlings growing under greenhouse conditions. Koch's postulates were completed via re-isolation and morphological identification of the pathogens in each trial.

***Inoculum preparation*:** All 27 isolates were gown on carrot discs, PDA, and sorghum grains for future use in this study. Isolates that were stored on media were single-spore isolated before storage. Single-spore cultures on solid media were stored at room temperature (25±2°C) for short-term storage and a duplicate of each culture was stored on sorghum grains at -4°C for long-term storage. Actively growing cultures on PDA were used as inocula for pathogenicity trials conducted in the greenhouse, while actively growing cultures on sorghum grains were used for pathogenicity trials conducted in the field. To infect grains, 2 kg of grains were distributed into 250-gram aliquots, and all aliquots were autoclaved and air-dried in a laminar flow hood to remove excessive moisture. Each fungal isolate was inoculated into individual aliquots. After complete colonization, grains were ground into powder form using a grinder and stored in the freezer at -4°C until inoculation day. To ensure the viability of the inocula after each field inoculation, infested grains were also plated on PDA and monitored for growth for 7-10 days at room temperature (25±2°C).

***Symptoms evaluation*:** The trees in each pathogenicity trial were monitored at various time points to develop disease symptoms such as yellowing, the water-soaked appearance of leaves, branch die-back (single or whole), bark splitting, gummosis and lesion development.

***Pathogenicity Trial 1*:** 365 seedlings were inoculated with 27 fungal strains previously isolated from dieback-affected shisham trees using the flap method described by (Poussio *et al*., 2010). Three methods were used in this trial: a) stem inoculation, b) soil inoculation, and c) a dual inoculation, where both stem and soil was inoculated with the same isolate (referred to as stem+soil). Five replicate trees per isolate were inoculated.

Method 1 (stem): One-hundred fifteen seedlings (60–120cm height) were mechanically injured using a disinfected (70% ethanol) scalpel at the stem at about 10cm from the basal root crown. Stems were inoculated with each isolate of *Ceratocystis* sp., placing a 5mm mycelial plug from 10-day-old culture. The inoculation point was wrapped with Parafilm to prevent desiccation and potential unwanted contamination. Stems inoculated with a sterile PDA 5mm plug were used as a negative control.

Method 2 (soil): One hundred thirty-five seedlings were inoculated at the soil level with all 27 fungal isolates. A 5mm mycelial plug from the 10-day-old culture of each fungus was placed directly onto the soil surface near the seedlings' root crown. Roots were slightly mechanically injured using a disinfected (70% ethanol) scalpel to promote infection. Small incisions were made to the secondary roots. Roots inoculated with a sterile PDA 5mm plug were used as a negative control.
Method 3 (dual inoculation): Combining the above mentioned methods, concurrent stem and soil inoculations were performed on one hundred and fifteen seedlings with only the *Ceratocystis* sp. isolates. Thus, each seedling had double the amount of inocula. All inoculated plants were kept in a greenhouse with an average day temperature of 26°C and remained under greenhouse conditions until symptoms developed. Stems and roots were inoculated with a sterile PDA 5mm plug as a negative control. Trees were monitored for symptom development and progression every week until 50% of the seedlings died. According to Koch's postulates, the outer bark of symptomatic seedlings was removed and wood samples from the stem of the seedlings were collected to re-isolate the inoculated fungi. Wood samples were taken from the margin of a lesion, where both stained and unstained wood could be visually observed. Stained tissue appeared as blue-grey streaks following the vascular system of the stem, a typical characteristic of infection with *Ceratocystis* sp. Wood samples were aseptically divided into four pieces, and all were placed between two carrot slices, each about 1cm in thickness (Figure 2C). Small hair-like projections (the sexual fruiting bodies, perithecia) that had emerged from wood pieces (Figure 2C) were transferred to plates containing PDA or MEA. After confirming the pathogen, trials were extended from the nursery to the field, where shisham plants had variable heights and stem diameters. On average, a) 1-year-old plants were 3-5 ft tall and 2.5cm in stem diameter, b) 2-year-old plants were 7-8 ft tall and 5-6cm in stem diameter, and c) 3-year-old plants were 9-10 ft tall and 8-9cm in stem diameter.

Pathogenicity trial 2: An experimental plot of 10,000 ft^2 (100×100 feet) was established at the University of Agriculture Faisalabad, Pakistan research area. Two hundred seventy shisham plants, 90 plants of each age class/group, a) 6-month-old nursery seedlings, b) 2-year-old plants, and c) 5-year-old trees, were collected from various experimental sites and arranged in a square block pattern (6 ft x 6 ft). All plants in the field were inoculated with 23 isolates of *Ceratocystis* sp. following the dual method described above in pathogenicity trial 1. Stem+soil inoculation was performed as described above. However, 10 g of infected sorghum grain powder was directly incorporated into the soil instead of the mycelial plug. The experiment was replicated three times. Observations were made weekly until 50% of the trees in the plot died. Re-isolation of pathogen isolates occurred according to the previously described protocol (pathogenicity trial 1-method 3).

Pathogenicity trial 3: A third pathogenicity test was conducted as described in pathogenicity trial 2 with the following exceptions: 1) one 10-15-year-old mature shisham plant naturally growing in the field was selected as an experimental unit, and 2) only stem inoculation as described in method 1 was used to infect the tree with only one isolate of *Ceratocystis* sp. strain FMB 0033 (Figure 3). Observations were taken at a 15-days interval.

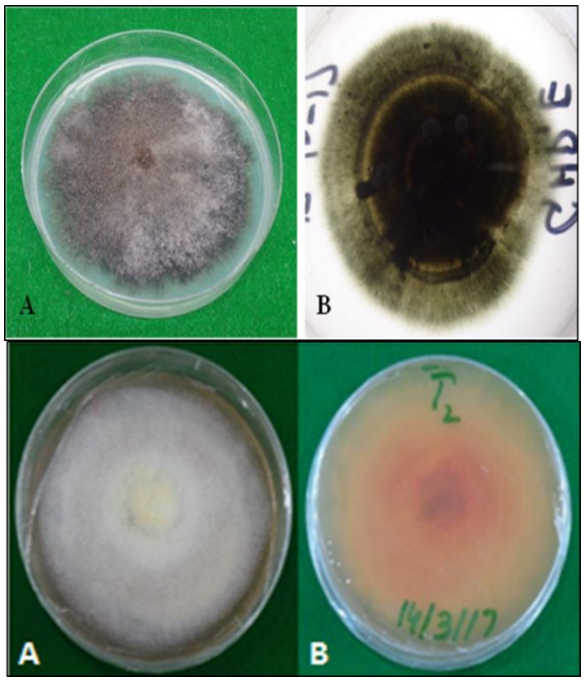

Figure 3. Pure culture of *Ceratocystis* sp. strain FMB 0033 (left side) and Fusarium sp. strain FMB 0063 (right side) growing on PDA left side A) frontal view, and B) backside view.

Pathogenicity trial 4: The fourth pathogenicity trial was conducted to check the synergistic effect of four *Fusarium* sp. isolates and one *Ceratocystis* sp. strain FMB 0033 (Figure 3). Stems of 12 nursery seedlings (6-12-month-old) were inoculated following the dual inoculation protocol described above (pathogenicity trial 1, method 3). However, *Ceratocystis* sp. was inoculated at the stem and *Fusarium* sp. was inoculated into the soil. Stems and roots were inoculated with a sterile PDA 5mm plug as a negative control. Observations and symptom development data were recorded weekly for the first month and after one month daily until all the seedlings were dead.

Disease management: Fungicides were evaluated for efficacy in controlling die-back disease using *in vitro* experiments and field trials. Four fungicides were tested for their effect on the mycelial growth of *Ceratocystis* sp.

In vitro evaluation of fungicide on *Ceratocystis* sp.: The inhibitory effect on colony growth caused by four systemic fungicides: 1) propiconazole, 2) difenoconazole, 3) hexaconazole, and 4) benzimidazole (Benomyl) at three concentrations of 100, 150, and 200 ppm was evaluated following the poison food technique described by (Sinclair and Dhingra, 2017). Suspensions of each fungicide were made at the listed concentrations by dissolving the appropriate gram weight of each fungicide in 10 mls of warm PDA. Petri plates containing solidified PDA amended with each fungicide were inoculated by placing a 5mm plug from 7-day-old pure cultures of each isolate. Three replicates were inoculated for each concentration of each fungicide. Inoculated PDA plates without fungicides served as controls. All plates were incubated at 26±2ºC. Data was recorded after 3, 5, and 7 days of treatment by measuring the distance from the colony center to the margin of mycelial growth. The average of four measurements was considered the colony growth rate for each data point. Percentage growth inhibition (PGI) was compared against the colony growth of the control plates (Ramesh Sundar *et al*., 1995).

PGI was calculated as follows: PGI=(C-T/C) x 100, where C is the fungus growth in the control plate and T is the fungus growth in the presence of fungicide.

In vivo evaluation of fungicides on shisham seedlings: The efficacy of 1) propiconazole, 2) difenoconazole, 3) hexaconazole, and 4) benzimidazole (Benomyl) was tested on 6-12-month-old shisham seedlings in greenhouse conditions. Thirty-six seedlings were artificially inoculated following the previously described dual inoculation method (pathogenicity trial 1, method 3) with *Ceratocystis* sp. Fungicides were applied at a rate of 200 ppm two weeks post-inoculation by the following methods: a) spraying leaves and stem with a hand sprayer, b) soil drenching, and c) a combination of a and b. Three replicates per application method were performed. In the first method, seedlings were sprayed thoroughly to wetness, avoiding a runoff, and in the second method, a 50 mL solution was uniformly applied to the soil around the base of the seedlings. A second dose of fungicides was applied at 10-12 days for both methods. Symptom development, disease progress, and seedling recovery were monitored for 3-6 months, depending upon the survival of the seedlings per treatment.

Field evaluation of fungicides: Twenty-four, 3-year-old shisham trees growing under field conditions in the experimental plot were selected to test the field efficacy of propiconazole and difenoconazole identified in the greenhouse study most effective. Three different concentrations of fungicides were tested: a) half of the suggested rate on the manufactures' documentation, b) the suggested rate, and c) double the suggested rate. Young trees were artificially inoculated with *Ceratocystis* sp. following the dual method described in pathogenicity trial 1, method 3. At four weeks post-inoculation, one-liter aliquots of each fungicide were applied to the soil surface around the stem. A second, third, and fourth application of each fungicide was conducted at one-month intervals. Symptom development, disease progress and plant recovery were monitored for six months.

RESULTS

Fungal Isolation: Isolation from symptomatic stem tissue using carrot baiting, moist chambers, MEA, PDA (Fig. 2), and WA yielded 188 isolates of *Ceratocystis*. Symptomatic root samples yielded four isolates of *Fusarium* species. The overall percentage of isolation of *Ceratocystis* sp. was 74% and 2.67% for *Fusarium* sp. The frequency of isolation of *Ceratocystis* sp. on carrot baits was 74.67%, moist chamber 28%, MEA 11.33%, PDA 5.33% and WA 0%. The isolates were identified as *Ceratocystis* or *Fusarium* sp. based on cultural morphology, including colony pigmentation, spore shape, structure and perithecia formation.

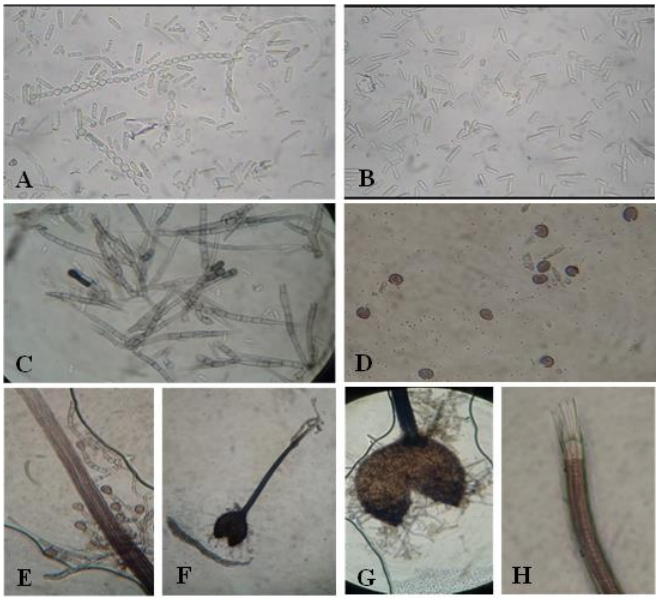

Figure 4. A) long chains of fungal ascospores, B) long cylindrical conidia, C) fungal hyphae, D) Chlamydospores, E) aleuriconidia, F) perithecia, G) opened perithecial neck releasing ascospores and H) ostiolate divergent hyphae.

The mycelia of *Ceratocystis* sp. isolates were septate and hyaline, but after 7-8 days, they turned greenish to dark brown and produced a banana-like odor. Pure colonies were olivaceous brown. All the isolates produced long chains of aleuriconidia. In addition, numerous brown to black perithecia were produced on PDA. These perithecia appeared globose with a straight rostrum ending in divergent, hyaline ostiolar hyphae. Ascospores were hat-shaped, hyaline, ovoid and abundant. Primary conidia were hyaline and cylindrical

and secondary conidia were hyaline and barrel-shaped, as Engelbrecht and Harrington described in 2005 (Figure 4). Morphological observations of *Ceratocystis* isolates from this study were consistent with morphological and molecular characterization previously published and confirmed as *Ceratocystis fimbriata* senu lato species complex by Ul Haq *et al*. in 2019, 2023. *Fusarium* sp. isolates produced hard resting structures such as chlamydospores and numerous amounts of micro and macro-conidia. Micro-conidia were aseptate and ovoid, while macro-conidia were lunate (crescentic) with thin walls and 5-6 septa. All the isolated *Ceratocystis* sp. were morphologically identical; thus, one representative isolate was selected from each district for pathogenicity trials.

Symptom development: Die-back symptom onset appeared to be gradual. However, all typical die-back symptoms were observed at some point throughout the study.

Pathogenicity trial 1: No visible disease symptoms were observed on seedlings inoculated with strains of *Fusarium* sp., but all 23 isolates of *Ceratocystis* sp. produced typical die-back disease symptoms. The seedlings inoculated with strains of *Ceratocystis* sp. began to show wilt about 21 days post-inoculation. Leaves would become completely chlorotic and brown within days (Figures 5A, 5B, and 5C).

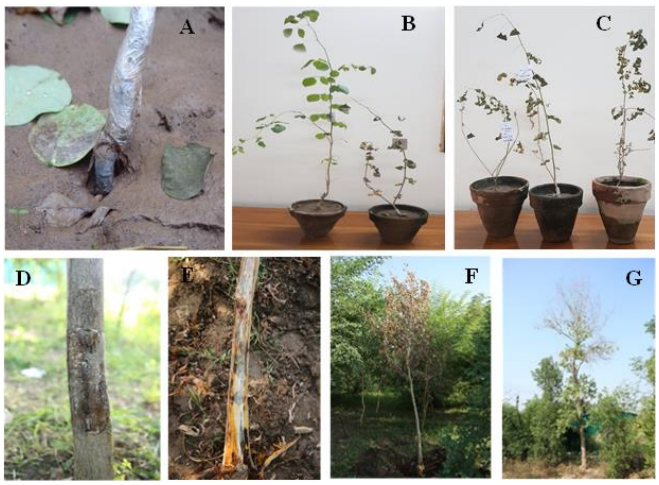

Figure 5. A) Seedling stem colonization by fungus using method 3 (stem+soil), B) expression of symptoms before and after inoculation; seedling on the left side represents no symptoms while the seedlings on the right side are diseased, C) variation of wilting observed on the inoculated seedlings, D) young field tree showing fungal colonization (white-greyish mycelia is observed) at the site of inoculation, E) blue staining along the vascular tissue observed after 58 days of inoculation, F) completely dead inoculated field plant and G) Mature shisham tree growing in natural conditions showing die-back symptoms typically.

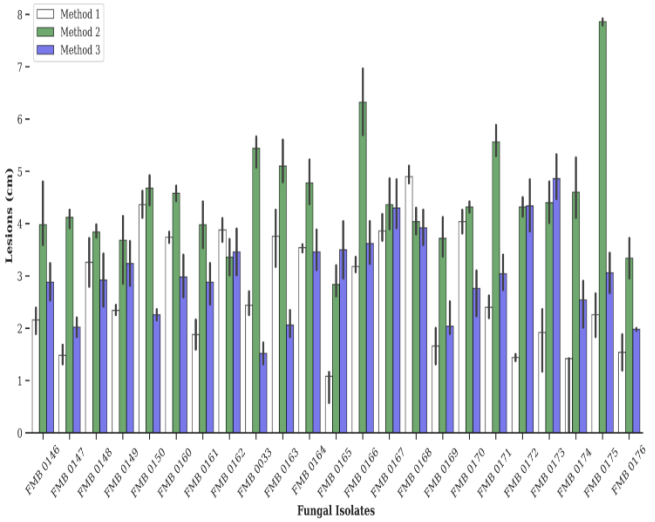

Figure 6. Graphical representation of lesions development on seedlings inoculated with 23 isolates of *Ceratocystis* sp. by three different methods: a) stem inoculation, b) soil inoculation, and c) a dual inoculation, where both stem and soil was inoculated with the same isolate (referred to as stem+soil).

When the bark at the inoculation site was removed, the pathogen's upward and downward progression (streaking) was observed. The appearance of streaking on the seedlings' stems was inconsistent (285 out of 365 total trees showed wood discoloration). Lesion length varied depending on the inoculation method. Lesions ranged from 1.44-7.02 cm, 2.34-7.86 cm, and 1.52-6.38 cm for methods 1, 2, and 3, respectively (Figure 6). Bark splitting was observed only in 2 out of 365 seedlings 42 days post-inoculation. Negative control seedlings showed only slight discoloration at the inoculation site, assumed to be a natural response to tissue wounding. The percentage of disease incidence (DI) observed was 67.82-74.07% and the percentage of disease severity (DS) observed was 41- 72%. Overall, the maximum incidence of 74.07% and severity of 72% of the disease was observed on seedlings who received a double inoculation according to methods described in pathogenicity trial 1, method 3. The onset of symptoms in these trees was earlier than in seedlings inoculated using methods 1 and 2.

Pathogenicity trial 2: All *Ceratocystis* sp. strains inoculated onto shisham trees growing under field conditions showed typical die-back disease symptoms regardless of age. Typical die-back symptoms observed in this study included yellowing, thinning of leaves and crown, branch die-back, wilting, vascular discoloration, and death (Figures 5E and 5F). Lesion length ranged from 4.8-24.23 cm, 9-25.66 cm, and 16.33-38.33cm for three age groups 1, 2, and 3, respectively (Figure 7).

Pathogenicity trial 3*:* The mature shisham tree inoculated with *Ceratocystis* sp. showed typical die-back symptoms within two years of inoculation. The symptom observed included yellowing and discoloration of the leaves, wilting from the crown to the base, and thinning of the crown. Die-back seen on the top branches at the crown of the tree gradually evolved into branch mortality. This partial die-back of the tree crown had a stag-headed appearance. After removing the bark, blue streaking along the xylem vessels and the tree roots was observed (Figure 5E).

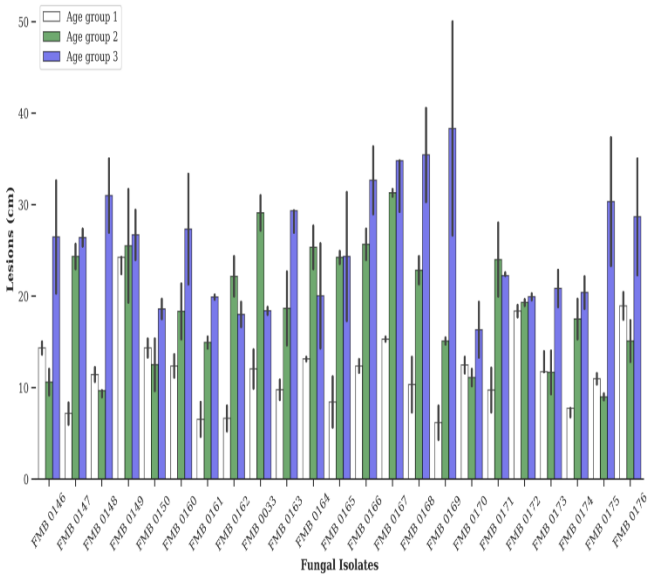

Figure 7. Graphical representation of lesions development on field-grown shisham trees belonging to different age groups a) 6-month-old nursery seedlings, b) 2-year-old plants, and c) 5-year-old trees inoculated with 23 isolates of *Ceratocystis* sp. following the dual inoculation, where both stem and soil was inoculated with the same isolate (referred to as stem+soil).

Pathogenicity trial 4: No synergistic effect was observed between isolates of *Fusarium* and *Ceratocystis* sp. *Fusarium* sp. did not produce any symptoms alone and no visible difference was observed.

Microscopy confirmation: All morphological characteristics expected for isolates classified as *Ceratocystis* sp. were observed for all isolates recovered from inoculated seedlings or trees (Figure 4).

Fungicide efficacy tests (In vitro and In vivo): All systemic fungicides tested against *Ceratocystis* sp. resulted in the inhibition of mycelial growth. However, results from the greenhouse experiment (*in vivo*) were inconclusive. *In vitro*, application of propiconazole application provided the maximum inhibition level (88.9%) of *Ceratocystis* sp. seven days post-application. At lower levels of inhibition, all of the fungicides tested did provide control at considerably similar efficacy levels as propiconazole, resulting in non-significant differences among the fungicides. The efficiency of these fungicides ranged from 72.8% to 84.7% inhibition (Figure 8). At seven days post-application, the lowest inhibition, 72.8%, was obtained with benomyl at 150 ppm. The second-lowest levels of inhibition at 80% and 80.1% were obtained with difenoconazole at 100ppm and hexaconazole at 100ppm, respectively.

Simultaneous application of fungicides (spraying and drenching) was the best method for inhibiting *Ceratocystis* sp. growth on shisham seedlings. *In vivo*, tests showed that most of the seedlings (27 out of 36) either sprayed or drenched with any of the four tested fungicides continued to develop die-back disease symptoms even after receiving subsequent doses. Lower levels of disease severity were observed on a few seedlings (9 out of 36) that received a double dose of propiconazole and difenoconazole. The double dose of these fungicides prolonged the seedlings' survival up to 65-140 days post-inoculation. Some seedlings (4 out of 36) re-sprouted after losing their leaves but did not survive for more than one month and, ultimately, died by the end of the 6-month observation period.

In the *In vivo* field trials, propiconazole and difenoconazole could not control the disease on 3-year-old trees, with two exceptions (2 plants out of 12) treated with propiconazole at the double the suggested application rate. In addition, disease severity differed between these two plants; one showed symptoms after two growing seasons, and the other showed symptoms in only 60% of the canopy.

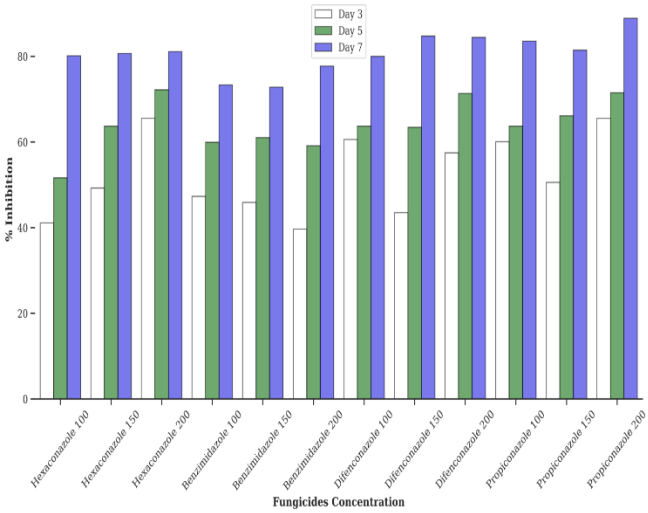

Figure 8. Percentage of inhibition of *Ceratocystis* sp. strain FMB 0033 by fungicides in laboratory conditions at three concentrations of 100, 150, and 200 ppm. Average of four measurements was considered as colony growth rate (distance from the colony center to the margin of mycelial growth) for each data point and was recorded after 3, 5, and 7 days of treatment.

DISCUSSION

This document addresses the operational taxonomy of fungal pathogen(s) responsible for dieback of shisham trees on the Indo-Pak subcontinent and possible disease management strategies to mitigate their impact in that region. In our efforts to determine the disease etiology and identify the fungal pathogen(s) associated with shisham die-back, we focused on infected trees in forests, urban areas, and tree farms. Trees showing yellowing of leaves, sparse foliage, branch death, crown die-back, the stag-headed appearance of branches, bark splitting, gum exudation, and vascular staining were sampled. Symptoms of shisham die-back disease like gum exudations, bark splitting, vascular discoloration, wilting and rapid death observed in field surveys and on artificially inoculated plants closely resemble mango decline caused by *Ceratocystis* sp. (Van Wyk *et al*., 2007; Al Adawi *et al*., 2009; Poussio *et al*., 2010). Previous reports by researchers studying this disease have described the same symptoms (Rattan, 2005; Latif *et al*., 2021).

There are three different types of disease (decline, dieback, and wilt) attributed to *Ceratocystis* based on symptomology (Al Adawi *et al.*, 2009; Poussio *et al*., 2010; Ul Haq *et al.*, 2019; Latif *et al*. 2021). However, there is no scientific consensus on what dieback is. Three different symptoms were noted on shisham trees with natural infections of dieback 1) Healthy-looking leaves showed rapid wilting, resulting in the death of the trees after approximately 15-20 days. 2) Wilting also occurred on isolated sides or affected tree branches. While some affected trees produced epicormic growth, they could not survive longer than 2-3 months, mainly if secondary infections occurred. 3) Affected trees showed yellowing, dehydration, and thinning of leaves and crowns. Despite variability in the appearance and severity of these symptoms, infections can always be identified through vascular streaking (longitudinal bluish to greyish streaks) just beneath the bark, were the unique symptoms noticed on shisham and identified as dieback disease. This streak may run several meters up and down the tree's stem from the site of infection, or just a portion of it. This distinctive symptom differentiates this disease from the often-confusing Fusarium wilt disease on the same plant and explains the dieback, a separate and significant disease of shisham in Pakistan (Latif *et al*., 2021). In contrast, Fusarium wilt produced internal pink streaking in the vascular tissue (Bakshi, 1954).

The results of surveys, isolations and pathogenicity trials illustrate three points clearly. First, *Ceratocystis* sp. is the dominant fungal species isolated from diseased shisham trees (74%). Second, carrot baiting is the most simple, robust, and cost-effective method for isolating *Ceratocystis* sp. from woody tissue samples. Third, surveys and pathogenicity trials proved that large-scale shisham mortality in Pakistan is attributed to *Ceratocystis* sp.

During this study, multiple pathogenicity trials were conducted on different age groups of shisham ranging from 6-12-month old seedlings, 1-3-year-old shisham trees, and 10-15-year old mature trees to identify disease-causing organisms accurately. All pathogenicity trials and field surveys support that *Ceratocystis* sp. is responsible for shisham die-back disease regardless of the tree age. Moreover, another pathogenicity trial was performed on 12 seedlings to investigate the synergistic effect of the *Ceratocystis* sp. and isolates of *Fusarium* sp. In all the cases, shisham seedlings were either inoculated with *Ceratocystis* sp. alone or in combination with *Fusarium* sp. However, disease symptoms were only observed when *Ceratocystis* sp. was present.

The fungal isolates from dieback-affected shisham trees were identified morphologically and phylogenetically as possible members of the *Ceratocystis fimbriata* sensu lato species complex (Ul Haq *et al*., 2019). The operational taxonomy supported species delineation for these *Ceratocystis* isolates as a new taxon of this complex. And the name *Ceratocystis dalbergicans* sp. nov. has been suggested to the fungus causing dieback disease in shisham (Ul Haq *et al*., 2023).

Data presented in this study supports that *Ceratocystis* sp. is the primary fungal pathogen responsible for widespread shisham die-back disease in the Indo-Pak subcontinent. Our research findings are supported by other studies that conclude that shisham mortality in Pakistan is due to a *Ceratocystis* sp. (Al Adawi *et al*., 2009; Poussio *et al*., 2010; Ul Haq *et al*., 2019, Ul Haq *et al*., 2023). However, previous research contradicts our findings, which claim organisms such as *Botryodiplodia theobromae* (Khan *et al*., 2004; Rehman *et al*., 2012), *Fusarium* spp. (Dayaram *et al*., 2003; Shakya and Lakhey, 2007; Rajput *et al*., 2008), *Ganoderma lucidum* (Parker, 1915; Troup, 1921; Sharma *et al*., 2000) and *Phytophthora cinnamomi* (Gill *et al*., 2002; Rehman *et al*., 2012) are responsible for shisham die-back. On the contrary, Ul Haq *et al*., 2023 have established that the novel species *C. dalbergicans* is the primary cause of dieback disease in shisham in Pakistan.

Although some researchers have identified host resistance and potential chemical control of shisham die-back in the Indian subcontinent, their work focused mainly on *F. solani*. However, *Ceratocystis* sp. has recently been reported from shisham growing areas of Pakistan, causing die-back, wilt, and decline (Al Adawi *et al*., 2009; Poussio *et al*., 2010; Ul Haq *et al*., 2019). Thus, there is an urgent need to understand the nexus of host-pathogen interaction, host genetic resistance sources and other disease management options such as chemical control with particular reference to *Ceratocystis* sp. Despite adopting IPM strategies, the die-back prevails at a higher degree of severity across the shisham-growing regions of Pakistan compared to adjacent countries. Furthermore, numerous reports cite the efficacy of fungicides against fungal growth under controlled conditions but rarely include

efficacy results in the field. Therefore, further studies evaluating chemical treatment options for shisham die-back in the field are critical in disease suppression and management. We explored this idea by conducting greenhouse and field trials of four potential fungicides to manage shisham die-back on seedlings and young trees.

All the tested fungicides could inhibit the fungus's mycelial growth *in vitro*. The efficacy of the four fungicides mentioned above was substantially lower in greenhouse trials. However, these fungicides could not prevent tree loss due to *Ceratocystis* sp. in the field. Propiconazole, hexaconazole and difenoconazole have provided promising inhibitory effects on the mycelial growth of *Ceratocystis fimbriata* (Sharma *et al*., 2010; Scruggs *et al*., 2017; Khan *et al*., 2017). Propiconazole could stop *C. fagacearum* growth in a controlled environment (Appel, 1992; Wilson and Lester, 1996). Triazole-based fungicides penetrate through the roots, stems and leaves, are absorbed in the xylem, and move acropetally in plants (Edgington, 1981). The systemic nature of these fungicides is believed to be the source of their efficacy against *Ceratocystis* spp., which are localized to vascular tissues in shisham trees.

***Conclusions*:** This paper summarizes our current understanding of *Ceratocystis* sp.'s role in shisham die-back disease. It also provides essential background information and an initial evaluation of chemical management options for the disease. We concluded that the shisham die-back disease's primary causal organism is *Ceratocystis* sp. This paper may be a resource for pathologists and foresters, especially when diagnosing shisham die-back based on symptoms observed in the field. This study broadens our understanding of the source of outbreaks of this disease in Pakistan and its detection and provides data to design timely response strategies that will reduce die-back epidemics in the future. Future studies can be focused on molecular-based detection assays, modes of pathogen dispersal and disease spread.

***Conflict of interest*:** All authors declare no conflict of interest.

***Authors' Contribution Statements*:** Imran ul Haq encouraged Muhammad Zunair Latif to investigate the etiology and management of shisham dieback. MZL executed research, write the original draft and performed the computation. Imran ul Haq, Siddra Ijaz, Amer Habib and Iqrar Ahmad Khan supervised field trials and the findings of this work, verified the analysis and help in editing and review the article.

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