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Antifungal susceptibility and characterization of *Alternaria alternata* causing leaf spot disease on *Aloe vera* (L.) Burm. f. in India

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ABSTRACT

Leaf spot is destructive disease of Aloe vera and the disease incidence during 2021-22 was varying 50-100%. The symptoms initially appeared as minute circular, water-soaked lesions on the adaxial surface of leaves, which later developed into depressed dark brown to black necrotic spots. The associated pathogen was isolated in pure culture on PDA exhibited rapid growth, initially light grey to olive green, which gradually turned dark brown to black with abundant sporulation. The colony surface typically showed concentric zonation, while the reverse side of the plate appears dark brown to black pigmentation. Further, ITS region was sequenced, and based on morphological and molecular sequences the pathogen was identified as Alternaria alternata. The pathogen found highly sensitive to the fungicides Difenoconazole under In-vitro conditions, and was found to be the most effective in inhibiting mycelial growth of the test pathogen Alternaria alternata.

Introduction

Aloe (*Aloe barbadensis* Miller) is a perennial succulent plant belonging to the family *Asphodelaceae* (formerly placed under *Liliaceae*), renowned for its medicinal, pharmaceutical and economic importance. Aloe gel is extensively utilized in the cosmetic industry for the manufacture of soaps, shampoos, hair-care products, toothpaste, body creams and several other personal care products. The plant is well adapted to hot and dry tropical climates and is widely distributed across Asia, Africa and other tropical and subtropical regions of the world. In India, it is commercially cultivated in Gujarat, Rajasthan, Maharashtra, Andhra Pradesh and Tamil Nadu (Surjushe *et al*., 2008; Sutaliya *et al*., 2025). More than 250 species of aloe are known worldwide and have very much important role in traditional medicine (Baby and Justin, 2010). Aloe have more than 200 compounds, among which 75 are biologically active compounds. The leaf of aloe made up of three layers, the outer most layer is protective layer, 15-20 cm cells thick in size and synthesize carbohydrates and proteins. The active compounds of aloe are anthraquinones, chromones, polysaccharides, and enzymes. (Sahu *et al*., 2013). It has laxative, antihelminthic, uterine stimulant properties and also effective for the treatment of sores and wounds, skin

disease, colds and coughs, constipation, piles, asthma, ulcer, diabetes and various fungal infections. In some part of the country, it is also used to prepare curry (Kumar and Yadav, 2014).

Despite of immense antimicrobial properties, the aloe plants reported susceptible to various fungal and bacterial phytopathogens. Leaf spots diseases are the most frequently and widely reported, wherever aloe crop grown (Kamalakannan *et al.*, 2008; Bajwa *et al*., 2010; Chavan and Korekar, 2011; Silva and Singh, 2012), whereas, bacterial soft rot disease reported predominantly during hot season in high humid areas (Meena *et al*., 2023). The fungal as well as bacterial pathogens are responsible for qualitative and quantitative degradation. The genus "*Alternaria*" is ubiquitous fungus found from temperate to tropical ecology in different habitats such as soil, plants and atmospheres (Woudenberg *et al*., 2015). Species of the genus *Alternaria* affect almost all types of the agriculture produces and leads substantially economic loss (Meena, 2012; Meena *et al*., 2020; Sun *et al*., 2023). The genus consisting of opportunist pathogenic species and causes disease on more than 380 host plant species (Chavan and Korekar, 2011).

The present study focusing on the identification of the associated phytopathogenic fungi, pathogenesis and study on the fungicidal susceptibility using commercial fungicides. During the farmers' field visits, symptoms such as water-soaked lesion, small brown to necrotic spots were observed in different part of Gujarat. The similar symptoms were also observed at different stages on the leaf of aloe in the experimental field at ICAR- DMAPR, Anand, Gujarat during 2021-22. Therefore, keeping all above in view a systematic study was carried out for better understanding the leaf spot disease of aloe.

Material and Methods

Sample collection and isolation

The symptom exhibited leaf samples were collected in aluminum foil from experimental field of ICAR-DMAPR, Anand, and farmers' fields of Gujarat. Further, to isolate the associated causal agent of disease, colonized tissues of leaves were cut into small pieces and surface sterilized with 70% alcohol followed by 2 min immersion in 2% sodium hypochlorite (NaOCl) and subsequently three washing with sterile distilled water. Then 2-3 small sections of leaf placed on solidified and sterilized potato dextrose agar (PDA) Petri plates, supplemented with 250 ppm chloramphenicol to prevent bacterial contamination. The inoculated cultures were incubated at 25 $\pm$ 2 ◦C in BOD incubator. Observation was recorded after 6-7 days of inoculation. After 7-8 days sub culture was carried out by transferring hyphal tip on new PDA plate. Microscopic slides were prepared by mounting in stain to examine morphological characteristics of pathogen for the 7 days old culture.

DNA extraction and PCR amplification

The genomic DNA was extracted from 3 days old fungal mycelium grown on PDA using the commercial DNA isolation kit (Qaigen). Further for characterization at molecular level the internal transcribed spacer (ITS) *ITS* region was amplified using ITS1 and ITS4 (White *et al*., 1990). Genomic PCR reaction was carried out using PCR master mix (Thermo scientific), 10mM forward and reverse primers of *ITS* region, DNA template and nuclease free water in the 25 µl reaction. The PCR cycle was performed at initial denaturation 94°C for 5 min followed by 35 cycles of PCR reaction (94°C for 30 second, 55°C annealing for 40 sec and extension at 72°C for 1 min) and final extension at 72°C for 10 mins as prescribed earlier (Meena *et al.*, 2019). Amplified products were separated on 1% low melting agarose gel. Expected size bands were excised from gel and purified using GeneJET Gel Extraction Kit (Thermo scientific). Purified PCR products were sequenced bi-directionally.

Bioinformatics analysis and identification of pathogen

Sequence similarity search was carried out using Blast*N* tool of NCBI for putative identification of pathogen. Multiple sequence alignment was performed using clustal W algorithm of Mega X with the *ITS* region homologues nucleotide sequences of genus *Alternaria* and one out group member *Stemphylium vesicarium.* Aligned sequences of *ITS* region were used to construct phylogenetic tree with the maximum likelihood tree algorithm using 1000 bootstrap replicates in Mega X software (Kumar *et al.*, 2018).

Pathogenicity test

To confirm pathogenicity of the isolated fungus from infected leaves of *Aloe vera*, a suspension culture of conidia (5×10^5 conidia/mL), collected from colonies on the purified fungal isolate AALS-1 were used to inoculate on aloe leaves. Three plants were used and three leaves per plant were inoculated, whereas the control group was treated with the sterile distilled water. After inoculation, the plants were covered with the clean transparent polythene bags to maintain the higher humidity and plants were transferred to green house for incubation and observations on disease development were recorded regularly. The experiment was repeated twice; the causative agent was reisolated from symptoms developed on

inoculated plant and was compared morphologically with the original strain.

Antifungal susceptibility assay

Eight commercially available fungicides were procured (Table 1) and their efficiency was analyzed against the isolated AALS-1 under *In-vitro* conditions. The experiment was based on mycelia growth inhibition using fungicide sensitivity assay. Optimized concentration of fungicides mixed in sterilized PDA medium and poured into petri plates. A fungal mycelium disk of 6 mm from the 7 days old pure culture was cut with cork-borer and placed at the center on fungicides amended PDA plates. The percent inhibition of the mycelium growth over the control was calculated by the formula (Vincent, 1947) described as per cent inhibition (PI) is equal to mycelium growth of the fungus in control treatment minus mycelium growth of the fungus with treatment multiplied by 100 and divided by mycelium growth of the fungus in control treatment (measured in mm or cm).

Statistical analysis

The data recorded from the experiments were subjected to Dunnett's statistical test for statistical analysis appropriate to the CRD by using the Microsoft Excel package. The results were presented at 5% level of significance (P = 0.05). The critical difference (CD) values were calculated to compare the various treatment means.

Results and Discussion

Symptoms and pathogenesis

Alternaria is a ubiquitous fungus found from temperate to tropical ecology in different habitats such as soil, plants and atmospheres. Diverse species of the genus *Alternaria* affect almost all types of the agriculture produces and leads substantially economic loss (Meena *et al*., 2012). Several studies showed the different causal agent associated with the leaf spot diseases on aloe. Avasthi *et al.* (2015 & 2018) reported the leaf spot disease of aloe caused by *Fusarium proliferatum* and *Curvularia* species. Similarly Ghosh *et al*. (2018) also reported *Alternaria brassicae* causing leaf spot disease on aloe, but the symptoms reported on the leaves were not consistent with the symptoms observed in the present study. In another study conducted by Ghosh *et al.* (2016), the results were in corroborating with the present study where identical symptoms and pathogen (*A. alternata*) were reported.

To resolve this, a systemic data was recorded on pathogenesis and *Alternaria alternata* was identified as causal agent of leaf spot disease on aloe based on the morphological as well as molecular keys. The survey results and field observations revealed that all the aloe plants were infected with leaf spot disease and the disease incidence was recorded up to 100% during mild winter season. The disease symptoms were initiated with minute, circular, transparent water-soaked lesions on adaxial surfaces of aloe leaves and within 3 days the lesions changed to light brown. The lesions were then in later stage after 7-8 days, became enlarged and transformed into dark brown to black spot. As disease progressed the spots became necrotic and the leaf surface finally developed into a saucer shaped depression on adaxial and the corresponding abaxial surfaces within one month (Fig.1, A-E). Under the sever condition the leaf surface covered with numerous such spots and each spot make the depression, which lead substantial yield reduction. Further, disease was initiated under control condition to establish the Koch's postulates and similar symptoms were recorded (Fig.1, G-I).

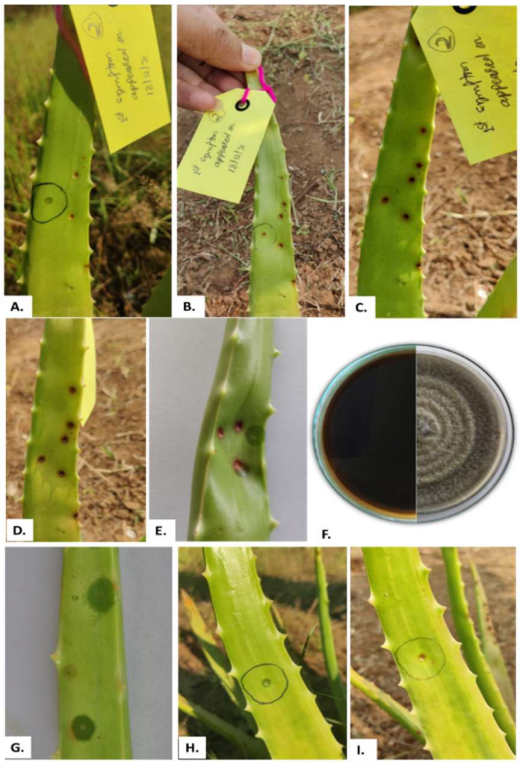

Fig.1. Pathogenesis of *Alternaria alternata* on *Aloe vera* where the progress was recorded at 3 days interval from (A-D) and depression form after 30 days of infection (E), typical colony morphology of *A. alternata* on PDA and Koch's postulates were also established (F-H).

Morphological and molecular characterization

The fungus produced olive green to dark brown color mycelium growth on PDA and the reverse pigmentation varied from dark brown to blackish color. The mycelium was grey-brownish, multi

celled, distinctly septate and produced irregular branches. The typical concentric zonation of fungal growth was distinctly visible in ten days old culture (Fig.1, F). The microscopic observation revealed the dark muriform type conidia, brown to black olivaceous color with the typical 18-36×8-16 µm size and the arises from the long-branched chains of conidiophores. The conidiophores were of 16-45×3-7 µm in size. Thus, based on the morphological features the fungus primarily identified as *Alternaria spp.* pathogen associated with leaf spot of aloe. The morphological features including the mycelium growth, conidial chain and conidia characteristics described here are consistent with those of *A. alternata* described by Simmons (Simmons, 1995; Sun *et al*., 2023). Further, approximately 550 bp *ITS* region of isolated pathogen sequenced. The retrieved sequence contigs of the *ITS* region assembled to generate the consensus sequences and submitted to NCBI GenBank (Accession number PZ374808). The BLAST*n* analysis showed highest 99.83% similarity with *Alternaria alternata* (Accession number PX068376.1). Further phylogenetic tree was constructed using maximum likelihood method using sequences of *ITS* region of *Alternaria* spp. including other closely related genera and it clustered with the *Alternaria alternata* (Fig. 2).

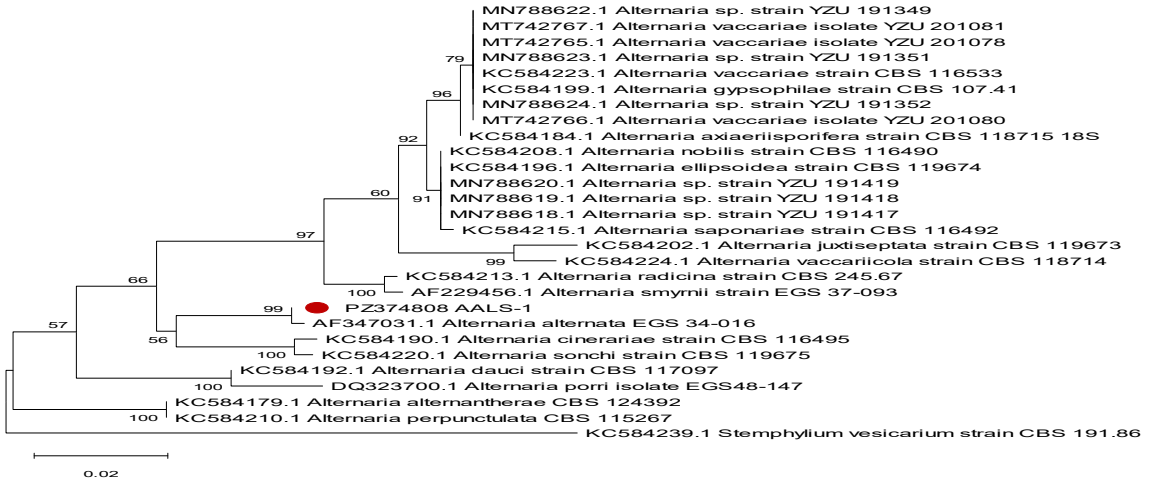

Fig. 2. The maximum likelihood (ML) tree was generated with 1000 bootstrap replications using the Tamura-3-parameter model. The ML tree is inferred from *ITS* sequences of genus *Alternaria* and one out group member *Stemphylium vesicarium.* Bootstrap support values greater than 50% are pointed out at the nodes. Isolate AALS-1 with red circle sequences in the present study. The bar indicates the substitutions number per position

In-vitro antifungal susceptibility

The results of *in vitro* efficacy of fungicides against *Alternaria alternata* presented in table 1. The mycelium growth of the test fungus significantly reduced by the most of them, however, among the tested fungicides Difenoconazole found most efficient to check the mycelium growth with the 98.04±1.96 % efficiency followed by Tebuconazole (50%) + Trifloxystrobin (25%) with 86.02±1.17% efficacy over the control. The mycelium growth of the fungus in difenoconazole and Tebuconazole (50%) + Trifloxystrobin (25%) treated Petri plats was insignificant, whereas in azoxystrobin and propineb the growth was recorded as 60.00±1.16 mm and 47.67±1.45 mm respectively after 7 days of incubation and were found least efficient against the *Alternaria alternata* under *In-vitro* conditions. Among the others Cuprous hydroxide inhibited the mycelium growth with 85.92±1.912 % efficacy, where 12.33±1.45 mm mycelium growth recorded as compare to the control treatment (88.00±1.53 mm). The results on the antifungal susceptibility were carried out under *In-vitro* conditions and supported by the several studies carried out on *A. alternata* (Meena *et al*., 2020).

Table 1. *In-vitro* efficacy of fungicides on the mycelium growth of *A. alternata* of leaf spot of aloe

S. No.	Treatment	Conc. (%)	Redial mycelium growth (mm)	Inhibition over control (%)
1.	Azoxystrobin	0.1	60.00±1.16	31.81±0.863
2.	Tebuconazole (50%) + Trifloxystrobin (25%)	0.1	12.33±1.20	86.02±1.17
3.	Difenoconazole	0.1	1.67±1.67	98.04±1.96

4.	Copper oxychloride	0.25	12.33±1.45	85.92±1.912
5.	Propineb	0.25	47.67±1.45	45.75±2.551
6.	Carbendazim (12%) + Mancozeb (63%)	0.25	18.33±1.20	79.15±1.436
7.	Metalaxyl (4%) + Mancozeb (64%)	0.25	22.33±1.76	74.56±2.328
8.	Metalaxyl (25%)	0.25	25.00±0.58	71.593±0.246
9.	Control	00	88.00±1.53	0
	SEm±		1.374	1.621
	CD (5%)		4.11	4.854
	CV (%)		7.44	4.41

*The redial growth of fungal isolate *AALS-1* is the mean value of three replicates.

Conclusion

Based on the results this study concluded that leaf spot disease of aloe caused by *Alternaria alternata*, is a destructive pathogen, and disease infection leads substantial quality and quantitative losses in aloe production. The pathogen was determined based on the morphological and molecular sequences of ITS region. Under *In-vitro* conditions, fungicide Difenoconazole found most efficient to restrict the mycelium growth followed by Tebuconazole (50%) + Trifloxystrobin (25%) against the test pathogen. These fungicides may be used for management of the disease in field conditions.

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Conflict of Interest

The authors declare that they have no conflict of interest about this manuscript and research.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data Sharing

All relevant data are within the manuscript.

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