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Full Length Paper

Destructive Leaf Rot Disease Caused by *Fusarium oxysporum* on *Aloe barbadensis* Miller in Bali.

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ABSTRACT

Aloe (*Aloe barbadensis* Miller) has been planted in Bali since 2006 with the total area of 170 hectares. There was a destructive leaf rot disease was found to be associated with aloe plants, however, there is no information available on the cause of the disease. This research was conducted in order to identify the causal agent that responsible for the leaf rot disease on aloe. The observation on the occurrence of the disease was done in five regencies in Bali, namely Buleleng, Karangasem, Bangli, Gianyar and Badung. Three observations point were determined in each regency by evaluating 20% of the total plant population in each observation point to determine the disease occurrences. Koch Postulate was applied to confirm the causal agent of the disease. Identification of the causal agent was done based on the morphological characteristics observed under Scanning Electron Microscope and molecular analysis of 18S rDNA. Results of this study showed that the leaf rot disease was found distributed in all five regencies of Bali, where the aloe is grown. The average incidence of the leaf rot disease was 7.35%. A pathogenic fungus, *Fusarium* sp. isolate Fo2010 was found to be the causal agent of the disease. Based on observation under scanning electron microscope and the analysis of 18S rDNA, the *Fusarium* sp. isolate Fo2010 was identified as *Fusarium oxysporum* with smooth surface macro conidia, intercalary, while the micro conidia and hypha were roughly. This is the first report of the leaf rot disease on *A. barbadensis* since its introduction to Bali in 2006.

Key words: destructive disease, *Aloe barbadensis*, *Fusarium oxysporum*, 18S rDNA.

INTRODUCTION

Aloe barbadensis Miller (most common name: *Aloe vera*) is a stemless or very short-stemmed succulent plant growing to 60-100 cm tall, belongs to the family Liliaceae. There are about four hundred species of Aloe, but the most common, well known and highest quality of them is *A. barbadensis.* The margin of the stem is serrated and has small teeth. The species are widely naturalized all over the world, occurring in temperate and tropical regions. Aloe leaves are filled with gel and the gel is the most important component of the plant and has great medicinal value. The *Aloe vera* contains amino acids, anthraquinones, enzymes, lignin, minerals, mono and polysaccharides, salicylic acid, saponins, sterols and vitamins (Barcroft and Myskja, 2009).

Many herbal drugs and drinks have been formulated from *A. vera* plants for the maintenance of good health. In cosmetic industries Aloe is used in the production of soap for bathing, shampoo, hair wash, tooth paste and body

creams (Daodu, 2000). Furthermore, *A. vera* gel has been reported to be very effective for the treatment of sore and wounds, skin cancer, skin disease, cold and cough, constipation, pile and fungal infection (Daodu, 2000; Djeraba and Quere, 2000; Olusegun, 2000), while

Davis and Moro (1989) reported *Aloe* plants can be used for treatment of asthma, ulcer and diabetes.

Aloe barbadensis has been cultivated in Bali since 2006 with the total area of 170 hectares and spread over 5 regencies namely Buleleng, Karangasem, Gianyar, Bangli and Badung. The aloe leaves then are processed into gel for further used such as medicinal and cosmetic purposes.

A destructive disease with leaf rot symptom was found for the first time on aloe plant in Bali in 2010. The symptoms appeared on the leaves, where the leaves become rotten and dried with dark brown color and crescent shape. Diameter of the infection was 1-4 cm and infection started from the leave edge and causing leaves became dried, rot and brownish in color, shrinking and eventually broken. The tips of the leaves become rotten and dried.

Since the aloe plants are sometimes used in medicine and cosmetics (Hegger, 1996; Olusegun, 2000), the contamination with fungal pathogen in the plants is of public importance. Some fungal pathogens and non-pathogens produce mycotoxins in their infected hosts and substrates on which they grow (Anthony et al., 2009). *Fusarium* produce mycotoxin trichothecenes which is very toxic for human (Miller and Trenholm, 1994). Alexoupolos et al. (1996) reported this toxin can cause cancer, hemorrhage, edema and immune deficiency. WHO (1979) reported that mycotoxins are hazardous to human and animal health. Base on this reason, the leaf rot disease on aloe plant should be controlled properly.

In order to control the disease, it is necessary to know and identify the causal agent of the disease; however there is no information and study done on leaf rot disease on aloe plant in Bali. This study was done in order to determine the incidence of the leaf rot disease on aloe plantation in Bali and to identify the causal agent of the disease.

MATERIALS AND METHODS

Survey for Disease Incidence and Samples Collection

Survey for disease incidence was done in five regencies in Bali, namely Buleleng, Karangasem, Bangli, Gianyar and Badung where aloe are cultivated. Three observation points were determined in each regency and 20% of the total plant population in each observation point was evaluated to determine the incidence of leaf rot disease. The disease incidence was determined based on the following formula: I(%) = 100 Ns/No ; where I(%): disease incidence in percentage, Ns: number of aloe plants showing leaf rot symptom and No: number of observed aloe plants.

From each observation point, 10 diseased leaves were collected randomly and placed into individually labeled plastic bags and sealed and were brought to the laboratory for isolation and identification.

Isolation of the causal agent

Isolation of the pathogen was done on 50 samples (10 samples from each observation sites) of diseased leaves brought from the field. The diseased leaf was cut into small pieces (3 mm length) and surface sterilized in 0.1% mercury chloride for 2 min and rinsed in three changes of sterile distilled water. These leaf cuttings were blotted between sterile filter papers and aseptically plated on potato dextrose agar (PDA). Two pieces were placed in a plate and incubated at room temperature ($25\pm2^{o}C$) for five days. Mycelia growth which occurred three days after inoculation was transferred on to fresh PDA to get pure culture. The pure cultures of the fungus were then inoculated into healthy aloe plants to confirm whether they can cause the same leaf rot disease as occurred in the field. For this purpose, the Koch's Postulate procedure was applied. The 30-day old aloe plants maintained in plastic pots were artificially injured with sterile needles and inoculated by spraying the spore suspension of the fungus at 1.5×10^{6} spores/ml. Three plants were inoculated with one isolate and three leaves were inoculated per plant. The plants injured with needles and sprayed with sterile distilled water were used as control. All of the tested plants were maintained in the green house with average daily temperature $28\pm2^{o}C$ with relative humidity $83\pm3\%$.

When the plant show the disease symptom, the symptom was compared with the naturally occurred disease symptom, and re-isolation of the fungus was done from the diseased-leaves. The pure fungus obtained from this re-isolation was then inoculated to the new healthy aloe plants with the same procedure as described previously. When the plants show the disease symptom, the re-isolation was done again to get the pure isolates of the fungus. These isolates can be considered as the causal agent of the leaf rot disease and were then subjected to the identification procedure.

Identification of the Fungus

The pure cultures were identified macroscopically and microscopically on the basis of their morphological characteristics on plates and pigmentation using identification keys of Barnett and Hunter (1972), and Pit and Hocking (1997). Observation of the morphological characteristic of the fungus was also done using scanning electron microscope (SEM) at the Laboratory of

Crop Science, College of Agriculture Ibaraki University, Japan. Molecular Identification of the fungus was done base on the analysis of 18S rDNA at the Laboratory of Plant Pathology, College of Agriculture Ibaraki University, Japan.

Preparation of materials for Scanning Electron Microscopy (SEM)

Representative isolate Fo2010 of *Fusarium* sp. was used for SEM observation. The fungus was grown on PDA medium on Petri dish for five days at 27±2°C. The specimens taken from the fungal colony were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 8 h and then at room temperature for 1 h. Fixed specimens were rinsed in sodium cacodylate buffer (pH 7.2). The specimens were post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at room temperature for 5 h. Following post fixation, the specimens were rinsed in distilled water, and then, the specimens were dehydrated in a graded series of ethyl alcohol. After dehydration, specimens were freeze cutting using freeze cutting device (TF-2, Eiko, Japan). After cutting, specimens were replaced by t-butyl alcohol, and then vacuum freeze-drying (ID-2, Eiko, Japan). The dried specimens were mounted on stubs and coated with osmium tetroxide (OPC 60A, Filgen, Japan) and platinum (JUC-5000, JEOL, Japan). The coated specimens were observed with a SEM (JSM-6701F, JEOL, Japan) with 5 kV of acceleration voltage (Hall, 1978; Hayat, 1981).

DNA Extraction

Fusarium sp. isolate Fo2010 was grown on PDA medium for 3 days at room temperature. A loopful of fungal mycelia taken from the edge of the colony was suspended in 100 µl PrepMan Ultra sample preparation reagent (PrepMan Ultra Protocol, Applied Biosystems, USA) in micro centrifuge screw-cap tube. The sample was then vortex for 30 seconds and placed in a heat block set at 95°C to 100°C for 10 minutes. The sample was then leaved under room temperature for two minutes. The tube was then centrifuged in the micro centrifuge at 10,000 rpm for 2 minutes and the pellet was collected as extracted DNA and used for further analysis.

PCR amplification

The extracted DNA was amplified by PCR sequencing of 18S rDNA gene using primers Internal Transcribed Spacer (ITS) 1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS 4 (5-TCCTCCGCTTATTGATATGC-3). The reactions was performed in Takara PCR Thermal Cycler Dice (Takara Bio, Otsu, Japan) with Ex Tag (Takara Bio, Otsu, Japan) under the following conditions: pre-denaturation 94°C (4 min.) followed by 35 cycles of denaturation 94°C (35 sec.), annealing 52°C (55 sec.), and elongation 72°C (2 min.) and post elongation 72°C for 10 min. (Nishizawa *et al*. 2010).

Sequencing of the ITS region and Computer Analysis of DNA sequences

Nucleotide sequences were determined with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the instructions supplied and with a PE Applied Biosystems Automated DNA Sequencer (model 3130xl, Applied Biosystems). The double-stranded DNA sequences were assembled and analyzed using Genetyx (version 11.0) and Genetyx-ATSQ (version 4.0) software (Genetyx, Tokyo, Japan), respectively, and compared with similar DNA sequences retrieved from the DDBJ/EMBL/GenBank data bases using the NCBI BLAST programs. The phylogenetic analysis was carried out using the multiple-sequence alignment tool from http://mafft.cbrc.jp/alignment/server/ (Nishizawa *et al*. 2010). Percent homology of DNA sequence of ITS region was compared with previously identified fungi such as *Fusarium solani* AM412635, *Fusarium* sp. EF453093, *F. oxysporum* isolate 1021 JF300168, *F. oxysporum* f.sp. lycopersici GU327639.1, *F. oxysporum* JN232196.1, and *Taphrina deformans* JF706574.1.

RESULTS AND DISCUSSION

Disease Incidence

The leaf rot disease on aloe plants was found in all five regencies of Bali where the aloe plants are grown. The disease showed leaf rot symptom on the leaves, where the leaves become rotten and dried with dark brown color and crescent shape. Diameter of the infection was 1-4 cm and infection started from the leave edge and causing leaves became dried, rot and brownish in color, shrinking and eventually broken. The tips of the leaves mostly become rotten and dried (Figure 1). The highest incidence of leaf rot disease was observed in Badung Regency with disease incidence by 21.60%, while the lowest one was observed in Buleleng Regency with disease incidence by 2.2% (Table 1). Badung regency was the first area for *A. barbadensis* cultivation in Bali, while Buleleng Regency was the latest one. This is the first report on the leaf rot disease occurred on aloe plant after its introduction to Bali in 2006.

Morphological Characteristics of the Causal Fungus

Figure 1. Symptoms of the leaf rot disease on *Aloe barbadensis* under field condition. Arrows: The leaf become rotten and curved and the tips of leaf become rotted and dried

Table 1. Incidences of leaf rot disease on *Aloe barbadensis* in five regencies of Bali in 2010.

No	Locations	Disease incidence (%)*
1	Badung	21.60 ± 3.78**
2	Bangli	5.55 ± 1.16
3	Buleleng	2.20 ± 0.96
4	Gianyar	3.62 ± 1.05
5	Karangasem	5.09 ± 2.11

*Average of three observation points in each regency.
**Standard deviation.

Fusarium sp. isolate Fo2010 grown on PDA and incubated at 25±2^{0}C produced white to cream colonies with aerial cottony mycelia. Observation under scanning electron microscope showed that the fungus had macroconidia curved in shaped with four septates and foot cell, 31µm length and smooth surface , whereas microconidia has raugh surface at 4.6 µm length as presented in Figure 2. Furthermore hypha has raugh surface, 13 µm diameter while clamydospora is intercalar has wavely surface and 37µm length. Pitt and Hocking (1979) reported that *Fusarium oxysporum* has 3 type of conidia namely macroconidia, microconidia and clamydospora intercalar, whereas some *Fusarium* sp. only has macroconidia and clamydospora. Surface structure of macroconidia, microconidia, clamydospora and hypha are important characteristics of *Fusarium oxysporum*. Study by Torres *et al.* (2003) showed that *Fusarium verticillioides* has smooth surface of it's hypha. Whereas Alberthini *et al*. (2003) showed that *Fusarium culmorum* has rough hypha. Alexopoulos et al. (1996) reported that species *Fusarium* may produce two types of conidia namely macroconidia and microconidia because of their respective size. Pitt and Hocking (1997) reported, macroconidia has 2-5 septates, fusiform, more or less curved, pointed at both ends with pedicellate basal cell, 20 – 46 µm x 3 – 4 µm. The smaller microconidia (0-2 septate) are ussually vary in shape and size , avoid ellipsoidal to cylindrical, straight or slightly curved, 5-12 x 2.2-3.5 µm. Clamydospores in hyphae or in conidia, hyaline, smooth or rough walled, sub globose, 5 – 15 µm in diameter, terminal or intercalary.

Some pathogenic fungi have been found to infect aloe plant such as *Alternaria alternata* causing leaf spot disease in *Aloe barbadensis* Mill. in India (Kamalakannan *et al*., 2008), *Fusarium phyllophilum* causing purple spot disease on *Aloe arboescens* Mill. (Kinshi *et al*., 1999) and *Haematonectria haematococca* (anamorph: *Fusarium* sp.) causing ring spot disease on *Aloe barbadensis* (Hirooka *et al.,* 2007). Furthermore Chavan and Korekan (2011) found that *Alternaria alternata* and *Fusarium* spp. caused the leaf spot disease in winter and rainy season on *Aloe barbadensis*.

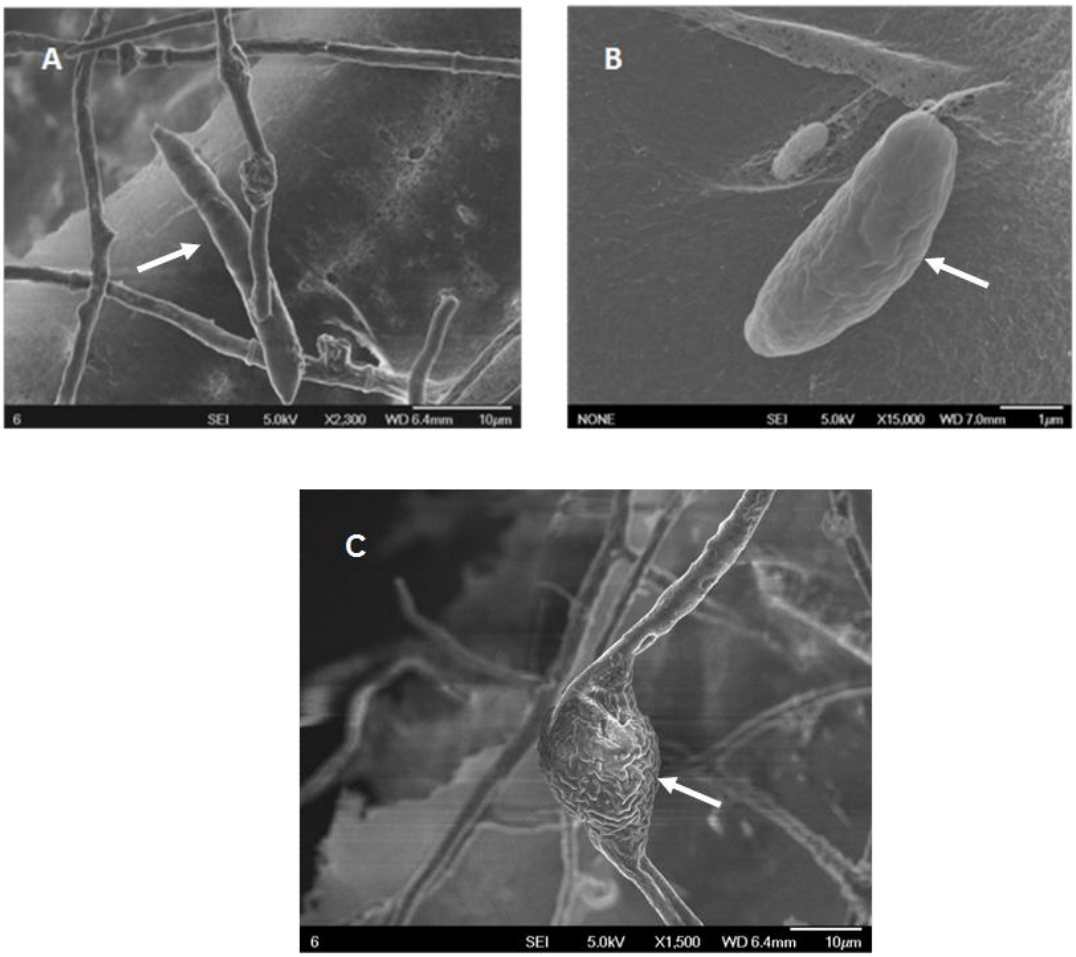

Figure 2. Scanning electron microscope photographs of *Fusarium* sp. isolate Fo2010. A.macroconidium (arrow); B. microconidium (arrow); C. clamydospora (arrow).

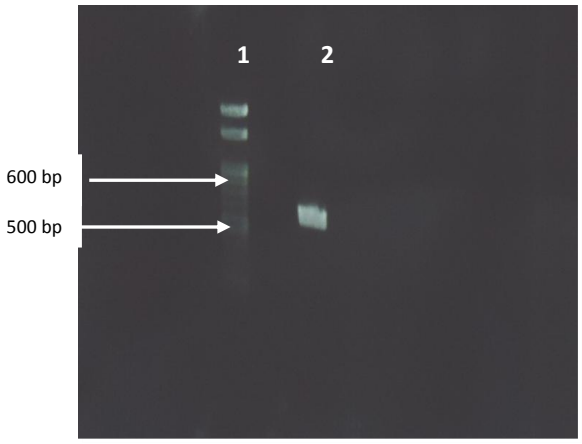

Figure 3. The Agarose Gel Electrophoresis of 18S rDNA of *Fusarium* sp. isolate Fo2010. Lane 1. Marker Gene Ladder 100, 0.1-2 kbp (Nippon Gene, Wako). Lane 2. Fragment of 18S rDNA *Fusarium* isolate Fo2010 (550 bp).

Ayodele and Ilondu (2008) found that *Plectosphaeralla cucumerina* causing *base* rot diseases on *A. barbadensis* Miller in Nigeria.

Genome Analysis

Separation pattern of 18S rDNA-specific fragments amplified with primers ITS1 and ITS4 is shown in Figure 3. Labelled band of appximately 550 bp is corresponding to 18S rDNA.

The fragment of 18s rDNA of *Fusarium* sp. isolate Fo2010 was then sequenced to determine the species of the fungus base on the percent homology with other references of identified species.

Based on the 18S rDNA analysis showed that *Fusarium* sp. isolate Fo2010 has 94% homology with *Fusarium oxysporum* JF300168 (Table 2). Genetic relationship of *Fusarium* sp. isolate F02010 with *Fusarium solani, Fusarium oxysporum* and *Taphrina*

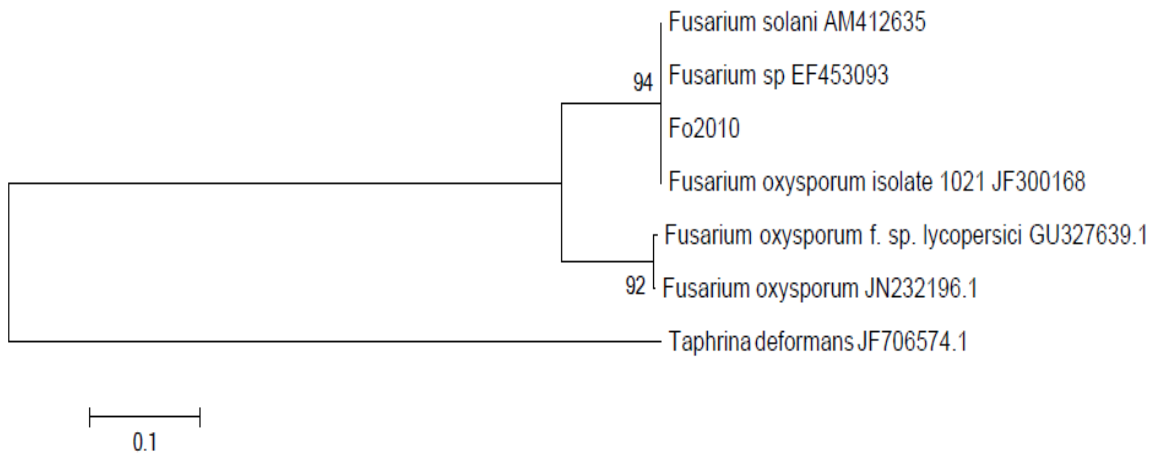

Figure 4: Phylogenetic relationship of the fungal internal transcribed spacer sequence of the characterized clone library of *Fusarium*. Bootstrap values greater than 50 are shown at branch points. The scale bar indicates 1% sequence divergence.

Table 2. Comparisons of 18S rDNA gene homology levels of Fusarium sp. isolate Fo2010 with multiple sequences in GenBank using the BLAST program

Isolates	Percent homology (%)
Fusarium solani AM412635	93
Fusarium sp EF453093	90
Fusarium oxysporum isolate 1021 JF300168	94
Fusarium oxysporum f sp *lycopersici* f sp GU327639	73
Fusarium oxysporum JN232196	73
Taphrina deformans JF706574	42

deformans is presented in Figure 4.

From the Figure 4 and Table 2 can be understood that *Fusarium* sp. isolate Fo2010 is closely related to the *Fusarium oxysporum* isolate 1021 JF300168. Luna and Berenice (2011) reported that *Fusarium oxysporum* isolate 1021 JF300168 infected chrysanthemum plants (*Dendranthema grandiflorum*) in Texcoco, Mexico and caused significant losses. Molecular detection and identification of human pathogenic fusaria showed that *Fusarium* sp. EF453093 can cause corneal infection in human.

The presence of *F. oxysporum* in *A. barbadensis* plants is of public health importance since the plants are sometimes used in medicine and cosmetics (Hegger, 1996; Olusegun, 2000). Some fungal pathogens and non-pathogens produce mycotoxins in their infected hosts and substrates on which they grow. *Fusarium* produce mycotoxin trichothecenes which is very toxic for human (Miller and Trenholm, 1994). Alexoupolos et al. (1996) reported this toxin can cause cancer, hemorrhage, edema and immune deficiency. WHO (1979) reported that mycotoxins are hazardous to human and animal health.

Based on the results of this study can be concluded that the leaf rot disease is the first destructive disease reported on *A. barbadensis* in Bali and the causal agent of the disease is identified as *Fusarium oxysporum*. Efforts must be done to control the disease in order to reduce the losses and the risk of mycotoxins contamination which are probably produced by *F. oxysporum*.

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References

Albertini MC, Citterio AB, Burattini S, Piacentin MP, Uguccioni F , Piatti E (2003). Morphological and biochemical modifications induced by a static magnetic field on *Fusarium culmorum.* Biochimie 85: 970-983. Available online at www.science direct.com.

Alexoupolos CJ, Mims CW, Blackwell, M (1996). Introductory Mycology. John Wiley and Sons. Inc. Singapore.

Anthony MH, Ayinla GT, Helnina AO, Ezekiel SA, Haruna OG (2009). Health implication of toxigenic fungi found in two Nigerian staples: guinea corn and rice. African J. Food Sci. 3(9): 250-256.

Ayodele SM, Ilondu EM (2008). Fungi assosiated with base rot desease of *Aloe vera* (*Aloe barbadensis*). Afr. J.Biotechnol., 7 (24): 4471-4474.

Barcroft A, Myskja A (2009). *Aloe vera* Nature's Silent Healer. BAAM Publishing Ltd. London.

Barnett HL, Hunter BB (1972). Illustrated genera of imperfect fungi, Burgess Publishing Co., New York.

Chavan SP, Korekan, SL (2011). A Survey of some Medicinal Plants for Fungal Diseases from Osmanabad District of Maharashtra State. Recent Research in Science and Technology 3(5):15-16

Daodu T (2000). *Aloe vera*, the miracle healing plant. Health Field Corporation, Lagos.

Davis RH, Moro NP (1989). *Aloe vera* and gibbrellin, anti-inflammatory activity in diabetes. J. Am.Paediatr. Med. Assoc. 79(1): 24-26.

Djeraba A, Quere P (2000). *In vivo* macrophage activation in Chicken with acemannan, a complex carbohydrate extracted from *Aloe vera.* Int. J.Immunopharmacol. 22(5): 365-372.

Donnell OK, Sarver B, Brandt M, Chang DC, Nobel-Wang J, Park BJ, Sutton D, Benjamin L, Lindsley M, Padhye A, Geiser DM, Ward TJ (2007). Phylogenetic diversity and microsphere array-based genotyping of human pathogenic Fusaria, including isolates from the multistate contact lens-associated U.S. keratitis outbreaks of 2005 and 2006. J. Clin. Microbiol., 45(7):2235-2248.

Hall JL (1978). Electron Microscopy and cytochemistry of plant cells. Elsevier/ North-Holland Biomedical Press. New York.

Hayat MA (1981). Fixation for Electron Microscopy. Academic Press. New York.

Hegger JP (1996). Beneficial effect of Aloe on wounds healing in anexcisional wound model. J. Altern. Complement Med., 2 (2): 271-277.

Hirooka Y, Kobayashi T, Takeuchi J, Ono T, Ono Y, Natsuaki, KT (2007). Aloe ring spot, a new disease of Aloe caused by *Haematonectia haematococca* (Berg.&Broome) Samuels and Nirenberg(anamorph:*Fusarium* sp.).J. Gen. Plant Pathol., 73:330-335.

Kamalakannan A, Gopalakrishnan C, Rebnuka R, Kalpana K (2008). First report of Alternaria alternate causing leaf spot on *Aloe barbadensis* in India. Australasian Plant Dis. Notes 3: 110-111.

Kinshi K, Furukawa T, Aoki T (1999). Purple spot of Aloe (*Aloe arborescens* Mill) caused by *Fusarium phyllophylum* Nirenberg et O'Donnell (New Disease). Ann.Phytopathol.Soc.Jpn. 65: 576-587.

Luna M, Berenice J. (2011). Bio-rational management of pests and diseases in chrysanthemum Dendranthema *grandiflorum* (Ramat.) Kitam 'Harman' . Master Thesis. Institute of Education and Research in Agricultural Sciences. Campus Montecillo, Texcoco, Edo Mexico. Cited on 10 January 2012. Available from http://hdl.handle.net/10521/352.

Miller JD, Trenholm HL (1994). Mycotoxins in grain: compounds other than aflatoxin. Eagan Press, USA.

Nishizawa T, Zhaorigetu, Komatsuzaki M, Sato Y, Kaneko N, Ohta H (2010). Molecular Characterization of Fungal Communities in Non-Tilled, Cover-Cropped Upland Rice Field Soils. Microbes Environ. 25 (3) : 204–210.

Olusegun A (2000). One hundred medicinal uses of *Aloe vera*. Good health Inc. Lagos.

Pitt JI, Hocking AD (1997). Fungi and Food Spoilage. Second Edition. Blackie Academic & Profesional Press.London.

Torres MR, Ramos AJ, Soler J, Sanchis V, Marı´n S (2003). SEM study of water activity and temperature effects on the initial growth of *Aspergillus ochraceus, Alternaria alternata* and *Fusarium verticillioides* on maize grain. Int. J.Food Microbiol., 81: 185– 193

World Health Organization (WHO) (1979). Mycotoxins, Environment, Health Criteria No. 11, Geneva.