First report on leaf spot of *Zizyphus mauritiana* caused by *Mycosphaerella arachidis* in Odisha, India

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Abstract

Leaf spot of Indian jujube (*Zizyphus mauritiana* Lam.) caused by *Mycosphaerella arachidis* Deighton has been recorded during winter seasons of recent years (2019–2024) in Odisha, India. Occurrence of the disease was first noticed in the month of September till continued up to January. *M. arachidis* turned to be an emerging pathogen under change in climatic condition and every time increasing their potentiality to infect a new host. A significant number of *Z. mauritiana* showed leaf spot symptom with approximately 55–60% average disease incidence. Leaf spots characterized with oval, circular, irregular patches which showed sequential color changes from light brown to reddish brown to pale brown encircling purple margin and ultimately grayish patches. The present research article describes the identification of the causal organism *M. arachidis,* its characterization and increasing host index. Optimum temperature (20–25 °C) coupled with moderate humidity (75–80%) during the pre-winter season helps in disease progress. To the best of our knowledge and review of literature study led to the conclusion that this is the first report of leaf spot of *Z. mauritiana* caused by *M. arachidis* from India.

Keywords: First report, India, *Mycosphaerella arachidis*, Rhamnaceae, *Zizyphus mauritiana*.

Introduction

Zizyphus mauritiana belongs to the family Rhamnaceae, is considered to be one of the most important horticultural crops in India. It is widely cultivated and has an immense pharmacological value. Fruits are edible, delicious and a rich source of triterpenes, tritarpene saponins, maslinic, oleanolic, urosolic, betulinic, components, 3-O-*cis*-*p*-alphitolic, 3-O-*cis*-*p*-caumaroylalphitolic, 3-O-*trans*-*p*-coumarylalphitolic, 3-O-*cis*-*p*-alphitolic, 3-O-*cis*-*p*-coumaroylalphitolic and jajuboside B, spinosin, 3-O-*trans*-alphitolic, 3-O-*trans*-*p*- coumarylaphitolic acids and swertisin (Pan *et al*., 2023). It showed antifungal, anti-tumor, antidiarrhoeal, antidiabetic activity (Pan *et al*., 2023). It is grown approximately on an area of 49,000 ha with an annual production of 481,000 ton (Anonymous, 2017). Even though the crop is extremely important, it suffers from considerable yield loss each year due to a large number of phytopathogen infection. Various disease symptoms like sooty mold, powdery mildew, *Alternaria* leaf spot, rust, Septoria leaf spot etc have been reported on this plant (Jamadar *et al*. 2009). Among the various biotic stresses, pests pose a significant threat, with insects like fruit flies (*Bactrocera* spp.) and jujube fruit fly (*Carpomya vesuviana*) inflicting damage by laying eggs on fruits, leading to premature fruit drop and quality deterioration (Amini *et al*. 2023). Moreover, aphids such as *Aphis gossypii* and *Aphis spiraecola* feed on the plant's sap, causing leaf curling and stunted growth. Additionally, mites like spider mites (*Tetranychus* spp.) and gall mites (Eriophyidae) contribute to foliage damage by feeding on plant tissues, leading to reduced photosynthetic efficiency (de Lillo *et al*. 2018). Furthermore, diseases like powdery mildew (*Oidium* spp.) and leaf spot caused by *Alternaria alternata* pose significant threat to *Z. mauritiana*, resulting in leaf necrosis, defoliation, and reduced fruit quality (Jamadar *et al*., 2009). Moreover, fungal pathogens like fruit rot (*Rhizopus stolonifer*) and anthracnose (*Colletotrichum* spp.) contribute to post-harvest losses by causing fruit decay during storage and transportation (Duan and Chen, 2022). These biotic stresses not only decrease the yield and quality of *Z. mauritiana* but also necessitate the implementation of integrated pest management strategies and disease control measures to ensure sustainable cultivation and improved resilience against future threats. *M. arachidis* caused leaf spot disease of *Z. mauritiana* and found to be a major biotic stress for jajube production in Indian agro-climatic condition. The disease is found to be severe during September to January. Optimum temperature (20–25 °C) coupled with high humidity (75–80%) and adequate rainfall makes a tri directional pool during the pre-winter season which triggers disease progress. The present study was undertaken to find out the emerging role of *Mycosphaerella arachidis* and its unveiled mechanism associated with *Z. mauritiana*

infestation.

Materials and Methods

Isolation and morphological characterization of the causal pathogen

Isolation of the pathogen was done on potato dextrose agar (PDA) from the infected plant samples by following the procedure of Rangaswami and Mahadevan (1998). The symptomatic portions along with some healthy tissues were cut in small pieces (2–5 cm) and surface sterilized with 0.1% NaOCl solution for 60 s followed by 3 consecutive washings with sterile distilled water. The cut plant samples were then transferred to PDA plates. The inoculated plates retain in BOD (biochemical oxygen demand) at 28±2 °C temperature. Pure culture colonies came by repeated sub-culturing from inoculated plate. Morphological characterization of the pathogen was done by visual symptomatic study, microscopic observation and comparative study with previously published *M. arachidis* reports.

Host range study

Host range study was done by collection of data from NCBI and CABI search. Year-wise progress of new host infection was recorded, compared and analyzed.

Molecular identification of the pathogen

A total of forty isolates were gathered from various regions throughout India. The CTAB DNA extraction method was used to confirm the isolates Zm 15, Zm 32, and Zm 40's molecular detection (Goodwin *et al.*, 2001).

Internal transcribed spacer (ITS) region sequencing was used to identify the isolated fungus molecularly (Khan *et al.*, 2021, 2023). The ITS region was chosen because to its diverse and multicultural makeup. ITS1 and ITS4 primer pairs were used to amplify the internal transcribed spacers of the ribosomal DNA of the chosen isolates (White *et al.*, 1990).

The final reaction mixture was prepared with 2.5 µL of 10x Taq buffer containing 15 mM $MgCl_2$, 1.5 U of Taq polymerase (3 U μL^{-1}), 1.0 µL of ITS1 primer (5 picomolar μL^{-1}), 1 µL of 25 mM dNTP mix each, and 3 µL (20–30 ng μL^{-1}) of sample DNA. The amplification was carried out using a BIORAD My CyclerTM thermal cycler (Bio Rad, USA); the final reaction mixture was prepared with these parameters. 30 s at 50 °C, 30 s at 95 °C, 1 min at 72 °C, and finally 10 min at 70 °C.

The amplified PCR product was screened on a 1.5% agarose gel in 1X TAE buffer and then stained with ethidium bromide to see it under a gel document unit (Bio Rad, USA). The 558 bp (Zm 15), 533 bp (Zm 32), and 604 bp (Zm 40) amplified, highly targeted, and diverse products underwent sequencing, trimming, and submission to the NCBI Genbank database.

Phylogenetic analysis

Phylogenetic analysis was done based on the ITS sequencing and analyzing closest or furthest proximity among isolates in different countries, through neighbor joining method by MEGA MEGA_X_10.1.6. (Tamura *et al.*, 2004, Kumar *et al.*, 2018). Phylogenetic analysis was done for each isolate specifically (with 14 randomly selected isolates from NCBI) as well as for all the three isolates in a group (42 isolates altogether from different countries).

Pathogenicity test

A pathogenicity test was conducted through detached leaf and twig assay. Approximately 6-8 cm long twig samples were cut from the top portion and kept in an empty plastic tray. The base of tray was covered with absorbent cotton, which helped to maintain *in vitro* moisture level. Cut twig parts were kept on sterile glass plates whose cut portions were dipped into 2% sucrose solution to get minimum nutrition level. Single mycelial discs were placed on the leaf surface. For easy entry of the pathogen with a fine pointed needle, a small wound was done by scratching of the leaf epidermal tissue. All the selected isolates were inoculated on separated twigs of *Z. mauritiana* plant. Observation was taken periodically for 7 days.

Results

Symptomatological expression of leaf spot disease of *Z. mauritiana*

Blackish brown round shaped spots appear on the leaf surface. Deep blackish external border line clearly observed (Fig. 1). Each spot was 2–3 cm in diameter. Sometimes spots appeared on the edges of the leaves.

Cultural and morphometric characterization of the pathogen

The full mycelia growth comes under 72–96 hours of sub-culturing and sporulation found at 25 °C within 120 h. *Mycosphaerella arachidis*, the causal agent of early leaf spot in *Z. mauritiana*, exhibits a distinctive cultural morphology that aids in its identification and understanding of its life cycle. When cultured on suitable media such as potato dextrose agar (PDA) (Fig. 2A-C) or V8 agar (Fig. 2D) under laboratory conditions, *M. arachidis* typically forms colonies with a velvet-like texture, initially appearing white or cream-colored, which later transitions to shades of gray, green, or brown to blackish as the colony matures. The colony surface may also become covered with a powdery or granular appearance due to the production of conidia, which are the asexual spores of the fungus. Under microscopic examination, the conidiophores of *M.*

arachidis are observed to arise directly from the mycelium, bearing conidiogenous cells that give rise to conidia in chains. These conidia are typically septate, cylindrical to ellipsoidal in shape, and possess a characteristic pigmented septum. The cultural morphology of *M. arachidis* is essential for accurate diagnosis and differentiation from other closely related fungi, aiding in disease management strategies and research efforts aimed at mitigating the impact of early leaf spot on peanut crops.

Pathogenicity test

All the host-specific isolates (Zm 15, Zm 32, and Zm 40) demonstrated pathogenicity within 5–6 days after the single mycelial disc inoculation strategy. All isolates demonstrated pathogenicity when sprayed with spore suspension; however, we chose the isolates specific to the host for higher virulence because they exhibited more damage (on a 0–9 point scale) than the other two. Similar symptoms were shown in pathogenicity tests conducted in field settings. Within seven to eight days of the injection, symptoms appeared when using the spore suspension spraying method. By repeating the pathogenicity test twice and isolating the same pathogen in culture media, Koch's postulate was proven.

Host range

Mycosphaerella arachidis has a wide host range. Gradual adaptation and emergence of the pathogen makes it more vigorous and pathogenic towords non-host or non-cultivated crops also. A diverse host range is depicted through Table 1.

Biology of *M. arachidis*

The biology and disease cycle of *M. arachidis*, the causal agent of leaf spot of *Z. mauritiana*, involve several key stages and processes. *M. arachidis* primarily survives between cropping seasons as mycelium in infected crop debris, seeds, or soil. When favorable environmental conditions, including high humidity and temperatures ranging from 20 to 30 °C, occur during the growing season, the fungus initiates infection.

The disease cycle begins with the release of airborne conidia from pycnidia, which are small, fruiting structures produced on infected plant debris or lesions from the previous season. These conidia are spread by wind and rain to nearby peanut plants. Upon landing on susceptible plant tissue, the conidia germinate, forming germ tubes that penetrate the leaf surface through stomata or wounds. Once inside the plant, the fungus colonizes the intercellular spaces, ultimately leading to the formation of characteristic circular to oval-shaped lesions on the leaves. These lesions start as small, water-soaked spots that later enlarge and turn brown, surrounded by a yellow halo. Within the lesions, *M. arachidis* produces pycnidia, which contain asexual spores called conidia. These conidia are released into the environment, perpetuating the cycle of infection and serving as a source of inoculum for further disease spread. In addition to asexual reproduction, *M. arachidis* can also undergo sexual reproduction, although this process is less common. Severe leaf spotting can cause early defoliation as the disease worsens, which lowers photosynthetic capability and ultimately affects productivity. Infected seeds can also serve as a source of primary inoculum for subsequent growing seasons, further perpetuating the disease cycle. In order to minimize the spread and impact of early leaf spot in peanut crops, effective management strategies, such as crop rotation, the use of resistant cultivars, timely fungicide applications, and sanitation practices, are dependent on an understanding of the biology and disease cycle of *M. arachidis*.

Molecular detection and phylogenetic analysis of the pathogen

The 558 bp (Zm 15), 533 bp (Zm 32), and 604 bp (Zm 40) sequences of the ITS - rDNA region exactly match the *Mycosphaerella arachidis* databases AF297224 (99%) and EF157732 (99%) similarity, according to a GenBank blast search of the publicly accessible fungal database. The resultant sequences, which were 558 bp (Zm 15), 533 bp (Zm 32), and 604 bp (Zm 40), were deposited in GenBank and given the accession numbers MT481910, MT482000, and MT481991.

Phylogenetic analysis carried out through maximum likelihood analysis which revealed that MT481910 and EF157735 (USA) are the closest taxa with 82% bootstrap value support, MT482000 and EF157739 (USA) with 80% bootstrap value support and MT481991 and EF157737 (USA) has 94% bootstrap value support, which indicates sequences were conspecific on the same branch node and the highest similarity among them under nuclear phylogenic model. In the summative model (neighbor joining method) of the phylogenetic tree (Fig. 3) three isolates showed three divergent ancestral origins from three clusters which indicate genomic variability among the isolates with three different ancestral origins.

Discussion

A major concern in agricultural ecosystems, *M. arachidis* appearance and severity on *Z. mauritiana* demand attention because of the possible consequences for plant health and food security. *M. arachidis*, commonly known as early leaf spot, is a fungal pathogen that primarily affects *Z. mauritiana* crops, causing leaf lesions that can lead to defoliation, reduced photosynthetic efficiency, and ultimately, yield losses (Kulkarni *et al.*, 2010). However, while *Z. mauritiana* is not a primary host of *M. arachidis*, emerging evidence suggests that this fungal pathogen can still pose a threat to its health

and productivity under certain conditions. The emergence of *M. arachidis* on *Z. mauritiana* is a multifaceted phenomenon influenced by various factors, including environmental conditions, host susceptibility, and agricultural practices. Climate change, with its associated alterations in temperature, humidity, and precipitation patterns, can create conducive conditions for the proliferation and spread of fungal pathogens like *M. arachidis*, thereby increasing the likelihood of their occurrence on non-traditional hosts such as *Z. mauritiana* (Meswaet *et al.*, 2021). Moreover, the globalization of trade and agriculture has facilitated the movement of pathogens across geographical boundaries, enabling their introduction to new host species and ecosystems (Jain *et al.*, 2019). Consequently, the emergence of *M. arachidis* on *Z. mauritiana* underscores the interconnectedness of global agricultural systems and the need for vigilance in monitoring and managing emerging plant diseases. The severity of *M. arachidis* on *Z. mauritiana* manifests through various symptoms and impacts that can compromise the health and productivity of the plant. Furthermore, the presence of *M. arachidis* on *Z. mauritiana* may exacerbate existing biotic and abiotic stresses, making the plant more susceptible to other pathogens, pests, and environmental fluctuations. Additionally, the economic implications of *M. arachidis* infection on *Z. mauritiana* cannot be overlooked, as reduced fruit quality and yield losses can negatively impact the livelihoods of farmers and disrupt local economies dependent on the cultivation and trade of this important fruit crop.

Addressing the severity of *M. arachidis* on *Z. mauritiana* requires a multi-faceted approach encompassing both preventive and management strategies. Proactive measures such as surveillance, quarantine protocols, and regulatory frameworks can help prevent the introduction and spread of *M. arachidis* to new host species and geographical regions, thereby reducing the risk of emergence and subsequent impacts on *Z. mauritiana*. Furthermore, research efforts focused on understanding the biology, epidemiology, and host-pathogen interactions of *M. arachidis* on *Z. mauritiana* are essential for developing targeted control measures and resistance breeding programs tailored to mitigate the severity of this fungal pathogen (Kalinganire *et al*., 2012). Integrated disease management (IDM) practices, including cultural, biological, and chemical control methods, can also play a crucial role in minimizing the impact of *M. arachidis* on *Z. mauritiana* while promoting sustainable agriculture and environmental stewardship (Maheswari and Haldhar, 2018).

Conclusion

M. arachidis infestation on *Z. mauritiana*, marks as a significant draft in plant pathological aspect. This report sheds light on a previously undocumented fungal pathogen affecting the Indian jujube, a vital crop in tropical and subtropical regions. Previously existing mild pathogens transformed and expressed as a major pathogen under change in climatic scenario. Complex adoptability and diverse host range makes it more vulnerable and severe to many agriculturally important crops. Economic and ecological perspective of *Z. mauritiana*, early detection, coupled with integrated disease management practices, is crucial. This report serves as a pivotal reminder of the dynamic nature of plant pathogens and the necessity for continuous research and adaptive management to safeguard agricultural sustainability and food security.

Contribution of authors

SP designed the experiment and collected data while framing of investigation, data analysis and interpretation were carried out by SD.

Conflict of interests

The author solemnly declares that they are not associated with any conflict of interest.

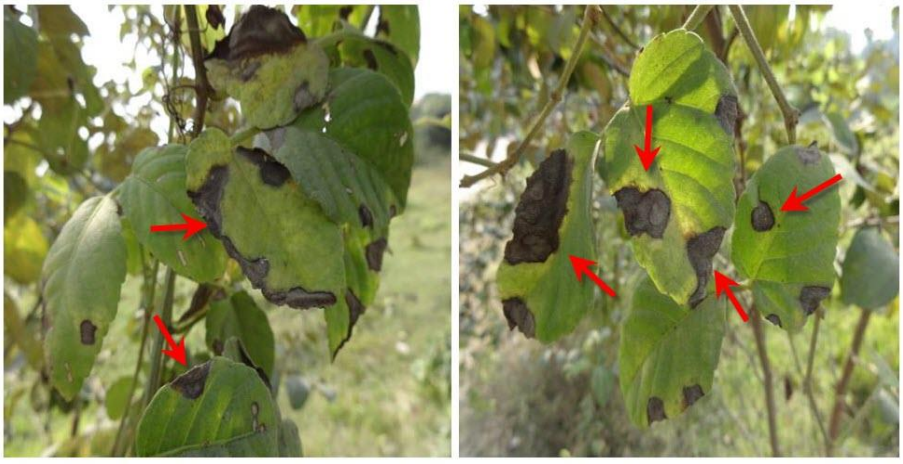

Fig. 1: Leaf spot disease symptom on *Zizyphus mauritiana.*

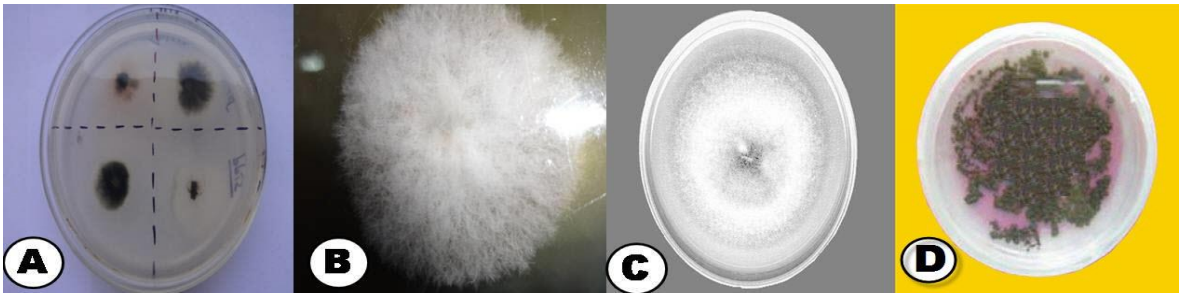

Fig. 2: Cultural characterization of *Mycosphaerella arachidis* on PDA medium (A-C), and V-8 agar medium (D).

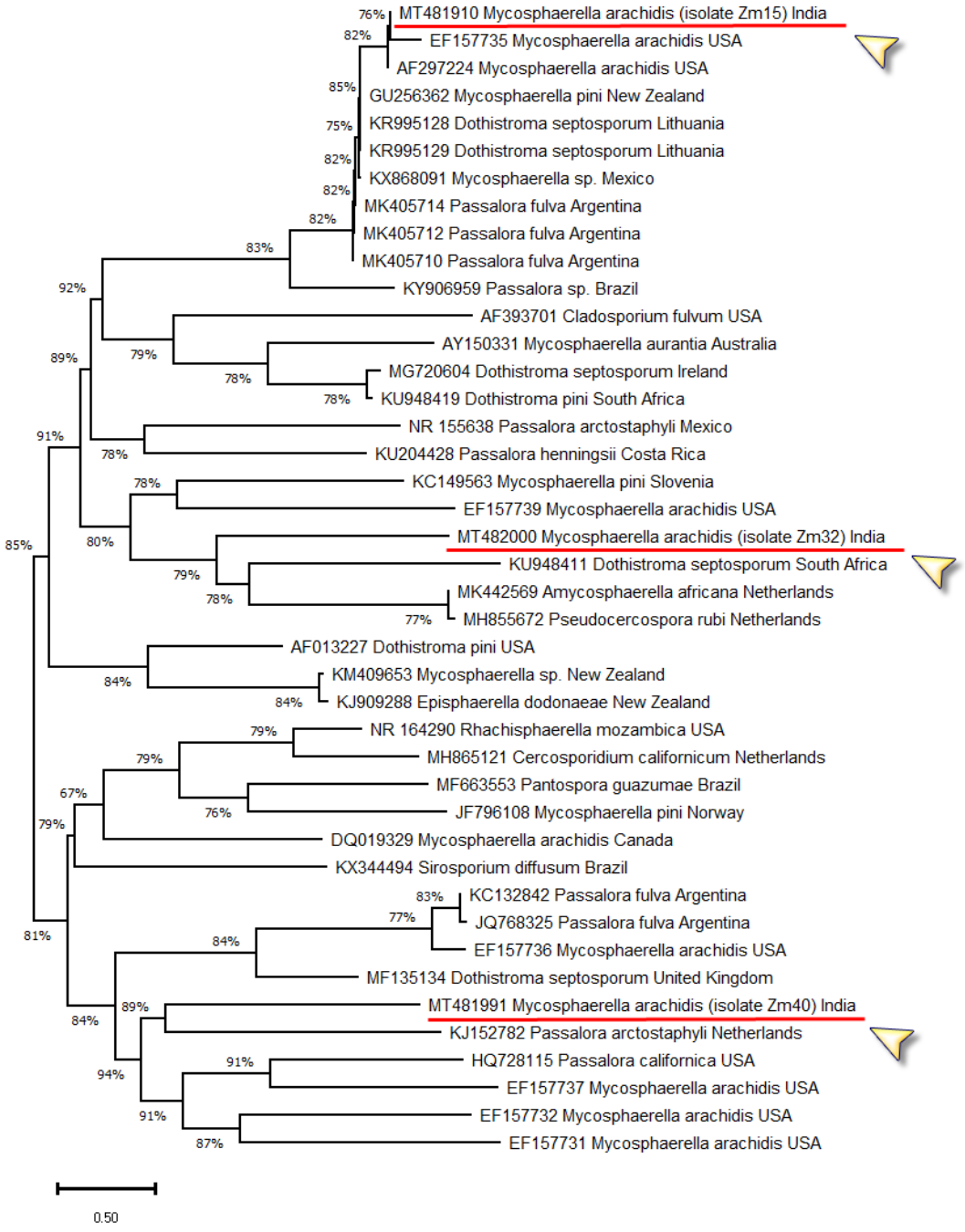

Fig. 3: Summative model of all three deposited sequences under neighbor joining method.

Table 1: Diverse host range, occurrence and pathogenic nature of *Mycosphaerella arachidis.*

Host Plant	Geographical Locations	Pathogenic nature of *M. arachidis*	References
Peanut (*Arachis hypogaea*)	Worldwide, particularly in tropical and subtropical regions	Primary host, causes early leaf spot	Nair *et al.* (2019); Denwar *et al. (*2021); Sun *et al.* (2023)
Soybean (*Glycine max*)	United States, Brazil, China, Argentina, India, among others	Secondary host, causes leaf spot, potential pathogen	Bhat *et al*. (2022)
Common bean (*Phaseolus vulgaris*)	United States, Brazil, Mexico, Africa, India	Secondary host, causes leaf spot	Simpson *et al*. (2003)
Chickpea (*Cicer arietinum*)	India, Ethiopia, Australia, Pakistan	Secondary host, causes leaf spot, potential pathogen	Nene *et al.* (1978); Vandana *et al*. (2020)
Lentil (*Lens culinaris*)	Canada, India, Australia, Turkey	Secondary host, causes leaf spot	Sudheesh *et al*. (2016)
Tobacco (*Nicotiana tabacum*)	United States, China, India, Brazil	Secondary host, causes leaf spot, mild pathogen	Clubreath *et al*. (2006); Oteng-Frimpong *et al*. (2023)

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