



Available Online at EScience Press

Plant Protection

ISSN: 2617-1287 (Online), 2617-1279 (Print)
http://esciencepress.net/journals/PP

Research Article

Biochemical and Molecular Characterization of *Zasmidium fructicola* Associated with Citrus Greasy Leaf Spot and Its Implications for Disease Management

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ARTICLE INFO

Article history

Received: 20th December, 2025

Revised: 27th March, 2026

Accepted: 29th March, 2026

Keywords

Zasmidium fructicola

Citrus greasy leaf spot

Molecular characterization

Biochemical defense

responses

Integrated disease

management

ABSTRACT

Citrus Greasy Leaf Spot (CGLS) is an emerging foliar disease that threatens citrus productivity in major growing regions of Pakistan. This study aimed to characterize the causal pathogen *Zasmidium fructicola*, assess its pathogenic variability, and evaluate host biochemical responses along with potential management strategies. Field surveys revealed that the highest disease severity index (41.13%) and disease incidence (56.01%) occurred in Sargodha, while the lowest values were recorded in Faisalabad. Grapefruit and mandarin were the most susceptible cultivars. Morphological characterization of fifteen fungal isolates confirmed their identity as *Z. fructicola*, exhibiting typical colony morphology and septate, pigmented conidia consistent with Mycosphaerellaceae. Molecular identification using ITS and TEF-1 α gene regions showed 98-99% sequence similarity with reference strains, and phylogenetic analysis grouped all isolates into a well-supported clade, confirming species identity. Pathogenicity assays demonstrated significant variability among isolates, with AKS-1 and AKM-1 exhibiting the highest virulence on grapefruit, mandarin, and sweet orange. Biochemical analyses revealed that resistant cultivars showed higher activities of antioxidant enzymes (SOD, POD, CAT), phenolics, proteins, and soluble sugars, whereas susceptible cultivars exhibited reduced defense-related metabolites following infection. *In vitro* assays indicated that carbendazim and tebuconazole significantly inhibited mycelial growth, with up to 95.7% and 92.16% inhibition, respectively. Among botanical extracts, *Syzygium aromaticum* showed the highest antifungal activity, achieving up to 97% growth inhibition. In conclusion, *Z. fructicola* was confirmed as the causal agent of CGLS, exhibiting notable pathogenic variability and wide geographic distribution. Integrated management using resistant cultivars, fungicides, and botanical extracts offers an effective strategy for its sustainable control in citrus orchards.

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Introduction

Citrus is a key fruit crop in tropical and subtropical regions, valued for its nutritional content (Liu et al., 2012; Lu et al., 2023). Belonging to the Rutaceae family, it encompasses around 1,300 species across 140 genera

(Hussain et al., 2021; Hazarika, 2023), with origins in Southeast Asia over 4,000 years ago (Zhong and Nicolosi, 2020). Initially recognized as four species, advancements in identification have expanded this number significantly (Ollitrault et al., 2020; Mujtaba and Firdous, 2025). Citrus

fruits, known for their unique aroma and high juice content, are rich in bioactive compounds like flavonoids, carotenoids, and essential vitamins (Saini et al., 2022; Khair et al., 2026). These compounds contribute to health benefits, including protection against cancer and cardiovascular diseases, with antioxidant-rich citrus linked to reduced risks of chronic illnesses and overall mortality (Salman and Imran, 2022; Borghi and Pavanelli, 2023; Lu et al., 2023). Globally, citrus production reached approximately 15.73 million tons across 1.1 million hectares. China leads as the largest producer, contributing 44.5 million tons, or 28.1% of the world's total, followed by Brazil (19.5 million tons), India (14 million tons), Mexico (8.9 million tons), and the USA (7.1 million tons) (FAO, 2021). In 2021, citrus fruits ranked second globally in fruit production, yielding 161.8 million tons from over 10.2 million hectares. Oranges led with 75.57 million tons (46.7% of total citrus), followed by tangerines at 41.95 million tons (25.9%). Limes and lemons produced 20.83 million tons (12.87%), while pomelos and grapefruits added 9.56 million tons. Other citrus varieties contributed 13.90 million tons. Pakistan's citrus sector produced 2.89 million tons (Sajid et al., 2022; Gonzatto and Santos, 2023).

Citrus production in Pakistan faces significant threats from various diseases, leading to severe economic losses (Irshad et al., 2012; Fateh et al., 2017; Saeed et al., 2019; Prompreing et al., 2025). Gummosis, characterized by rotted roots, cracked bark, and leaf yellowing, can ultimately kill trees (Van Tran et al., 2023; Noor et al., 2024). Citrus scab, a fungal infection, causes scab-like lesions on fruit, leaves, and twigs, primarily affecting lemons (Shin et al., 2021). Citrus melanose results in dark spots on leaves and fruit, leading to yellowing and premature leaf drop (Anwar et al., 2021). Citrus canker, a bacterial disease, causes defoliation and fruit drop, marked by elevated lesions on leaves and fruit (Naqvi et al., 2022; Das, 2023; Ali et al., 2023; Jamil et al., 2025). Management strategies include exclusion, sanitation, and targeted chemical treatments (Behlau et al., 2021). Citrus Greening, caused by bacteria transmitted by the citrus psyllid, leads to various symptoms and remains a significant threat despite control measures (Li et al., 1994, 2020; Hussain et al., 2022).

Citrus greasy leaf spot, caused by the fungus *Mycosphaerella citri*, poses a significant threat to citrus production, leading to severe economic losses. This disease impacts leaf health, causes premature fruit

drops, and contributes to tree decline. Its adaptability to various climates and resistance to traditional fungicides complicate management efforts, with grapefruit losses reported at 45% and sweet orange losses at 25% in Florida (Aguilera-Cogley and Vicent, 2020). In Brazil, a survey across 19 towns indicated 100% occurrence in groves and plants, with 70% prevalence on leaves (Laranjeira et al., 2020). Citrus greasy leaf spot, caused by *Zasmidium citrigriseum* (formerly *Mycosphaerella citri*), leads to significant defoliation in citrus trees, especially from December to April (Aguilera-Cogley et al., 2023). Initial symptoms include irregular brownish-black oil-like spots surrounded by a greenish-yellow halo. The disease also affects fruit, causing unattractive blemishes that reduce commercial value. The fungus can survive as both a saprophyte and a pathogen, overwintering on fallen leaves and infecting new growth in spring (Abdelfattah et al., 2017). This serious disease is prevalent in warm, humid climates, causing defoliation, stunted growth, and diminished fruit quality (Huang et al., 2015). DNA barcoding offers a precise method for identifying fungal samples (Deka, 2022).

To manage citrus greasy leaf spot, cultural practices such as careful irrigation and pruning are essential, along with copper-based fungicides and difenoconazole + azoxystrobin sprays before and after flowering (Liu et al., 2022). Strobilurin fungicides like Abound, Amistar Top, and Pristine can be applied anytime to control the disease on leaves and fruit, particularly from late May to early June, as they effectively manage both melanose and greasy spot while minimizing fruit damage from copper fungicides (Dewdney, 2021). Fenbuconazole has shown promise in reducing disease severity and preventing yield loss, with three applications proving more effective than one (Aguilera-Cogley and Vicent, 2020). Other fungicides, including Carbendazim, Tebuconazole, Captan, and Mancozeb, along with plant extracts like *Mentha*, *Azadirachta indica*, *Cinnamom zeylanicum*, and *Zingiber officinale*, are also being tested for their effectiveness in controlling the disease.

In light of the negative impacts of citrus greasy leaf spot, a survey was conducted across various locations in Punjab, Pakistan, including Sargodha, Mandi Bahauddin, Toba Tek Singh, Layyah, and Faisalabad. Samples were collected and analyzed in the lab, revealing limited research on this disease in Pakistan (Malik et al., 2021). This suggests that citrus greasy leaf spot is emerging as a significant threat to the citrus industry in Pakistan. Given its prevalence in

Punjab's citrus-growing regions, addressing this issue is crucial for the future of the industry. The study aims to characterize the fungi associated with citrus greasy leaf spot using molecular approaches, to analyze the biochemical alterations induced in citrus plants by the pathogen associated with greasy leaf spot, and to develop integrated management strategies for the isolated pathogen through the evaluation of various chemicals and plant extracts under *in vitro* conditions.

Materials and Methods

Site selection, sample collection, and sample preparation

The survey study was conducted to assess the greasy spot symptoms and its effects on citrus. For this purpose, citrus orchards in major citrus growing districts of Punjab, Pakistan, including Sargodha 32.0740° N, 72.6861° E, Toba Tek Singh 30.9709° N, 72.4826° E, Faisalabad 31.4504° N, 73.1350° E, Mandi Bahauddin 32.4105° N, 73.3709° E and Layyah 31.0998° N, 71.0022° E, were selected as study sites (Figure 1). Diseased leaf samples showing greasy spot symptoms were collected from citrus trees aged between

10 and 22 years in the selected orchards. GPS coordinates of each infected plant were recorded using a GPS camera. A total of eight trees per orchard, including both healthy and affected trees, were sampled. Leaf samples were collected and stored in sterile polyethylene bags, labeled with location, date, sample numbers, host variety, and collection sites (Table 1). The samples were hermetically sealed and transported to the Molecular Plant Pathology Laboratory, Department of Plant Pathology, University of Agriculture Faisalabad for isolation of the causal organism. Samples were then stored at 4°C in a refrigerator for future use.

Disease incidence, prevalence, and percent disease index calculation

The current experiment was conducted to determine disease incidence (Zhang et al., 2019), prevalence (Vitale et al., 2021) and percent disease index (Chiang and Bock, 2022) in each orchard using the following formulas (Abedin and Chowdhury, 1982; Bock et al., 2020):

$$\text{Disease Severity (\%)} = \frac{\text{No of effected leaf per plant}}{\text{Total no leaf per plant}} \times 100$$

$$\text{Disease Incidence (\%)} = \frac{\text{Number of infected plants per field}}{\text{Total number of plants per field}} \times 100$$

$$\text{Disease Prevalence (\%)} = \frac{\text{No of orchards infected per district}}{\text{Total no of orchards surveyed per district}} \times 100$$

$$\text{Disease disease index (\%)} = \frac{\text{Total sum of numerical ratings}}{(\text{No of observation}) \times (\text{Maximum grade in rating scale})} \times 100$$

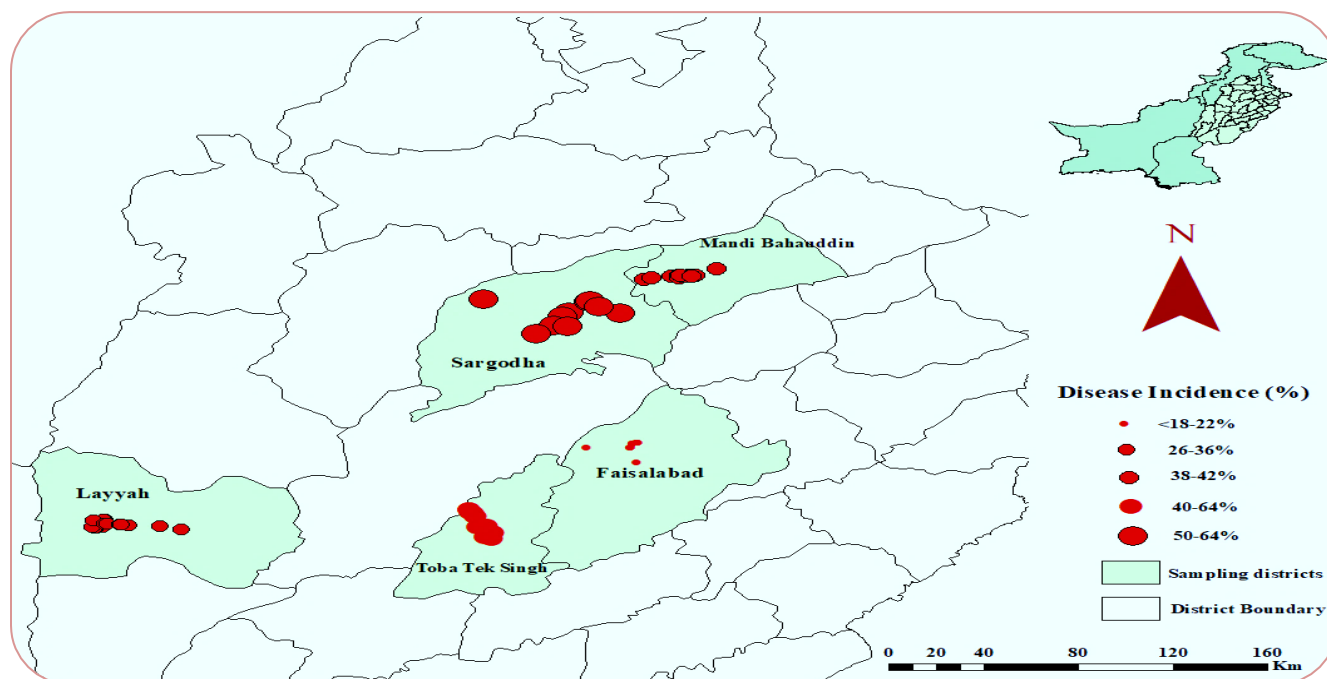


Figure 1. Geographical map showing the mean incidence of citrus greasy leaf spot disease in surveyed locations of Punjab, Pakistan.

Table 1. Labeling of citrus samples collected from different locations in Pakistan.

Isolates	GPS coordinates		Local area name	Isolates	GPS coordinates		Local area name
	Latitude	Longitude			Latitude	Longitude	
AKS_1	32.251225	72.89243	Chenab Bazar-Kot momin	AKL_9	30.967441	71.058771	Chowk Azam
AKS_2	32.224576	72.933227	Sargodha	AKL_10	30.966661	71.067526	Chowk Azam
AKS_3	32.183013	73.012405	Bhalwal	AKL_11	30.961839	71.115242	Chowk Azam
AKS_4	32.224598	72.933283	Sargodha	AKL_12	30.958426	71.149809	Chobra
AKS_5	32.183013	73.012405	Bhalwal	AKL_13	30.961235	71.120852	Chobra
AKS_6	32.224598	72.933283	Bhalwal	AKL_14	30.951504	71.264622	Chobra
AKS_7	32.251225	72.89243	Sargodha	AKL_15	30.934065	71.346675	Layyah
AKS_8	32.183013	73.012405	Bhalwal	AKT_1	31.044595	72.443432	Toba Tek Singh
AKS_9	32.224597	72.933282	Bhalwal	AKT_2	31.044541	72.443317	Rajana
AKS_10	32.251219	72.892437	Bhalwal	AKT_3	30.901618	72.522305	Kamlia
AKS_11	32.18875	72.816233	Sargodha	AKT_4	30.88198	72.526133	Rajana
AKS_12	32.166031	72.7967708	Bhalwal	AKT_5	30.888098	72.516792	Toba Tek Singh
AKS_13	32.251402	72.892571	Nai Basti, Bhalwal	AKT_6	30.893909	72.518329	Toba Tek Singh
AKS_14	32.254177	72.898622	Bhalwal	AKT_7	30.890038	72.502158	Toba Tek Singh
AKS_15	32.224624	72.933336	Sargodha	AKT_8	30.911119	72.532018	Toba Tek Singh
AKM_1	32.380233	73.1059918	Head Faqreean	AKT_9	30.903427	72.514465	Kamlia
AKM_2	32.388476	73.130981	Malkwal	AKT_10	30.926832	72.502121	Toab Tek Singh
AKM_3	32.397814	73.210656	Malkwal	AKT_11	30.949631	72.510226	Peer Mahal
AKM_4	32.402	73.238388	Malkwal	AKT_12	30.946077	72.47389	Toba Tek Singh
AKM_5	32.39003	73.238592	Malkwal	AKT_13	31.008956	72.465222	Toba Tek Singh
AKM_6	32.396555	73.281282	Malkwal	AKT_14	31.026882	72.455368	Toba Tek Singh
AKM_7	32.404853	73.277593	Malkwal	AKT_15	31.033107	72.447988	Toba Tek Singh
AKM_8	32.399343	73.267212	Mandi Bahauddin	AKF_1	31.43095	73.06062	HFG
AKM_9	32.398526	73.249292	Mandi Bahauddin	AKF_2	31.43103	73.06037	HFG
AKM_10	32.401827	73.242008	Mandi Bahauddin	AKF_3	31.43049	73.06031	HFG
AKM_11	32.402069	73.300021	Mandi Bahauddin	AKF_4	31.43048	73.06089	HFG
AKM_12	32.440258	73.381329	Mandi Bahauddin Gojra	AKF_5	31.43797	73.07691	HFG
AKM_13	32.439978	73.380314	Gojra	AKF_6	31.43821	73.07691	Botanical
AKM_14	32.402234	73.299298	Gojra	AKF_7	31.43795	73.07788	Botanical
AKM_15	32.399195	73.284778	Gojra	AKF_8	31.43756	73.07837	Botanical
AKL_1	30.948826	71.047699	Layyah	AKF_9	31.437	73.07707	Botanical
AKL_2	30.968832	71.056891	Layyah	AKF_10	31.32074	73.07701	Botanical
AKL_3	30.987404	71.057651	Layyah	AKF_11	31.40677	72.88291	AARI
AKL_4	30.990078	71.054215	Layyah	AKF_12	31.40677	73.05153	AARI
AKL_5	30.942527	71.022522	Layyah	AKF_13	31.40672	73.05072	AARI
AKL_6	30.940751	71.018268	Layyah	AKF_14	31.40643	73.05168	AARI
AKL_7	30.944854	71.010465	Layyah	AKF_15	31.40672	73.05072	AARI
AKL_8	30.984819	71.014384	Layyah				

HFG = Horticulture Fruit Garden, AARI = Ayub Agricultural Research Institute.

Fungal isolation, purification, and identification

Fungal pathogens were isolated from infected leaf samples using the method described by Ricker and Ricker (1936). Leaf pieces (approximately 3-4 mm) were excised from the leading edge of greasy spot lesions using a sterile scalpel. The pieces were washed in running tap water, surface sterilized with 2% sodium hypochlorite solution, and rinsed twice in sterile distilled water. After drying on sterilized blotting paper, the leaf pieces were aseptically transferred to Potato Dextrose Agar (PDA) plates supplemented with streptomycin sulfate. Plates were incubated at 24-28°C with a 12-h photoperiod for 7-15 days and examined periodically under a stereo-zoom microscope for fungal growth.

Colonized fungi were purified by sub-culturing hyphal tips emerging from infected leaf pieces onto fresh PDA plates. Purified cultures were transferred to PDA slants, incubated at 24°C for 15 days, and stored at 4°C for further studies. Morphological identification of fungal isolates was performed by preparing lactophenol cotton blue mounts from 7-15 days old cultures and examining them under a compound microscope. Size, shape, and septation of conidia were used as key parameters for identification. Further purification was done by the hyphal tip transfer method. Identification of purified fungal cultures was based on morphological characteristics (Barnett and Hunter, 1998).

Pathogenicity test

The study used one-year-old citrus cultivars from the Citrus Nursery at the University of Agriculture Faisalabad. Plants were grown in formalin-sterilized loam soil (72% sand, 17% silt, 8% clay) in 10-12 inch diameter pots under greenhouse conditions (20-25°C). Pathogenicity of *Zasmidium* spp. was tested following Koch's postulates by preparing a spore suspension (1×10^7 spores/ml) and spray-inoculating the plants. Control plants were sprayed with sterile distilled water, and all plants were covered with polyethylene bags to maintain humidity. Greasy spot symptoms were monitored for 45-60 days, and the pathogen was re-isolated from symptomatic leaves and confirmed morphologically. Pathogens were also re-isolated from infected fruits and cultured on PDA medium. Disease incidence and severity were calculated based on lesion diameter, with the experiment arranged in a completely randomized design (CRD) (Sukorini et al., 2013).

Fungal inoculation preparation

Pure cultures were replicated on PDA plates and incubated for seven days. Conidia and mycelia were harvested from these cultures and suspended in 500 ml of sterilized water containing 5 ml of Triton X-1000 (5%). The suspension was shaken vigorously for 10-12 min using a mechanical shaker. The suspension was then filtered through a double layer of cheesecloth. The concentration of the spore suspension was adjusted to 1×10^7 spores/ml using a hemocytometer (Sivakumar et al., 1997).

Fungal culture, identification, frequency calculation

Colony morphology

Colonies of *Zasmidium* spp. were grown on common cultural media and observed for color, growth rate, and size.

Microscopic examination

Conidia were examined for septation, shape, color, and size using lactophenol cotton blue mounts.

Fruiting structures

The presence and morphology of pseudothecia on decomposing leaves were observed.

Fungal identification

Fungal isolates were identified based on their morphological characteristics using the methods described by Gilman (1945) and Domsch et al. (1980).

Fungal frequency (Gautam and Avasthi, 2019) was calculated using the following formula:

$$\text{Relative density of fungi (\%)} = \frac{\text{No. of pieces colonized by a pathogen}}{\text{Total number of pieces}} \times 100$$

Sample preparation and assessment of antioxidant enzyme activities and H₂O₂ concentration

Leaf samples (0.5 g) were homogenized in potassium phosphate buffer for antioxidant enzyme and protein analyses, whereas methanol was used for the extraction of soluble sugars and phenolic compounds. The homogenates were centrifuged at 12,000 rpm for 5 min, and the resulting supernatants were used for subsequent biochemical analyses.

Superoxide dismutase (SOD) activity was determined using a reaction mixture containing enzyme extract, methionine, nitro blue tetrazolium (NBT), Triton X-100, potassium phosphate buffer, and distilled water. The reaction mixture was exposed to ultraviolet (UV) light for 15 min following the addition of riboflavin, and absorbance was measured at 560 nm according to the method of Giannopolitis and Ries (1977).

Peroxidase (POD) activity was assayed using a reaction

mixture consisting of 100 μL enzyme extract, 100 μL guaiacol (20 mM), 800 μL potassium phosphate buffer, and 100 μL H_2O_2 (40 mM). Absorbance was recorded at 470 nm using an ELISA plate reader following the method of Liu et al. (2009).

Catalase (CAT) activity was estimated by mixing 100 μL enzyme extract with 100 μL hydrogen peroxide (H_2O_2 , 5.9 mM). Absorbance was measured at 240 nm using an ELISA plate reader according to the method described by Liu et al. (2009).

Hydrogen peroxide (H_2O_2) concentration was determined following the method of Velikova et al. (2000). Fresh citrus leaf tissue (50 mg) was homogenized in trichloroacetic acid (TCA) buffer and centrifuged at 12,000 rpm for 15 min. The supernatant was filtered and mixed with potassium phosphate buffer (pH 7.0) and potassium iodide, followed by incubation for 5 min. Absorbance was measured at 390 nm using a BioTek 800TS microplate reader, and H_2O_2 concentration was expressed as $\mu\text{mol g}^{-1}$ fresh weight (FW).

Biochemical estimation of total soluble sugars, phenolics, and proteins

Total soluble sugars were quantified using the Anthrone reagent method described by Yemm and Willis (1954). Briefly, 100 mg of leaf tissue was hydrolyzed with 5 mL of 2.5 N HCl in a boiling water bath for 3 h, cooled, and neutralized with sodium carbonate. The final volume was adjusted to 100 mL, followed by centrifugation. Aliquots of 0.5 and 1 mL were used for analysis, while glucose standards of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg mL^{-1} were prepared. Distilled water was added to make the final volume up to 1 mL, followed by the addition of 4 mL Anthrone reagent. The reaction mixture was boiled, cooled, and absorbance was recorded at 630 nm using a spectrophotometer.

Total phenolic content was estimated using the Folin-Ciocalteu method with gallic acid as the standard, following Ainsworth and Gillespie (2007). Approximately 20 mg of plant tissue was homogenized in 2 mL of 95% methanol and incubated for 96 h. The extract was centrifuged at $16,128 \times g$ for 10 min, and 100 μL of the supernatant was mixed with 200 μL of 10% Folin-Ciocalteu reagent and 800 μL of 700 mM Na_2CO_3 . The mixture was incubated for 2 h, and absorbance was measured at 765 nm. Total phenolic content was expressed as mg gallic acid equivalents (GAE) g^{-1} sample. All analyses were performed in triplicate.

Total soluble protein content was determined according

to the method of Bradford (1976). Briefly, 40 μL of enzyme extract was mixed with potassium phosphate buffer (pH 5.0) and vortexed thoroughly. After centrifugation, 160 μL of Bradford reagent was added to the supernatant and incubated for 5 min. Absorbance was measured at 595 nm using an ELISA plate reader.

Molecular identification of fungal species

Extraction of fungal genomic DNA

Fungal genomic DNA was extracted using a modified CTAB method described by Moller et al. (1992). Approximately 50 mg of fungal mycelium obtained from a 10-day-old potato dextrose agar (PDA) culture was transferred into 1.5 mL Eppendorf tubes containing 500 μL of TES lysis buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; and 2% SDS). The mycelial mat was homogenized using a sterile micro-pestle, followed by the addition of 3 μL Proteinase K and incubation at 60°C for 60 min. Subsequently, 140 μL NaCl and 64 μL of 10% CTAB were added, and the mixture was further incubated at 60°C for 10 min.

The lysate was extracted with chloroform:isoamyl alcohol (24:1) and centrifuged at 14,000 rpm for 10 min. The aqueous upper phase (approximately 500 μL) was carefully transferred to a fresh tube, and genomic DNA was precipitated by adding 800 μL of absolute ethanol followed by incubation at -20°C for 15 min. The samples were centrifuged to pellet the DNA, washed with 70% ethanol, air-dried at 37°C for 20 min, and finally dissolved in 50 μL deuterium oxide ($\text{d}_3\text{H}_2\text{O}$). The extracted DNA was stored at -20°C until further analysis.

DNA electrophoresis and quantification

Extracted DNA samples were analyzed by electrophoresis on 1.0% (w/v) agarose gels prepared in 0.5 $\times$ Tris-Borate-EDTA (TBE) buffer. Electrophoresis was carried out at 90 V for 40-45 min. DNA bands were visualized by staining with ethidium bromide (100 $\mu\text{g mL}^{-1}$). Gel images were documented under ultraviolet (UV) illumination using the Gel Doc™ EZ Imager, and image analysis was performed using Gel Lab™ software (Bio-Rad, USA). DNA concentration and purity were further quantified using a Thermo Scientific NanoDrop 8000 UV-Vis Spectrophotometer.

PCR amplification and sequencing

PCR amplification was performed using universal primers targeting the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) and the translation elongation factor 1-alpha (EF1 α) gene. The ITS region was amplified using primers ITS1-F and ITS4-R

described by White et al. (1990) and Li (1994). Amplification of the EF-1 α gene was performed using EF1-728F and EF1-986R primers.

Each 25 μ L PCR reaction mixture consisted of 12.5 μ L of 2 $\times$ DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 1 μ L of each primer, 1 μ L of fungal genomic DNA template, and 9.5 μ L of nuclease-free water. PCR amplification was carried out under the following thermal cycling conditions: an initial denaturation at 94°C for 2

min; followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50-55°C for 30 sec, and extension at 72°C for 1 min; with a final extension at 72°C for 10 min.

The amplified PCR products were sequenced, and the obtained sequences were submitted to the NCBI GenBank (<http://www.ncbi.nih.gov>) database through the BankIt online submission portal. Each fungal isolate was assigned a unique accession number for future reference and sequence retrieval (Table 2).

Table 2. Primers used in this study.

Sr No.	Primer	Sequence	Reference
1	ITS1F	CTTGGTCATTTAGAGGAAGTAA	(White <i>et al.</i> , 1990)
2	ITS4	TCCTCCGCTTATTGATATGC	(White <i>et al.</i> , 1990)
3	EF1	ATGGGTAAAGGARGACAAGAC	(Geiser <i>et al.</i> , 2004)
4	EF2	GGARGTACCAGTSATCATGTT	(Geiser <i>et al.</i> , 2004)

Sanger sequencing

Purified PCR products were sequenced using the Sanger sequencing method (Sanger et al., 1977) with specific internal primers. Nucleotide sequencing was performed commercially by MCLAB, California, USA, and MACROGEN, Republic of Korea.

Phylogenetic analysis of isolates

DNA sequences obtained from all isolates were aligned and subjected to phylogenetic analysis using homologous sequences retrieved from the NCBI database (www.ncbi.nlm.nih.gov). GenBank accession numbers and isolate information were obtained from the NCBI database. Phylogenetic relationships were inferred using MEGA7 software (<https://www.megasoftware.net/>) following the method described by Kumar et al. (2016). Neighbor-joining phylogenetic trees were constructed according to the method of Saitou and Nei (1987), and evolutionary distances were calculated using the p-distance model (Nei and Kumar, 2000). Sequence alignments were generated using ClustalW, manually refined, and ambiguous gaps were excluded from the

analysis. The robustness of the phylogenetic trees was evaluated by bootstrap analysis with 1,000 replications following Felsenstein (1985).

Management of citrus greasy spot

The management of citrus greasy spot was evaluated using five fungicides, Carbendazim, Tebuconazole, Captan, Mancozeb, and Copper Oxchloride, and five plant extracts derived from *Zingiber officinale*, *Azadirachta indica*, *Mentha*, *Syzygium aromaticum*, and *Cinnamomum zeylanicum* at concentrations of 100, 300, and 600 ppm to determine their efficacy against *Z. fructicola*.

In vitro evaluation of fungicides

The antifungal efficacy of the fungicides was assessed *in vitro* using the poisoned food technique in the Virology Laboratory, Department of Plant Pathology, under controlled conditions. Different concentrations were prepared by dissolving the required quantities of each fungicide in distilled water to obtain a final volume of 100 mL, using the recommended dose (R) as the standard reference (Grover and Moore, 1962) as shown in Table 3.

Table 3. List of fungicides used against *Zasmadium* spp.

Sr. No.	Trade name	Main active ingredient	Recommended Dose
1	Nativo	Trifloxystrobin+Tebuconazole	0.2g/100 ml
2	Tecto	Carbendazim	2.5g/10 L water
3	Sportak	Captan,	0.055 ml/100 ml
4	Scholar	Mancozeb	0.2g/100 ml
5	Cabrio top	Copper Oxchloride	0.25g/100 ml

Preparation and inoculation of potato dextrose agar

Potato dextrose agar (PDA) medium was prepared and amended with predetermined concentrations of fungicides (R, R/2, and R/4) before being poured into sterilized 9-cm Petri plates. A sterile inoculating needle was used to place a mycelial disc of *Zasmidium* spp. at the center of each plate. PDA plates without fungicide served as the control treatment. Each treatment was replicated five times in a completely randomized design (CRD). The inoculated and labeled plates were incubated at $28 \pm 2^\circ\text{C}$. After 10 days of incubation, mycelial growth was measured along two perpendicular diameters to determine fungal colony growth.

Preparation of plant extracts

Five plant extracts, *Z. officinale*, *A. indica*, *Mentha* spp., *S. aromaticum*, and *C. zeylanicum*, were evaluated for their antifungal activity against *Zasmidium* spp. Fresh plant materials were collected, washed thoroughly, surface-sterilized with 70% ethanol, and oven-dried at 70°C for 48 h before being ground into fine powder. For crude extract preparation, 300 g of powdered material from each plant species was soaked in 400 mL of 96% ethanol for three days with intermittent shaking. The extracts were filtered through Whatman No. 1 filter paper and concentrated using a rotary evaporator. The concentrated extracts were further mixed with dichloromethane (1:3) for 30 min and filtered again. The resulting filtrates were dried, reconstituted in 20% ethanol, and stored at -20°C until further use (Sukorini et al., 2013).

Effect of plant extracts on the growth of *Zasmidium* spp.

The antifungal activity of plant extracts against *Zasmidium* spp. was evaluated using the poisoned food technique. PDA medium was amended with crude plant extracts at concentrations of 5, 10, and 15 g L^{-1} . A 5-mm mycelial disc taken from a 7-day-old actively growing culture was inoculated onto the treated PDA plates. Plates without plant extracts served as controls. Colony growth was recorded after incubation, and fungal morphology was examined using lactophenol cotton blue staining.

Each treatment was replicated 10 times and the experiment was repeated twice. Percent inhibition of mycelial growth was calculated using the formula described by Skidmore and Dickinson (1976). Following incubation, colony diameter was measured to evaluate the inhibitory effects of the tested treatments. The recorded data were subjected to statistical analysis to determine the significance of treatment effects on fungal growth inhibition.

Statistical Analysis

The experimental data were statistically analyzed using Statistix 8.1. Analysis of variance (ANOVA) was performed to evaluate significant differences among treatments and their interactions. Treatment means were separated using Tukey's Honest Significant Difference (HSD) test at a significance level of $P \leq 0.05$.

Results

Disease severity index and disease incidence

The Disease Severity Index (DSI) of Citrus Greasy Spot (CGS) was assessed in each surveyed area by counting the number of lesions on infected leaves of susceptible citrus cultivars. The highest DSI was recorded in the citrus orchards of Sargodha at 41.13% (Table 4, Figure 2A), whereas the lowest DSI was observed in Faisalabad at 33.24%. Disease severity in Toba Tek Singh (36.44%) and Layyah (36.30%) did not differ significantly. Among the citrus cultivars, grapefruit and mandarin were found to be highly susceptible to CGS, exhibiting a greater number of lesions on both the abaxial and adaxial leaf surfaces.

Disease incidence was determined by recording the number of infected plants in each surveyed location. The highest disease incidence was observed in the orchards of Sargodha at 56.01%, followed by Mandi Bahauddin at 53.30%, where a substantial number of infected cultivars, particularly grapefruit and mandarin, were recorded. In contrast, the lowest disease incidence was documented in Faisalabad at 19.52%. An average disease incidence of 29.93% was recorded in Layyah (Table 4, Figure 2B).

Morphological characteristics of fungi

Morphological studies were conducted for the preliminary identification and validation of the causal fungal pathogen based on colony characteristics, including colony size, shape, and color, as well as asexual conidial morphology such as shape, size, color, and septation. A total of fifteen fungal isolates were obtained from citrus-growing orchards in Punjab, Pakistan, including three isolates each from Sargodha, Toba Tek Singh, Layyah, Mandi Bahauddin, and Faisalabad. However, saprophytic fungi, including *Alternaria*, *Aspergillus*, and other non-target fungal species that co-occurred with the original fungal isolates, were excluded from further morphological and molecular analyses because they were not related to the objectives of the present study.

Colony characteristics

Each isolate cultured on PDA exhibited slow growth and formed flat colonies with sparse to smooth aerial

mycelia, reaching approximately 4 cm in diameter after 14 days of incubation. The upper colony surface initially displayed a gray center with a dark green margin, whereas the reverse side gradually turned completely black. These distinct colony characteristics facilitated the identification and characterization of the fungal isolates.

Micromorphological characteristics

Micromorphological examination revealed that the fungal isolates produced verruculose, septate, branched mycelia that ranged from light brown to brown in color, with

hyphal diameters ranging from 1.5 to 2.0 mm. The conidiophores bearing conidia were medium brown, straight to geniculate, sub-cylindrical, erect, and possessed 0–2 septa. Conidia were brown, subobtusate at the apex, sub-cylindrical to fusoid-ellipsoidal, verruculose, and contained 0–1 septa. Based on both macro- and micromorphological characteristics, the representative fungal isolates were preliminarily identified as *Zasmidium fructicola*. The observed morphological features were consistent with those reported in previous studies by Huang (2015) and Park et al. (2017).

Table 4. Assessment of disease severity (%) and disease incidence (%) in different districts of Punjab, Pakistan.

Disease severity index (%)				
Cultivars				
Districts	Sweet orange	Mandarin	Grapefruit	Average
Sargodha	41.10	41.17	41.16	41.13 ^a
Mandi Bahauddin	38.02	37.71	38.04	37.92 ^b
Toba Tek Singh	36.20	36.56	36.60	36.44 ^{bc}
Layyah	36.96	36.14	35.70	36.30 ^c
Faisalabad	33.30	32.95	33.46	33.24 ^d
Disease incidence index (%)				
Sargodha	50.00	62.50	55.55	56.02 ^a
Mandi Bahauddin	41.66	62.50	55.55	53.24 ^a
Toba Tek Singh	38.46	41.66	38.46	39.52 ^b
Layyah	26.31	35.71	27.77	29.93 ^{bc}
Faisalabad	19.23	20.83	18.51	19.52 ^c

Mean value showing superscript letter are not significantly different regarding Tukey's HSD test at probability ($P \leq 0.05$).

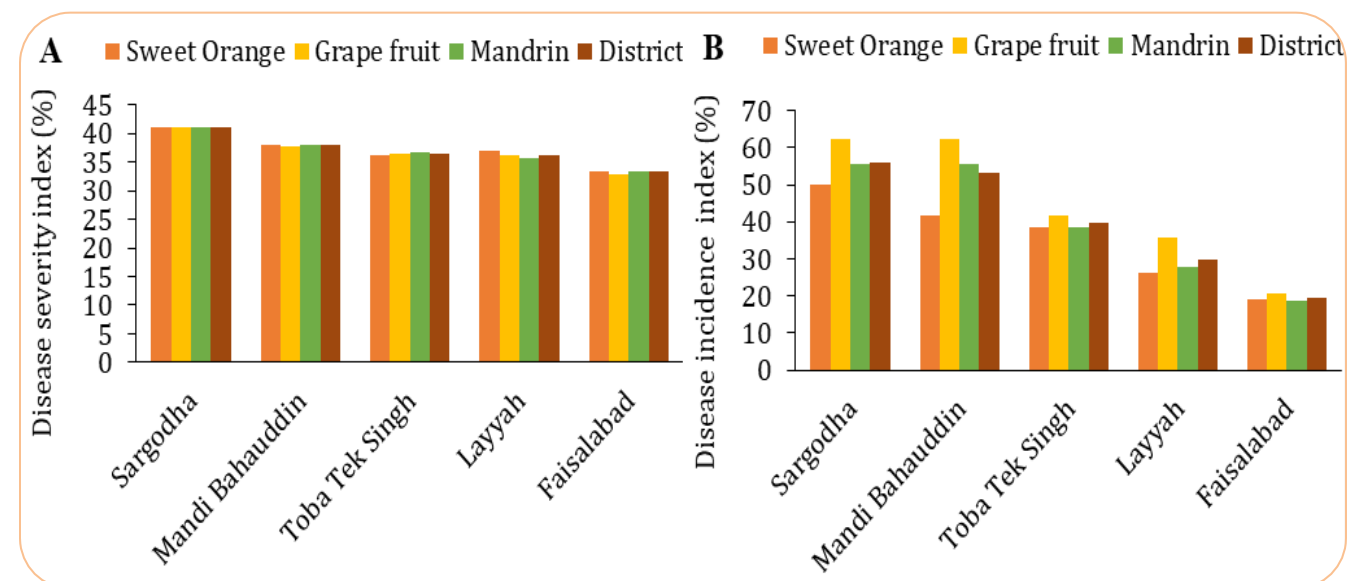


Figure 2. Disease severity (A) and disease incidence (B) of citrus greasy leaf spot in different districts of Punjab during 2022. Means followed by the same superscript letters are not significantly different at $p \leq 0.05$ according to Tukey's HSD test.

Molecular identification of fungi

PCR analysis

Genomic DNA from each fungal isolate was subjected to PCR amplification using universal primer sets (Figure 3 and 4). The entire ITS rDNA region was amplified using the ITS1 and ITS4 primers, whereas the translation elongation factor 1-alpha (TEF-1 α) region was amplified using the EF1-728F and EF1-986R primers. Gel electrophoresis analysis revealed that the amplified PCR products of the fungal isolates

were approximately 545 bp for the ITS region and 296 bp for the TEF-1 α region.

A total of fifteen PCR amplicons representing two gene regions were recovered from isolates collected from Sargodha (5 amplicons), Mandi Bahauddin (3 amplicons), Layyah (2 amplicons), Faisalabad (2 amplicons), and Toba Tek Singh (3 amplicons). NCBI BLASTn analysis demonstrated that all fungal isolates shared 99% sequence identity at the ITS and TEF1- α loci with reference isolates/strains of *Z. fruticola*.

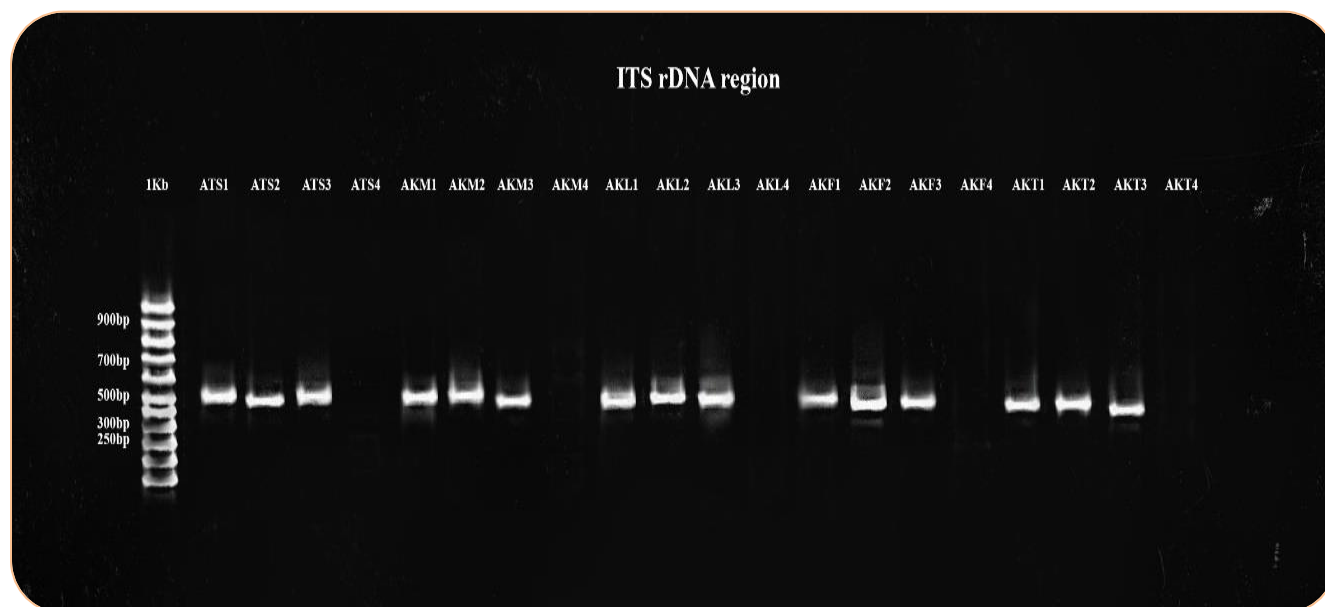


Figure 3. PCR amplicons of *Z. fruticola* isolates showing a 550 bp ITS region fragment, compared with a 1 kb DNA ladder, as revealed by gel electrophoresis analysis.

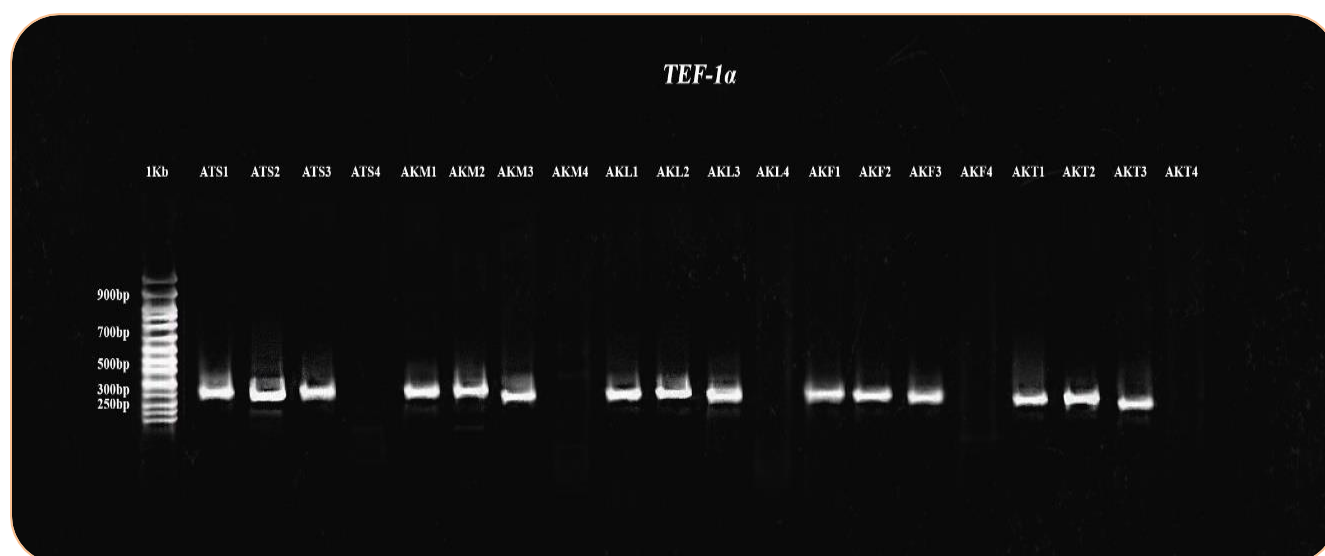


Figure 4. PCR amplicons of *Z. fruticola* isolates showing a 300 bp fragment of the TEF-1 α gene, as compared with a 1 kb DNA ladder, as determined by gel electrophoresis analysis.

Phylogenetic analysis of *Z. fructicola*

Fifteen isolates obtained from three citrus varieties were identified as *Zasmidium* spp. through multi-gene phylogenetic analysis. BLASTn analysis indicated that all isolates exhibited 98% sequence similarity with reference strains of *Zasmidium* spp. The combined dataset consisted of 475 characters from the Internal Transcribed Spacer (ITS) region (positions 1–475) and 198 characters from the TEF1- α gene (positions 476–673).

The maximum parsimony analysis generated a phylogenetic tree with a tree length (TL) of 190, a consistency index (CI) of 0.92, a retention index (RI) of 0.97, and a composite index of 0.89. Of the 673 total characters analyzed, 512 were constant, 66 were parsimony-uninformative, and 95 were parsimony-informative. The partition homogeneity test produced a value of 0.21, indicating that the ITS and TEF1- α datasets were sufficiently congruent to be combined for phylogenetic reconstruction.

The phylogenetic tree generated from the combined ITS and TEF1- α dataset using maximum parsimony (MP) and maximum likelihood (ML) analyses grouped all isolates into a distinct clade. The TEF1- α locus exhibited a higher proportion of variable sites (18%) compared with the ITS region (7%). The isolates formed a well-supported cohesive clade with MP/ML bootstrap support values of 99/–, indicating genetic divergence from other reference strains of *Zasmidium* spp., likely due to nucleotide substitutions, deletions, and insertions.

Pathogenic variability of *Z. fructicola* isolates

Five isolates of *Z. fructicola* were collected from different locations, namely Sargodha (SAK-1), Mandi Bahauddin (AKM-1), Layyah (AKL-1), Toba Tek Singh (AKT-1), and Faisalabad (AKF-1). The isolates exhibited significant

pathogenic variability when inoculated onto one-year-old healthy plants of susceptible citrus cultivars. Among the tested isolates, SAK-1 and AKM-1 proved to be the most virulent, producing the highest number of yellow pustules on the leaves of Grapefruit, Mandarin, and Sweet Orange cultivars, with maximum disease severities of 66.9% and 64.87%, respectively. In contrast, isolate AKL-1 exhibited moderate virulence, with an average disease severity of 33.6% across the inoculated citrus cultivars (Table 5, 6 and Figure 5). Conversely, isolates AKT-1 and AKF-1 displayed low virulence, producing the fewest greasy leaf spots and resulting in only 32% disease severity after 30 days of inoculation. No significant differences were observed among the citrus cultivars, indicating that all tested cultivars were highly susceptible to CGS infection. To fulfill Koch's postulates, the pathogen was successfully re-isolated from the inoculated leaves. The pathogenicity assay confirmed that *Z. fructicola* was the causal agent of CGS.

Biochemical characterization

In non-inoculated leaves, Sweet Orange exhibited the highest superoxide dismutase (SOD) activity associated with susceptibility to citrus greasy leaf spot (CGLS), whereas Red Blood exhibited the lowest SOD content. The SOD activities in Mandarin (2.20 $\mu\text{g/g}$) and Grapefruit (2.21 $\mu\text{g/g}$) were statistically similar (Figure 6). In inoculated leaves, Sweet Orange (0.56 $\mu\text{g/g}$) and Grapefruit (0.55 $\mu\text{g/g}$) recorded the highest SOD activities, while Kinow exhibited the lowest value (0.33 $\mu\text{g/g}$). Overall, significant variations in SOD activity were observed between non-inoculated and inoculated genotypes, with higher enzymatic activity detected in non-inoculated leaves, indicating a marked response to *Z. fructicola* infection.

Table 5. Comparative efficiency of *Z. fructicola* isolates on disease severity 30 days after inoculation during the pathogenicity trial.

Isolate of <i>Z. fructicola</i>	Disease severity scale	Number of spots observed / leaf			Disease severity index (%) $\pm$ S.E
		Grapefruit	Mandarin	Sweet	
(AKS_1)	5	12	14	11	66.9 ^a $\pm$ 9.56
(AKM_1)	5	13	11	12	64.87 ^{ab} $\pm$ 11.15
(AKL_1)	4	8	8	10	33.60 ^{bc} $\pm$ 2.50
(AKT_1)	4	9	8	9	29.10 ^c $\pm$ 3.50
(AKF_1)	3	5	6	6	12.90 ^c $\pm$ 0.53

Mean value $\pm$ S.E (Standard error) indicating similar superscript alphabets are not statistically varied based on Tukey's HSD test at probability ($P \leq 0.05$). *Category of disease rating scale: 0 = no lesion appearance per leaf, 1 = 1–2 lesions per leaf, 2 = 3–5, 3 = 5–6 lesions per leaf, 4 = 8–10 lesions per leaf, >11 lesions per leaf.

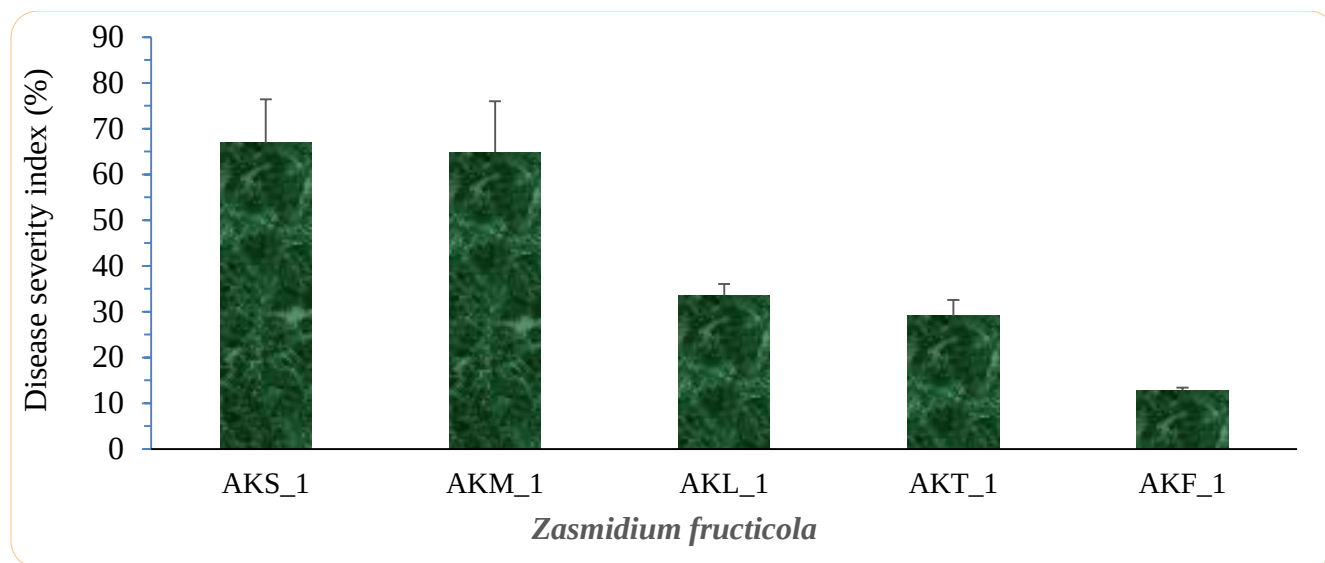


Figure 5. Pathogenicity analysis of *Z. fructicola* isolates based on disease severity index of citrus greasy leaf spot at 30 DAI. Mean values $\pm$ S.E. (standard error) followed by similar superscript letters are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

Table 6. Analysis of variance (ANOVA) for the virulence potential of *Z. fructicola* isolates on disease severity index (%) of citrus greasy leaf spot at 30 DAI.

Source	DF	SS	MS	F _{cal}	P _{value}
Inoculated isolates	4	6673.07	1668.27	11.9	0.0008*
Error	10	1406.67	140.67		
Total	14	8079.73			
Grand Mean = 41.464		CV = 28.60			

All the inoculated isolates have significant effect on disease severity index (%) of citrus greasy spot at 30-days after inoculation as represented in ANOVA.

Peroxidase (POD) activity was highest in Grapefruit (3.26 $\mu\text{g/g}$), followed closely by Sweet Orange (3.22 $\mu\text{g/g}$) and Mandarin (3.18 $\mu\text{g/g}$), all of which were statistically at par. In contrast, the resistant Kinow cultivar exhibited lower POD activity (2.81 $\mu\text{g/g}$). Overall, POD activity was significantly higher in susceptible cultivars than in resistant cultivars. In inoculated leaves, susceptible cultivars again showed elevated POD activity, with Sweet Orange (1.46 $\mu\text{g/g}$) and Grapefruit (1.42 $\mu\text{g/g}$) recording the highest values, whereas Red Blood exhibited comparatively lower POD activity (1.22 $\mu\text{g/g}$).

Significant variations in catalase (CAT) activity were also observed between non-inoculated and inoculated leaves of susceptible and resistant citrus cultivars. In non-inoculated leaves, susceptible cultivars exhibited CAT activity ranging from 5.12 to 5.52 $\mu\text{g/g}$, with Sweet Orange recording the highest value (5.52 $\mu\text{g/g}$). In

contrast, resistant cultivars showed comparatively lower CAT activity ranging from 4.13 to 4.65 $\mu\text{g/g}$, with Kinow exhibiting the lowest value (4.13 $\mu\text{g/g}$). In inoculated leaves, Sweet Orange retained the highest CAT activity (3.13 $\mu\text{g/g}$), whereas Kinow recorded the lowest value (1.63 $\mu\text{g/g}$). Overall, Sweet Orange exhibited significantly greater CAT activity than the other cultivars in both non-inoculated and inoculated conditions (Figure 6).

Significant differences in hydrogen peroxide (H_2O_2) concentrations were observed between non-inoculated and inoculated leaves of citrus cultivars. In non-inoculated leaves, resistant cultivars exhibited H_2O_2 concentrations ranging from 1.68 to 2.11 $\mu\text{g/g}$, whereas susceptible cultivars ranged from 1.37 to 2.11 $\mu\text{g/g}$. Following inoculation, H_2O_2 concentrations declined in all cultivars, with susceptible cultivars ranging from 0.30 to 0.35 $\mu\text{g/g}$ and resistant cultivars ranging from 0.25 to 0.30 $\mu\text{g/g}$ (Figure 6).

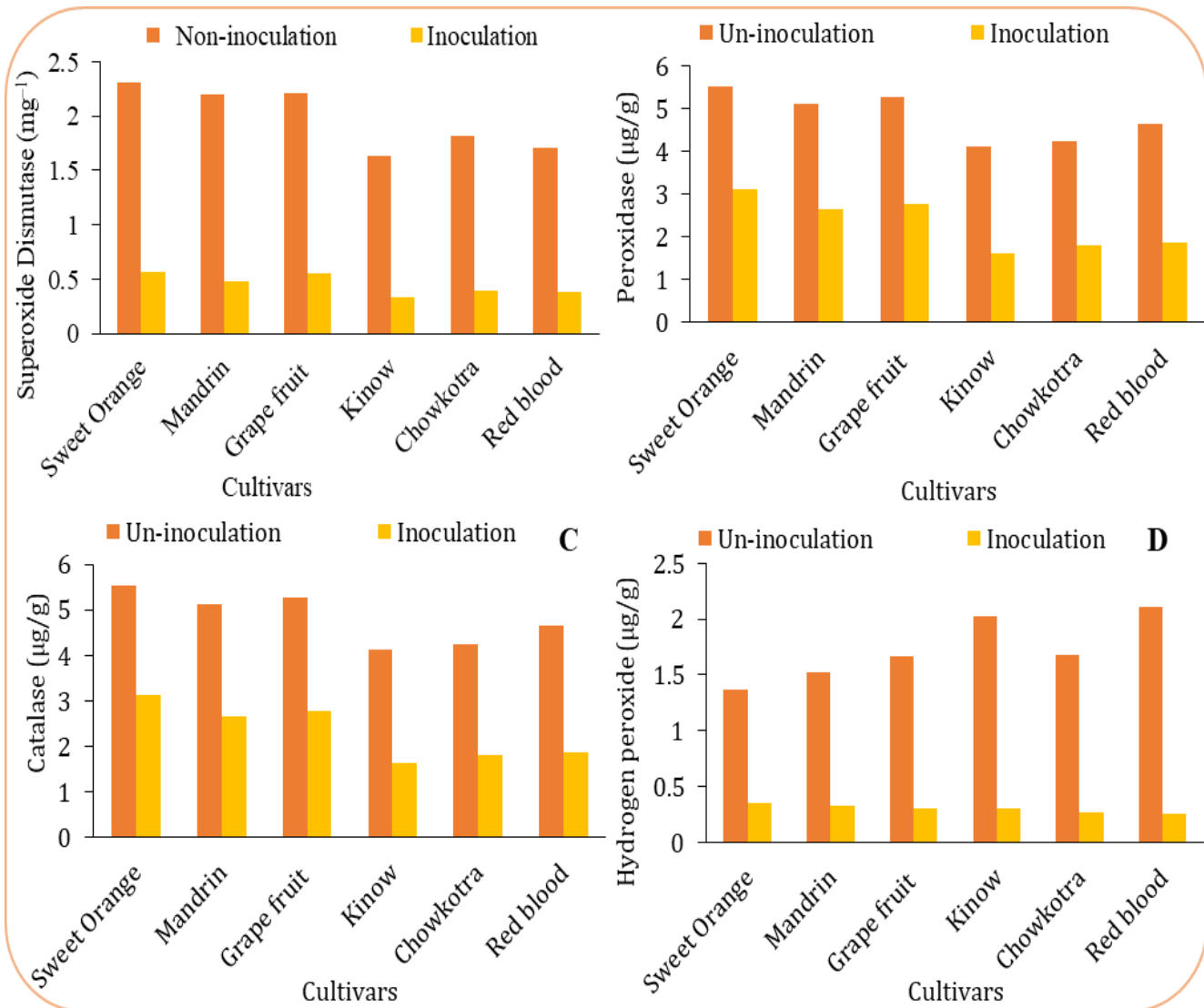


Figure 6. Determination of SOD (A), POD (B), CAT (C), and hydrogen peroxide (D) content ($\mu\text{g/g}$) in citrus cultivars inoculated with *Z. fructicola*. Mean values $\pm$ SE (standard error) with similar superscript letters are not significantly different according to Tukey's HSD test ($P \leq 0.05$).

Response of total soluble sugars, phenolics, and proteins

Significant variations in total soluble proteins, phenolics, and sugars were observed in both susceptible and resistant citrus cultivars under non-inoculated and inoculated conditions. Susceptible cultivars exhibited comparatively lower protein contents, ranging from 2.52 to 2.77 $\mu\text{g/g}$ in non-inoculated leaves and 0.76 to 0.97 $\mu\text{g/g}$ in inoculated leaves. In contrast, resistant cultivars recorded higher protein concentrations ranging from 3.67 to 4.13 $\mu\text{g/g}$ in non-inoculated leaves and 1.27 to 1.37 $\mu\text{g/g}$ in inoculated leaves, indicating that *Z. fructicola* inoculation significantly reduced protein

content in susceptible cultivars, as shown in Figure 7.

Similarly, resistant cultivars exhibited higher total phenolic contents (3.91–4.18 $\mu\text{g/g}$) compared with susceptible cultivars, which showed lower phenolic concentrations ranging from 1.37 to 1.67 $\mu\text{g/g}$. Total soluble sugar (TSS) contents followed a similar trend, with resistant cultivars recording higher sugar contents ranging from 3.14 to 3.23 $\mu\text{g/g}$ in non-inoculated leaves and 2.40 to 2.63 $\mu\text{g/g}$ in inoculated leaves. Conversely, susceptible cultivars exhibited comparatively lower sugar contents ranging from 2.36 to 2.57 $\mu\text{g/g}$ in non-inoculated leaves and 2.11 to 2.23 $\mu\text{g/g}$ in inoculated leaves.

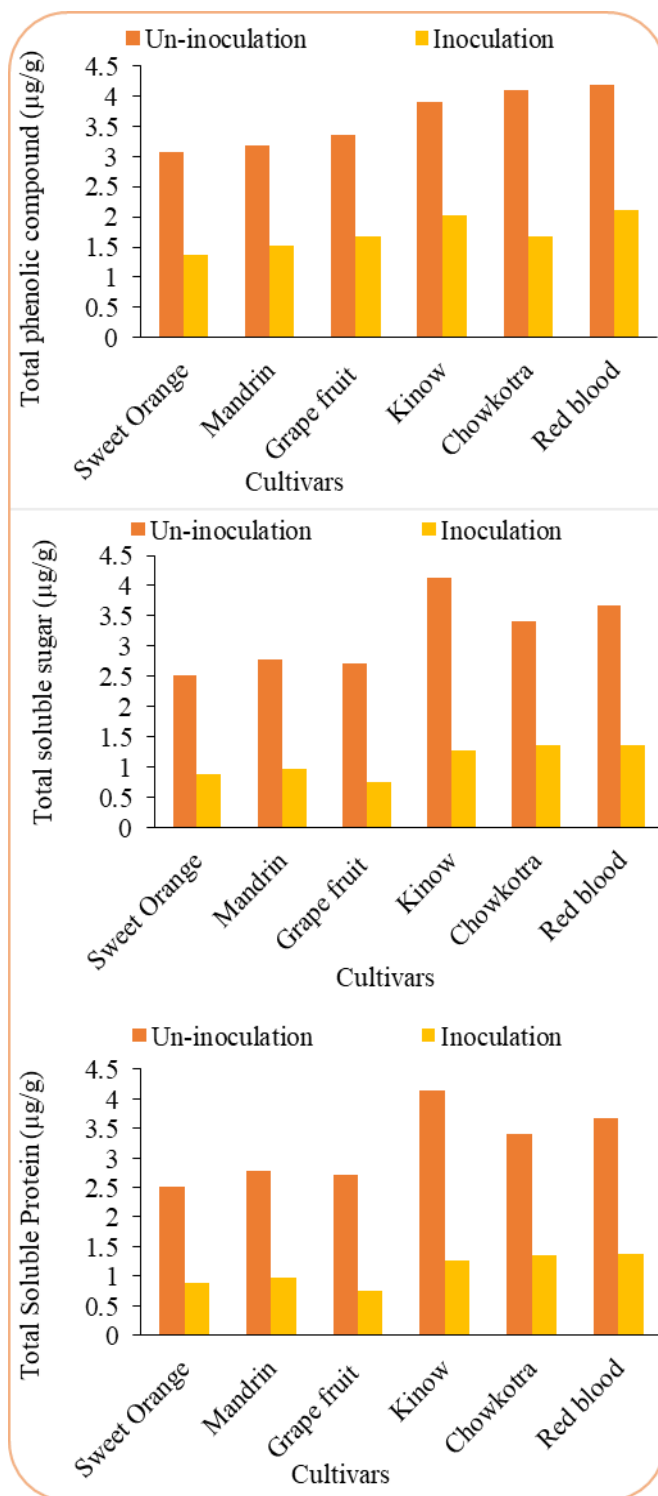


Figure 7. Estimation of total soluble sugars, proteins, and phenolic compounds ($\mu\text{g/g}$) in citrus cultivars following inoculation with *Z. fructicola*. Mean values $\pm$ S.E. (standard error) followed by the same superscript letters are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

In vitro efficacy of chemical fungicides for disease management

The mycelial growth inhibition of *Z. fructicola* was evaluated *in vitro* using five fungicides at concentrations of 100, 300, and 600 ppm. All tested fungicides exhibited a significant effect ($P \leq 0.05$) compared with the control at 3, 6, and 9 days after inoculation (DAI). Among the fungicides, carbendazim showed the highest efficacy at 600 ppm, achieving 95.7% mycelial growth inhibition at 9 DAI, followed closely by tebuconazole (92.16%). In contrast, captan at the lowest concentration (100 ppm) showed comparatively lower and inconsistent performance, inhibiting only 24.1% of mycelial growth at 3 DAI. Mancozeb exhibited a moderate response, with 51% inhibition at 9 DAI. Overall, the efficacy of all fungicides increased progressively with incubation time, demonstrating enhanced suppression of mycelial growth over the experimental period as shown in Figure 8.

In vitro efficacy of botanical extracts

Five botanical extracts were evaluated at concentrations of 100, 300, and 600 ppm for their ability to suppress radial colony growth of *Z. fructicola* under *in vitro* conditions. All extracts showed significant effects ($P \leq 0.05$) compared with the control at 3, 6, and 9 days after inoculation (DAI). *S. aromaticum* was the most effective treatment, exhibiting consistently high radial growth inhibition with mean values of 95.1%, 96.0%, and 97.0% at 100, 300, and 600 ppm, respectively, across the observation periods. In contrast, *Z. officinale* showed comparatively lower and less consistent efficacy, with inhibition rates of 63.24%, 65.2%, and 67.1% at the respective concentrations. Overall, the inhibitory effect of all botanical extracts increased with time and concentration, indicating improved suppression of fungal growth over the incubation period, as shown in Figure 9.

Discussion

In this study, the highest disease severity index and disease incidence of CGLS were recorded in citrus orchards of Sargodha. Such elevated levels of disease severity and incidence in specific regions are often attributed to a combination of epidemiological factors (Mondal et al., 2003), compatible host-pathogen interactions (Hammond-Kosack and Rudd, 2008), and prevailing agricultural practices (Mengeshaw et al., 2023).

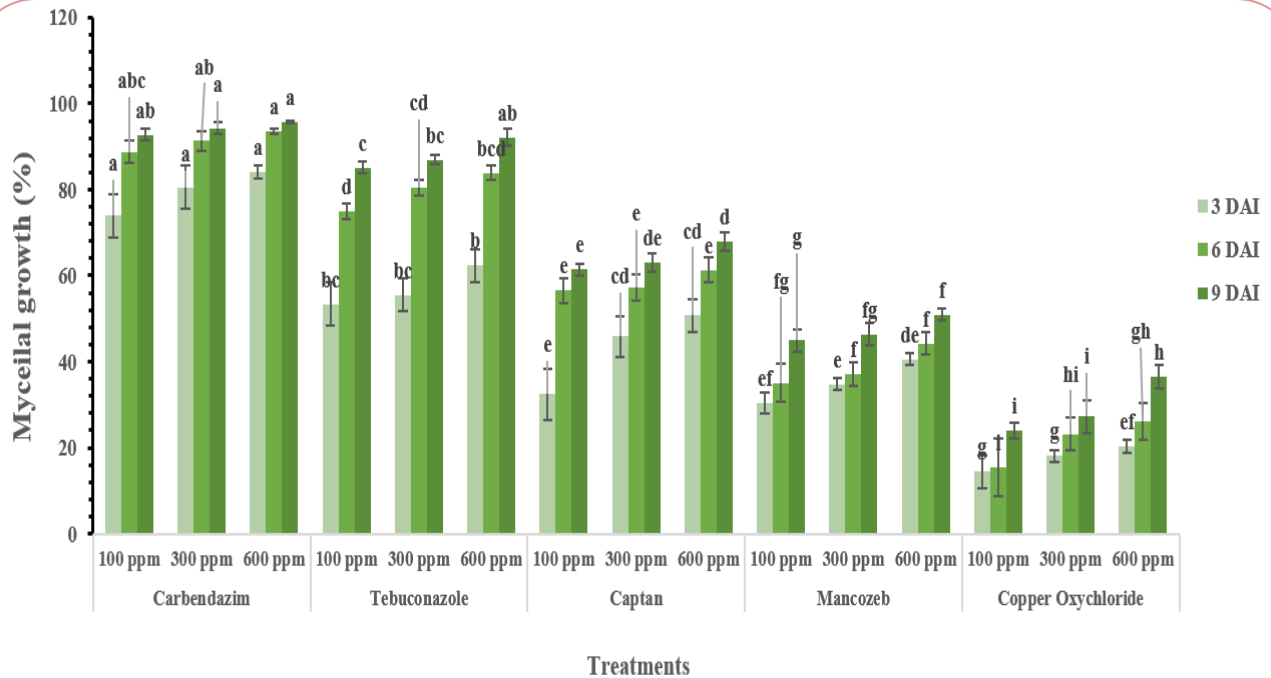


Figure 8. Antifungal activity of five fungicides at three different concentration levels on mycelial growth inhibition (%) after 3, 6, and 9 days of incubation. Mean values $\pm$ S.E. (standard error) followed by similar superscript letters are not significantly different according to Tukey's HSD test ($P \leq 0.05$).

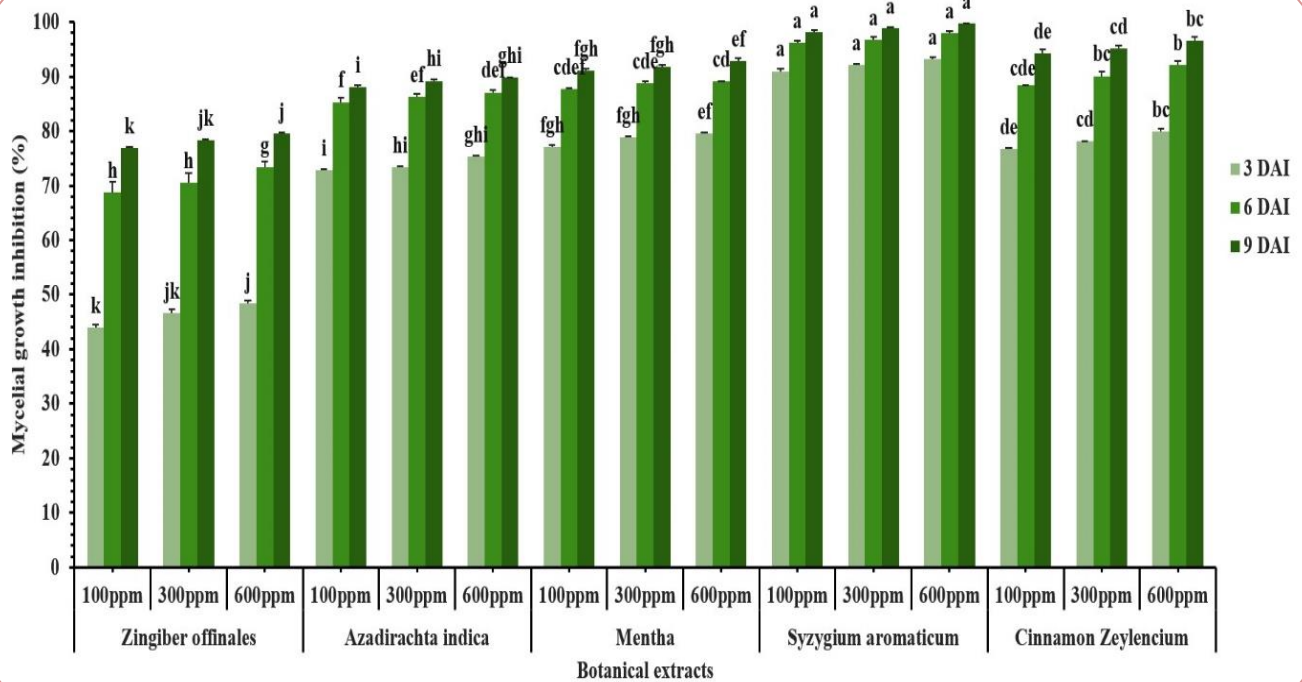


Figure 9. Antifungal activity of five botanical extracts at three different concentration levels on mycelial growth inhibition (%) at 3, 6, and 9 days of incubation. Mean values $\pm$ S.E. (standard error) followed by similar superscript letters are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

Morphological and phylogenetic analyses identified the representative isolates as *Z. fructicola*, which belongs to the family Mycosphaerellaceae. Species within this family are widely recognized as contributors to CGS globally, particularly in tropical and subtropical regions (Mondal and Timmer, 2006; Huang et al., 2015). For instance, *Mycosphaerella hori* has previously been isolated in Japan, where it caused typical CGS symptoms and is considered a synonym of *Cercospora* (imperfect stage fungi). Similarly, in Australia, another *Mycosphaerella* species has been reported as a causal agent of CGS (Wellings, 1981). Further investigations in China and Indonesia have identified *Z. fructicola*, *Z. fructigenum*, and *Z. indonesianum*, all of which induce greasy spot symptoms on citrus leaves and fruits (Huang et al., 2015). Moreover, in Spain and Panama, the *Z. citri-griseum* complex and *A. africana* have been identified as causal agents based on pathogenicity assessments and molecular analyses (Aguilera-Cogley et al., 2017). These findings highlight the global distribution and taxonomic diversity of pathogens associated with CGS, emphasizing the need for region-specific management strategies tailored to local environmental conditions.

The etiological agents of citrus greasy spot have undergone significant taxonomic revision since their initial identification as *Cercospora citri-grisea* in the USA (Fisher, 1961). The pathogen was later reclassified as *Mycosphaerella citri* (sexual stage) and *Stenella* (asexual stage) based on comprehensive epidemiological studies (Whiteside, 1970a). The name *Zasmidium citri* was subsequently adopted following taxonomic revisions (Crous et al., 2009), and this nomenclature was standardized under the Melbourne Code (2011).

In the present study, typical CGS symptoms were observed on sweet orange, mandarin, and grapefruit. Similar symptoms have been reported on sweet orange and other citrus species in Argentina (Marco and Whiteside, 1986). Greasy spot symptoms are particularly common on grapefruit, Tahiti lime (*C. latifolia*), and sweet orange (Honger, 2004). In Ghana, high susceptibility was reported in satsuma (*C. unshiu*), rough lemon, sweet orange, and mandarin (Aguilera-Cogley and Vicent, 2015). In this study, *Z. fructicola* isolates were frequently recovered from infected leaves of grapefruit, mandarin, and sweet orange. This fungus, synonymous with *Mycosphaerella fructicola*, has been identified as a causal agent of CGS

based on morphological and phylogenetic evidence from China. Notably, infection by this pathogen is largely restricted to members of the Rutaceae family (Mondal and Timmer, 2006), although it has also been reported as an endophyte in *Pinus densiflora* in Korea (Park et al., 2017).

Morphological characterization confirmed that *Z. fructicola* belongs to the Mycosphaerellaceae family, consistent with previous reports (Huang et al., 2015). However, reliance solely on morphological traits is insufficient for accurate genus- and species-level identification. In contrast, DNA barcoding provides a more reliable approach for resolving Mycosphaerellaceae taxonomy. The combined use of ITS rDNA and TEF-1 α markers has proven particularly effective in delineating species within *Zasmidium* (Huang et al., 2015). In this study, phylogenetic analysis clearly separated *Z. fructicola* isolates from reference strains, forming a well-supported clade consistent with previously reported phylogenies (Huang et al., 2015; Aguilera-Cogley et al., 2017).

Pathogenicity assays demonstrated that isolates of *Z. fructicola* from Sargodha (SAK-1) and Mandi Bahaudin (MAK-1) were highly aggressive, inducing large yellow-brown lesions on susceptible cultivars, particularly grapefruit and mandarin, using mycelial suspension inoculation. Similar methods have been previously used in pathogenicity studies (Whiteside, 1970b; Aguilera-Cogley et al., 2017). The observed aggressiveness may be attributed to genetic variability in pathogen populations and favorable environmental conditions enhancing pathogen fitness and virulence (Arif et al., 2025). To the best of our knowledge, recent pathogenicity studies of *Z. fructicola* on these citrus cultivars are lacking in Pakistan and globally. Therefore, further research is required to elucidate pathogenic variability and host-pathogen compatibility.

Biochemical analyses of inoculated and non-inoculated citrus genotypes revealed patterns consistent with previous studies on foliar diseases. Superoxide dismutase (SOD) activity was significantly higher in resistant genotypes than in susceptible ones (Hameed et al., 2021; Iqbal et al., 2023). In contrast, some studies have reported elevated SOD activity in resistant inoculated plants, indicating variability in defense responses among cultivars (Mahawer et al., 2023). Peroxidase (POD) activity was also higher in resistant cultivars, supporting its role in defense responses

(Hameed et al., 2021; Iqbal et al., 2023). Catalase (CAT) activity was associated with regulation of hydrogen peroxide (H_2O_2) levels, where differential activity patterns were observed between resistant and susceptible genotypes. These findings are consistent with previous reports suggesting that CAT is often upregulated in susceptible cultivars (Kumar et al., 2011; Iqbal et al., 2023). Reactive oxygen species (ROS), particularly H_2O_2 , play a key role in plant defense through programmed cell death and restriction of pathogen growth (Roemmelt et al., 1998; Shetty et al., 2007).

Total phenolic content (TPC) was higher in resistant cultivars, indicating strengthened structural barriers against pathogen invasion (Lattanzio et al., 2006; Meena et al., 2017; Hameed et al., 2021; Iqbal et al., 2023). In contrast, total soluble sugars (TSS) were reduced in susceptible cultivars following infection, supporting previous findings that pathogen infection reduces sugar accumulation (Hameed et al., 2021; Iqbal et al., 2023).

Total soluble proteins (TSP) are essential for regulating structural and enzymatic processes involved in plant growth and defense. They also contribute to limiting pathogen colonization and spread within host tissues (Nafees et al., 2019). Following pathogen infection, protein concentrations generally decline (Martin et al., 2003). In this study, inoculated and susceptible cultivars exhibited significantly lower protein levels compared with resistant and non-inoculated plants, consistent with earlier reports (Hameed et al., 2021; Iqbal et al., 2023).

Among the fungicides evaluated, carbendazim and tebuconazole showed strong inhibitory effects against *Z. fructicola* at higher concentrations. Carbendazim, a methyl benzimidazole carbamate (MBC), inhibits mitosis by disrupting β -tubulin assembly (Kim et al., 2007; Potočník et al., 2009). Tebuconazole inhibits sterol biosynthesis by targeting lanosterol 14α -demethylase, thereby compromising fungal membrane integrity (Yin et al., 2009). Although no previous studies have specifically reported their effects on *Z. fructicola*, demethylation inhibitor (DMI) fungicides are generally effective against *Mycosphaerellaceae* species (Aman and Rai, 2015; Hanada et al., 2015; Kumar et al., 2020). For example, carbendazim has shown strong inhibition of *Mycosphaerella musicola* and *M. fijiensis*, causal agents of Sigatoka leaf spot in banana (Kumar et al., 2020; Holdings and Lanka, 2023).

In addition, botanical extracts were evaluated for antifungal activity. Clove (*S. aromaticum*) exhibited the highest inhibitory effect against *Z. fructicola*. This activity is attributed to bioactive compounds such as eugenol, eugenyl acetate, α -humulene, and caryophyllene, which possess strong antifungal properties (Hernández et al., 2007; Costa et al., 2011). Previous studies have also demonstrated the antifungal efficacy of clove against *Mycosphaerella fijiensis* (Adirano-Anaya et al., 2018), although its activity against *Z. fructicola* has not been previously reported.

Conclusions

This study provides important insights into the interaction between *Zasmidium fructicola* and citrus cultivars, highlighting biochemical and physiological mechanisms associated with host resistance and susceptibility. Total soluble proteins (TSP) were significantly reduced in infected susceptible cultivars, indicating their role in disease resistance. Enzymatic antioxidants, including SOD, POD, and CAT, were differentially regulated, with higher SOD and POD activities observed in resistant cultivars, suggesting enhanced ROS scavenging capacity.

Pathogenicity results confirmed the high aggressiveness of *Z. fructicola* isolates, particularly from Sargodha and Mandi Bahauddin. Among the tested fungicides, carbendazim and tebuconazole exhibited strong antifungal activity, while clove extract showed promising biocontrol potential. These findings support the integration of chemical and botanical strategies within sustainable disease management programs for CGS.

Future research should focus on elucidating the genetic basis of resistance in citrus cultivars and exploring synergistic interactions between fungicides and plant-derived compounds for improved disease control strategies.

Author's contributions

AK and LA designed the research and wrote the paper. AK and LA performed the survey and collected the material. AK performed molecular characterization and biochemical analysis with LA and SAK interpreted the results. AK, SAK and AN contributed to data analysis.

Research Funding

This research did not receive any grant from funding agencies.

Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger

SDG 12: Responsible Consumption and Production

SDG 15: Life on Land

Policy Addressed

This study aligns with Pakistan's National Food Security Policy and FAO's Integrated Pest Management (IPM) framework by promoting sustainable management of citrus greasy leaf spot through resistant cultivars, fungicides, and botanical extracts. Molecular characterization of *Zasmidium fructicola* also supports plant health surveillance under the International Plant Protection Convention (IPPC).

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