Research Article

Diagnosis and *In-Vitro* Management of Leaf Blackening Disease: An Emerging Threat to Cotton Crop

Mushahid Sattar[1], Safdar Ali[1]*, Fahad Hussain G Mohammed Yar[2], Saba Farooq[3], Muhammad Ahmad Zeshan[4], Muhammad Usman Ghani[5,6], Yasir Iftikhar[6] and Salma Malik[7]

[1]Department of Plant Pathology, University of Agriculture Faisalabad, 38000, Pakistan; [2]Department of Biology, Jouf Univeristy, Al Jouf Sakaka, Saudi Arabia; [3]Department of Environmental Sciences, Government College University Faisalabad. Pakistan; [4]Department of Plant Pathology, College of Agriculture, University of Sargodha, Sargodha, Pakistan. 40100; [5]Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan, 38000; [6]National-Regional Joint Research Center for Soil Pollution Control and Remediation in South China, Guangdong Key Laboratory of Integrated Agro-Environmental Pollution Control and Management, Institute of Eco-Environmental and Soil Sciences, Guangdong Academy of Sciences, 808 Tianyuan Road, Tianhe District, Guangzhou, Guangdong, China, 510650; [7]Plant pathology Barani Agricultural Training Institute Rawalpindi, Pakistan.

Abstract | Cotton (*Gossypium hirsutum* L.) belongs to *Malvaceae* family that is cultivated as a source of fiber for raw material of textile industries. Cotton leaf blackening (Sooty mould) is causing huge losses mainly in cotton growing areas of Punjab, Pakistan and a very minute research work was performed on this disease. This research was planned to identify the causes of cotton leaf blackening and its suitable control measures. During survey symptomatic leaves and whitefly were collected from the diseased fields. The plants grown under controlled conditions in a completely randomized design (CRD) were inoculated with fungi and whitefly in 4 groups i.e., T_1= Control, T_2= Fungal spores, T_3= Whitefly, T_4= both fungal spores and whitefly with 3 replications of each treatment. The pathogenicity trial indicated a major synergistic effect of fungi and whitefly because the plant group inoculated jointly showed maximum disease symptoms i.e. more than 90%. The plant group inoculated with fungi and whitefly alone was stood 2nd and 3rd for the symptom development in that order. Under *in-vitro* studies, all fungicides effectively reduced the growth of fungi and their efficacy increased with concentration. There was maximum fungal growth reduction due to fungicides at highest does (150 ppm) followed by 100 ppm and 50 ppm. Amistar Top was the most effective fungicide by restricting fungal growth upto 95% followed by Ready Super, Miravis, Electus Super and Pyrazole Super. The causal organism for cotton leaf blackening was identified as *Alternaria macrospora*, and sooty mould growth was increased due to whitefly infestation.

Received | September 17, 2025; **Accepted** | October 28, 2025; **Published** | March 28, 2026
***Correspondence** | Safdar Ali, Department of Plant Pathology, University of Agriculture Faisalabad, 38000, Pakistan; **Email:** safdar147m@uaf.edu.pk
Citation | Sattar, M., S. Ali, F.H.G.M. Yar, S. Farooq, M.A. Zeshan, M.U. Ghani, Y. Iftikhar and S. Malik. 2026. Diagnosis and *in-vitro* management of leaf blackening disease: An emerging threat to cotton crop. *Pakistan Journal of Agricultural Research,* 39(1): 242-248.
DOI | https://dx.doi.org/10.17582/journal.pjar/2026/39.1.242.248
Keywords | *Alternaria macrospora*, Cotton leaf blackening, Fungal disease, *In-vitro* management, Fungicide efficacy, Whitefly

Introduction

The economic system of Pakistan is mainly based on agriculture, which has a significant contribution in national income with 19.2% share in GDP and 70% workforce engagement (GOP, 2021). Cotton (*Gossypium hirsutum* L.) is the most valuable cash crop that is grown on a large area worldwide and its production in Pakistan was 4.5 Million Metric Tons (USDA, 2020).

Biotic stresses: The dual threat of whitefly and fungal pathogens

Although cotton is an economically important crop, however its quantitative yield and quality of the fiber is badly affected by numerous biotic and abiotic interruptions (Aini *et al*., 2022). The major yield losses of cotton crop are attributed to pests and diseases which are almost 30% (Tarazi *et al*., 2019). One of the emerging biotic constraints for cotton production is blackening disease that significantly hinders with photosynthetic frequency and efficiency that contributes towards lowering the yield and quality of the produce (Rodriguez *et al*., 2021). Cotton leaf blackening is caused by a necrotrophic fungus *Alternaria macrospora* that infects the leaves aggressively. The affected plants depicts dark brown to black necrotic lesions on older leaves which after merging cause leaf drop and ultimately lowering plant vitality (Broughton *et al*., 2021). This disease is not prevalent in many parts of the world as compared to other major cotton diseases; however, it's an emerging threat in cotton crop with more than 15% incidence and species of *Alternaria* are associated with it (Omyma *et al*., 2019). A significant genetic diversity was found in isolates of the main causal organism *A. macrospora* when subjected to molecular characterization using ITS and SSR primers suggetsing evoultion and variablity in pathogen (Sampathkumar and Raghavendra, 2024). The blackening disease intensity increased when the cotton crop was infested with whitefly as it releases honeydew on the backside of the leaves during phloem feeding which later on covered with fungal growth and cause sooty mold. Whitefly infestation weaken the plant that becomes vulnerable to other fungal infections like *Alternaria macrospora* (Safdar *et al*., 2019). The honeydew functions as a growth medium for sooty mould growth that blackens leaf surfaces, rigorously impairs photosynthesis and reduces yield quality and quantity (Faiza *et al*., 2015). *A. macrospora* has dark pigments and filamentous colony and it thrives best in hot and dry environment making it a suitable pathogen for cotton (Omyma *et al*., 2019).

Current management strategies and knowledge gaps

Blackening disease being a synergistic combination of fungus and whitefly is mostly managed by neonicotinoids, organophosphates, and insect growth regulators (Wang *et al*., 2017). Whitefly *Bemisia tabaci* develop resistance against the synthetic insecticides making it a challenge to control blackening disease (Sparks and Nauen, 2015). The detection, identification, diagnosis and management of synergistic blackening disease have been studied very rare that necessitates handling this emerging threat.

Keeping in view the above mention features and management strategies, the current study was planned with these objectives.

- To survey various cotton fields in Faisalabad, Pakistan to collect disease samples
- To identify the causes of cotton leaf blackening
- To check the effects of different fungicides against cotton leaf blackening (sooty mould)

Materials and Methods

Survey and sampling

Samples were collected by conducting various surveys around the cotton growing areas of Faisalabad, Pakistan. Diseased leaves were selected on the basis of characteristic symptoms in sterilized zipper bags for sampling and transportation.

Media preparation and pouring

Potato Dextrose Agar (PDA) media was used for culturing of fungal pathogen *(Alternaria macrospora)* Potato starch was prepared by taking 250g peeled potato slices and boiled in 500mL of water followed by sieving and added into the flask containing 500mL distilled water, 20g glucose and 20g agar. The volume of flask was made up to 1000mL by adding distilled water, covered with cotton plug and parafilm followed by autoclave. The prepared media was poured into the sterilized petri dishes under laminar flow chamber and were left for cooling.

Isolation and purification of causal organism

All the disease samples were stored in the Plant Virology laboratory, Department of Plant Pathology,

UAF (Pakistan). The disease-causing pathogen (*Alternaria)* was isolated by following standard procedure by cutting diseased tissues and sterilize with 70% ethanol followed by consecutive washing with distilled water. Small pieces were placed on the PDA plates aseptically and then incubated for fungal growth. The fungal colonies that were grown on PDA media around small bits, the hyphal tip from each colony were obtained and transfer to further PDA containing Petri plates for purification. These plates having pure culture (Figure 1) were incubated at 26-28 °C.

Identification on the basis of morphological characters of isolated fungus

Two weeks old purified fungal cultures were used for slide preparation. Slides were prepared and fungus was identified on the basis of morphological characteristics (colony, colour, spore size and growth pattern) under 40x of light microscope (Plant Pathology lab, UAF). Morphology of isolated fungi was compared with consistent key for identification of specie.

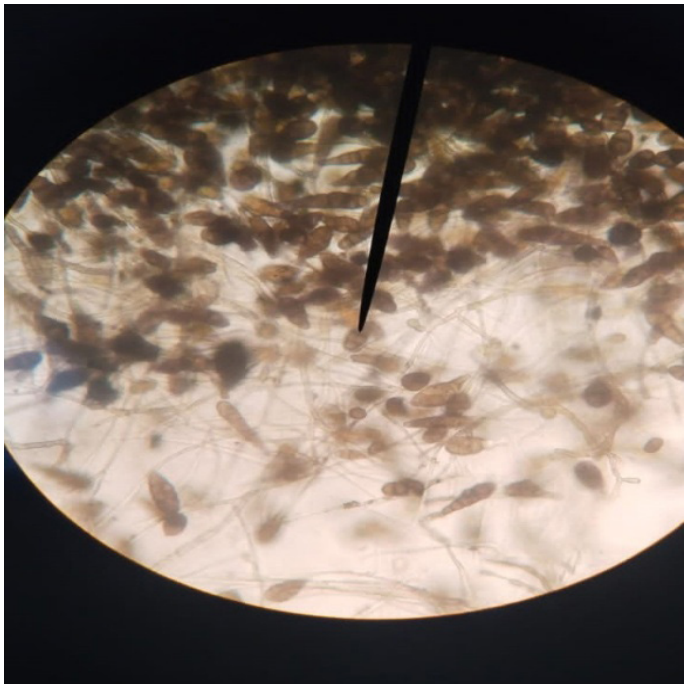

Figure 1: *(A) Microscopic view of fungal pathogen.*

Collection of whitefly population and its inoculation on healthy plants

Whitefly population was collected from infected cotton plants with the help of aspirator. Cotton plants were grown in pots in net house Department of Entomology, UAF. Whiteflies were applied on the cotton plants and a population of 5 whiteflies per leave was maintained during treatment period.

Pathogenicity test

The cultured fungal spores were introduced on the young healthy cotton plant leaves and disease development was observed. The fungal spores were re-isolated from the inoculated host plants and were identified as being identical to original fungal pathogen or not. The leaves showing blackening symptoms were collected with 24 hours interval.

Preparation of fungicides concentrations

Fungicides (Ready Super, Electus Super, Pyrazole Super, Amistar Top and Miravis) were used against fungal pathogen (*Alternaria)* and water as a control. Three concentrations (50ppm, 100ppm, 150ppm) of each fungicide were used. Higher concentration of each fungicide was diluted by adding equal volume of distilled water by following serial dilution method. Each tube was labeled with name, date and concentration with permanent marker.

In vitro *evaluation of different fungicides against isolated fungi*

Evaluation of different fungicides was done against isolated fungi by using poison food technique. Potato Dextrose Agar (PDA) was used for this purpose. The fungicides were diluted by using serial dilution method (parts per million). Different fungicides were used (Figure 3) as a poison against given fungal pathogen (*Alternaria)* and each fungicide was used at 3 different dilution levels (50ppm, 100ppm, 150ppm). 3 replications of each concentration were used.

Results

Impact of fungal spores and whitefly infestation on cotton blackening disease

The impact of fungal spores and whitefly infestation was checked on the development of blackening disease of cotton. These cotton plants were grown under controlled conditions and significant differences in the impact of both fungal spores and whitefly infestation was assessed by analysis of variance (ANOVA). The ANOVA results showed a statistically significant effect of both inocula (fungi and wf) on the blackening of cotton leaves ($F = 12.3$, $P < 0.01$). This indicates that both of the inocula contribute towards disease development (Table 1).

The specific impact of each inoculation was assessed by using post-hoc analysis (Tukey's HSD) and it was noted that the maximum blackening development

was observed under treatment T_4 (both fungus and whitefly) that was 22.8 leaves/plant, followed by T_3 (whitefly) 9.26 leaves/plant, T_2 (fungus) 6.13 leaves/plant and T_1 (Control) 4.1 leave/plant,. The lowest blackening development was observed in T_1. The significantly higher blackening development was observed under T_4 as compare to all other treatments (Figure 2).

Table 1: *ANOVA for the development of blackening of cotton.*

Source of variance	D.F	S.S	M.S	F	P
Treatment	3	5.5393	1.3847	12.3	<0.01
Error	8	4.5136	0.1133	--	--
Total	11	10.124	--	--	--

ANOVA revealed a highly significant effect of inoculumn on cotton leaf blackening after (F = 12.3, P < 0.01).

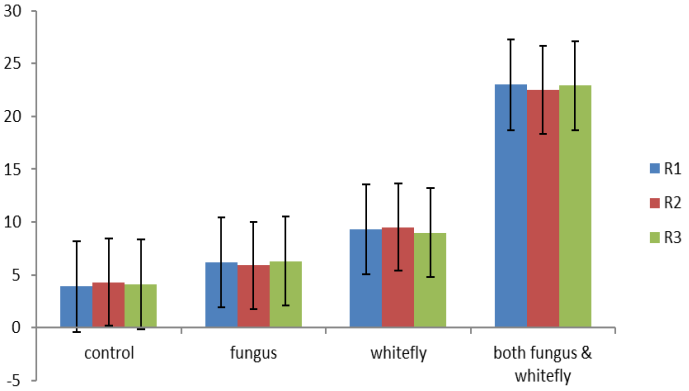

Figure 2: *Assessment of cotton blackening disease as a result of inoculation by fungi and whitefly.*

Disease development in cotton plants over time

The potential of inoculum establishment in the host cotton plants grown under controlled conditions was assessed by recording disease development at different time intervals i.e. 4th DAI, 8th DAI, 12th DAI and 16th DAI (Table 2). There was significant progression in disease development in all the inoculations with time. The combined effect of whitefly infestation and fungal inoculation was more than their individual effect.

Table 2: *Cotton blackening disease progression over time.*

DAI	Fungal spores	Whitefly	Fungus + Whitefly
4	2.0 a	1.3 a	3.4 a
8	4.5 b	2.4 b	7.8 b
12	7.6 c	4.3 c	16.5 c
16	9.28 d	6.22 d	22.4 d

Different letters within the same row are significantly different at P < 0.05 according to Tukey's HSD test.

Effectiveness of fungicides against cotton blackening disease

A two way factorial ANOVA was performed to assess the impact of fungicidal treatments and their concentration on the management of cotton blackening disease. The comparison of treated plots with untreated check revealed that all the fungicides were effective in controlling disease, moreover the efficacy was also significantly improved at different concentrations. The two way interaction of fungicides and concentration was significant and positive against the disease (Table 3).

Table 3: *Impact of fungicides on the management of cotton blackening disease.*

Source of variance	D.F	S.S	M.S	F	P
Treatment	5	11340.2	2321.2	41.76	<0.001
Concentration	2	3588.1	1724.5	29.54	<0.001
Treatment × Concentration	10	5408.6	514.3	17.29	<0.001
Error	36	2088.9	57.31	--	--
Total	53	22715.2	--	--	--

The efficacy of 5 fungicides was assessed against cotton blackening disease and the percent disease reduction was noted. The bar graph is depicting the percent disease reduction on y-axis and the fungicides used on the y-axis (Figure 3). Amistor was the most effective fungicide in controlling disease under field conditions while pyrazole was least effective in that order. Reddy and Miravis were 2nd and 3rd most effective in disease control after Amistor. There was gradual increase in disease reduction with increase in concentration of the fungicides in all the treatments.

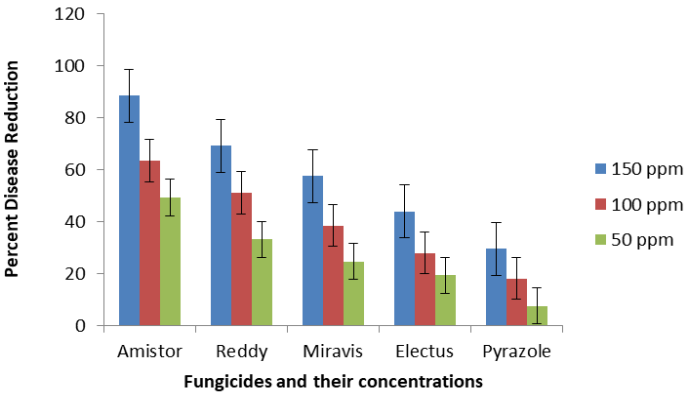

Figure 3: *Efficacy of fungicides against cotton blackening disease.*

Efficacy of fungicides under in-vitro conditions

The effect of different fungicides were found signif-

icant on the growth/mm of *Alternaria* ($F_{5, 17}$=19.3; p <0.01). The maximum growth/mm (57mm) of *Alternaria* after 12th day of treatment applied was observed under control group. The minimum growth/mm (1.3 mm) of *Alternaria* was observed under 150ppm of Amistor Top, followed by Ready Super (2.1 mm), Miravis (5.4 mm), Electus Super (8.2 mm) and Pyrazole Super (12.3 mm). Statistically no difference of growth/mm was observed under Amistor Top and Ready Super (Figure 4).

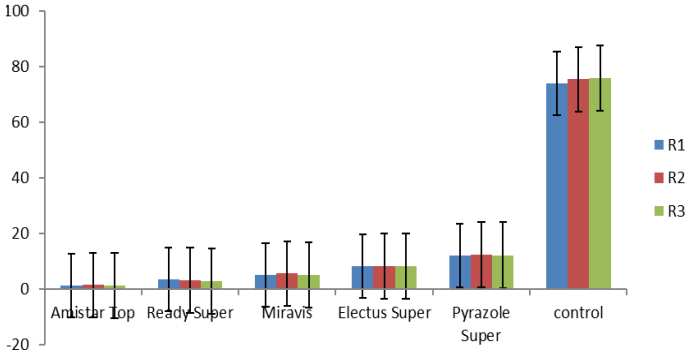

Figure 4: *Parameters for the growth/mm of Alternaria against 150ppm of fungicides (Ready Super, Electus Super, Pyrazole Super, Amistar Top and Miravis) after 12 days of application.*

Discussion

The present study unveils the details about the etiology and disease control strategies by providing a comprehensive interaction between fungal pathogen, whitefly infestation and their effect on disease intensity. The study hypothesized that whitefly has a significant role in describing the disease development along with fungal invasion. The synergistic effect of whitefly and fungi was significantly higher towards disease development than their individual effect. The reason for pronounced disease intensity was the honey dew secreted by the whiteflies and injuries caused by the insect infestation. A similar finding was provided by (Sain *et al*., 2021); as there was more than 60% disease intensity in combined infection of whitefly and *Alternaria* while 45% disease recorded in fungal infection. Another study from Guru Kashi University revealed that American cotton cultivars were more prone to disease development as a result of late sowing and combined infection by whitefly and fungi (Selvaraj *et al*., 2024). Black and non-parasitic sooty mould is a superficial fungus growth on plant surfaces. There was an interaction between sap-feeding insects and non-parasitic fungi that causes the sooty mold. The temperate and tropical regions are favorable for the development of sooty mold (Scot Nelson, 2008). Pan *et al*. (2024) presented a lightweight deep learning framework (CDDLite-YOLO model) which was used to detect cotton blackening disease with almost 90% accuracy. The causal organism of cotton blackening disease *Alternaria* has many isolates with difference in colony texture, conidia dimensions, and level of virulence according to which a variation in disease intensity can be noted (Sampathkumar and Raghavendra, 2024).

A significant efficacy of tested fungicides was recorded against the causal organism under laboratory conditions and in reducing the intensity of blackening disease under field conditions. Amistor Top was the most effective among all the tested fungicides as it contains Azoxystrobin which is a QoI (Quinone outside Inhibitor), and inhibits electron transport at the cytochrome bc1 complex to disrupt respiration of mitochondria. Disruption in respiration further limits the germination of fungal spores and the growth of its mycelia (Achilonu *et al*., 2023). The second component of Amistor Top is difenoconazole, which is a DMI (Demethylation Inhibitor) that compromise the integrity of fungal cell membrane interfering the biosynthesis of ergosterol (Ekabote *et al*., 2024). There was a significant difference in the performance of Amistor Top and Ready Super fungicides although these have ingredients which share a same mode of action i.e. QoI and DMI. Azoxystrobin in Amistor Top has more translaminar mobility and residual activities as compared with Pyraclostrobin while difenaconazole has deeper penetration. Moreover, Azoxystrobin is highly stable due to its formulation and can tolerate warm and humid environment (Aguiar, 2021). Miravis Duo was also among the effective fungicidal treatments as it contains Pydiflumetofen that acts as SDHI (Succinate Dehydrogenase Inhibitor) and inhibits fungal respiration at a different site from QoIs (Hospital *et al*., 2023). In Electus Ultra, the inclusion of dimethomorph might diluted the formulation against *Alternaria* spp. (Yang *et al*., 2021).

It has been observed that fungicides with almost similar active ingredients but different brands gave significantly different results against *Alternaria* spp. and cotton blackening disease. The efficacy may be affected by the type of adjuvants i.e., surfactants, stickers and penetrants. The other reasons for varied

performance of fungicides might be carrier used i.e., water, oil and other solvents; it may be also be affected by particle size, concentration of active ingredients, application timing and quality.

Conclusions

Conclusion of present research work regarding the causes of cotton leaf blackening is that the cotton whitefly in association with any fungal (*Alternaria)* is a main cause of cotton leaf blackening (Sooty mold). The presence of both pathogens is necessory for the sucessful develpoment of this disease. The disease is saprophytic, but it indirectly damage the production of cotton. The crop show least symptoms when treated with only single pathogen like whitefly or fungal spores. Amistor Top and Ready Super gave most significant result against *Alternaria* in *in vivo* conditionds. Miravis shown significant result. While Electus Super and Pyrazole Super gave medium to signicicant result. All these fungicides (Amistor Top, Ready Super, Miravis, Electus Super and Pyrazole Super) are recommended against *Alternaria* and other fungus of same group.

Acknowledgement

The authors acknowledge Chairman Department of Plant Pathology, University of Agriculture Faisalabad. Pakistan for provision of research area to conduct trials.

Novelty Statement

Leaf blackening disease is an emerging and serious threat to cotton which have not been studied before in Pakistan.

Author's Contribution

Mushahid Sattar: Conceptulization, writing original draft.
Safdar Ali: Supervision, methodology.
Fahad Hussain G Mohammed Yar, Saba Farooq and Salma Malik: Software.
Muhammad Ahmad Zeshan: Investigation, visulization, editing.
Muhammad Usman Ghani: Formal analysis.
Yasir Iftikhar: Methodolgy.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

References

Achilonu, C.C., M. Gryzenhout, S. Ghosh, S. and G.J. Marais. 2023. *In vitro* evaluation of azoxystrobin, boscalid, fentin-hydroxide, propiconazole, pyraclostrobin fungicides against Alternaria alternata pathogen isolated from *Carya illinoinensis* in South Africa. Microorganisms, 11(7): 1691. https://doi.org/10.3390/microorganisms11071691

Aguiar, N.F.B., 2021. Tebuconazole and azoxystrobin: Understanding the fungicide potential of the combination used in a commercial formulation. Master's thesis, Universidade de Aveiro (Portugal).

Aini, N., A.N. Jibril, S. Liu, P. Han, Z. Pan, L. Zhu and X. Nie. 2022. Advances and prospects of genetic mapping of Verticillium wilt resistance in cotton. J. Cotton Res., 5: 5-11. https://doi.org/10.1186/s42397-021-00109-0

Broughton, K.J., P. Payton, D.K.Y. Tan, D.T. Tissue and M.P. Bange. 2021. Effect of vapour pressure deficit on gas exchange of field-grown cotton. J. Cotton Res., 4: 30-36. https://doi.org/10.1186/s42397-021-00105-4

Ekabote, S.D., B. Patil, A.N. Ramesh and S. Onkarappa. 2024. *In vitro* and *in vivo* evaluation of fungicides against anthracnose disease on pomegranate (*Punica granatum* L.) caused by *Colletotrichum gloeosporioides*. Crop Protection, 178: 106598. https://doi.org/10.1016/j.cropro.2024.106598

Faiza, I., B. Sarra, M. Kenza, E.H. Imad and G.B. Nassira. 2015. The role of phenolic compounds in the defense of sooty mould of olive leaves (*Olea europea* L.). Afr. J. Microbio. Res., 9: 1075-1081. https://doi.org/10.5897/AJMR2014.7254

GOP, 2021. Pakistan economic survey 2020-21. Finance and Economic Division, Ministry of Finance, Govt. of Pakistan, Islamabad, Pakistan.

Hospital, C.D., A. Tete, K. Debizet, J. Imler, C. Tomkiewicz-Raulet, E.B. Blanc and S. Bortoli. 2023. SDHi fungicides: An example of mitotoxic

pesticides targeting the succinate dehydrogenase complex. Environ. Int., 180: 108219. https://doi.org/10.1016/j.envint.2023.108219

Lin, J. and Y. Zhu. 2024. *Alternaria* leaf spot in cotton: Identification and control. Mol. Microbiol. Res., 14. https://doi.org/10.5376/mmr.2024.14.0020

Omyma, E.M., M.M. Beshir and N.E. Ahmed. 2019. Cotton leaf blight disease caused by *Alternaria alternata* in Sudan. J. Plant. Prot. Res., 3: 412-417.

Pan, P., M. Shao, P. He,, L. Hu, S. Zhao, L. Huang and J. Zhang. 2024. Lightweight cotton diseases real-time detection model for resource-constrained devices in natural environments. Front. Plant Sci., 15: 1383863.

Rodriguez, D., A.I. Tabar, M. Castillo, M. Martinez-Gomariz, I.C. Dobski and R. Palacios. 2021. Changes in the sensitization pattern to *Alternaria alternata* allergens in patients treated with Alt a 1 Immunotherapy. J. Fungi (Basel), 7: 974-977. https://doi.org/10.3390/jof7110974

Safdar, M.Z., M. Nacem, M. Mamoon-ur-Rashid, F. Dhapra, H. Parwaiz and G. Murtaza. 2019. Effect of abiotic factors on population dynamics of whitefly and jassid on BtCotton. Acta Sci. Agric., 3: 118-121.

Sain, S.K., D. Monga, N.S. Hiremani, D.T. Nagrale, S. Kranthi, R. Kumar and V.N. Waghmare. 2021. Evaluation of bioefficacy potential of entomopathogenic fungi against the whitefly (*Bemisia tabaci* Genn.) on cotton under polyhouse and field conditions. J. Invert. Pathol., 183: 107618. https://doi.org/10.1016/j.jip.2021.107618

Sampathkumar, A. and K.P. Raghavendra. 2024. Molecular identification and genetic diversity of alternaria isolates causing leaf spot disease in cotton from major cotton growing areas of south zone of India. Indian J. Agric. Res., 58(3): 532-538. https://doi.org/10.18805/IJARe.A-6104

Scot Nelson. 2008. Sooty mold. In: Plant disease by cooperative extension service, college of tropical Agriculture and human resources. University of Hawaii. 14: 52-56.

Selvaraj, K., B.V. Sumalatha, R. Sundararaj, T. Venkatesan, A.N. Shylesha and S.N. Sushil. 2024. Guide on diagnosis of invasive whiteflies and their natural enemies. Tech. Bull., 1: 2021.

Sparks, T.C. and R. Nauen. 2015. IRAC: Mode of action classification and insecticide resistance management. Pesti. Biochem. Physiol., 12: 122-128. https://doi.org/10.1016/j.pestbp.2014.11.014

Tarazi, R., J.L.S. Jimenez and M.F.S. Vaslin. 2019. Biotechnological solutions for major cotton (*Gossypium Hirsutum*) pathogens and pests. Biotec. Res. Innov., 3: 19-26. https://doi.org/10.1016/j.biori.2020.01.001

USDA, 2020. United States Department of Agriculture. Foreign agricultural service. Global Market Analysis . Accessed March 20.

Wang, S., Y. Zhang, X. Yang, W. Xie and Q. Wu. 2017. Resistance monitoring for eight insecticides on the sweetpotato whitefly (Hemiptera: Aleyrodidae) in China. J. Econ. Entomol., 110: 660-666. https://doi.org/10.1093/jee/tox040

Yang, L., X. Zhou, Y. Deng, D. Gong, H. Luo and P. Zhu. 2021. Dissipation behavior, residue distribution, and dietary risk assessment of fluopimomide and dimethomorph in taro using HPLC-MS/MS. Environ. Sci. Pollut. Res., 28(32): 43956-43969. https://doi.org/10.1007/s11356-021-13713-z