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Full Length Research Paper

Co-infection of *Tylosema esculentum* (Marama bean) seed pods by *Alternaria tenuissima* and a *Phoma* spp.

Uzabakiriho, J. D., Shikongo, L. and Chimwamurombe, P. M*

Department of Biological Sciences, University of Namibia, Private Bag 13301, 340 Mandume Ndemufayo Avenue, Windhoek, Namibia.

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Marama bean, *Tylosema esculentum,* is an endemic, perennial wild tuberous Fabaceae, widely distributed in the Southern Kalahari (Namibia, Botswana and to a lesser extends in South Africa). It is well adapted to the deep sandy soils of the Kalahari. It is drought avoiding. It has been valued by the indigenous people of the semi arid land of the Kalahari for its nutritional and medicinal qualities. The aim of the study was to determine the identity of fungal pathogens that occur on marama bean. Diseased pods presenting necrotic spots with dense sporulation in the centre were collected. They were subjected to fungal isolation using potato dextrose agar (PDA) at room temperature under fluorescent light for five days. Two fungi with different cultural conidial morphology were isolated. Single spores from the two cultures were separately inoculated on PDA to obtain a pure culture. Genomic fungal DNA was extracted from fresh mycelium using the cetyl trimethylammonium bromide (CTAB) method and was used as a template in internal transcribed spacer (ITS) PCR amplification. Based on the sequence analysis of the 5.8S ribosomal DNA and the ITS, the two isolates associated with necrotic Marama pods were identified as *Alternaria tenuissima* and *Phoma* spp. Isolate Po72 due to the high sequence homology, which was 99% in both cases. To our knowledge, this is the first report describing the presence of these two fungi on Marama bean.

Key words: Marama, ITS, fungal pathogen, *Tylosema esculentum*.

INTRODUCTION

Tylosema esculentum (Burchell.) *A. Schreiber* (Fabaceae), known as marama or marama bean is an endemic, perennial wild tuberous legume widely distributed in the Southern Kalahari (Namibia, Botswana and to a lesser extends in South Africa). It is well adapted to deep sandy soils of the Kalahari Desert (Southern Africa) (Castro et al., 2005; National Academy of Sciences (NAS), 2006). Marama bean has been highly valued for its nutritional and medicinal values by the San people ("Bushmen") from time immemorial (Mmonatau, 2005; Musler and Schonfeld, 2006).

The protein and oil content of Marama is comparable to soya and peanuts (Hartley et al., 2002). Amarteifio and Moholo (1998) reported 34.1% of protein and 33.5% of fat content and Keegan and van Staden (1981) reported 30 to 39% of proteins and 36 to 43% of fat. Its fatty acids are unsaturated and 87% are a combination of oleic acid, linoleic acid and palmitic acid. According to Musler and Schonfeld (2006), Mmonatau (2005) and Bower et al. (1988), the sulfur containing amino acid such as methionine and cysteine are limiting amino acids.

Marama is not only a key part of diet of Khoisan, Batswana, Herero and some other Bantu speaking people but also a delicacy as well. It is believed that "marama may have been in our diet as long as any food in existence" (NAS, 2006). Marama is now being considered as a new alternative crop where it occurs. Up to date, no disease investigation has been reported on it. During the rainy season in February and March 2008, samples presenting necrotic spots with dense sporulation in the center were observed and collected in the Sandveld area in the Omaheke region (Eastern Namibia) where marama

***Corresponding author. E-mail: pchimwa@unam.na.

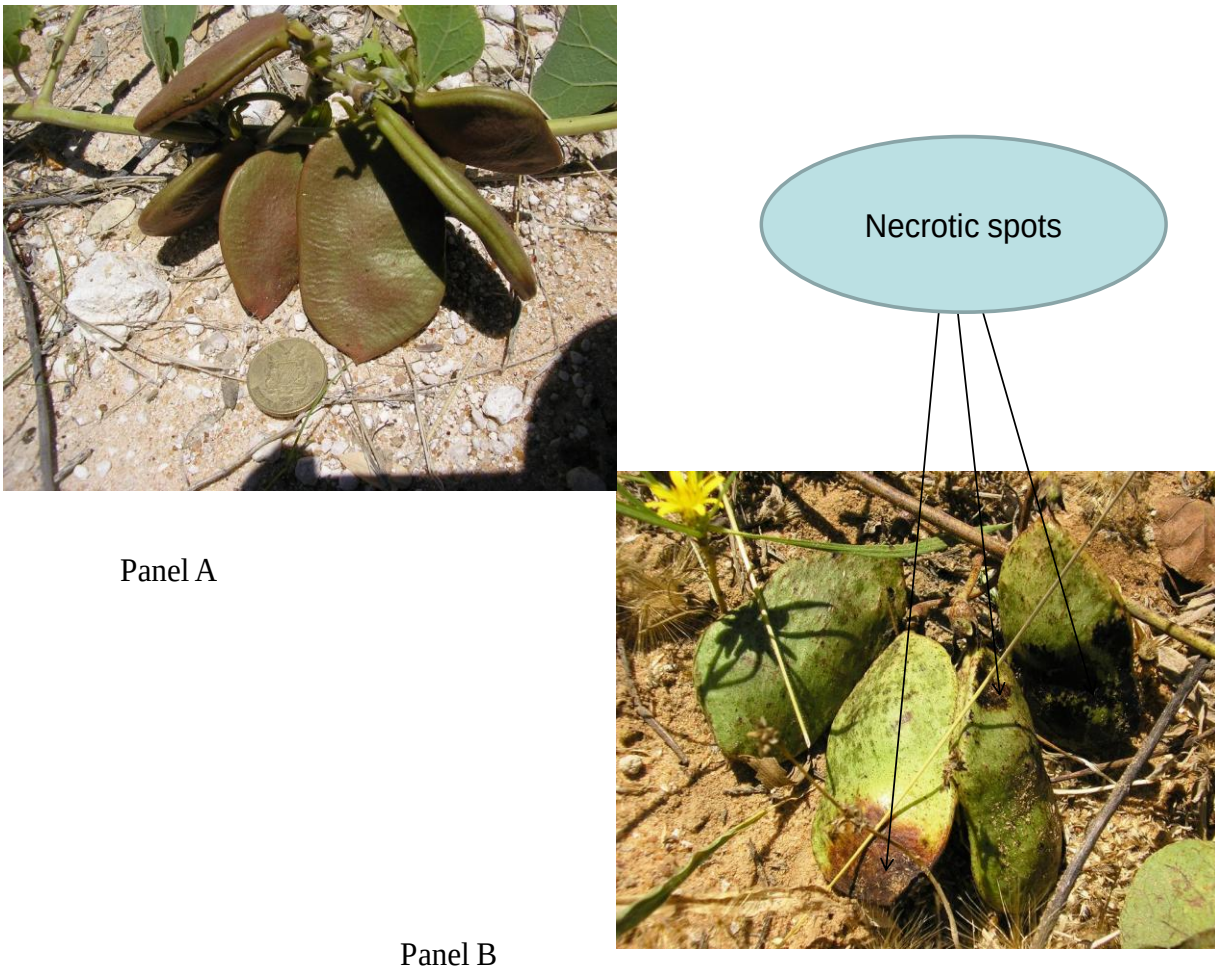

Figure 1. Young healthy marama pods in Panel A and infected pods in Panel B.

bean grows.

Plants are host to many diseases caused by several pathogens such as bacteria, viruses, nematodes and Fungi. Only around 10% the fungi can successfully infect plants but they account for 80% of the damage (Morocko et al., 2006; Agrios, 1997). Our knowledge of pathogens in indigenous plants is limited and unfortunately acquired after biological disasters. Failure to anticipate and control the pathogens can be costly (Ridley et al., 2000). Most of the research has only been focusing on species with an economic or commercial value which may be more susceptible to diseases neglecting indigenous plant species that are an integral component of the environment. As a new crop candidate, it is imperative to assess marama's natural resistance to pests and pathogens for its success. The screening of fungi pathogens which can potentially affect marama should be regarded as a major crop protection component towards its domestication process. Reliable unequivocal molecular diagnosis tests are required to guarantee and give assurance not only to increase the understanding but to also contribute to an effective control and management of these indigenous plant pathogens. This present work aimed at isolating and characterizing fungal pathogens observed on *T. esculentum* using molecular methodologies based on polymerase chain reaction (PCR) and sequence analysis of the ribosomal internal transcribed spacer (ITS).

MATERIALS AND METHODS

Sample collection

Samples of diseased Marama pods presenting necrotic spots with dense sporulation in the centre were collected at different locations in the Sandveld area in the Omaheke region (Eastern Namibia) during the rainy season (February and March 2008). They were kept in an ice packed cooler before being transported to the laboratory and kept at 4°C awaiting fungal isolation procedures (Figure 1).

Pure culture isolation

To obtain isolates from diseased pods, pieces of 0.5 x 0.5 cm were removed from the leading edge of the lesion; surface sterilized several times with 70% ethanol and washed three times with sterile distilled water. They were then plated onto Petri dishes containing

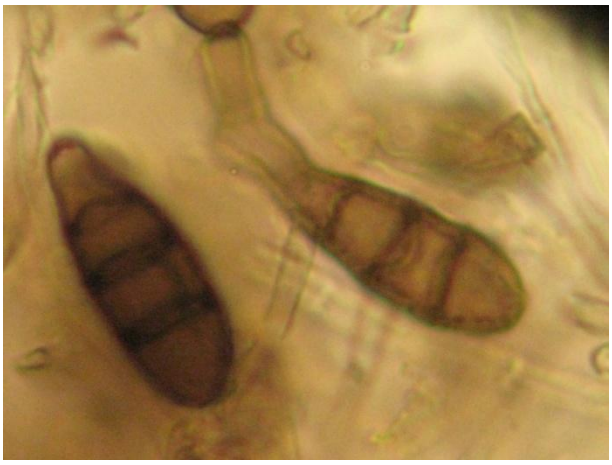

Figure 2. Club-shaped conidia of fungal isolates from marama pods.

potato dextrose agar (PDA). They were incubated at room temperature (25°C) under white fluorescent lights for 5 days. The hypha tips from the edges of each colony were inoculated to a new medium (PDA) and incubated at 25°C until pure cultures were obtained. Single spore cultures were made from the respective pure cultures. Two morphologically distinct colonies were obtained. They were all inoculated into sterile conical flasks containing malt extract broth (MEB) for 14 days until the mycelium has fully grown. The resulting mycelium was filtered in Buchner Funnel, dried for two days and collected on filter paper (Whatman 1) in the HEPA filtration cabinet.

DNA extraction

The genomic fungal DNA was isolated from 2 g of mycelium of individual isolates using the modified CTAB methods (Chimwamurombe and Kanyomeka, 2008). DNA samples were stored at 4°C for further use. DNA was resolved in 1% agarose gel in Tris-borate-EDTA (TBE) buffer and visualized by ethidium bromide staining and UV illumination.

PCR amplification

The ITS regions of the fungal isolates were amplified by PCR using the following primers ITS1 (5'TCCGTAGGTGAACCTGCGG3'), ITS2 (5'GCTGCGTTCTTCATCGATGC3') and ITS4 (5'TCCTCCGCTTATTGATATGC3'). Amplification reactions were performed in 12.5 µl of Go-green buffer (dNTPs, Taq polymerase, $MgCl_2$ and 10x PCR buffer), 2 µl of ITS1/2 or ITS1/4 primer pairs, 3 µl of double distilled water and 2 µl of the DNA template. The PCR amplification conditions consisted of an initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 65°C; the initial extension for 10 min at 72°C and finally 1 min at 72°C. The samples were then kept at 4°C awaiting downstream processing. The ITS amplicons PCR were held at 4°C. PCR amplified DNA was separated by electrophoresis in 1% agarose gel and viewed under ultraviolet (UV) light.

DNA sequencing and sequence analysis

DNA sequences of PCR amplified products were done by a commercial company using an automated sequencer ABI 377. Primers used for sequencing are ITS1/2 using forward and reverse primers

Six sequences of each isolate were obtained and were aligned. Then one of each was used for Basic Local Alignment Search Tool (BLAST) interrogation of the NCBI Sequence Database.

RESULTS

Cultural and morphological characterization of fungal isolates

Isolate I produced colonies that grew rapidly, flat spreading, powdery to velvety often largely submerged in the medium. At the start, the colour is always white and becomes olive brown at a later stage. The underneath of the mycelial mat looked dark brown to black. Club-shaped asexual spores or conidia were observed under microscope. They were pigmented and multi-celled (Figure 2).

Isolate II produced a flat colony with downy to wooly texture covered by grayish and short hyphae in time. At the beginning, the colony's surface looks grayish white which later darkens to greenish black to olive brownish spot like with a light border. The underneath of the mycelial mat looked dark brown to black due to pigment production.

Molecular characterization

DNA extracted from isolates I and II was amplified by PCR using ITS1 and ITS2 primers which resulted in expected amplicons (data not shown). These two products were sequenced in both directions and analyzed for homology with those of other species using BLAST. Percentage similarity with sequences in the NCBI database was determined (Figures 3 and 4)

Sequence analysis for homology of the ITS regions of Isolate I and II revealed high homology with the ITS region of rDNA from *Alternaria tenuissima* with 99% nucleotide homology for Isolate I while the isolate II showed the same percentage of 99% with *Phoma* sp. *Po72.*

DISCUSSION

In this study, the molecular characterization of the ITS regions of rDNA of fungi isolate I and II revealed that the association of the samples of diseased Marama pods presenting necrotic spots with dense sporulation in the centre that were collected at different locations in the Sandveld area in the Omaheke region (Eastern Namibia) are *A. tenuissima* as fungi I and *Phoma* spp. isolate po72 as fungi II. They both belong to the Ascomycota family. Both fungi were fast growing on PDA. Due to the high (99%) ITS sequence homology in both cases, the diagnosis that the fungi are *A. tenuissima* and *Phoma* spp. isolate po72 stands.

We cannot rule out the fact that these fungal species may be highly specific for the marama host. Previous

Fungus I

Alternaria tenuissima genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence.

Length =527 base-pairs

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Query 1      GCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTA 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject2   GCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTA 61

Query61     CTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTG 20
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject62   CTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTG  121

Query 121   CAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 122 CAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTG  181

Query    181  GCATCGATGAARAACGCAGC  200
              |||||||||| ||||||||
Subject  182  GCATCGATGAAGAACGCAGC  201
```

Figure 3. *Alternaria tenuissima* sequence aligned against the subject sequence in the Genbank database, with 99% similarity using BLAST searches program. The letter 'R' indicates the 1 mismatch of the sequences aligned.

Fungus II

Phoma sp. Po72 ITS1, 5.8S ribosomal RNA and ITS2, complete sequence

Length = 500 base-pairs

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Query    1    TAGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTTCGCCC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject  469  TAGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTTCGCCC  410

Query    61   GCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGT  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject  409  GCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGT  350

Query    121  TACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAARAACGCAG  171
              ||||||||||||||||||||||||||||||||||||||| |||||||
Subject  349  TACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG  299
```

Figure 4. *Phoma* sp. *Po72* sequence aligned against the subject sequence in the Genbank database, with 99% similarity using BLAST searches program. The letter 'R' indicates the 1 mismatch of the sequences aligned.

phylogenetic sequence analyses have shown that *Alternaria* spp. cluster together according to their conidia size and morphology (Mirkova and Konstantinova, 2003). The lack of a 100% sequence homology in our isolate can suggest that this particular isolate in our study may be highly specialized for marama bean host (Gannibal and Klemsdal, 2007). Besides, according to Pryor and Michailides (2002) and Simmons (1992), *Alternaria* spp, depending on cultural conditions and even within a single culture, can exhibit morphological plasticity which makes their classification very complicated. A more accurate species identification based on sporulating habits combined with sensitive molecular methods would clear this ambiguity. Taking into account the molecular analysis data, we can confirm that the isolate I infecting Marama is a strain of *A. tenuissima.*

Despite being a plant and human pathogen, *A. tenuissima* is a cosmopolitan and seasonal fungus that has developed a highly adjustable mechanism to different environmental stresses (Feng et al., 2007; Peever et al.,

2004; Rashman et al., 2002). To date, *A. tenuissima* is known to infect various parts of economically important plants belonging to different families and in different parts of the world (Mirkova and Konstantinova, 2003; Peever et al., 2004; Gannibal et al., 2007). For instance, Pryor and Michailides (2002) showed that *A. tenuissima* induces late blight in Pistachio in California (USA) while Serdani et al. (2002) established that it is a major cause of apple dry core rot in South Africa. According to Anderson et al. (2002), Gannibal and Klemsdal (2007) and Rotem (1994), it can infect cereal grains and be a source of food contamination. In humans, it appears to be a cutaneous opportunistic pathogen associated with the increasing use of immuno-suppressants, immunological impairment due to organ transplants or neoplastic diseases (Feng et al., 2007). Its pathogenicity is mainly associated with the production of melanin and host specific toxin (Kishore et al., 2005). It has been established that *A. tenuissima* plays a role in deteriorating and degrading synthetic polymeric material pollutants in the environment especially vulcanized rubbers considered as pollutants (Lugauskas et al., 2004).

Phoma is a ubiquitous ascomycota that include plant pathogenic and saprophytic fungi and contains more than 200 putative species found on plants and in the soil (Sutton, 1980). Their classification based on cultural morphology is a mammoth task. They can cause necrotic lesion on various plants that can range worldwide from potato, to rice, and to lupines (Reddy et al., 1996). Phoma is enhanced in wet and cool conditions (Phaneuf, 1998) which were prevailing in the area when the diseased pods were collected. In Namibia, in 1988, 10 million hectares of *Acacia melifera* were infected and dying back. The causative agent was later identified as four strains of the fungus *Phoma glomerata* (Schreuder, 1988).

In recent studies, it has been shown that endophytic fungi such as *Alernaria* sp. and *Phoma* sp. are neither incidental residents nor latent pathogens of the plant hosts but highly correlated with humidity and precipitation (Suryanarayanan et al., 2005). The Sandveld area known for its severe desert conditions had experienced particularly abundant rainfall which might have favored the dispersal of spore and infections when we collected the diseased Marama pods. Furthermore, marama grow in patches that can be far distant from each other and rainfall and humidity can often be unequally distributed in Kalahari Desert (Mendelsohn et al., 2002). This situation might have contributed to restriction of diseased pod to this area but further investigations are needed to confirm this.

During our study, we have only isolated two types of endophytic fungi in contrast to tropical and temperate species which can easily harbor 20 species (Suryanarayanan et al., 2005). The low diversity can be associated to the arid environment where marama occurs where low precipitations and persistent drought alternate in comparison to temperate and tropical areas. In addition, marama is drought avoiding plant that only spouts and accomplishes its life cycle during the rainy season. This adaptation constitutes a barrier that reduces the number of infections of endophytic and parasitic fungi (Suryanarayanan et al., 2005).

Conclusion

We isolated and identified two fungal species that infect marama seed pods. We, however, cannot conclude that these two isolates are equally pathogenic. Further studies of pathogenicity test (Koch postulates) are necessary to determine whether they are equally or differentially pathogenic on marama. We suggest that additional work should be done on both fungi and include an evaluation of larger samples sizes and isolates from different locations.

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REFERENCES

Agrios GN (1997). Plant Pathology. 4th Edition. Academic Press Inc, New York.

Amarteifio JO, Moholo D (1998). The chemical composition of four legumes consumed in Botswana. J. Food Compos. Anal. 11:329-332.

Anderson B, Kroger E, Roberts R.G (2002). Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. Mycol. Res. 106:170-182.

Bower N, Hertel K, Oh J, Storey R (1988). Nutritional evaluation of Marama bean (*Tylosema esculentum,* Fabaceae): Analysis of the Seed. Econ. Bot.. 42:533-540.

Castro S, Silveira P, Pereira C, Fiqueiredo E (2005). Systematic studies in *Tylosema esculentum* (Leguminosae). Bot. J. Linnaean Soc. 147:99-115.

Chimwamurombe PM, Kanyomeka L (2008). Investigation of report of *Ampelomyces sp*. associated with twig yellowing in Southern and Eastern Namibia occurring on Namibian *Acacia erioloba.* J. Pure Appl. Microbiol. 2 (2):333-336.

Feng F, Qiu D, Jiang L (2007). Isolation of cDNA sequences encoding the MAP kinase *HOG1* and the MAP kinase kinase *PBS2* genes of the fungus *Alternaria tenuissima* through a genetic approach. J. Microbiol. Methods 69:188-19.

Gannibal PB, Klemsdal SS (2007). AFLP analysis of Russian *Alternaria tenuissima* populations from wheat kernels and other hosts. Eur. J. Plant Pathol. 119:175-182.

Hartley ML, Tshamekeng E, Thomas SM (2002). Functional heterostyly in *Tylosema esculentum* (Caesalpinioideae). Annals of Botany 89:67-76.

Keegan AB, van Staden J (1981). Marama bean, *Tylosema esculentum*, a plant worth of cultivation. South Afr J. Sci. 77:387.

Kishore KH, Kanjila S, Minsra S, Reddy CR, Murty US (2005). Comparative chemical characterization of pigmented and less pigmented cell walls of *Alternaria tenuissima.* Current Microbiol. 51:399-401.

Lugauskas A, Prosychevas I, Jaskelevicius B (2004). Physical and chemical aspects of long-term bio-deterioration of some polymers and composites. Environ. Toxicol.19:318-328.

Mendelsohn J, Jarvis A, Roberts C, Robertson T (2002). Atlas of Namibia. A portrait of the land and its people. David Philip Publishers. Cape Town. South Africa.

Mirkova E, Konstantinova P (2003). First Report of *Alternaria* Leaf Spot on Gerbera *Gerbera jamesonii* H. Bolux ex J. D. Hook in Bulgaria. J. Phytopathol.151:323-328.

Mmonatau Y (2005). Flour from the Morama bean: Composition and sensory properties in a Botswana perspective. (Master's Thesis). University of Pretoria.

Morocko I (2006). Characterization of the strawberry pathogen *Gnomonia fragariae,* and Biocontrol possibilities (Ph.D. Thesis). Swedish University of Agricultural Sciences.

Musler DL, Schonfeld HC (2006). The nutrient content of Marama bean (*Tylosema esculentum*) and underutilized legume from Southern Africa. Agricola 16:7-13.

National Academy of Sciences (2006). Lost crops of Africa. Vol II: Vegetables .Development, Security, and Cooperation Policy and Global Affairs. National research Council of the National Academies. The National Academies Press. Washington, D.C.

Phaneuf E (1998). Description and study of a Phoma sp., a new fungal pathogen of lupines (Lupinus albus L.) in Québec (MSc. Thesis). McGill University.

Peever TL, Su G, Carpenter-Boggs L, Timmer LW (2004). Molecular systematic of citrus-associated *Alternaria* species. Mycologia 96:119-134.

Pryor BM, Michailides TJ (2002). Morphological, pathogenic and molecular characterization of *Alternaria* isolates associated with late blight of Pistachio. Phytopathology 92:406-416.

Rashman MZ, Honda Y, Arase S (2002). Red-light-induced Resistance in Broad bean (*Vicia faba*,L) to leaf spot disease caused by *Alternaria tenuissima. J*. Phytopathol. 151:86-91.

Reddy MV, Atlin G, Paulitz TC (1996). Response of White lupine cultivars to *Phoma sp.* and *Colletotrichum gloeosporioides.* Canadian J. Plant Pathol. 18:278-278.

Ridley GS, Bain J, Bulman LS, Dick MA, Kay MK (2000). *Threats to New Zealand's indigenous forests from exotic pathogens and pests*. (Science for Conservation No. 142). Department of Conservation, Wellington, New Zealand.

Rotem J (1994). The genus *Alternaria*. Biology, epidemiology and pathogenecity. St. Paul Minnesota: APS Press.

Serdani M, Kang JC, Andersen B, Crous PW (2002). Characterization of Alternaria species-groups associated with core rot of apple of South Africa. Mycol. Res. 106:561-569.

Schreuder W (1988). Dieback of blackthorn (*Acacia mellifera* subsp. *detinens*) in South West Africa (MSc. Agric. Thesis). University of Stellenbosch.

Simmons EG (1992). *Alternaria* taxonomy: Current status, viewpoint, challenge. In: *Alernaria* Biology, Plant Diseases and Metabolites. Chelkowski J and Visconti A (Eds). Elsevier Science Publishers, Amsterdam.

Suryanarayanan TS, Wittlinger SK, Faeth S (2005). Endophytic fungi associated with cacti in Arizona. Mycol. Res. 109:635-639.

Sutton BC (1980). The coelomycetes. Kew, United Kingdom: Common wealth Mycological Institute.