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ORIGINAL ARTICLE

First report of leaf spot disease of *Aloe vera* caused by *Fusarium proliferatum* in India

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Abstract

Severe leaf spot disease was observed on *Aloe vera* plants in the winters of 2011 and 2012 during a survey of various nurseries of Gwalior, India. Irregular, sunken, dark creamish brown spots having reddish brown margin were noticed on both surfaces of the leaves. The causal organism was consistently isolated from symptomatic leaves on potato dextrose agar media (PDA). A total 59 isolates of fungi were recovered from diseased *A. vera* leaves, and 37 isolates were identified as belonging to the genus *Fusarium*. On the basis of morphological characteristics and internal transcribed spacer (ITS) region of rDNA amplified using the primers ITS4/ITS5 the pathogen was identified as *Fusarium proliferatum* (Matsushima) Nirenberg and pathogenicity of the isolate was confirmed by using Koch's postulates. To the best of our knowledge, this is the first report of leaf spot disease caused by *Fusarium proliferatum* on *A. vera* plants in India.

Key words: *Aloe vera*, *Fusarium proliferatum*, leaf spot, pathogenicity

Introduction

Aloe vera (L.) Burm. f., a unique and important medicinal plant, belongs to the family Aloeaceae. It is a tropical plant of dry climates and is widely distributed in Asia, Africa and other warm regions of the world. It can utilize crassulacean acid metabolism (CAM) and has the capacity to store large amounts of water in its tissues (Alves *et al.* 2004). It has a very short stem, fleshy leaves, orange tubular flowers and fruits that contain numerous seeds. Its long, green, fibrous leaves are composed of three layers i.e. an outer thick green cuticle or rind, a middle layer with pericyclic cells located at the top of the vascular bundles which contain yellow latex, and the inner fillet aloe gel. The gel contains a diverse array of biologically active compounds namely vitamins, minerals, amino acids, enzymes, mono and polysaccharides, phenolics, saponins, lignin and salicylic acid (Boudreau and Beland 2006). It has been extensively used for medicinal purposes in several cultures like Greece, Egypt, India, Mexico, Japan and China. It is very effective in wound healing, burns, eczema, rashes, herpes lesions, insect bites, stings, psoriasis and sunburn (Rajeswari *et al.* 2012). It reduces high blood pressure, heart attack, inflammation of joints and arthritis pain. It improves immunity and is widely used as a major ingredient of various beauty products (Sharma *et al.* 2013).

Aloe vera is attacked by a number of fungal and bacterial pathogens which affect its growth as well as also alter its therapeutic properties. Lack of scientific farming techniques and abiotic factors such as temperature, high moisture and humidity favours the growth of microbial infection in leaves, collars and roots of the plant. *Aloe vera* plantations have been reported to suffer from several fungal diseases (Roy and Bilgrami 1975; Roy 1976; Harsh *et al.* 1990; Gupta and Masood 2003; Dubey and Pandey 2007; Majumdar *et al.* 2007). To explore the availability of diseased *A. vera* plants a survey was conducted during the winters of 2011 and 2012. Severe spotting and drying were noticed on *A. vera* leaves grown in nurseries and botanical gardens of

various areas in Gwalior. The present investigation was done to determine the incidence of leaf spot disease on *A. vera* and to identify the causal agent of the disease.

Materials and Methods

Survey area and sample collections

A systematic survey was done in five major areas of Gwalior during the winters i.e. (January-February) of two consecutive years. From each selected area, 15 samples were collected randomly, kept in individually labelled plastic bags and brought to the laboratory for isolation, culture and identification of the pathogen. The percent of disease incidence was calculated by applying the formula given by Ginting and Maryono (2009):

$$\text{Disease Incidence (DI)} = \frac{\text{Number of infected plants}}{\text{Total number of plants in nursery}} \times 100.$$

Isolation and identification of the causal organism

Isolation was performed from the collected diseased leaves of *A. vera* brought from the nurseries and botanical gardens. Symptomatic leaves were thoroughly washed with running tap water to remove other unwanted contaminants and then cut into small pieces (0.5–1.0 mm). These pieces were surface sterilized with 1% sodium hypochlorite (NaOCl) solution for 2 min and rinsed 3–4 times in sterile distilled water for one minute. The sterilized leaf pieces were then aseptically transferred to petriplates containing potato dextrose agar (PDA) media supplemented with chloramphenicol 50 $\mu g \cdot ml^{-1}$. A single piece was placed on each plate and incubated at 27 ± 2°C for 5–6 days. Fungal colonies developed from the tissue pieces were sub cultured on fresh PDA and incubated for 8–10 days for sporulation. The isolated fungus was identified on the basis of culture and morphological characteristics as described by Leslie and Summerell (2006).

Molecular analysis

The genomic DNA of a fungal isolate was extracted by the cetyltrimetyl ammonium bromide (CTAB) method (Maheshwar and Janardhana 2010). Isolated fungus was grown on PDA medium for 7 days. Two to three mycelial plugs were inoculated aseptically into 100 ml of potato dextrose broth (PDB) and incubated for 7 days at 28 ± 2°C. The actively growing mycelium mat on broth was filtered through a double layer of sterile muslin cloth and washed with sterile distilled water. About 200 mg of mycelia were homogenized into fine powder with sand using mortar and pestle. The mixture was transferred to 1.5 ml micro tubes and centrifuged at 10,000 rpm for 10 min and the supernatant was removed. The mycelium was then re-suspended in CTAB solution (2.0 g CTAB, 10 ml of 1 M Tris, pH 8.0, 4 ml of 0.5 M EDTA, 28 ml of 5M NaCl, 40 ml of distilled water and made up the volume 100 ml with distilled water), incubated at 65°C and mixed with chloroform isoamyl alcohol (24 : 1). The epiphase was collected in a microtube and isopropanol was added. After centrifugation, the pellet was collected, air dried, and finally dissolved in 20 µl of sterile deionized water.

The internal transcribed spacer (ITS) regions of rDNA were successfully amplified using universal pri mers ITS4 and ITS5. The polymerase chain reaction (PCR) was carried out sequentially in a final volume of 15 µl containing 1.0 µl of each primer (20 pmol), 0.2 µl of Taq DNA polymerase (5 $U \cdot \mu l^{-1}$), 1.5 µl of 10× PCR buffer, 1.8 µl of $MgCl_2$ (25 mm), 0.2 µl of (25 mm) dNTPs and 50 mg of template DNA. An initial denaturation step for 3 min at 95°C was followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50°C and extension for 1 min at 72°C, with a final extension step of 10 min at 72°C. The PCR product was visualized by agarose gel electrophoresis. The portion of ITS gene region was then sequenced at the National Fungal Culture Collection of India (NFCCI), Pune Maharashtra.

Fungal pathogenicity

Pathogenicity was carried out by using the pin prick method on *A. vera* leaves. Healthy 8–9 month old *A. vera* plants were selected to check the pathogenicity of the causal organism. Three healthy leaves of each plant were selected and pierced with sterilized pins. Conidial suspension of the test organism ($1 \times 10^7 \cdot ml^{-1}$) was prepared in sterile distilled water by using a hemocytometer. The fungal suspension was then sprayed on pricked leaves and wrapped with moist blotting paper. Leaves sprayed with sterilized distilled water served as control. Three replicates for each treatment and control were maintained. Both inoculated and control leaves were then placed in plastic bags and incubated at 27 ± 2°C for 8–10 days. Re-isolation of fungus from artificially inoculated leaves was also carried out to confirm the Koch's postulates.

Results

Incidence and symptoms of diseases

Survey results revealed that leaf spot disease on *A. vera* was recorded from all the five areas during the winters

Fig. 1. Symptoms of leaf spots on *Aloe vera* caused by *Fusarium proliferatum:* A – initial symptoms of disease; B – later symptoms of disease

Table 1. Disease incidence of *Fusarium proliferatum* isolated from *Aloe vera* during 2011 and 2012

Serial number	Collection sites	Disease incidence [%]	
		2011	2012
1	Morar	19.25 ± 3.89	37.86 ± 4.09
2	City Centre	16.99 ± 9.12	28.75 ± 10.54
3	Chetakpuri	36.12 ± 3.93	21.95 ± 4.34
4	Lashkar	30.18 ± 2.6	38.18 ± 3.62
5	Kampoo	32.21 ± 3.93	27.76 ± 3.16

Mean ±SD of five observation sites

of 2011 and 2012. In 2011, the highest incidence of leaf spot disease was recorded in the Chetakpuri area (36.18 ± 3.9%) while in 2012, it was (38.12 ± 3.62%) in the Lashkar area (Table 1). The disease began as mostly circular to irregular lesions on the adaxial and abaxial surfaces of leaves. In an earlier stage of infection, spots were sunken and creamish in colour. Gradually, these spots became enlarged, embedded, dark, creamish brown in colour having reddish brown margin, varying in size from 1.2–4.0 × 1.0–3.8 cm in diameter. As the disease progressed dark brown sporulation was observed in the centre of the spots. As the severity increased, spots frequently coalesced, became dry and necrotic. Eventually leaves were broken down (Fig. 1A–B).

Morphological and cultural characteristics

Of the 59 isolates of *Fusaruim* collected from infected *A. vera* leaves, 37 isolates were identified as *F. proliferatum* (Matsushima) Nirenberg based on morphological and cultural characteristics. The fungi produced white to light cream floccose aerial mycelium on PDA. Reverse pigmentation varied from light yellowish to reddish purple with concentric rings. Aerial conidiophores at first unbranched, became loosely to densely branched, typically sympodial and some irregularly verticillate. Aerial conidia i.e. microconidia were hyaline, oval to clavate, 0–1 septate and borne in chains on mono and polyphalides, measuring 7–10 × 2.5–3.2 μm in diameter. Sporodochial conidia i.e. macroconidia were few, 3–7 septate, hyaline, smooth, slightly sickle shaped to almost straight with curved apical cells produced abundantly from monophialides, measuring 31–58 × 2.7–3 μm in diameter. Chlamydospores were absent (Fig. 2A–D).

Molecular characterization

The internal transcribed spacer region of ribosomal DNA was amplified using fungal-specific primer pair ITS4/ITS5 and sequenced. The identified fungal isolate showed 100% sequence similarity with *F. proliferatum* (NFCCI Accession No. 3640). The resulting sequence of an isolate was submitted to GenBank (accession No. KJ767073.1). Sequence alignment of *F. proliferatum*

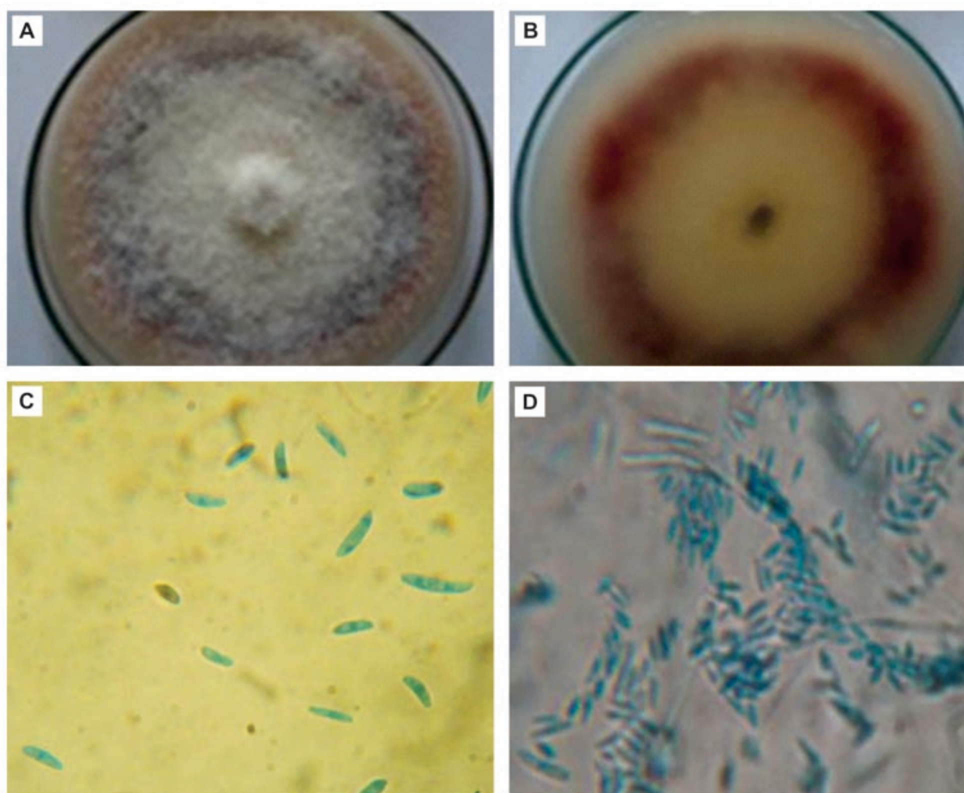

Fig. 2. Adaxial (A) and abaxial (B) side of culture of *Fusarium proliferatum* on potato dextrose agar (PDA) medium; C – microconidia; D – macroconida

Table 2. Top five hits upon Basic Local Alignment Search Tool analysis

Gene Bank Accession No.	Description	Max score	Query cover	E value*	Identity [%]
KM265507.1	Fungal sp. E14418B	836	836	0.0	100
KJ767073.1	*Fusarium proliferatum* isolate A2S1-D96	836	836	0.0	100
KJ767072.1	*Fusarium proliferatum* isolate A1S1-D30_1	836	836	0.0	100
KJ767071.1	*Fusarium proliferatum* isolate A1S1-D12	836	836	0.0	100
KF293357.1	*Fusarium* sp. Fs025TNW-T	836	836	0.0	100

*E value – expect value

is shown in Figure 3. Based on the BLAST search, all the sequences showed 100% similarity with the isolated fungi (Table 2).

Pathogenicity test

The symptoms of leaf spot infection which occured during the greenhouse experiment were similar to the natural infection. Initial symptoms appeared in the form of water soaked, small circular spots on the fourth day of inoculation. Progressively, spots enlarged as the infection increased and turned light brown. As the maturity spots became dry, sunken, brown with reddish maroon margins appeared. Spots on artificially inoculated leaves ranged from 0.5 × 1.0 cm to 0.8 × 2.1 cm. The pathogen which appeared on inoculated leaves was consistently re-isolated from symptomatic parts whereas, control leaves remained symptomless. The pathogen was re-isolated from inoculated leaves, successfully completing Koch's postulates.

Discussion

The genus *Fusarium* is one of the most economically important groups of fungi infecting various medicinal plants, as well as and horticultural crops worldwide. Among the various species, *F. proliferatum* is a common ubiquitous pathogen associated with a large number of crops and plants including date palm,

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Query  1    GCTTGGCCGCGCCGCGTACCAGTTGCGAGGGTTTTACTACTACGCAATGGAAGCTGCAGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  477  GCTTGGCCGCGCCGCGTACCAGTTGCGAGGGTTTTACTACTACGCAATGGAAGCTGCAGC  418

Query  61   GAGACCGCCACTAGATTTCGGGGCCGGCTTGCCGCAAGGGCTCGCCGATCCCCAACACCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  417  GAGACCGCCACTAGATTTCGGGGCCGGCTTGCCGCAAGGGCTCGCCGATCCCCAACACCA  358

Query  121  AACCCGGGGGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357  AACCCGGGGGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGC  298

Query  181  GGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  297  GGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA  238

Query  241  TCGCATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  237  TCGCATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTT  178

Query  301  GATTTATTTATGGTTTTACTCAGAAGTTACATATAGAAACAGAGTTTAGGGGTCCTCTGG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  177  GATTTATTTATGGTTTTACTCAGAAGTTACATATAGAAACAGAGTTTAGGGGTCCTCTGG  118

Query  361  CGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAACAATTGGTATGTTCA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117  CGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAACAATTGGTATGTTCA  58

Query  421  CAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCAG  463
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  57   CAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCAG  15
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Fig. 3. Alignment statistics of *Fusarium proliferatum*: query length – 466, score – 836 bits (926), identities – 463/463 (100%), gaps – 0/463 (0%), strand – plus/minus

banana, mango, maize, rice, asparagus and garlic (Jurado *et al.* 2010). *Fusarium proliferatum*, one of the destructive species, causes diseases like foot-rot of corn (Farr *et al.* 1989), leaf spot of asparagus (Elmer *et al.* 1999), leaf spot of *Cymbidium* (Ichikawa and Aoki 2000), root rot of soybean (Arias *et al.* 2011), bakanae of rice (Zainudin *et al.* 2008; Quazi *et al.* 2013), wilt of date palm (Khudhair *et al.* 2014), tomato wilt (Chehri 2016) and tomato fruit rot (Murad *et al.* 2016). A number of fungal pathogens are also found to be associated with leaf spot of *A. vera* such as *F. phyllophilum* (Kishi *et al.* 1999), *Colletotrichum gloeosporioides* (Avasthi *et al.* 2011), *F. oxysporum* (Kawuri *et al.* 2012), *Nigrospora oyrzae* (Zhai *et al.* 2013), *Phoma betae* (Avasthi *et al.* 2013), *Alternaria alternata* (Abkhoo and Sabbagh 2014), *Sphaeropsis sapinea* (Kamil *et al.* 2014), *Curvularia lunata* and *C. ovoidea* (Avasthi *et al.* 2015), *Cladosporium sphaerospermum* (Avasthi *et al.* 2016), *Polyrostrata indica* (Avasthi *et al.* 2017a) and *Phoma eupyrena* (Avasthi *et al.* 2017b). To the best of our knowledge, this is the first report of *F. proliferatum* causing leaf spot disease in *A. vera* in India.

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