Studies on *Ceratocystis* spp. associated with mango die-back in Oman

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by

Ali Obaid Al Adawi

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Supervisor:	Prof. Michael J. Wingfield
Co-supervisors:	Prof. Brenda D. Wingfield
	Prof. Michael Deadman
	Dr. Irene Barnes

DECLERATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Philosophiae Doctor* to the University of Pretoria, contains my own independent work. This work has hitherto not been submitted for any degree at any other University.

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Ali Obaid Al Adawi

November 2011

DEDICATED TO

My father and my wife Hoda

TABLE OF CONTENTS

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PREFACE

Mango wilt disease was first reported in Brazil in 1938 causing devastating damage to mango trees. After 60 years, the disease was reported in Oman followed by confirmed reports in Pakistan and United Arab Emirates. The disease has consequently become a major concern in mango growing countries in the Middle East. In early investigations, many difficulties were experienced diagnosing the cause of mango wilt disease in Oman. However, improved isolation techniques and the ability to apply molecular diagnostic methods have facilitated the identification of the pathogen, now recognized as *Ceratocytis manginecans*. This thesis provides the results of the first studies to better understand mango wilt disease in Oman and Pakistan.

The first chapter of this thesis provides a review of mango wilt disease wherever it occurs in the world. This chapter commences with a summary regarding mango production around the world with special attention being given to the situation in Oman. Details dealing with previous investigations on mango wilt disease including disease symptoms, aetiology, epidemiology and mode of disease dispersal are also provided. Furthermore, the taxonomy of *Ceratocystis* spp. associated with mango wilt disease is discussed and possibility of species associated of mango wilt disease jumping to co-geographic hosts is considered.

In second chapter, the incidence of mango wilt in Oman was examined in several areas in Al Batinah region. In addition, the aetiology of mango wilt disease was considered by collecting samples from infected mango trees and bark beetles associated with the disease and isolation from these samples using different methods developed to isolate primary pathogens. Two different *Ceratocystis* species were isolated during course of this study. In two related and published studies outside this thesis, these species were identified and characterized using morphology and DNA sequencing techniques. They were identified as *Ceratocystis*

manginecans and *Ceratocystis omanensis*, the former was shown to cause mango wilt disease by satistfying Koch's postulates.

The role of the bark beetle *Hypocryphalus mangiferae* in mango wilt disease was examined in chapter three of this thesis. Surveys were conducted in two areas in Al Batinah region to investigate the extent of bark beetle association with mango wilt disease. Furthermore, living specimens of *H. mangiferae* were tested for their ability to infest healthy mango seedlings and thus to inoculate the mango wilt pathogen into these seedlings and result in disease.

The fourth chapter of this thesis deals with the population structure of *C. manginecans* associated with mango wilt disease in Oman and Pakistan. Ninetysix isolates of *C. manginecans* associated with mango wilt disease and *H. mangiferae* were collected from different areas in both countries. Two sets of microsatellite primers were employed to determine whether the pathogen is native in the area or an alien invasive in both countries.

Concurrent with epidemic of mango wilt disease, two wilt diseases emerged associated with Ghaf (*Prosopis cineraria*) and shisham (*Dalbergia sissoo*) trees in Oman and Pakistan. A *Ceratocystis* sp. associated with both of these leguminous hosts was compared with isolates of mango wilt pathogen (*C. manginecans*) based on morphological and DNA sequence comparisons. In addition, inoculation experiments were undertaken to determine whether the pathogen could infect the various hosts.

The last chapter of this thesis focussed on obtaining genetic mango genetic stock resistant to the mango wilt pathogen. This is considered to be the single most critical option in disease management. Thirty mango cultivars were assessed for resistance to infection by *C. manginecans* using three inoculation trials. Furthermore, performance of some of these

cultivars in the face of natural mango wilt disease infection was examined during an epidemic of mango wilt disease in two areas in Al Batinah region.

The studies presented in this thesis are the first to be undertaken on mango wilt disease after *C. manginecans* was discovered and shown to cause the disease in Oman. These studies will hopefully have contributed significantly to a better understanding of this devastating disease. Furthermore, they will hopefully also lead to improved management options and reduced losses in the future.

CHAPTER 1

Mango wilt disease: A serious threat to the mango industry worldwide

1.1. INTRODUCTION

Mango (*Mangifera indica* L.) is a member of the Ancaradiaceae that includes 73 genera (Bompard 2009). Mango is an important fruit crop in the tropical and subtropical areas of the world. Southeast Asia or the Malay Archipelago are believed to represent the centre of origin of mango (Mukherjee & Litz 2009). The tree has been cultivated in India for an estimated 4000 years (Mukherjee & Litz 2009) and is evergreen and can live for more than a hundred years. The height of fully mature trees ranges between 3 - 10 metres and in some situations they can reach over 40 m (Mukherjee & Litz 2009; Bally 2006).

Mango cultivars can either be monoembryonic, where the seeds have a single embryo, or polyembryonic, where the seeds produce more than one embryo. The progeny from monoembryonic cultivars have characters different from those of the mother as all of their embryos are zygotic. Seeds of polyembryonic cultivars contain one zygotic embryo and the rest of the embryos produce true to-type progeny via asexual reproduction (Bally 2006). Monoembryonic mango varieties are found mainly in India while polyembryonic varieties have a south-East Asian origin. Many polyembryonic mango cultivars are characterized by fruits that are green when ripe in contrast to monoembryonic cultivars that have fruits with highly coloured skins (Morton 1987; Mukherjee & Litz 2009).

In India, there are more than 1000 mango cultivars. Many of these have different common names but their morphological characteristics are identical (Morton 1987; Negi 2000). Of these, only 24 cultivars are regarded as being of commercial value and they are grown widely in India and in many other countries around the world. These cultivars are grouped into those that fruit early in the season (e.g. Pairi, Suvarnarekha), early to mid season cultivars (e.g. Langra, Rajapuri), mid season (e.g. Alampur Baneshan, Alphonso, Bangalora, Banganapally, Dasheheri) and late season cultivars (e.g. Fazli, Mulgoa, Neelum) (Morton

1987; Mukherjee & Litz 2009; Knight *et al*. 2009). Additional information regarding the origin of approximately 63 different mango cultivars of superior quality, their different common names and current distribution have been provided by various authors (Morton 1987; Knight *et al*. 2009; Mukherjee & Litz 2009).

Introductions of mango trees from India to East Africa are believed to have been by the Portuguese during the 15th and 16th centuries (Mukherjee & Litz 2009). However, the role of the Persians and Arabians, especially the Omani traders in introducing mangoes to Africa during the 10th Century might have been overlooked (Purseglove 1972; Knight & Schnell 1993; Iyer & Schnell 2009). This is supported by the fact that mango trees were already present in East Africa during the 14th century. The traveller, Ibn Batuta (1325-1349 AD), described the role of mango fruits in the diets of rich householders in Mogadishu. Given the fact that the shelf life of mango fruits is a maximum of about two weeks, and the difficulty of travelling during 14th Century, it might be concluded that mango trees were either grown in these areas or fruit were only transported short distances from surrounding areas (Garu *et al*. 1995; Iyer & Schnell 2009).

Mango trees were introduced into Brazil from West Africa during the 16th and 17th centuries by the Portuguese and were further spread from Brazil to the West Indies during the 18th century (Mukherjee & Litz 2009). The Spanish introduced polyembryonic mango cultivars from the Philippines into Mexico in the early 19th century (Morton, 1987). In 1861, a polyembryonic mango cultivar was introduced into Florida, USA, from Cuba and this represented the first mango introduction into the USA (Morton, 1987). This was followed by multiple introductions of mango into Florida from India and the West Indies during the 19th and 20th centuries. All these introductions led to the development of mango cultivation in

Florida and the emergence of new hybrid cultivars resulting from crosses between various cultivars (Iyer & Schnell 2009).

Recently, molecular analysis using microsatellite markers has made it easier to trace the origins of mango hybrid crosses. For example, it was shown that “Turpentine”, the polyembryonic cultivar originally from West Indies is the paternal parent of the Haden variety while Mulgoa, from India, is the maternal parent. Furthermore, Haden is considered the parent of several commercial cultivars such as Cogshal, Eldon, Glenn, Lippens, Osteen, Parvin, Palmer, Smith, Springfels, Tommy Atkins and Zill (Campbell 1992; Knight & Schnell 1993; Schnell *et al*. 2006). The variety Haden and its progeney have become very popular and are distributed and planted in many parts of the world. This variety was introduced into Brazil from Florida in 1931 and is now widely cultivated in that country (Morton 1987). Haden is unfortunately also considered amongst the most susceptible varieties to the fungal pathogen, *Ceratocystis fimbriata sensu lato* (*s.l*), the causal agent of mango wilt disease in Brazil (Rossetto *et al*. 1996a).

According to FAO (FAO STAT 2008), mango ranked fifth in total production amongst major fruit crops worldwide. World production of mango exceeded 28 million tons in 2008. India is one of the largest producers with an annual production of over 13.6 t, representing 40% of the world’s production. Other major mango producers include China (3,976,716 t), Thailand (2,374,165 t), Indonesia (2,013,123 t), Mexico (1,855,359 t), Pakistan (1,753,686 t), Brazil (1,154,649 t), Philippines (884,011 t), Bangladesh (802,750 t), Nigeria (734,000 t), Egypt (466,436 t) and Yemen (387,906 t).

This review provides a brief introduction to mango cultivation in Oman prior to the emergence of mango wilt disease. This is followed by a comprehensive review of mango wilt disease in terms of aetiology, economic impact, epidemiology and management. The review

also includes an examination of the current taxonomic status of *C. fimbriata s.l*, its population biology and the role of bark beetles in fungal dissemination.

1.2. MANGO CULTIVATION IN OMAN

1.2.1. History

The history of Oman is deeply rooted in it's connection with other countries linked to its ancient trading routes. Ancient cultivation of annual and perennial crops seen across the country is testament to a long history of importation of germplasm from distant countries via these trading routes (Hammer *et al*. 2009). Wheat, for example, is an ancient crop in Oman that was introduced around 3000 BC. The long history of cultivated crops in Oman can also be seen from the diversity of other cultivated crops such as barley (*Hordeum vulgare* L. *s.l.*), date palm (*Phoenix dactylifera* L.), field bean (*Vicia faba* L.) and mango (Hammer *et al*. 2009).

Mango was first brought into Oman from India around 1568-1575 AD when it was planted in the Maqnaiat area in Wilyat Ibri in the Dhairaya region (Al Salmi 1997; Al Busaidi 2008). The introduction of mango germplasm from India and other countries continued through trading routes and resulted in the extension of the mango growing areas in Oman (Miles 1896; Miles 1910). Many travellers including Kaempfer (1688), Miles (1896; 1910) and Zwemer (1902) briefly described the cultivation of mango in Oman. In the early periods of mango cultivation, many of the introduced varieties were of good quality and had desirable tastes (Wisegerber 1984). Before the first appearance of mango wilt disease in 1998, many ancient and very large mango trees could be found around the country, especially in the Quriayat and Sohar areas (Miles 1896; Zwemer 1902).

1.2.2. Planted areas and production

Mango is one of the most important fruit crops in Oman and is ranked fourth in the area of cultivation after date palm, citrus and banana. The total population of mango trees in Oman was estimated at around 493,568 trees in 2005 among these, 93,000 were found in the yards of private homes (Ministry of Agriculture and Fisheries 2007; 2009a).

According to the 2004/2005 census, 75% of mango cultivation was concentrated along the northern coast of the country in the Batinah region followed by the Sharqiya region (7.9%), Dahira (7%), Muscat (4.8%), Dakhilya (2.6%), Musandam (2.4%) and Dhofar (0.2%). Mango is grown all over the Batinah region, but mainly in the coastal areas of Sohar (228 ha), Shinas (125 ha), Barka (109 ha), Suwaiq (94 ha), Saham (91 ha) and Khabourah (43 ha) (MAF 2009a).

In 2008, mango production in Oman was estimated at 10,000 ton with a value over 3.5 million Omani Rials (1 R.O ≈ 2.5 US$). The average mango production ranged from 6 to 9 tons per hectare (MAF 2009b). Most of the mangoes produced were consumed locally and around 100 tons (1%) was exported to the Gulf countries (Royal Oman Police (ROP) 2008). However, due to the increase in mango consumption from 3 kg per capita in 1998 to 10 kg in 2008, mango has had to be imported into the country (MAF 2009b; Ministry of National Economy (MNE) 2004; ROP 2002; 2004; 2006; 2008). Mango fruit is mainly imported from countries such as India, Pakistan, South Africa, Australia, Thailand, Indonesia and Kenya (ROP 2002; 2004; 2006; 2008).

1.2.3. Propagation and cultivars

The mango germplasm that was introduced into Oman (a long time ago) was almost all of the Indian monoembryonic type. The generations of progeny from these mango trees

varied greatly due to uncontrolled pollination between different mango varieties. In general, the indigenous mango varieties today are very large trees, non-regular fruit bearing and usually fibrous, insipid in flavour and with a sub-acid to acid taste (MAF 1990).

Evaluation programmes to test mango varieties have been conducted since the late 1970's. Several local mango varieties with very good taste were selected for propagation and testing in an evaluation programme (Al Jabri 2003). Furthermore, 22 mango varieties obtained mainly from India and some from Egypt were evaluated based on the quantity and quality of fruit production (MAF 1983b). Early evaluation considered Langra, Dushahri, Alfounso and Hindi Businarah as excellent mango cultivars for Oman (MAF 1990). More extensive evaluations later suggested that the Baneshan, Allumpur Baneshan, Rose, Neelum, Pairi, Zafran and Mulgoa cultivars were of good quality with variability in fruit production (MAF 1983b; 1990; 1991; 1992). Among those cultivars, 16 with good yield and quality were propagated through grafting over rootstocks and distributed to farmers (MAF 1989). Previously, mango trees were propagated through direct planting of seeds of unknown monoembryonic type trees. Since the 1980s, mango trees were mostly propagated through grafting selected mango varieties onto local rootstocks. On a yearly basis since 1993, the Ministry of Agriculture and Fisheries has distributed over fifteen thousand grafted mango plants to farmers, free of charge and in order to encourage farmers to grow selected mango cultivars with high yield and quality.

1.2.4. Pests

Mango trees are attacked by many pests and diseases. More than twenty three species of insects belonging to nineteen families have been reported associated with mango trees in Oman (MAF 1992). The most economically important pests recorded in Oman are fruit fly (*Bactrocera dorsalis*) (Diptera: Tephritidae), thrips (*Frankliniella* sp.) (Thysanoptera:

Thripidae), the leaf gall midge (*Procontarinia matteiana*) (Diptera: Cecidomyiidae), the stem borer (*Sphenoptera arabica*), mites (*Aceria mangiferae*) (Acari: Eriophyidae) and the most recently recorded bark beetle (*Hypocryphalus mangiferae*) (Coleoptera: Scolytid) (MAF 1983a; MAF 1992; Al Adawi *et al*. 2006).

Control measures for insects are usually applied when their populations reach an economic threshold and different management combinations are used for each mango pest. For example, management of the mango fruit fly (*B. dorsalis*) includes using pheromone traps and protective spraying with chemicals during the fruiting stages (MAF 1989). However, mechanical and chemical control of the mango leaf gall midge (*P. Matteiana*) has been found to be ineffective due to the overlapping generations throughout the year (Sankaran & Mjeni 1989). Subsequent to 1984, the parasitoid (*Chrysonotomyia pulcherrima*) was introduced from India and released in Oman as a biological control agent to reduce the leaf gall midge infestation (Sankaran & Mjeni 1989; MAF 1993).

1.2.5. Diseases

Several important diseases are associated with mango trees in Oman. These include die-back (*Lasiodiplodia theobromae*) (Waller & Bridge 1978; Moghal *et al*. 1993), mango malformation (*Fusarium mangiferae*) (Kvas *et al*. 2008), anthracnose (*Colletotrichum gloeosporioides*) (Moghal *et al*. 1993); bacterial black spot (*Xanthomonas axonopodis* pv. *mangiferaeindicae*) (Al Rawahi *et al*. 1998) and the topic of this thesis, *Ceratocystis* wilt (*C. fimbriata s.l.*).

Besides mango trees, *L. theobromae* has been reported associated with over twenty-six different host plants in Oman causing mainly symptoms of die-back disease (Waller & Bridge 1978; Moghal *et al*. 1993). Recently, malformed inflorescences of the mango flowers

with enlarged and many branched panicles were reported in Oman (Kvas *et al*. 2008). The causal agent was identified as *F. mangiferae* based on morphology and DNA sequence comparisons (Kvas *et al*. 2008). Anthracnose (*Colletotrichum gloeosporioides*) is frequently observed in the Batinah region causing moderate losses of mango trees. This pathogen causes damage to the inflorescences, leaves and fruit (Moghal *et al*. 1993). Bacterial black spot (*Xanthomonas axonopodis* pv. *mangiferaeindicae*) has been reported in Oman to cause mango fruit lesions (Al Rawahi *et al*. 1998).

A variety of management strategies can be used to control these diseases including pruning of affected inflorescence and branches, spraying systemic or copper chemicals and planting resistant mango cultivars (Ploetz & Prakash 1997). The application of any of these control strategies depends on the type of disease and the age of the mango organ affected. Combinations of control measures are commonly necessary, to reduce the impact of these diseases of mango trees and their impact on mango production (Ploetz & Freeman 2009).

1.3. MANGO WILT DISEASE

Mango wilt disease was first reported relatively recently from Oman killing thousands of mango trees (Al Adawi 2002; Al Adawi *et al*. 2003; Al Adawi *et al*. 2006). Initially, mango wilt disease was reported in only a few trees in the Barka area in the south of the Al Batinah region in 1998. By 2002, the disease epidemic had covered the whole of the Al Batinah region with differences in disease incidence occurring amongst areas.

1.3.1. Disease history

Mango wilt disease with symptoms similar to those recorded in Oman, Pakistan and UAE was first reported in 1938 in Recife in the state of Pernambuco in Northeast Brazil

(Pyenson 1938). After four years, similar disease symptoms were detected in the state of São Paulo. In that country, the disease is locally known as 'seca', murch or mango blight disease (Viegas 1960; Rossetto *et al*. 1980; Ribiero 1980; Ploetz & Prakash 1997; Ploetz 2003).

Mango decline disease has been reported to be a serious and destructive disease in India, since 1939 (Das Gupta & Zachariah 1945) and in other mango growing countries of the world (for comprehensive reviews see Ploetz & Prakash 1997; Ploetz 2003). The symptoms of diseased mango reported from India are typical to what has been reported in Oman, Pakistan and Brazil. Reference to the disease as 'dieback' in these countries has caused some confusion but the term is clearly used in a wide sense in India and Pakistan, which not only includes the mango disease but also a similar wilt disease of shisham (*Dalbergia sisso*) (Prakash & Srivastava 1987; Khanzada *et al*. 2004). For the purpose of this review, we refer to the disease as "mango wilt". The disease has become severe in mango orchards of the Western Uttar Pradesh (U.P.), which is the worst affected state in India. In this area, about 30-40% of the orchards are found to be affected with this disease, creating a serious problem in the region (Prakash & Srivastava 1987; Ploetz & Prakash 1997; Ploetz & Freeman 2009).

1.3.2. Symptoms

Usually disease symptoms on mango begin with 1 mm shot-hole signs in the trunks of healthy trees. This is an indication of infestation by the bark beetle, *H. mangiferae*, burrowing into the bark of the mango trees (Fahim *et al*. 2002; Al-Adawi 2002; Al-Adawi *et al*. 2006; see Chapter 2). Subsequently, fungal infection becomes established in susceptible trees through the entry holes and wounds created by *H. mangiferae*. This is normally followed by mass attack of bark beetles (*H. mangiferae*) in response to the pheromones released by the pioneer beetles (Byers 2000). Shortly after infestation, the trees normally start to exude an amber coloured gum that turns a dark reddish colour. The amount of gum exudation varies

between mango cultivars. When the bark is peeled back from the stems of affected trees, a brown to dark brown and streaked discolouration can be observed in the vascular tissues (Al-Adawi 2002; Al-Adawi *et al*. 2006; see Chapter 2). The disease symptoms include wilt of parts, or the entire tree. Partial wilt symptoms are usually reported in local mango cultivars propagated directly from seeds or on scion cultivars susceptible to the wilt disease pathogen (Al Adawi *unpublished data*). Sudden wilt of the whole mango tree is usually noticed in grafted trees. This is as a result of the tolerant or resistant scion variety propagated on a susceptible rootstock (Al Adawi *unpublished data*). In grafted trees, disease is reflected by vascular discoloration that is restricted to the rootstock and lower levels or no vascular discolouration symptoms are seen in the scion. Due to severe infection in the rootstock part, wilt of the entire tree canopy can occur in a short period of time.

1.3.3. Aetiology

Initially, when mango wilt disease first appeared in Brazil, it was assumed that *Diplodia recifensis* Batista (morphologically similar to *L. theobromae*) was the primary causal agent (Batista 1947). In India, mango decline disease was attributed to *L. theobromae* with high temperature as an important predisposing factor (Das Gupta & Zachariah 1945). However, the predominant isolation of *L. theobromae* from diseased mango trees with similar symptoms to those mentioned above, was misleading in several cases where mango wilting had been reported (Batista 1947; Al Adawi 2002; Khanzada *et. al*. 2004). A re-examination of the disease aetiology and re-isolations of diseased material recovered a *Ceratocystis* species from mango trees affected with the disease in Brazil, Oman and Pakistan (Ribiero 1980; Malik *et al*. 2005; Al-Adawi *et al*. 2006).

1.3.4. Characterization of *Ceratocystis* spp. associated with mango wilt disease

In order to control or manage diseases caused bypathogens, it is important to understand the evolutionary biology of the causal agents (McDonald & Linde 2002; McDonald 2004). This involves investigations concerning macro-evolution through phylogenetic analyses and micro-evolution through population genetics (Taylor *et al*. 1999; McDonald & Linde 2002; McDonald 2004). Data inference from phylogenetic and population genetics will help to elucidate the precise identity of the pathogen isolated and this will indicate if the pathogen outbreak is the result of alien invasion or whether it has arisen from a host jump from a residential species (Taylor *et al*. 1999; McDonald & Linde 2002; Ploetz 2007). In the following section, the current status of the taxonomy of the genus *Ceratocystis* is briefly reviewed. In addition, knowledge regarding the phylogenetic and population genetics of *Ceratocystis* species in the Fimbriata clade; a group that includes several species associated with mango wilt disease will also be considered.

1.3.4.1. Taxonomy of *Ceratocystis* spp.

The genus *Ceratocystis* is a member of the Ascomycota and resides in the Microascales (Zhang *et al*. 2006; Schoch *et al*. 2009). *Ceratocystis* includes saprotrophic and pathogenic species and infection is mainly associated with wounds on plants (Kile 1993). Species of this genus are characterized by globose ascomatal bases with long necks (Zhang *et al*. 2006) and sticky exudates of ascospore droplets from the tips of the ascomatal necks (Ingold 1961; Upadhyay 1981; Wingfield *et al.* 1993; Roux & Wingfield 2009). Previously, the identification of *Ceratocystis* species was mainly based on morphological features (Hunt 1956; Upadhyay 1981; Wingfield *et al.* 1993).

Contemporary taxonomic treatments based on DNA sequence analyses support the delineation of *Ceratocystis* into at least three distinct lineages or clades (Wingfield *et al*. 2006; Harrington 2009). These include the *Ceratocystis coerulescens* clade, the *Ceratocystis moniliformis* clade and the *Ceratocystis fimbriata* clade. Member of the clades are easily recognised by their morphology, ecological niches and DNA sequence data (Wingfield *et al*. 2006; Harrington 2009). The *Ceratocystis* species in the Coerulescens and Moniliformis clades, with the exception of *C. fagacearum* (Bretz) Hunt, do not produce chlamydospores and those in the Moniliformis clade have spines on their ascomatal bases (Wingfield *et al*. 1997; Harrington & Wingfield 1998; Van Wyk *et al*. 2006a; Harrington 2009). Species that reside in the *C. fimbriata* complex produce chlamydospores and they lack spines on their bases (Webster & Butler 1967a; Upadhyay 1981; Wingfield *et al*. 1993). The species within the *C. fimbriata* and *C. moniliformis* clades are characterised by having hatshaped ascospores (Upadhyay 1981; Wingfield *et al*. 1993) and produce fruity aromas (Malloch & Blackwell 1993; Hanssen 1993). Consequently, species in these two groups attract casual insects forming loose associations (Moller & De Vay 1968b; Malloch & Blackwell 1993; Kile 1993; Heath *et al*. 2009b). Species in the *C. coerulescens* clade do not produce fruity aromas and form mutulistic relationships with bark beetles aiding their dispersal (Harrington & Wingfield 1998; Heath *et al*. 2009b). The *C. moniliformis* clade includes wound inhabiting saprophytic or weak pathogenic species that affect mainly gymnosperms and are associated with sap stain diseases (Kile 1993; Witthuhn *et al*. 1998; 1999; Van Wyk *et al*. 2006a; Heath *et al*. 2009a).

The species in the *C. fimbriata* group include mainly pathogenic species of world wide economic importance causing diseases mainly in angiosperms (Kile 1993; CAB International 2001). Examples of pathogenic species belonging to this group include *C. fimbriata* Ellis & Halsted *sensu stricto* (*s.s*) which was the first species described in this

genus and causes black rot disease in sweet potato (*Ipomaea batatas*) and is designated as the type species of the genus *Ceratocystis* (Halsted & Fairchild 1891; Engelbrecht & Harrington 2005), *C. albifundus* M.J. Wingf., De Beer & Morris, causing wilt disease of *Acacia mearnsii*, *C. cacaofunsta* Engelbrecht & Harrington, a serious wilt disease of *Theobroma cacao*, and *C. platani* Engelbrecht & Harrington, the causal agent of a canker wilt disease of *Platanus* sp. (Wingfield *et al.* 1996; Engelbrecht & Harrington 2005).

The type species of *Ceratocystis fimbriata* falls within the Fimbriata clade. Since it was first reported on sweet potato in the United States, many strains of *C. fimbriata* have been reported from a broad host range in many parts of the world causing wilts, vascular discolouration, canker, stem and root rot disease symptoms (Kile 1993; CAB International 2001; Roux & Wingfield 2009). Based on host specialization of some of these isolates from different hosts and geographical areas, but especially based on phylogenetic inference, it has been recognised that *C. fimbriata s.l.* encompasses many cryptic species that are not clearly distinguishable based on morphology (Webster & Butler 1967a; Barnes *et al*. 2001; Baker *et al*. 2003; Barnes *et al*. 2003; Engelbrecht & Harrington 2005; Van Wyk *et al*. 2006c). Recent molecular techniques have allowed researchers to resolve many species from the *C. fimbriata s.l.* complex (Wingfield *et al*. 1996; Barnes *et al*. 2003; Engelbrecht & Harrington 2005; Johnson *et al*. 2005; Van Wyk *et al*. 2007a & b; Rodas *et al*. 2008; Van Wyk *et al*. 2009a & b; Van Wyk *et al*. 2010; Van Wyk *et al*. 2011 a & b). Species that have recently been described in the *C. fimbriata s.l* complex and their hosts are listed in Table 3.

1.3.4.2. *Ceratocystis* species associated with mango wilt disease

In Oman, isolations from mango trees affected with mango wilt disease yielded two species of *Ceratocystis* in addition to *Lasiodiploida theobromae* (Al Adawi *et al*. 2006). Based on morphological characteristics and DNA sequence comparisons for the internal

transcribed spacer region (ITS), beta-tublin (BT) and translation elongation factor (TEF) gene regions, both *Ceratocystis* species were shown to be new to science (Al Subhi *et al*. 2006; Van Wyk *et al*. 2007a). One species was named *Ceratocystis omanensis* Al Subhi, M.J. Wingf. M. Van Wyk & Deadman and resides in the *Ceratocystis moniliformis* clade, which is recognized to include non-pathogenic species (Al Subhi *et al*. 2006; Al Adawi *et al*. 2006). The second species resolved in the *Ceratocystis fimbriata s.l* clade and was named *Ceratocystis manginecans* M. Van Wyk, A Al-Adawi, & M.J. Wingf (Van Wyk *et al*. 2007a). *C. omanensis* and *C. manginecans* can be easily differentiated based on differences in culture growth and various morphological characteristics (Table 4). *C. omanensis* was isolated only from diseased mango trees in Oman. Both *C. manginecans* and *L. theobromae* on the other hand, were recovered from diseased mango tissues and directly from bark beetles (*H. mangiferae*) associated with the mango wilt disease in Oman and Pakistan (Al Adawi *et al*. 2006; Van Wyk *et al*. 2007a). Inoculation trials on mango seedlings conducted in Brazil, Oman and Pakistan proved that *L. theobromae* is a weak pathogen and that it is *C. manginecans* in Oman or *C. fimbriata s.l* in Brazil that are responsible for mango wilt disease (Batista 1947; Ribiero 1980; Al Adawi *et al*. 2006; Malik *et al*. 2010).

The causal agent of mango blight disease in Brazil has been attributed to *Ceratocystis fimbriata s.l* (Ribeiro 1980). This pathogen was first reported in Brazil in 1934 causing wilt disease in *Crotalaria juncea* (Costa & Krug 1935). Morphological, molecular and population data for available isolates of *C. fimbriata* from Brazil have shown that the Brazilian mango isolates represent distinct taxa, but are closely related to *C. manginecans* (Van Wyk *et al*. 2007a; Ferreira *et al*. 2010). Recent molecular analysis of a Brazilian *C. fimbriata s.l* population isolated from various hosts, including mango, revealed a highly differentiated population within mango isolates (Ferreira *et al*. 2010; Harrington *et al*. 2011). Further

characterization of *Ceratocystis* mango isolates from Sao Paulo in Brazil, revealed the presence of two new species that are now treated as *C. mangicola* M Van Wyk & MJ Wingf. sp. nov. and *C. mangivora* M Van Wyk & MJ Wingf. sp. nov. (Van Wyk *et al*. 2011).

1.3.4.3. Host specificity of *Ceratocystis* species within the Fimbriata clade

Several studies have demonstrated that various species within the *C. fimbriata* clade are restricted to specific host plants while others are not. Host specificity has been shown for *C. fimbriata* isolates from sweet potato, rubber, coffee, sycamore and cacao on their respective hosts (Olson & Martin 1949; Pontis 1951; Ribeiro & Coral 1968; Coral *et al*. 1984; Ribeiro 1993; Baker *et al*. 2003). However, host specialization is apparently not the case for all species in the *C. fimbriata s.l*. group (Ribeiro *et al*. 1987; Ribeiro *et al*. 1988; Vogelzang & Scott 1990; Baker *et al*. 2003; Silveira *et al*. 2006; Van Wyk *et al*. 2010). In cross inoculation experiments in Brazil, isolates of *C. fimbriata s.l* from *Cassia renigera* and mango were found pathogenic on a wide host range including: *Acacia decurrens*, *Crotalaria juncea*, *Cajanus cajan*, *Ficus carica*, *Mangifera indica*, *Hevea brasiliensis*, *Cassia* sp., *C. carnaval*, *C. ferruginea*, *C. moschata*, *C. multijuga*, *C. nodosa*, *C. renigera*, *C. siamea* and *C. speciosa*. These same isolates were, however, non-pathogenic on *Theobroma cacao*, *Gmelina arborea* and *C. grandis* (Ribeiro *et al*. 1987). Cross inoculation between isolates of *C. fimbriata* from mango and *Acacia decurrens* showed that both isolates were pathogenic on both hosts (Ribeiro *et al*. 1988). An isolate of *C. fimbriata* associated with basal rot of *Syngonium* was also found to be pathogenic on *Caladium*, *Aiocasia*, *Crotalaria* and *Xanthosoma* spp. (Vogelzang & Scott 1990). Isolates of *C. fimbriata* from Eucalyptus were found also to be pathogenic on mango and *Crotalaria juncea* (Baker *et al*. 2003). In addition, *C. fimbriata s.l* isolates associated with sugar apple (*Annona squamosa*) blight in Brazil were reported as able to cause similar disease symptoms in both sugar apple and mango trees

(Silveira *et al*. 2006) and isolates from sugar apple and mango have similar morphology and identical ITS sequences (Silveira *et al*. 2006).

Recent population analyses using microsatellite primers and phlyogenetic analysis using ITS-rDNA and MAT1-2 sequences showed extensive variation within Brazilian *C. fimbriata s.l* isolated from different hosts including mango trees (Ferreira *et al*. 2010; Harrington *et al*. 2011). Furthermore, inoculation experiments using Brazilian *C. fimbriata s.l* isolates from mango, fig (*Ficus carica*), *Eucalyptus* sp., inhame (*Colocasia esculenta*) and *Gmelina arborea* showed high variability in their aggressiveness within and among populations when cross inoculated into cultivated hosts or relatives of native hosts species (Harrington *et al*. 2011). Therefore, there is no evidence of host specificity within Brazilian *C. fimbriata s.l* populations collected from various hosts (Harrington *et al*. 2011). Recently, *C. manginecans* was isolated in Indonesia associated with wilt and die-back disease of *Acacia mangium* (Tarigan *et al*. 2011). This represents the first report of *C. manginecans* on a host other than mango. All of the above mentioned evidence illustrates lack of strict host specificity in *C. fimbriata s.l* and *C. manginecans* and thus their potential to jump hosts (Slippers *et al*. 2005).

Generally, the emergence of a new infectious disease such as those caused by species of *C. fimbriata s.l.* on a specific host plant is caused by anthropogenic introduction, climate change, changes in farming techniques, or recombination (Anderson *et al*. 2004). Once the epidemic of a newly emerged disease is established, the associated pathogen might also cause devastating damage to wild or cultivated sympatric hosts through host jump (Roy 2001; Anderson *et al*. 2004; Slippers *et al*. 2005; Woolhouse *et al*. 2005). Therefore, it is critical to improve surveillance strategies to prevent introduction of newly invasive pathogens through

quarantine entries besides monitoring disease occurrence in co-geographic hosts for already established alien invasive pathogens (Anderson *et al*. 2004).

1.3.4.4. Population structure and reproductive mode of *Ceratocystis fimbriata s.l*

The assessment of genetic structure for populations of certain plant pathogenic fungi is critical for developing better disease management strategies (McDonald 1997; McDonald & Linde 2002). The evaluation of genetic variation within populations can lead to an understanding of whether populations are clonal or recombining. Consequently, this can assist plant pathologists and breeders to select representative genotypes from pathogen populations to be used for evaluation of fungicides or germplasm (Milgroom & Peever 2003). Furthermore, comparison between different populations can help to elucidate if pathogen populations are native or recently introduced. This is critical for quarantine measures and to track the source of introductions in cases of recently introduced pathogens in order to prevent further introductions of new genotypes (McDonald 1997).

The genetic structure of populations is governed by evolutionary forces including mutation, population size and random genetic drift, gene and genotype flow, reproduction and mating systems and selection (McDonald & Linde 2002). The mode of reproduction is one of the major evolutionary forces affecting genetic variation in populations (Milgroom 1996; McDonald 1997; Taylor *et al*. 1999). Therefore, an understanding of the reproduction mode of certain plant pathogenic fungi is very important for disease management (Milgroom 1996). Fungi that reproduce predominantly through asexual means will yield clonal population structures. Populations that undergo sexual reproduction, on the other hand, or that include both reproduction strategies, are more likely to yield more diverse populations (Milgroom 1996; Taylor *et al*. 1999).

Both asexual and sexual reproduction shapes the population structure found in *Ceratocystis* species. *Thielaviopsis* Went is an anamorph of *Ceratocystis* represents (Paulin-Mahady *et al*. 2002). Sexual reproduction in *Ceratocystis* species such as *C. fimbriata*, *C. coerulescens*, *C. pinicola* and *C. virescens* is generally considered homothallic (i.e. self fertile) (Harrington & McNew 1997; Witthuhn *et al*. 2000). In other species such as *C. paradoxa*, *C. radicicola*, *C. fagacearum* and *C. eucalypti*, reproduction is heterothallic (i.e. obligate sexual outbreeding) (Olson 1949; Webster & Butler 1967a; Kile *et al*. 1996; Harrington & McNew 1997; Witthuhn *et al*. 2000). *C. manginecans* is homothallic like other species in the *C. fimbriata s. l* clade (Harrington & McNew 1997; Witthuhn *et al*. 2000). Essentially, they exhibit mixed sexual and asexual reproduction and reproduce sexually through selfing (Webster 1967; Webster & Butler 1967a & b; Harrington & McNew 1997; Witthuhn *et al*. 2000). In self-fertile cultures of *C. fimbriata s.l*, the perithecia produce ascospores that have both self-sterile (MAT-1) and self-fertile (MAT-2) progenies in a 1:1 ratio (Harrington & McNew 1997). The self fertility of MAT-2 strains is due to uni-directional mating type switching (Harrington & McNew 1997; Witthuhn *et al*. 2000). The self fertile reproduction in a haploid species produces clonal progeny identical to those produced during asexual reproduction resulting in clonal populations (Milgroom 1996; Taylor *et al*. 1999). Although self fertility is more common, outcrossing in homothallic *Ceratocystis* species can occur (Milgroom 1996; Witthuhn *et al*. 2000; Engelbrecht *et al*. 2004). Population analyses are, therefore, needed to assess the role of the reproduction strategy in shaping the population structure of *Ceratocystis* species (Milgroom 1996; Taylor *et al*. 1999).

The assessment of population structure within certain fungal populations has relied mainly on the application of molecular techniques (Milgroom & Peever 2003). Different

molecular markers have been utilized to assess the genetic diversity of species within the *C. fimbriata* clade. Dominant markers that have been used include random amplified polymorphic DNA (RAPD), direct amplification of minisatellite region DNA (DAMD) (Santini & Capretti 2000), nuclear genome fingerprinting using $(CAT)_5$ and mitochondrial genome fingerprinting using *Hae*III (DeScenzo & Harrington 1994; Harrington *et al*. 1998; Roux *et al*. 2001).

Co-dominant microsatellite markers that have been found to be a superior marker of choice for population analysis (Selkoe & Toonen 2006), have also been developed for *C. fimbriata* (Barnes *et al.* 2001; Steimel *et al.* 2004). Intraspecific variation within *C. fimbriata s.l* populations have been assessed using these markers. These markers clearly differentiate between populations of *C. fimbriata* collected from various hosts and different geographical origins (Barnes *et al.* 2001; Steimel *et al.* 2004). In addition, these markers have also been used for population analyses in closely related species such as *C. platani*, *C. albifundus*, *C. cacaofunesta* and *C. pirilliformis* (Engelbrecht *et al*. 2004; Barnes *et al*. 2005; Van Wyk *et al*. 2006b; Engelbrecht *et al*. 2007; Kamgan *et al*. 2009; Ferreira *et al*. 2010).

In general, pathogens that have been recently introduced into an area are expected to have lower genetic diversities compared to the native populations (McDonald 1997; Linde *et al*. 2002; Grunwald & Flier 2005; Hunter *et al*. 2008). The population analysis of *C. fimbriata s.l* isolated from Eucalyptus in South Africa demonstrated low gene diversity (H = 0.36) in this country and suggested their establishment through founder events (Van Wyk *et al*. 2006b). Relatively high gene diversities, however, were found in populations of *C. albifundus* from South Africa (H = 0.38) and Uganda (H = 0.41), indicating that this species is most likely indigenous to the African continent (Barnes *et al*. 2005). On the other hand, the relatively high gene diversity of an Eastern United States *C. platani* population (H = 0.2178),

compared with a European population (*H* = 0.0191), suggested the species is indigenous to the United States and more recently introduced into Europe (Engelbrecht *et al*. 2004; Ocasio-Morales *et al*. 2007). Population analysis for *C. cacaofunesta* revealed low gene diversity values of this pathogen in Costa Rica (*H* = 0.0386), Colombia (*H* = 0.0938) and Bahia in Brazil (*H* = 0.0308) indicating recent introductions into those countries (Engelbrecht *et al*. 2007). The population analyses of *C. cacaofunesta* from Rondonia (*H* = 0.1979) and Ecuador (*H* = 0.1020) on the other hand, suggested these populations were in their native region due to the relatively high gene diversity observed (Engelbrecht *et al*. 2007). Although the value of 0.1 was interpreted as being high (Engelbrecht *et al*. 2007) and thus indicating a native population this is at variance with the report of the introduced population which has a diversity value of 0.3 (Van Wyk *et al*. 2006b). The diversity values reported are probably not comparable as different techniques were used.

In Brazil, the population genetics of *C. fimbriata s.l* isolated from different hosts and specifically from mango were previously evaluated by using microsatellite markers (Ferreira *et al*. 2010). In addition, 41 of these isolates of *C. fimbriata* collected from various hosts and different locations in the state of Sao Paulo in Brazil were evaluated through their pathogenic reaction in five mango cultivars including Alda, Vitoria, Itaparica, Coquinho and Jasmim (Ribeiro 1993). The population consisted of isolates obtained from infected *Mangifera indica* (29 isolates); *Hypocryphalus mangiferae* (2 isolates); *Crotalaria juncea* (4 isolates); *Cajanus cajans* (2 isolates); *Acacia decurrens* (3 isolates) and *Ficus carica* (1 isolate). *C. fimbriata s.l* isolates from *M. indica* and *H. mangiferae* grouped into seven different pathotypes according to the differential response of susceptibility or resistance of the inoculated mango cultivars (Ribeiro 1993). The analysis of genetic diversity for the Brazilian *C. fimbriata s.l* populations collected from five hosts, including Eucalyptus, mango, fig, inhame and *Gmelina arborea*,

revealed, with exception of the first two hosts, highly differentiated populations. Furthermore, populations of *C. fimbriata s.l* collected from diseased mangos in different areas in Brazil had higher gene diversities compared to populations collected from other hosts. The mango populations of *C. fimbriata s.l* collected from Campus (Rio de Janeiro), Sao Fidelis (Rio de Janeiro) and Sao Paulo, had 10 (from 14 isolates), 6 (from 19 isolates) and 5 (from 8 isolates) different genotypes respectively (Ferreira *et al*. 2010). Considering only the population from mango from Sao Paulo, and the number of isolates used in both population studies, the results of the population studies is relatively concordant with a pathotype study of a number of genotype in Sao Paulo (Ribeiro 1993; Ferreira *et al*. 2010).

Recently, Rizzato *et al*. (2010) developed a new set of microsatellite markers using *C. fimbriata s.l* isolates from diseased mango trees in Brazil. They indicated the new marker set would yield a higher resolution compared with the currently available microsatellite primers that were developed (Barnes *et al.* 2001; Steimel *et al.* 2004) using isolates of *C. fimbriata* from hosts other than mango. The new set of markers were found to be highly informative producing 6.65 allele per locus from 13 *C. fimbriata s.l* isolates from diseased mangos in Sao Paulo (Rizzato *et al*. 2010).

1.4. EPIDEMIOLOGY AND MODE OF MANGO WILT PATHOGEN DISPERSAL

1.4.1. Epidemiology and economic impact

The occurrence and distribution of mango wilt disease in Batinah, Oman, was investigated during June 2000. This survey showed that the general distribution of the disease in the region was high in the south (e.g. Barka and Suwaiq) and lower disease incidence was found in the northern regions (e.g. Sohar and Shinas). The average disease incidence in Batinah was estimated at over 54% of mango trees. Almost five percent (19,797) of all mango trees were dead (Al Adawi 2002). In 2001, the Ministry of Agriculture and Fisheries in Oman initiated an integrated management programme for mango wilt disease in the Batinah region. The management programme included the removal of dead and infected mango trees, painting tree trunks with gypsum (Calcium sulphate dehydrate) mixed with fungicides and insecticides and spraying the trees with systemic insecticides and fungicides (Al Adawi 2002). During 2001, around 7.24% (26,980 mango trees) were eradicated due to mango wilt in this region (Al Adawi 2002; Al Adawi *et al*. 2006). In the following year (2002), the number of dead mango trees increased dramatically to 16.48% (67,136 trees) in the northern areas and the disease incidence increased significantly. During 2002, high levels of wilt were found in Saham (41.3%), Khaborah (21.6%) and Masina (16.4%) (MAF 2003). Between 2000 and 2002, the average percentage of mango tree mortality due to mango wilt disease in surveyed areas in the Batinah region exceeded 28% (113,913 trees) (Table 2.). An evaluation of mango wilt disease incidence in thirty farms from different localities in the Barka area showed that the disease was distributed throughout these areas and that there were no significant differences in disease incidence among localities . However, it was noted that local mango cultivars (seed propagated) were more severely affected, with higher disease incidence, compared to exotic mango cultivars (graft propagated) (Al Adawi 2002).

The spread of mango wilt disease throughout Oman from the initial outbreak in the Barka area took less than 5 years. Severe mango wilt disease incidence was observed in Qurayiat (160 km from Barka) in 2004. In this area, mango wilt disease caused 24% tree mortality and affected 66.6% of the total mango tree population (MAF 2004). During 2003, wilt incidence of mango trees in the Dahira (3.1%) and Dakliyia (2.2%) regions were relatively lower compared with the Batinah region (MAF 2005; 2006a). Furthermore, comparisons between the two censuses carried out in the seasons of 1992/93 and 2004/05 revealed a 6% (25 652 trees) reduction in the total number of mango trees in Oman. The number of mango trees did, however, fluctuate within regions between the two censuses. In the Muscat and Batinah regions, the mango population dropped by 32.8% (8,649 trees) and 22.5% (81,882 trees) respectively while tree populations increased in the regions of Musandam (5,938 trees), Dahira (14,250 trees), Dakhilya (11,787 trees), Sharqiya (32,754 trees) and Dhofar (150 trees) (Table 1) (MAF 2009a).

From the data collected during the mango wilt disease surveys, the integrated mango decline management programme implemented during 2001, and the data from the agriculture census in 2004/2005, an estimated death of over 210,000 mango trees occurred during the period from 2000 to 2005 in the Batinah region alone. Assuming that the average mango production is 25kg/tree, the average mango yield reduction can be estimated at 5,250 tons/year. The approximate average mango fruit price during the season is 0.35 O. R/kg. Therefore, the cost of yield lost per year can be estimated at more than 1.8 million R.O. Consequently, the quantity of mango imported into Oman increased significantly following the mango wilt disease epidemic and reached its peak during 2003 with 25,000 tons of mango being imported (ROP 2002; 2004; 2006; 2008).

Concurrent with the emergence of mango wilt disease in Oman, a mango wilt disease was also observed for the first time during 1998, killing mango trees in the Sindh and Punjab areas in Pakistan (Malik *et al*. 2005). After three years, a disease with similar symptoms to those of mango wilt disease was detected on Shisham (*D. sisso*) trees in Punjab in Pakistan (Kazmi *et al*. 2005).

As mentioned above, Pakistan is one of the largest producers of mango in the world. More than 250 mango cultivars are grown in Pakistan (Ahmad *et al*. 2008). The majority of these cultivars are originally of the Indian monoembryonic type and their fruit quality is characterized by high total soluble solid (TSS) and aroma (Collins *et al*. 2006). In 2008, the mango area under cultivation exceeded 165 thousand hectares, producing 1.7 million tons with a value of over 427 million US$ (FAOSTAT 2008). In addition, Pakistan was ranked the fourth highest mango exporter in the world (FAOSTAT 2008). During 2007, over 62,000 tons of mango produced in Pakistan was exported mainly to the Middle East with an export value exceeding 20 million US$ (FAOSTAT 2007). Punjab and Sindh were considered as major mango producing areas and contributed 67% and 32%, respectively, to the total mango production in Pakistan (Collins *et al*. 2006).

The economic loss due to mango wilt disease in Pakistan can be estimated by compiling data from different sources. During 2004, the disease incidence was assessed in several different areas in the Punjab and Sindh districts. Mango wilt disease incidence ranged from 2.4% to 10.3% in Punjab and from 3% to 15% in Sindh (Kazmi *et al*. 2005). From these data, it was estimated that the average disease incidence in Pakistan was 5.6% and around 4.3% and 8% in Punjab and Sindh, respectively. In Pakistan, mango planting densities range between 55 to 70 trees/ha (Saifullah *et al*. 2007). In cases where an average of 70 trees/ha is planted, the total number of wilted mango trees during 2004 was estimated at over 403,181.

The average mango production from these trees is equivalent to 9 ton/ha with a value of 323 US$/ton (Bakhsh *et al*. 2006; FAO stat 2007). The economic losses in 2004 can be estimated at over 16.7 million US$ (Table 3). These calculations of economic losses in Oman and Pakistan only take the losses due to yield reduction into consideration. However, economic losses could also include loss of production costs for mango trees, the loss of valuable genetic germplasm resources and earlier ineffective management treatments.

In the United Arab Emirates (UAE), mango wilt disease was first noticed during 2004 (Wafa Khoury, FAO *personal communcation*). The emergence of the disease in UAE was synchronous with a high mango wilt disease incidence in the northern part of the Batinah region in Oman, especially in the Shinas area which shares a border with UAE (Al Adawi *personal observation*). This suggests that mango wilt disease most likely moved from Oman into UAE.

1.4.2. Infection court and dispersal mode

Fresh wounds are a prerequisite to initiate infection of susceptible host tissues by *C. fimbriata s.l* (Teviotdale & Harper 1991; Wingfield *et al*. 1993; Kile *et al*. 1993). Different factors involved in wound development in trees include human mediated factors, such as pruning and harvesting practices, and non human mediated factors, such as wind, hail damage and insects (Kile *et al*. 1993; Roux & Wingfield 1997 & 2009). In Colombia and Venezuela, coffee canker disease caused by *C. fimbriata s.l* was found associated with pruning and accidental injuries created by farmers (Pontis 1951; Marin *et al*. 2003). In the same way, almond canker disease caused by *C. fimbriata s.l* in the United States was found associated with wounds created accidentally on tree trunks during harvest (Teviotdale & Harper 1991). Likewise, mortality of *Acacia* spp. in Africa caused by *C. albifundus*, is normally associated with wounds that have developed through pruning practices or hail damage (Roux &

Wingfield 1997 & 2009). In Uruguay, infection and staining of *Eucalyptus grandis* by *C. fimbriata s.l* was also initiated through pruning wounds (Barnes *et al*. 2003). Recently, several new *Ceratocystis* species such as *C. eucalypti*, *C. pirilliformis*, *C. neglecta* and *C. fimbriatomima* were isolated and described from trees that were artificially wounded (Kile *et al*. 1996; Barnes *et al*. 2003; Rodas *et al*. 2008; Van Wyk *et al*. 2009a).

Ceratocystis species can be dispersed long distance through human activity by transferring and using contaminated materials such as soil, pruning tools, wood cuttings and infected seedlings (CABInternational 2001). Furthermore, dispersal can be through natural means such as wind, water streams and root grafting. The *Mal do machete* is a common name of a serious cacao wilt disease caused by *C. cacaofunesta* which reflects the name of the cacao harvesting tool, the machete, which is responsible for spreading the disease (Engelbrecht *et al*. 2007). Besides pruning tools, *C. platani*, the pathogen responsible of canker and wilt disease of *Platanus* spp., is spread short distances to adjacent healthy trees through root grafting (Kile 1993). Long distance movement of this pathogen also occurred from the eastern United States into Europe during World War II through contaminated wood cuttings (Engelbrecht *et al*. 2004).

Besides the above mentioned natural and human mediated factors, the dispersal of the *C. fimbriata s.l* species complex is also mediated by certain insects (Moller & De Vay 1968a & b). Insects attacking healthy trees act as a source of pathogen inoculum transmitting the pathogen from infected trees to uninfected trees. As in other species in *Ceratocystis*, species in the *C. fimbriata* group are disseminated by interactions with certain insect types such as bark beetles (Coleoptera: Scolytidae) (Crone & Bachelder 1961; Graham 1967; Moller & De Vay 1968a & b; Upadhyay 1981). Fruity, aromatic volatiles that are produced by species in the *C. fimbriata* group, attract casual insects to the infected plant tissues (Kile 1993).

Rossetto *et al.* (1980) carried out olfactometer tests and found that the bark beetle, *H. mangiferae*, was attracted to *C. fimbriata s.l* either grown in cultures or in infected mango twigs.

The spores of the *C. fimbriata* group are well adapted to dispersal by beetles (Malloch & Blackwell 1993). They produce mucilaginous sticky spores at the tips of long and exposed perithecial necks (Malloch & Blackwell 1993; Hanssen 1993; Christen *et al*. 1997). The mucilage is a sticky material that holds spores together, aids in sticking the spores to the exoskeleton of beetles, protects the spores from desiccation and also from digestion materials in the gut of the beetles. Spore dissemination by beetles may be either epizoic (outer) and/or endozoic (inside) of the beetle body (Beaver 1989).

The fruity aroma produced by species of *Ceratocystis* residing in the *C. fimbriata* and *C. moniliformis* clades attracts different types of casual insects that can aid in its dispersal (Malloch & Blackwell 1993; Kile 1993). Consequently, species of both groups form loose associations with different type of insects. In this regard, their dispersal does not strictly rely on a specific insect vector for their dissemination (Heath *et al*. 2009b). Recently, *C. albifundus*, the casual agent of a serious wilt of *Acacia mearnsii* was found to be associated with three different species of nitidulid beetles (Heath *et al*. 2009b). Furthermore, species of the *C. fimbriata* group have been found to be associated with insects other than bark beetles, such as picnic beetles (Coleoptera: Nitidulidae), flies (Diptera) and mites (Crone & Bachelder 1961; Graham 1967; Moller & De Vay 1968b; Upadhyay 1981).

1.4.2.1. Bark beetles associated with mango wilt disease

Pyenson (1938) recorded several species of Scolytid beetles associated with symptoms of serious wilt mango disease in Recife in the state of Pernambuco in Brazil. Furthermore, Batista (1947), from the same area, suggested *Diplodia recifensis* Batista as the causal agent of mango wilt disease and that it was vectored by *Xyleborus affinis*. However, Silva *et al.* (1959) found through several inoculation tests that *Xyleborus affinis* was not able to infest healthy mango trees and suggested that it is a secondary phytophagous beetle. Later, Castro (1960) identified *H. mangiferae* as a major beetle associated with the disease in Recife and published detailed studies on the history, taxonomy and biology of the beetle. During the same period, Viegas (1960) reported a beetle closely related to the coffee borer *Hypothenemus plumeriae*, associated with mango wilt disease in the State of Sao Paulo in Brazil. However, Medeiros and Rossetto (1966) reported *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytidae) as vector of *C. fimbriata s.l* and considered the previous report of *H. plumeriae* as a misidentification of the same beetle. Infestation by secondary bark beetles like *Xyleborus* spp. and *Platypus* spp. often occurs after infestation by *H. mangiferae* and where infection by *C. fimbriata s.l* has already taken place and has progressed into the main trunk of the mango tree (Medeiros and Rossetto 1966; Rossetto & Medeiros 1967; Rossetto *et al.* 1980).

In Pakistan, four bark beetles have been found associated with mango wilt disease including *H. mangiferae*, *Sinoxylon* sp., *Xyleborus* sp. and *Nitidulidae* sp. (Van Wyk *et al.* 2007a; Masood *et al*. 2008). However, it was only *H. mangiferae* that was found in the early stages of disease on mango trees; the other beetles colonised the already infected mango trees or those that were already dead (Masood *et al*. 2008).

Normally *H. mangiferae* attacks mango trees stressed by biotic or abiotic factors. It was shown in Brazil, however, that infection of mango trees by *C. fimbriata s.l.* is not crucial for the onset of infestation by *H. mangiferae* (Rossetto *et al*. 1980). In fact, bark beetles are attracted to mango trees in response to visual stimuli and chemicals released from mango trees (Byers 1995; Pena & Mohyuddin 1997). It was found that the females of *H. mangiferae* are attracted to susceptible cultivars by geraniol and isobutyl acetate compounds (Da Silva 2006). In Oman, *H. mangiferae* attack was found mostly associated with local mango cultivars and less so with the exotic cultivars (Al Adawi 2002). This type of preference was also reported by recent detailed studies in Brazil which showed the tendency of these beetles to attack mainly the Espada mango variety (Da Silva 2006). Re-infestation by *H. mangiferae* does, however, increase in the presence of previous attacks by *H. mangiferae* and also in trees already infected *C. fimbriata s.l* (Da Silva 2006). Once bark beetle infestation has been established in mango trees, they release species-specific aggregation pheromones (Byers 1995; Pena & Mohyuddin 1997; Pena *et al*. 2009). Thereafter, due to the increase in beetle populations, healthy trees in the area become infested (Pena *et al*. 2009).

C. manginecans in Oman and *C. fimbriata s.l* in Brazil have been consistently isolated from adult *H. mangiferae* beetles collected from infected mango trees during the early stages of disease development (Ribeiro & Rossetto 1971; Al Adawi *et al*. 2006). These fungi can then be isolated from beetles by using a carrot isolation technique described by Moller & Devay (1968a). In Brazil, only 1% of *H. mangiferae* were contaminated with *C. fimbriata s.l* (Ribeiro & Rossetto 1971), while in Oman, the same isolation technique yielded *C. manginecans* from *H. mangiferae* at frequencies between 13 and 83% (Al Adawi *et al*. 2006). Generally, Scotylid beetles are known for their ability to fly long distances and can travel as far as 45 km per day for several days (Hill 1994; Byers 2000). Consequently, *H. mangiferae*

can be considered as an active carrier of pathogen inoculum from infected to healthy sites, contributing to the spread of disease in very short periods of time (Al Adawi 2002; Al Adawi *et al*. 2006). This reflects the crucial role that *H. mangiferae* plays as a vector for the mango wilt pathogen, facilitating pathogen entry into healthy mango trees (Castro 1960; Rossetto *et al*. 1980; Ribeiro 1993; Al Adawi *et al*. 2006; Van Wyk *et al*. 2007a).

1.4.2.2. Role of *Hypocryphalus mangiferae* in dispersal of mango wilt disease

1.4.2.2.1. Distribution

H. mangiferae was first described from India in 1903 and is believed to be native to south East Asia (Butani 1993). It is mainly reported in tropical areas of the world where mango is grown, including Malaysia, Indonesia, Brazil, Mexico and Florida. *H. mangiferae* is phloeophagous (phloem feeding) and monophagous (restricted to one host) and attacks the main stems and woody branches of mango trees (Castro 1960; Butani 1993; Atkinson & Peck 1994; Pena & Mohyuddin 1997).

1.4.2.2.2. Morphology and life cycle

In Oman, twelve overlapping generations of *H. mangiferae* are produced per year (Fahim *et al*. 2002). Each generation takes between 27 to 34 days with an average of 31.2 days (Fahim *et al*. 2002). In Pakistan and Brazil, the average life cycle is completed after 25.2 (±4.2) and 23.2 days respectively (Castro 1960; Masood *et al*. 2009). The differences in the average life cycle might be due to the differences in the climatic conditions between the countries. Immature adults of *H. mangiferae* are light brown and turn dark brown upon maturation (Fahim *et al*. 2002; Masood *et al*. 2009). Adults are approximately 1.5 mm long and 1 mm wide. The male is similar to the female with the only difference being that the posterior margin of the abdominal sternum is more broadly rounded in the male (Wood

1982). The females of *H. mangiferae* initiate the attack of the mango trees by boring through the bark to mine for egg galleries. The males arrive thereafter and after mating, the females lay their eggs inside the egg galleries. Neither sex leaves the breeding gallery after mating. Females lay between 41 to 139 eggs with an average of 75 eggs (Fahim *et al*. 2002). The eggs hatch and produce creamy white larvae (2 mm when mature), which start to make their own feeding galleries in the phloem. The larval stage lasts for approximately 20 days. The pupation stage lasts for five days and occurs near the exit hole (Fahim *et al*. 2002; Masood *et al*. 2009).

To determine the levels of infestation per tree, logs of mango trees were cut (40 cm long, 4 cm diameter) and used as traps for the bark beetles and placed in a mango field (Fahim *et al*. 2002). The average number of entrance holes of bark beetles counted after four, eleven and fifteen days were found to be 141, 555 and 109 respectively. Logs were placed in cages after bark beetle infestation and beetles exit holes ranged between 167 to 710 holes per each log. This indicated the efficiency of using log traps in monitoring the population of *H. mangiferae* in infested mango fields (Fahim *et al*. 2002). Furthermore, log traps can be part of an integrated management programme through attracting *H. mangiferae* and destroying traps before the emergence of new populations from the logs (Fahim *et al*. 2002). In Oman, a study of the bark beetle population in 2002 revealed that the highest population trapped using logs of mango or yellow sticky traps was during the months of March and April (Fahim *et al*. 2002). Log trap monitoring in Pakistan revealed the highest activity of *H. mangiferae* was in May where relative humidity values are at their lowest during the year (Saeed *et al*. 2010). The difference in peak periods can be attributed to differences in climatic conditions between the countries. The population of *H. mangiferae* was found to be affected negatively by relative humidity (Saeed *et al*. 2010).

1.5. MANAGEMENT OF MANGO WILT DISEASE

1.5.1. Eradication and chemical treatment

Mango wilt disease management includes different control measures aimed at breaking the life cycle of the pathogen. In Brazil and Oman, sanitation and pruning represent an essential control measure as they reduce the impact of the disease in an already infected mango field through reducing pathogen inoculum and reducing bark beetle populations (Rossetto & Ribeiro 1990; MAF 2006b). Sanitation includes eradication of dead trees and burning of infected or dead branches of the affected mango trees. Pruning tools are also disinfected with sodium hypochlorite and pruning wounds are dressed with Valsa wax (Thiophanate methyl) to prevent entry of the pathogen into these open wounds (Rossetto & Medeiros 1967; Rossetto & Ribeiro 1983; Rossetto & Ribeiro 1990; MAF 2006b).

Different combinations of insecticides and fungicides have been tested to reduce the economic impact of mango wilt disease (Al Adawi 2002). In Oman, painting the trunks of healthy mango trees with gypsum was found to reduce bark beetle infestation (Fahim *et al*. 2002). In experiments with mango logs, a Marshall (carbosulfan) insecticide was found to be more effective as a protectant against bark beetle infestation compared to Superacid (Methidathion) (Fahim *et al*. 2002). Furthermore, spraying with systemic fungicides such as Topsin (Thiophanate methyl) was found to slow the progress of the disease for a short period of time. However, these control measures are considered as a preventive, rather than curative (Ploetz & Freeman 2009). This is because beetle and pathogen are normally protected by the thick bark layers which are impossible to reach through spraying. In addition, it seems as though the preventive efficacy of these control measures is not long lasting (Ploetz & Freeman 2009).

In Oman, injecting infected mango trees with systemic fungicides and insecticides was found to be helpful, especially in the case of trees in the early stages of infection (MAF 2006b & c). Microinjection using a mixture of both systemic fungicides and insecticides formulated in Imisol capsules (active ingredient consists of Debacarb, Carbendazim and Imidacloprid) were found to be effective in suppressing the progress of mango wilt disease in infected mango fields (MAF 2006b & c). This type of injection is less costly and time consuming but the correct timing of the applications and their residues in fruits needs further investigation in order to be widely distributed and recommended (MAF 2006b & c).

1.5.2. Cultivars resistant to mango wilt disease

Selection of mango cultivars resistant to the mango wilt pathogens will most likely represent the best effective control measure against the disease (Rossetto & Ribeiro 1983). In Brazil, screening mango cultivars has shown a range of responses to the disease (Ploetz & Freeman 2009). Several mango cultivars were evaluated for resistance against either infection of *C. fimbriata s.l* isolates or infestation by *H. mangiferae* (Zaccaro *et al*. 1984). The evaluation program involved both monoembryonic and polyembronic mango cultivars in order to obtain resistant cultivars that can be used as scion only, in the case of the former group of cultivars, and used either as scion or rootstock, in the case of the latter group of cultivars (Rossetto *et al*. 1996a & b). Zaccaro *et al*. (1984), evaluated six Brazilian mango cultivars and sixteen exotic cultivars for their resistance against *C. fimbriata s.l* and *H. mangiferae*. The result of this study showed Familia, Joe Welch, Palmer, Pele de Moca and Tommy Atkins as the least susceptible cultivars to infestation by *H. mangiferae*. Irwin, Keitt, Manga D Agua, Oliveira Neto, Ruby and Tommy Atkins were the cultivars most resistant to inoculation with *C. fimbriata s.l* (Zaccaro *et al*. 1984). The resistance of cultivars such as Tommy Atkins to both infestation of *H. mangiferae* and infection of *C. fimbriata s.l* would be

an excellent option for mango growers (Zaccaro *et al*. 1984). However, using cultivars resistant only to infection by *C. fimbriata s.l* would also be suitable as *H. mangiferae* infestation does not necessarily lead to the death of mango trees (Pena & Mohyuddin 1997).

Further screening of mango cultivars in Brazil identified variable response to inoculation with isolates of *C. fimbriata s.l* (Ribeiro 1993; Ribeiro *et al*. 1995; Rossetto *et al*. 1996a & b). Mango cultivar evaluation in Brazil used two selected *C. fimbriata s.l* isolates, FITO 334-1 and FITO 4905 (Ribeiro 1993; Ribeiro *et al*. 1995; Rossetto *et al*. 1996a & b). Isolate FITO 334-1 was selected as a predominant pathotype (16 isolates: 39%) found in the population and the second selected isolate (FITO 4905) was the only isolate pathogenic to the Jasmim (=Ubá) cultivar (Ribeiro 1993). Different mango inoculation trials using both pathotype isolates revealed that cultivars Sao Quirino, Irwin, Edwards, Carabao, Manga d Agua, Pico and Van Dyke were resistant. The cultivars Rosa, Sabina, Oliveira Neto, Sensation, IAC100 Bourbon and Tommy Atkins were moderately resistant. However, cultivars Coquinho, Glenn, Joe Welch, Zill and Haden were susceptible to both isolates. The cultivar Kent performed similarly to Jasmim and responded differentially to the isolates tested as described above (Ribeiro *et al*. 1995; Rossetto *et al*. 1996a & b; Ploetz & Freeman 2009). Therefore, evaluation of resistance in mango cultivars to infection by the mango wilt pathogen should consider testing variability in aggressiveness of the pathogen population and selecting representative isolates for inoculation experiments.

Disease incidence surveys in Oman and Pakistan have revealed that the local mango cultivars, either grafted or propagated directly through seeds, were highly susceptible to mango wilt disease, compared to exotic cultivars (Al Adawi *et al*. 2006; Panhwar *et al*. 2008). Local cultivars are seed-derived from selected trees showing good characteristics. In addition, from field observations in Oman, the degree of susceptibility in this group of

cultivars is subtle and about 60% of infections and dead trees were in local mango cultivars (Al Adawi *et al.* 2006). Screening for resistance is further complicated by the use of rootstocks of local origin onto which exotic scions are grafted. Local cultivars are particularly susceptible and heavy infection of the rootstocks leads to the rapid death of whole trees (Al Adawi *et al.* 2006). In Pakistan, the highest disease incidence was found in the indigenous mango varieties (16.4%), which are seed propagated, followed by grafted cultivars including Sindhri (11.28%) and Langra (10.89%) cultivars (Panhwar *et al*. 2008). Rootstocks of grafted mango trees were frequently affected compared with the scion, which were more commonly asymptomatic. No symptoms of the disease were observed in the scion part of the mango cultivars Chonsa, Black Chonsa and White Chonsa. Infection was restricted to the rootstock part of these trees (Al Adawi *unpublished data*). In Oman and Pakistan, seed-propagated cultivars are almost all descended from Indian monoembryonic cultivars, which have single zygotic embryos yielding hybrid offspring (Iyer & Schnell 2009). Therefore, extensive variability would be expected for seedlings propagated through seeds of monoembryonic cultivars. However, common genotypes susceptible to the mango wilt pathogen might be shared in those local monoembryonic cultivars. The future outcrossing of those cultivars with resistant cultivars would yield hybrid cultivars with less susceptibility to infection by the mango wilt pathogens. Through breeding programs in Brazil, several mutant progenies, resistant to two isolates of *C. fimbriata s.l* have been bred from relatively susceptible cultivars. The selected cultivars include IAC101 Coquinho, IAC102 Touro, IAC103 Mococa, IAC104 Dura, IAC106 Jasmim and IAC108 Pindorama (Rossetto *et al*. 1996b).

1.6. CONCLUSIONS

Mango wilt disease was first reported in Brazil in the 1930s. By the end of 20th century, it had been reported in Pakistan and Oman, killing thousands of mango trees in both countries. Recently, the disease has been observed in the United Arab Emirates (UAE) (Al Adawi, *personal observation*) and the same symptoms are being reported from Yemen (Shaher Al Absi, *personal communication*) and Bangladesh (Mosharraf Hossain, *personal communication*). Since its first report in Brazil, the aetiology of mango wilt disease has been thoroughly investigated and after twenty years, fungal pathogens in the *Ceratocystis fimbirata s.l,* complex are accepted as the primary causal agents of the disease. The aetiology of the disease was investigated in Oman and Pakistan and several causal agents were reported associated with the disease. These agents include abiotic (e.g. salinity) and biotic factors (e.g. bark beetle, fungi, phytoplasma). Among the biotic factors found to be intimately associated with the disease was a fungus belonging to the *C. fimbriata s.l* group, now recognized as *C. manginecans,* and a bark beetle, *Hypocryphalus mangiferae*. Mango wilt disease has been investigated in Oman and Pakistan. Results of these investigations showed that *C. manginecans* was the primary pathogen of the disease in these countries (Al Adawi *et al*. 2006; Malik *et al*. 2010). The genetic diversity of isolates of the fungus from Oman and Pakistan need to be investigated. This will make it possible to determine the probable source population of the pathogen. Such a study will help to elucidate whether the population of *C. manginecans* is an alien invasive or if it is native on other hosts.

Although it is clear that *H. mangiferae* is associated with *C. manginecans* in Oman, the insect has not been shown to be a vector of the pathogen. Given the very short period of time that it has taken the diseases to spread in Oman and Pakistan, it seems likely that *H.*

mangiferae is involved in the epidemiology of *C. mangniecans*. Answering this question is an important component of the studies that make up this thesis.

Mango wilt disease management includes chemical treatments which are expensive, effective for only short periods of time and hazardous to human health and the environment. Selection of resistance cultivars represents an attractive option for long term effective control of the disease. In this regard, comprehensive inoculation trials involving as many mango cultivars as possible for testing are needed. Studies to accomplish this goal form part of this thesis.

The research in this thesis will investigate the topics mentioned above through field surveys, inoculation trails, DNA sequence comparisons for taxonomic purposes and population genetic studies. It is intended that the research that makes up this thesis will contribute at least in part to resolving the controversies regarding the causal organisms of mango wilt disease in Oman and Pakistan. It should also contribute to knowledge of the population structure of *C. manginecans* to help understand the unprecedented spread of mango wilt disease. Taken together, it is hoped that the studies presented in the chapters of this thesis will lead to a much improved understanding of Mango wilt disease in Oman. Moreover, they will promote strategies to reduce the devastation that has been caused by it.

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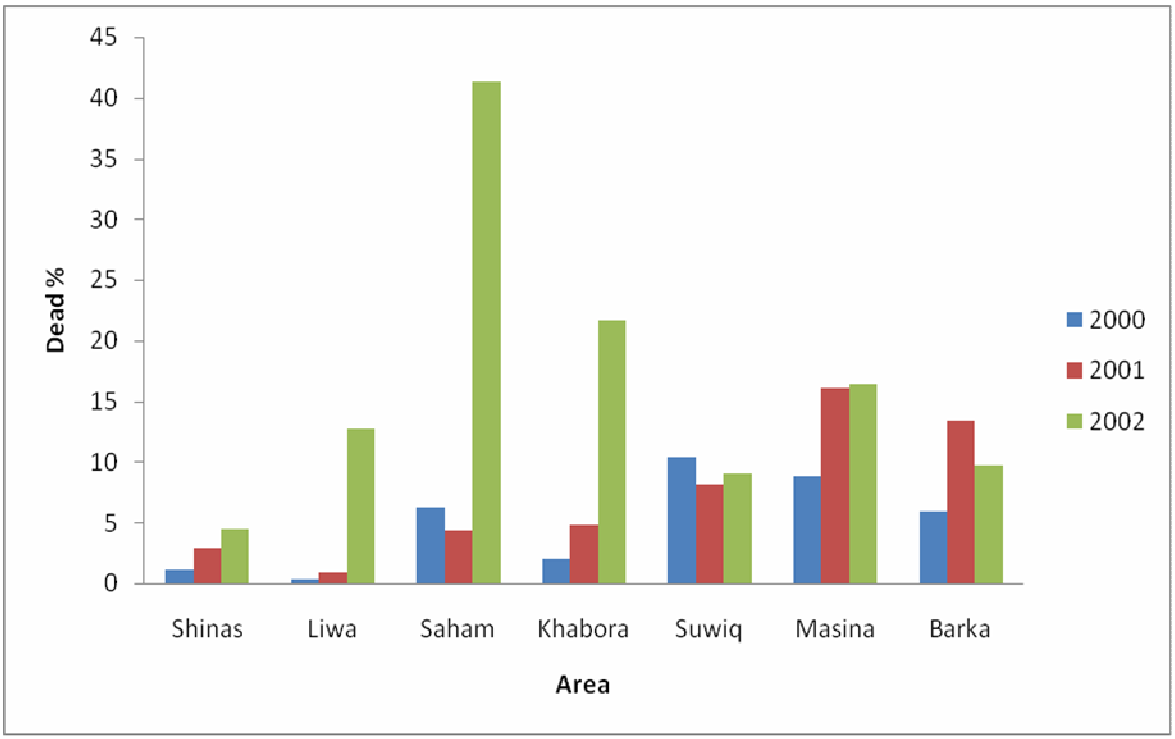

Fig. 1. The incidences of Mango wilt disease in Al Batinah region in Oman during 2000-2002 (data was adapted from different sources: disease incidence in 2000 (Al Adawi, 2002), 2001 (MAF, 2002) and 2002 (MAF, 2003).

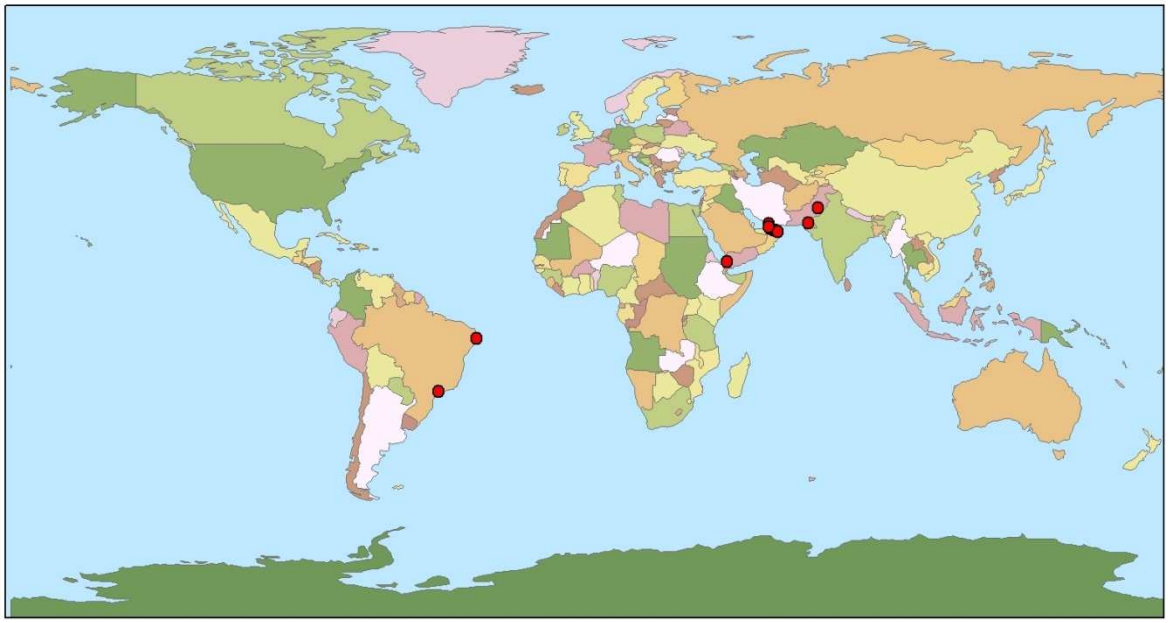

Fig. 2. Current distribution map of mango wilt disease in the world.

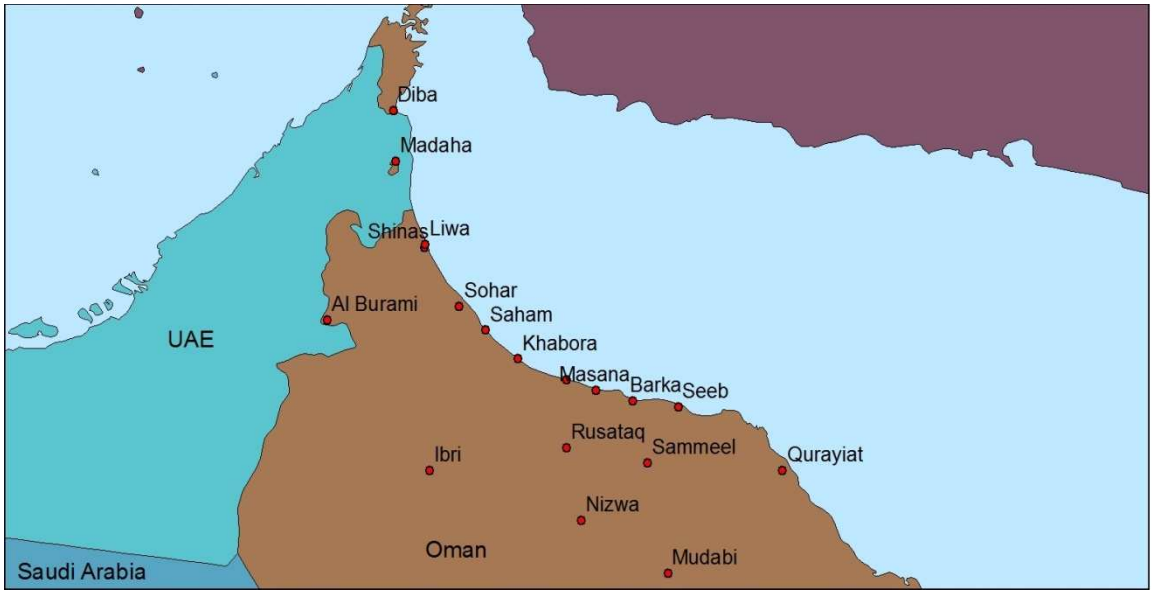

Fig. 3. Distribution of mango wilt disease in Sultanate of Oman.

Table 1. Number of mango trees in different areas of Oman recorded during agriculture census.

Region	Total number of mango trees		Difference between the two censuses
	1992/93[a]	2004/05[b]	
Muscat	26334 (6.18%)	17685 (4.41%)	-8649[c]
Batinah	364189 (85.45%)	282307 (70.48%)	-81882[c]
Musandam	3778 (0.89%)	9716 (2.43%)	5938
Dahira	22127 (5.19%)	36377 (9.08%)	14250
Dakhilya	2426 (0.57%)	14213 (3.55%)	11787
Sharqiya	6613 (1.55%)	39367 (9.83%)	32754
Dhofar	753 (0.18%)	903 (0.23%)	150
Sultanate of Oman	**426220**	**400568**	**-25652**

[a] MAF, 1994.

[b] MAF, 2009a.

[c] These differences are mostly likely due to the mango wilt disease in addition to abiotic factors including salinity stress.

Table 2. Incidences of Mango wilt disease in Pakistan and the economic losses due to the disease during the 2004 season.

District/area	Production	Total	DI %[c]	Wilted	Reduction in		Loss
	Area (ha)[a]	Tree[b]		tree	Area	Yield (ton)[d]	Value (US$)[e]
Punjab	**54 313**	**3 801 910**	**4.33**	**164 623**	**2351.8**	**21165.8**	**6 836 545.7**
Khanewal	2 752	192 640	3	5 779	82.6	743.0	240 001.9
Multan	7 891	552 370	4.18	23 089	329.8	2968.6	958 855.9
Muzaffar garh	3 359	235 130	3.69	8 676	123.9	1115.5	360 314.2
Jhang	1 757	122 990	2.44	3 001	42.9	385.8	124 625.4
T. T. Singh	1 038	72 660	2.7	1 962	28.0	252.2	81 471.6
Sahiwal	1 942	135 940	3.19	4 336	61.9	557.5	180 088.1
Faisalabad	1 376	96 320	6.85	6 598	94.3	848.3	274 002.2
Lodharn	372	26 040	2.66	693	9.9	89.1	28 765.3
Shujabad	ND	ND	10.26	ND	ND	ND	ND
Sindh	**47 088**	**3 296 160**	**8.098**	**266 923**	**3 813.2**	**34318.7**	**11084932.4**
Hyderabad	12 621	883 470	7.89	69 706	995.8	8 962.2	2 894 781.6
Sangher	7 337	513 590	3	15 408	220.1	1 981.0	639 859.8
Omer Kot	ND	ND	5.6	ND	ND	ND	ND
Karachi	205	14 350	15	2 153	30.8	276.8	89 390.3
Mirpur Khas	11 876	831 320	9	74 819	1 068.8	9 619.6	3 107 117.9
Pakistan[f]	**103 110**	**7 217 700**	**5.586**	**403 181**	**5759.7**	**51 837.5**	**16 743 519.4**

[a] Data from MINFAL, 2006.

[b] The average number of tree is 70 tree/ha according to Saifullah, *et al*. 2007.

[c] Disease incidence data from Kazmi *et al*. 2005.

[d] The average mango production is 9 ton/ha according to Bakhsh *et al*. 2006.

[e] The average mango fruit value is 323 US$/ton according to FAO stat, 2007.

[f] The total reflects overall mango production in Pakistan, including districts with minor mango production.

Table 3. Species described in the *Ceratocystis fimbriata* complex with their hosts and distribution. The majority of these species have been described within the last six years.

Species	Host	Geographical origin	Reference
C. acaciivora	*Acacia mangium*	Indonesia	Tarigan *et al.* 2011
C. albifundus	*Acacia mearnsii*	South Africa, Uganda	Wingfield *et al.* 1996
C. atrox	*Eucalyptus grandis*	Australia	Van Wyk *et al.* 2007b
C. cacaofunsta	*Theobroma cacao*	Costa Rica, Ecuador, Brazil	Engelbrecht and Harrington, 2005
C. caryae	*Carya* spp., *Ulmus* spp. and *Ostrya virginiana*	USA	Johnson *et al.* 2005
C. colombiana	*Coffea arabica*, *Citrus* sp., and *Schizolobium parahybum*	Colombia	Van Wyk *et al.* 2010
C. curvata	*Eucalyptus deglupta*	Ecuador	Van Wyk *et al.* 2011
C. diversiconidia	*Terminalia ivorensis*	Ecuador	Van Wyk *et al.* 2011
C. ecuadoriana	*Eucalyptus deglupta*	Ecuador	Van Wyk *et al.* 2011
C. fimbriata senso stricto	*Ipomaea batatas*	USA, Papua New Guinea,	Engelbrecht and Harrington, 2005
C. fimbriatomima	*Eucalyptus* sp.	Venezuela	Van Wyk *et al.* 2009a
C. larium	*Styrax benzoin*	Indonesia	Van Wyk *et al.* 2009b
C. manginecans	*Mangifera indica*	Oman	Van Wyk *et al.* 2007a
C. neglecta	*Eucalyptus* sp.	Colombia	Rodas *et al.* 2008
C. obpyriformis	*Acacia mearnsii*	South Africa	Heath *et al.* 2009a
C. papillata	*Annona muricata*, *Citrus* sp., *Coffea arabica*, *Schizolobium parahybum* and *Theobroma cacao*	Colombia	Van Wyk *et al.* 2010
C. pirilliformis	*Eucalyptus nitens*	Australia	Barnes *et al.* 2003
C. platani	*Platanus* sp.	USA, Europe	Engelbrecht and Harrington, 2005
C. polychrome	*Syzygium aromaticum*	Indonesia	Van Wyk *et al.* 2004
C. polyconidia	*Acacia mearnsii*	South Africa	Heath *et al.* 2009a
C. populicola	*Populus* spp	USA	Johnson *et al.* 2005
C. smalleyi	*Carya* spp. and *O. virginiana*	USA	Johnson *et al.* 2005
C. tanganyicensis	*Acacia mearnsii*	Tanzania	Heath *et al.* 2009a

Species	Host	Geographical origin	Reference
C. tsitsikammensis	*Rapanea melanophloeos*	South Africa	Kamgan *et al*. 2008
C. zombamontana	*Eucalyptus* sp.	Malawi	Heath *et al*. 2009a

Table 4. Morphological characteristics of the different *Ceratocystis* species infecting mango.

Characteristics	*C. mangicola*[a]	*C. mangivora*[a]	*C. manginecans*[b]	*C. omanensis*[c]
Geographical distribution	Brazil	Brazil	Oman, Pakistan, UAE, Indonesia	Oman
Other hosts recorded	None	None	*Acacia mangium*	None
Culture colour	Brown (15"k)	Brown (15"k)	Greyish olive	Wood brown
Odour	Banana	Banana	Banana	None
Growth rate	Opt. at 25 °C No growth at 5ºC and 35ºC	Opt. at 25 °C No growth at 5ºC and 35ºC	Opt. at 20-25°C No growth at 35°C	Opt. at 30-35 °C No growth at 4 °C
Hyphae	Smooth	Smooth	Smooth	Smooth and granular walls
Ascomatal bases				
Colour	Dark-brown to black	Dark-brown to black	Black	Dark brown to black
Shape	Globose to sub-globose	Globose to obpyriform	Globose	Globose
Length	(115-)136-192(-236) µm	(174-)192-256(-310) µm	(153-)192-254(-281) µm	(154-)206-254(-279) µm
Width	(125-)139-199(-230) µm	(171-)188-244(-295) µm		
Ascomatal necks				
Disc form at base	Absent	Absent	Absent	Present
Length	(541-)766-980(-1103) µm	(394-)437-575(-654) µm	(514-)557-635(-673) µm	(385-)443-819(-1097) µm
Width (base)	(21-)26-36(-46) µm	(21-)26-34(-40) µm	(25-)32-42(-48) µm	(30-)43-57(-64) µm
Width (tip)	(15-)19-27(-33) µm	(16-)19-29(-35) µm	(14-) µm 16-22(-26) µm	(14-)16-22(-26) µm
Ostiolar hyphae				
Shape	Divergent and convergent	Divergent and convergent	Divergent	Divergent
Length	(47-)57-73(-79) µm	(60-)75-91(-96)	(42-)45-59(-69) Mm	(10-)18-36(-50) µm
Ascospores				
Length	3-4 µm	3-5 µm	3-4 µm	2-4 µm

Characteristics	*C. mangicola*[1]	*C. mangivora*[1]	*C. manginecans*[2]	*C. omanensis*[3]
Width (excluding sheath)	3-4 µm	4-6 µm	4-5 µm	4-6 µm
Width (including sheath)	5-6 µm	5-8 µm	7-8 µm	5-7 µm
Primary phialides				
Length	(59-)71-119(-140) µm	(70-)78-106(-124) µm	(72-)81-109(-144) µm	(19-)22-36(-56) µm
Wide at base	(3-)4-6(-7) µm	(3-)5-7 µm	5-7(-9) µm	(1-)2-4(-5) µm
Width (broadest point)	5-7(-8) µm	5–7(-8) µm	6-8(-9) µm	NA
Width (tip)	3-5(-8) µm	3-5 µm	3-6 µm	1-3 µm
Secondary phialides				
Length	(53-)72-114(-148) µm	(42-)62-100(-118) µm	(59-)65-77(-84) µm	NA
Width (base)	4-6(-7) µm	(3-)4-6 µm	5-8 µm	NA
Width (tip)	6–8(-9) µm	(4-)6-8(-9) µm	(5-)6-8 µm	NA
Primary conidia				
Length	(15-)18-24(-29) µm	(12-)16-24(-31) µm	(15-)23-29(-33) µm	6-8 (-9) µm
Width	(3-)4-6 µm	2-5 µm	3-6 µm	2-3 µm
Secondary conidia				
Length	(6-)7-9(-11) µm	(8-)9-13(-15) µm	(8-)9- 11(-12) µm	(5-)6-8(-10) µm
Width	6-8 µm	(5-)6-8(-9) µm	5-7(-8) µm	3-5 µm
Chlamydospores	Present	Absent	Present	Absent
Shape	Globose to sub-globose		Globose to sub-globose	
Length	(12-)14-16(-17) µm		(11-)12-14 µm	
Width	(9-)11-13(-14) µm		9-11(-12) µm	

[a] Van Wyk *et al*. 2011.

[b] Van Wyk *et al*. 2007.

[c] Al Subhi *et al*. 2006.

CHAPTER 2

Aetiology and causal agents of mango sudden decline disease in the Sultanate of Oman

(Al Adawi *et al*. 2006; *European Journal of Plant Pathology* **116**: 247-254)

ABSTRACT

Mango sudden decline is a recently introduced, economically serious disease in Oman. Affected mango trees have wilting symptoms that usually begin on one side and later spread to involve the entire tree. Trees exude amber-coloured gum from the bark of their trunks or branches and vascular tissues are discoloured. Having entered Oman in the recent past, survey data are presented that show the disease to have spread throughout the northern part of the country. Evidence is presented that the vascular wilt pathogen *Ceratocystis fimbriata* causes mango sudden decline disease in Oman, possibly in concert with *Lasiodiplodia theobromae* and the recently described *Ceratocystis omanensis*. Isolates of these fungi from affected trees, cause infection and can be recovered from inoculated seedlings. Bark beetles (*Cryphalus scabrecollis*) are shown to carry *C. fimbriata* and *L. theobromae* and are presumably responsible for transmitting both pathogens to healthy mango trees. Acting as a wounding agent and vector, the bark beetle is likely to have assisted the rapid spread of the disease across Oman.

2.1. INTRODUCTION

Mango, date, lime and banana are the most important perennial fruit crops in the Sultanate of Oman. The area of mango production in 2004 was 2500 ha with production of over 8600 t, concentrated in the Al Batinah region along the northern coast of the country. Cultivation is based on local Omani varieties and exotic scions grafted onto Omani rootstocks.

During 1998, many mango trees began dying in the southern part of the Al Batinah region. The disease was locally called sudden decline in recognition of the rapid death of affected trees. It was first reported in the Barka area in the south of the Al Batinah region. The disease spread northwards and was subsequently reported in Masanah, Suwaiq, Khabora, Saham, Sohar, Liwa, and Shinas (Fig 1, Al Adawi 2002). Subsequently, sudden decline has increased in severity, threatening mango cultivation in the country. To limit the spread of the disease, in 2001 the Ministry of Agriculture and Fisheries (MAF) embarked on an eradication programme to remove infected trees. More than 13% of the trees were removed from some districts of the Al Batinah region (Table1).

In 2000, during preliminary studies on the disease, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (CAB International reference W6341) was frequently isolated from mango trees affected by sudden decline. *Ceratocystis fimbriata* Ellis and Halst. (culture collection CMW, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa, see Van Wyk *et al*. 2005) was recovered more frequently, as was *C. omanensis* Al Subhi, Wingfield, van Wyk & Deadman (culture collection CMW, see Al Subhi *et al*. 2006). In addition, the bark beetle *Cryphalus scabrecollis* Eichhoff. (Coleoptera: Scolytidae) (International Institute of Entomology, identification reference 24214) appeared to be

consistently associated with the disease, possibly acting as a wounding agent and as a vector of spores.

Because of the importance of mango sudden decline in Oman and the consequent threat to local and regional mango production, research was conducted to establish by survey the distribution of the disease in northern Oman, to investigate the role of *C. fimbriata*, *C. omanensis* and *L. theobromae* in the aetiology of sudden decline disease, and to consider the role of the bark beetle in disease development.

2.2. MATERIALS AND METHODS

2.2.1. Disease distribution and symptom development

The field distribution of mango sudden decline was determined in 2000. The survey covered 8 districts of the Al Batinah region where mango production is concentrated (Fig 1). Detailed examinations were made of trees at all stages of disease development in each of the areas visited.

2.2.2. Pathogen isolation and identification

Samples were collected from several trees at each farm surveyed. Most isolations were made from stem tissue. Wood samples (0.5 – 1.5 x 2.00 – 3.00 cm) were chiselled from the margins of infected tissue after bark removal. Plant tissue was cut from the leading edges of lesions and washed with tap water, surface sterilized in 1% NaOCl for 1 min., rinsed in sterile distilled water, blotted on sterile filter paper, and aseptically placed between carrot discs (Moller & DeVay 1968) or transferred to Petri dishes containing malt extract agar

(MEA). Plates were incubated at room temperature (22 – 25 ^{o}C) and after 24 hr subcultured to fresh MEA plates.

Carrot discs were incubated for 5 – 7 days at room temperature under high humidity. After ascomata developed, ascospore masses were transferred to MEA supplemented with streptomycin (500 mg/l). Isolations made directly onto MEA were purified by transferring hyphal tips onto new MEA plates. Isolated cultures are maintained at the Ghadafan Agriculture Research Station (GCC), Ministry of Agriculture and Fisheries, Oman.

2.2.3. Pathogenicity tests

Pathogenicity tests were conducted on stems of young mangoplants (24 months old) of local variety growing in 13 cm diameter pots, containing a mixture of peatmoss and loamy soil. The trees were inoculated with 4 isolates of *C. fimbriata*, *C. omanensis* and *L. theobromae*. Five seedlings per treatment were inoculated by inserting an agar disc (3 mm diameter) bearing mycelium taken from the leading edge of actively growing colonies on MEA, under the bark that had been lifted away from an I-shaped incision (10 mm long) made with a sterile scalpel (Mullen *et al*. 1991). Seedlings inoculated with uncolonized MEA served as controls. Moistened, sterile cotton pads were placed over wounds that were then wrapped loosely with Parafilm to maintain a humid environment. Parafilm wraps were removed one week after inoculation.

Inoculated seedlings were assessed weekly to record symptom development. As lesions developed, pieces of stem were plated on MEA to verify the presence of inoculated pathogens; fungi were recovered and re-cultured to confirm identity. Analysis of variance of lesion length values taken 42 days after inoculation was done using the GLM procedure in

SAS (v 8.2, SAS Institute, Cary, NC, USA). Means were compared with Tukey's Studentized Range (HSD) at $P \leq 0.05$.

2.2.4. Isolations from bark beetles

Over 700 *C. scabrecollis* bark beetles, adults and larvae, were caught using aspirator from different locations in the Al Batinah region. Bark beetles were also taken from 3 mango log traps taken from healthy mango trees and placed, for 30 days, within an infested orchard in Barka. As a control, three similar logs were placed under polyester fleece tents within the same orchard for the same duration.

Insects were immersed in 1% NaOCl for 1 min., rinsed with sterile distilled water, blotted dry on sterile filter paper, and aseptically placed on potato dextrose agar (PDA). Plates were incubated at room temperature for 3 days, after which colonies emerging from beetles were sub-cultured and identified. Bark beetles were also placed in a cavity made on the inner surface of a pair of carrot discs and incubated at room temperature for 4 days. For isolation from plant tissue and insects, the carrot disc technique was later modified to enhance *C. fimbriata* recovery by soaking carrot discs in streptomycin (100 mg/l) before incubation. In all cases, *C. fimbriata* was identified based on culture morphology, distinctive perithecia and culture aroma (Upadhyay 1981).

2.3. RESULTS

2.3.1. Disease distribution and symptom development

The distribution of the disease across the different districts of Al Batinah region showed the same pattern as the distribution of numbers of affected trees subsequently

removed by the Ministry of Agriculture and Fisheries. This data (Table 1) indicates highest disease levels in the east of the region (76% in Barka, location 9 in Fig 1), decreasing closer to the border with the United Arab Emirates (12.5% in Shinas, location 1 in Fig 1).

Initial disease symptoms were gummosis from the bark and branch death (Fig 2a) on affected trees. The affected trees usually displayed other symptom, including vascular discoloration beneath the gummosis (Fig 2b). Tree death usually occurred within 6 months of first symptom appearance. Diseased trees always showed signs of damage caused by the bark beetle *C. scabrecollis* (Fig 2c, 2d). The majority of diseased trees had developed large, inconspicuous trunk cankers where the bark appeared darker than normal. Beneath the affected bark underlying tissues were discoloured brown to black. Rootstocks of grafted trees were frequently severely affected compared with the scion, which was commonly asymptomatic (Fig 2e). Cankers located near ground level often resulted in death of the entire tree, especially with grafted trees. However, local varieties appeared to be more severely affected than exotic scions on grafted trees.

2.3.2. Pathogen isolation and identification

From April 2000 to May 2004, samples were collected from 9 areas of the Al Batinah region. Of the 294 fungal isolates made from a random selection of plant tissue and beetles, the majority (193 = 65.6%) were recovered from plant tissue. Fewer successful isolations (101 = 34.4%) were made from beetles. *L. theobromae* represented 137 (46.6%) of the isolates, while *C. fimbriata* represented 83 (28.2%) and *C. omanensis* represented 8.2%. Fifty isolates (17%) remained unidentified, although some were possibly *L. theobromae* that failed to produce pycnidia in culture. *L. theobromae* and *C. fimbriata* were isolated in approximately equal frequency from wood; *C. omanensis* was isolated predominantly from wood. Some samples appeared to yield two or more of the pathogens and it is possible that

excessive growth of one, especially *L. theobromae*, masked the appearance of *C. fimbriata*, causing an underestimation of the frequency of isolation of this pathogen.

2.3.3. Pathogenicity tests

Mango plants inoculated with *C. fimbriata* developed gummosis and extensive lesions on all treated seedlings (Table 2). Wilting progressed over a few days into a permanent wilt, with leaves still attached. Longitudinal sections under the bark revealed dark brown discoloration extending above and below the inoculation site.

Lesions also developed on plants inoculated with *C. omanensis* and *L. theobromae*. However, mean lesion length was significantly longer on stems inoculated with *C. fimbriata* (29.4 cm) compared with *C. omanensis* (1.8 cm) and *L. theobromae* (1.8 cm). Control seedlings did not display lesions. In each case the fungus used as inoculant was reisolated from infected seedlings.

2.3.4. Isolation from bark beetles

Both *C. fimbriata* and *L. theobromae* were isolated from adult beetles. Between 2000 and 2002 *C. fimbriata* was isolated at relatively low frequency (0 – 13.2%) from beetles, even when the carrot baiting method was used. When this method was modified by treating carrot slices with streptomycin to reduce bacterial contamination, the recovery percentage of *C. fimbriata* from adult beetles improved significantly (Table 3).

The frequency of *L. theobromae* isolation from beetles ranged from 1.3 to 72% for adults and 71 to 100% for larvae (Table 3). In 2000, *L. theobromae* was isolated from 72% of adult beetles collected from Barka, and from 100% of larvae collected in the same orchard. In 2001, beetles were collected from the same area and 80% of larvae and adults yielded isolates of *L. theobromae*. The fungus was isolated from 43% and 23% of bark beetles collected from

Khabora and Sohar respectively during 2002. However, the rate of recovery of this fungus was lower for samples collected from Masanah (1.3%) and Seeb (3.9%).

Where isolations were made from adult beetles collected from three log traps, 3/15 (20%), 25/60 (42%) and 6/48 (13%) of the insects yielded *L. theobromae*. However, *L. theobromae* was isolated from 27/38 (71%) larvae collected from these traps. Removal of the bark of the log traps showed that tissue directly beneath the beetle infestation holes was discoloured. There was no discoloration below the bark on logs without insect injury. Isolation of fungi from the margins of the discoloured tissue yielded *L. theobromae*. No insects were found on log traps placed under polyester fleece and no vascular discoloration was evident. *C. fimbriata* was not isolated from log traps.

2.4. DISCUSSION

The disease was locally called sudden decline in recognition of the rapid death of affected trees. It was first reported in the Barka area in the south of the Al Batinah region in 1998. The disease spread northwards and was subsequently reported in Masanah, Suwaiq, Rustaq, Khabora, Saham, Sohar, Liwa and Shinas (Al Adawi 2002). Results of this study show that three fungi, *C. fimbriata*, *C. omanensis* and *L. theobromae* are closely associated with sudden decline disease of mango in Oman. This conclusion is based on the consistent isolation of the three species from stems of affected trees, the ability of these fungi to cause lesions in inoculated seedlings, and their recovery from the diseased tissue of inoculated plants. The results show a close association of the disease and the bark beetle, *C. scabrecollis*, with the disease and its ability to transmit these fungi. Although *C. fimbriata* was not isolated from beetles collected in the logs placed in infected orchards, this was

primarily because the optimized protocol for isolating *Ceratocystis* spp. had not been developed at the time.

The roles of the three fungi associated with mango sudden decline are uncertain. All three fungi were able to cause lesions on inoculated seedlings and they might all contribute to symptom development. However, since *C. fimbriata* was the most pathogenic fungus in inoculation tests, it may be the most important component in disease development. This fungus has caused a similar devastating disease of mango in Brazil known as *Seca*, since the late 1930s (Ribiero, 1980). Symptoms of that disease are similar to symptoms in Oman, including wilting, vascular discoloration, gummosis, blighting and tree death (Ploetz & Prakash 1997). This supports the hypothesis that *C. fimbriata* is the primary factor associated with sudden decline of mango in Oman. The fungus is a well known vascular wilt pathogen of many tree species (Kile 1993). On mango, before the present study, *C. fimbriata* had only been recorded in Brazil.

The most extensive lesion development in this study occurred following inoculation with *C. fimbriata*. Thus, *L. theobromae* may act as a secondary pathogen, colonizing lesions produced by *C. fimbriata*. The low frequency of isolation of *C. fimbriata* when specialized techniques are not used, and the relative ease with which *L. theobromae* is isolated, could have led researchers to conclude that *L. theobromae* was the causal agent of the disease in initial studies on mango sudden decline.

L. theobromae is nonetheless closely associated with sudden decline of mango in Oman. This fungus was consistently isolated from symptomatic tissue on dying trees and it also gave rise to distinctive lesions in inoculation tests. *L. theobromae* is a well known opportunistic tree pathogen in the tropics and sub-tropics (Punithalingam 1980). The fungus is the asexual state of *Botryosphaeria rhodina* (Sutton 1980) and like many other species of

Botryosphaeria, exists as an endophyte in asymptomatic tree tissue (Johnson 1992; 1994). It is a well documented pathogen of mango (Johnson 1992; 1994; Ploetz & Prakash 1997; Ploetz 2003). *L. theobromae* has been in Oman for many years, it has been recorded on mango causing dieback disease (Moghal *et al*. 1993). *L. theobromae* may contribute to tree death in Oman after insect damage and infection by *C. fimbriata*.

The relationship between *C. fimbriata* and *L. theobromae* in mango decline aetiology needs further investigation. In Brazil, symptoms associated with *C. fimbriata*, causing *Seca* disease are identical to those associated with *Diplodia recifensis* Batista (morphologically similar to *L. theobromae*) causing Recife sickness; both diseases are found in the same area (Ploetz & Prakash 1997). It is possible that Recife sickness and *Seca* may be the same disease, but the literature on these diseases is confused and difficult to interpret (R.C. Ploetz, *personal communcation*).

This study provides clear evidence for the role of the bark beetle *C. scabrecollis* in sudden decline of mango in Oman. Scolytid beetles are also thought to play key roles in the development of Recife sickness and *Seca* diseases of mango in Brazil (Ribiero 1980). In Brazil, the related beetle *Hypocryphalus mangiferae* is reported as the primary species responsible for disseminating *C. fimbriata* (Ribiero 1980). *Ceratocystis* spp. are typically vectored by bark beetles and other insects (Graham 1967). More specifically, *C. fimbriata* produces a fruity aroma that is attractive to insects and its sticky spores, produced at the apices of long and exposed perithecial necks. The pathogen is ideally suited to transport by Scolytid beetles (Hanssen 1993; Christen *et al*. 1997). The tunnelling of these beetles into the stems of mango trees provides rapid access to host tissue.

The random distribution and rapid progress of mango sudden decline disease across northern Oman suggests the involvement of an insect vector. In Oman, the disease was first

observed in Barka in 1998. By 2001 it had spread to Shinas (Table 1), approximately 200 km distant; an apparent spread rate of 60 km per year.

The involvement of *C. omanensis* in mango sudden decline requires further investigation. The results of this study suggest that it is a weaker pathogen than *C. fimbriata*, but is nonetheless capable of causing lesions. The pathogenicity of a larger number of isolates of all three fungi associated with sudden decline, an analysis of differences in susceptibility between local and exotic varieties and investigations into the role of the bark beetle in disease development are being considered. Furthermore, isolation of *C. fimbriata* from mango for the first time outside of South America suggests a serious lapse in local quarantine procedures that requires investigation.

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Table 1. Number of mango trees in the Al Batinah region eradicated by the Ministry of Agriculture and Fisheries, in 2001 and incidence of mango wilt recorded during a survey covering the period June 2000[a].

District[b]	Number of trees prior to epidemic	Number of trees removed		Incidence (%) recorded during survey
Barka	74794	10154	(13.6%)	76.0
Masanah	24012	591	(2.5%)	89.4
Suwaiq	55282	7465	(13.5%)	60.2
Khabora	35969	1212	(3.4%)	54.5
Saham	95918	4182	(4.4%)	76.1
Sohar	n/a[c]	n/a[c]	n/a[c]	41.9
Liwa	36246	321	(0.9%)	19.9
Shinas	65670	189	(0.3%)	12.5

[a] Source: MAF, 2002.

[b] District order reflects increasing distance from Barka, see Fig 1.

[c] Data not available.

Table 2. Lesion development (mean of 5 replicates) on mango seedlings 42 days after inoculation with *C. fimbriata*, *C. omanensis* and *L. theobromae*.

Isolate	Pathogen	Source	Location	Mean lesion length (cm)[a]
GCC55	*C. fimbriata*	Mango	Sohar	19.8 a
GCC58	*C. fimbriata*	Bark beetle	Sohar	24.5 a
GCC120	*C. fimbriata*	Mango	Shinas	27.1 a
GCC138	*C. fimbriata*	Mango	Shinas	46.1 a
GCC43	*C. omanensis*	Mango	Sohar	1.4 b
GCC48	*C. omanensis*	Mango	Sohar	3.8 b
GCC49	*C. omanensis*	Mango	Sohar	1.0 b
GCC52	*C. omanensis*	Mango	Sohar	1.0 b
GCC134	*L. theobromae*	Mango	Shinas	2.0 b
GCC100	*L. theobromae*	Mango	Sohar	1.7 b
GCC174	*L. theobromae*	Mango	Sohar	2.4 b
GCC356	*L. theobromae*	Bark beetle	Liwa	1.0 b

[a] Values not having a letter in common are significantly different at $P = 0.05$ according to Tukey's Studentized Range (HSD)

Table 3. Recovery of *C. fimbriata* and *L. theobromae* from adult bark beetles isolated using potato dextrose agar and carrot baiting techniques.

Location[a]	**Isolation date**	**Isolation medium**					
		PDA				**Carrot**	
		C. fimbriata		***L. theobromae***		***C.fimbriata***	
Barka	October 2000	0/25	(0.0)[b]	18/25	(72.0)[b]	0/18	(0.0)[b]
Barka	October 2000	0/10	(0.0)	10/10	(100.0)[c]	4/52	(7.7)
Barka	January 2001	0/20	(0.0)	16/20	(80.0)		
Barka	January 2001	0/10	(0.0)	8/10	(80.0)[c]		
Masanah	March 2002	0/78	(0.0)	1/78	(1.3)		
Rustaq	January 2004					2/3	(66.7)[d]
Khabora	February 2002	0/100	(0.0)	43/100	(43.0)		
Saham	April 2001					5/38	(13.2)
Sohar	March 2002	0/100	(0.0)	23/100	(23.0)		
Sohar	November 2003					23/40	(57.5)[d]
Liwa	January 2004					13/24	(54.2)[d]
Liwa	January 2004					1/4	(25.0)[d]
Liwa	May 2004					2/10	(20.0)[d]
Quriyat	March 2004					5/6	(83.3)[d]
Seeb	March 2002	1/76	(1.3)	3/76	(3.9)	0/26	(0.0)

[a] See Fig 1.

[b] Numbers in parentheses represent percent recovery

[c] Isolated from larval stage

[d] Indicates use of streptomycin pretreatment of carrot slices

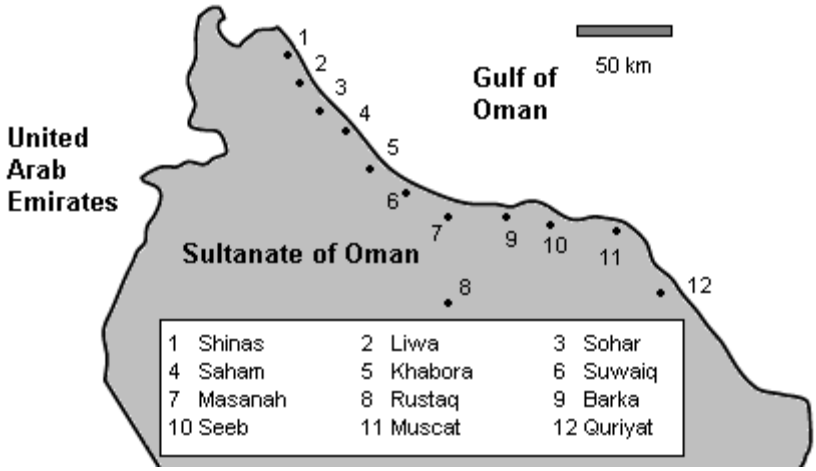

Fig. 1. Map of northern Oman showing locations for districts covered by the survey quantifying the incidence of mango sudden decline disease.

Fig. 2. Symptoms of mango sudden decline, (a) gummosis on bark, (b) branch death, (c) vascular discolouration, (d) adult bark beetle, (e) bark beetle damage, (f) dead tree with severely affected rootstock but asymptomatic scion.

CHAPTER 3

The mango wilt pathogen *Ceratocystis manginecans* vectored by *Hypocryphalus mangiferae* (Coleoptera: Scolytinae) in Oman

ABSTRACT

In Oman, the bark beetle *Hypocryphalus mangiferae* is closely associated with trees affected by mango wilt disease caused by *Ceratocystis manginecans*. Although it has previously been assumed that this beetle plays a role in the dispersal of the pathogen, this has not been established experimentally. The aim of this study was to determine whether *H. mangiferae* vectors *C. manginecans* from infected to healthy mango trees. A survey conducted in northern Al Batinah region of Oman revealed that *H. mangiferae* was closely associated with mango wilt disease symptoms and was found in the early stages of the disease. Healthy, two-year-old mango seedlings were exposed to *H. mangiferae* collected from diseased mango trees. Seedlings were infested by the bark beetles and after six weeks, typical mango wilt disease symptoms were observed. *Ceratocystis manginecans* was isolated from the wilted mango seedlings while uncolonized control seedlings remained healthy. The results indicate that *H. mangiferae* vectors *C. manginecans* in Oman and is, therefore, an important factor in disease aetiology and epidemiology.

3.1. INTRODUCTION

A serious mango disease epidemic has recently appeared in Oman that has led to the death of thousands of trees. The disease was first noted during 1998 in the Barka area, in the southern part of the Al Batinah region (Al Adawi *et al*. 2003). The death of mango trees was initially attributed to various factors but is now known to result from infection by the virulent fungal pathogen *Ceratocystis manginecans* M. Van Wyk, A. Al Adawi & M.J. Wingf. (Al Adawi *et al*. 2006, Van Wyk *et al*. 2007).

When mango wilt disease was first discovered in Oman, the cause was initially attributed to heavy infestations of bark beetles. Consequently, insecticide sprays were recommended to minimize its impact (F. Fahim & S. Al Khatri, *personal communication*). Bark beetles collected from diseased mango trees were identified as *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytinae) (Florida Department of Agriculture, E2005-1780-701). This insect is native to southern Asia including India, Malaysia and Indonesia (Pena & Mohyuddin 1997). It has been introduced into many areas where mango is grown, including Brazil and southern Florida (Atkinson and Peck 1994, Butani 1993). The beetle is phloeophagous and monophagous (restricted to mango), and infests the main stems and branches of trees (Atkinson & Peck 1994; Butani 1993, Pena & Mohyuddin 1997).

Examination of mango trees suffering from mango wilt revealed that symptoms of the disease begin on healthy trees at the sites of *H. mangiferae* entrance holes. Bark beetle infestation is followed, in most cases, by the exudation of gum at the sites where insects enter the stems. Brown to black vascular discolouration develops in wilted and severely affected trees (Al Adawi *et al*. 2006). Once external symptoms become apparent, *C. manginecans* can readily be isolated from discoloured woody tissues.

Recently, a mango wilt disease with symptoms similar to those seen in Oman was reported in Pakistan (Malik *et al*. 2005, Van Wyk *et al*. 2007). Furthermore, the disease

symptoms were associated with *H. mangiferae* infestation and *C. manginecans* was isolated from adult beetles (Van Wyk *et al*. 2007). A very similar mango wilt disease has been known in Brazil since 1938 (Viegas 1960). *Ceratocystis fimbriata sensu lato* is consistently isolated from diseased mango trees and has been confirmed as the causal agent of the disease in Brazil (Viegas 1960, Ploetz 2003, Ferreira *et al*. 2010). *H. mangiferae* is also closely associated with the disease in Brazil and *C. fimbriata s.l.* has been isolated from adult beetles (Castro 1960, Rossetto *et al*. 1980, Ribeiro 1993).

During the past 12 years, mango wilt disease in Oman has spread rapidly from the south to the north of the Al Batinah region and to other regions including Muscat, Musandam, Dakhiliyah and Sharqiyah. This rapid spread is consistent with a pathogen that has an insect vector such as bark a beetle (Al Adawi *et al*. 2006). Furthermore, the disease in Oman is randomly distributed within each area and the isolation rate for *C. manginecans* from *H. mangifera* associated with the disease was over 80% (Al Adawi *et al*. 2006). *Ceratocystis manginecans* isolates from diseased mango trees or from *H. mangiferae* were confirmed to be pathogenic and produce vascular discolouration and wilt symptoms (Al Adawi *et al*. 2006). Although it has been assumed that *C. manginecans* is disseminated by *H. mangiferae*, this has not been proven experimentally (Al Adawi *et al*. 2006, Van Wyk *et al*. 2007). The aim of this study was to follow Leach's principles (Leach 1940) and to confirm the role of *H. mangiferae* in the mango wilt disease epidemic through transmission of *C. manginecans* from infected to healthy mango trees.

3.2. MATERIAL AND METHODS

3.2.1. Survey and isolations

During 2004, mango trees on seventeen farms in the Sohar and Liwa areas, located in the northern part of the Al Batinah region, were assessed for the presence of *H. mangiferae*

and their association with symptoms of wilt disease (Fig 1). At each farm, the salinity of irrigation water was measured and the percentage of healthy trees, dead trees and those infested with *H. mangiferae* was calculated. Percent bark beetle infestation was calculated based on whether trees were seed-propagated or grafted. Disease incidence was also calculated by counting infected trees with visible signs of disease such as gummosis, vascular discolouration under the bark and partial wilt. Data from the survey were statistically assessed using analysis of variance. Mean *H. mangiferae* infestation levels and disease incidence in different areas or types of propagation were separated by Tukey's test using SAS software. Correlation between salinity of irrigation water and *H. mangiferae* infestation levels, and disease incidence, were analysed using SAS.

3.2.2. Inoculation of mango seedlings with live *H. mangiferae*

Fifteen 2-year-old mango plants propagated from local Omani varieties (unnamed, monoembryonic) were grown in 13 cm diameter pots containing a mixture of peat moss and loamy soil (50: 50). The plants had an average height of 96 cm and stem diameter of 1.9 cm, 10 cm above the soil level (Table 1). The stem of each mango plant was enclosed in a 1.5 l transparent plastic container, 10cm above the soil level (Fig 2a); the top of the container was covered with nylon mesh to allow gas exchange.

During March 2004, 225 individual *H. mangiferae* beetles were collected from mango trees affected by wilt, using an aspirator. Twenty five of these insects were randomly selected and used for pathogen isolation using carrot baiting (Moller & DeVay 1968) to verify the presence of *C. manginecans* on the insects. Another twenty bark beetles were randomly extracted from the sample and placed in the plastic containers surrounding 10 plants. Five containers surrounding seedlings were left empty to serve as controls. All plants were maintained at 25 ± 2^{o}C.

The number of *H. mangiferae* individuals infesting the plants was estimated by subtracting the number of dead beetles found at the base of each container from the total number of bark beetles added to the container. Gummosis and wilt symptoms were recorded for each plant every seven days and vascular discolouration was recorded after 64 days when fungal isolations were made from all plants.

As soon as seedlings showed symptoms of wilt, samples of discoloured vascular tissue were removed and surface sterilized with 70% ethanol and placed between fresh carrot slices (Moller & DeVay 1968). Carrot slices were checked after seven days for the presence of *Ceratocystis* perithecia. Ascospore droplets from the tip of perithecial necks were transferred to 2% MEA (Malt Extract Agar) supplemented with streptomycin sulphate (500 mg/l) and incubated at 25 ± 2^{o}C.

3.2.3. Pathogen identification

Ceratocystis isolates were identified by growth pattern on MEA and morphological characteristics (Van Wyk *et al*. 2007). Two isolates were randomly selected representing isolates of *Ceratocystis* sp. from mango seedlings exposed to *H. mangiferae*. Total genomic DNA was extracted from pure cultures of each isolate (Barnes *et al*. 2001). The concentration of extracted DNA was measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, Delaware) and diluted to a concentration of 5-20 ng/µl with sterile distilled water. The primers ITS1 and ITS4 were used to amplify part of ribosomal DNA operon (White et al. 1990). PCR reactions were prepared in a total volume of 25 µL which included 1 µL of diluted genomic DNA, 2.5 µL of 10x PCR buffer including 1.5 mM of $MgCl_2$, 2 µL of dNTPs (10 mM), 1 U of *Taq* polymerase, 0.5 µL of each primer, and 18.2 µL of sterile distilled water.

PCR reactions were performed as described by Van Wyk *et al*. (2007). PCR amplification products were purified using 6% Sephadex G-50 columns (1 g Sephadex in 15

ml sterile water, Sigma-Aldrich, Steinheim, Germany). Sequencing reactions were prepared in 10 µL total volumes containing 2 µL purified PCR product, 1 µL of the same primers used for the first PCR amplification, 2 µL 5x dilution buffer and ABI Prism Big Dye Terminator mix, v. 3. 1 (Applied Biosystems Inc., Foster City, California). Sequencing PCR cycles consisted of 25 repetitions of 96 ºC for10 s; 50 ºC for 4s; 60 ºC for 4 min. Sequencing reactions were cleaned using Sephadex G-50. Sequences were analysed using an ABI Prism 3100 DNA sequencer (Applied BioSystems, Foster City, California, USA). Resulting sequences were subjected to a BLAST search in order to compare with available ITS sequences of *Ceratocystis* spp. in GeneBank (National Center for biological Information; http://www.ncbi.nlm.nih.gov).

3.3. RESULTS

3.3.1. Surveys and isolations

Approximately 1090 mango trees in Sohar and Liwa were inspected for the presence of *H. mangiferae*. More than 50% of the trees examined were found to be infested with *H. mangiferae*, 40% showed wilt symptoms and 18% of trees had died (Fig 3). All trees with disease symptoms were infested with *H. mangiferae*. Furthermore, 11% of the trees were free of wilt symptoms but were infested with *H. mangiferae*. Survey data revealed higher *H. mangiferae* infestation levels in Liwa (55.9%) compared to Sohar (43.7%). Wilt disease incidence was significantly ($P < 0.05$) higher in Liwa (49.5%) than in Sohar (33.9%). Differences in mortality levels between Sohar (19.4%) and Liwa (12.4%) were not significant.

Hypocryphalus mangiferae infestation and disease incidence in local seed- propagated mango trees was significantly higher (60.3%, 48.9%) than in grafted trees (37.1%, 31.7%). Percent mortality was higher in seed-propagated trees (21%) than in grafted trees (11.9%).

The salinity levels of irrigation water ranged from 0.630 to 2.07 dS m^{-1} in Liwa and from 0.480 to 1.94 dS m^{-1} in Sohar. There was no correlation between *H. mangiferae* infestation levels or disease incidence with salinity. Results of all correlation analyses for parameters identified in the survey are shown in Table 2.

3.3.2. Inoculation of mango seedlings with *H. mangiferae*

Isolations from *H. mangiferae* yielded *Ceratocystis manginecans* from 12% of the beetles. Eighty percent (159 of 200) of the *H. mangiferae* individuals added to containers entered the bark of healthy plants. Infestation levels ranged from 40 to 100% of individual beetles for the different plants (Table 1). Colonization by *H. mangiferae* was mainly initiated at the leaf scars and towards the bases of the plants where the stems were thickest. Longitudinal cuts into stems revealed that beetle tunnels were restricted to the bark, did not extend into the xylem, were short, and in only one instance, contained eggs.

Gummosis, initially brown, but later black-brown, was observed in 80% of the exposed mango seedlings. No gummosis was observed on the control plants (Fig 2b). The amount of gummosis varied among seedlings: four seedlings developed gummosis within 2 weeks and a further four showed gummosis after 4 weeks (Table 1). Wilt symptoms were first observed after 42 days (Table 1). Four exposed seedlings developed wilt symptoms (Fig 2c) and no wilt was observed in the control seedlings.

Longitudinal sections through exposed stems revealed vascular discolouration radiating from beetle entry points (Fig 2b). Discolouration was not observed in the control seedlings or in the roots of any plant.

3.3.3. Pathogen identification

Isolations from exposed seedlings (Fig 2d) yielded *Ceratocystis*-like, grey-olive colonies on MEA. The pathogen was isolated from the four wilted mango seedlings and from two that developed gummosis. No control seedlings yielded *Ceratocystis* isolates after

attempted isolation. Perithecia produced hat-shaped ascospores typical of *C. fimbriata s. l.* All isolates produced secondary and primary conidiophores in addition to cylindrical and barrel- shaped conidia that were indistinguishable from those of *C. manginecans* (Van Wyk *et al*. 2007). BLAST searches of ITS sequences in GenBank for three *Ceratocystis* isolates isolated in this study (CMW15384, CMW15386, CMW15389) showed that they had a 98% similarity with all sequences of *C. manginecans* from Oman (CMW13851= AY953383.1, CMW13852= AY953384.1, CMW13854= AY953385.1). Furthermore, all *Ceratocystis* isolates isolated in this study had identical microsatellite allele sizes to those of *C. manginecans* from *H. mangiferae* and diseased mango in Oman and Pakistan (Chapter 4).

3.4. DISCUSSION

Results of this study provide clear evidence that *H. mangiferae* acts as the vector of the mango wilt pathogen, *C. manginecans*. Survey data demonstrate an intimate association between infestation by *H. mangiferae* and mango wilt disease. Moreover, *H. mangiferae* was found to infest healthy mango trees in the surveyed areas. There was no significant correlation between irrigation water salinity and level of *H. mangiferae* infestation and mango wilt disease incidence. Healthy mango plants exposed to *H. mangiferae* showed symptoms typical of the disease, including gummosis, vascular discolouration and wilt. In addition, *C. manginecans* was re-isolated from the symptomatic tissue.

In the ten years since the disease was first observed in Oman, mango wilt has spread rapidly from the south to the north across the Al Batinah region (more than 250 km), as well as to other regions of the country. Such rapid spread of disease by a fungus widely recognized to be insect-vectored (Webber & Gibbs 1989, Storer *et al*. 1999) would require an active mechanism of dispersal such as a wood boring beetle. Results of the survey in this study demonstrated a clear association between *H. mangiferae* and mango wilt disease in all

infected mango trees. However, infestation by *H. mangiferae* was also found in 11% of mango trees free of disease symptoms. Healthy mango trees attacked by *H. mangiferae* might represent an early stage of disease development. This supports the view that *H. mangiferae* is the primary vector with an ability to attack non-stressed and healthy mango trees. In Brazil, *H. mangiferae* has been reported as common on mango trees without disease symptoms and on those at the start of disease development (Castro 1960, Rossetto *et al*. 1980).

High *H. mangiferae* infestation levels and mango wilt disease incidence in Liwa compared to Sohar support previous observations that both moved progressively from south to north through the Al Batinah region of northern Oman, beginning in 1998 (Al Adawi *et al*. 2006). Results of the present study also show that non-grafted local mango varieties are highly susceptible to the disease compared to grafted varieties comprising a local rootstock and exotic scion. *H. mangiferae* was most commonly associated with local mango varieties and less so with exotic, grafted varieties. This is consistent with previous observations that mango wilt disease is significantly more common on local mango varieties compared to those grafted with non-native scions (Al Adawi *et al*. 2006). The reason for the greater susceptibility in local varieties is unclear but it is also consistent with the fact that large numbers of bark beetle tunnels were observed on the rootstocks of grafted trees. This could be due to genetic differences between exotic and local varieties, expressed in differences in defense responses to bark beetle infestation and *C. manginecans* infection (see also Al Sadi *et al*. 2010). Research is currently underway to select varieties resistant to mango wilt disease and this should also include investigations into susceptibility to infestation by *H. mangiferae*.

Farms included in the survey varied in the salinity of irrigation water. Although salinity is a major limiting factor for agriculture in Oman, only one farm out of the 17 surveyed, had irrigation water salinity levels unsuitable (EC > 2 dS/m) for mango cultivation (Meurant *et al*. 1999). This farm represents 7.4% of all mango trees included in the survey.

There was no correlation between infestation by *H. mangiferae*, disease incidence and irrigation water salinity and this factor appears not to be involved in the development of mango wilt disease. Nonetheless, future research should consider the relationship between infestation by *H. magiferae* and stress including salinity, nutritional or environmental predisposition.

The results of this study clearly demonstrate the ability of *H. mangiferae* to colonize healthy mango seedlings and they confirm that the beetles are able to establish infections of *C. manginecans* in health mango plants. The pathogen was easily isolated from plants exposed to insects subsequent to symptom development and the majority of sampled insects were shown to carry the fungus. These data provide clear evidence that the pathogen is spread to the healthy seedling by *H. mangiferae*. This is also consistent with the fact that mango wilt caused by *C. fimbriata s.l.* in Brazil is widely accepted to be vectored by introduced *H. mangiferae* (Castro 1960, Rossetto *et al*. 1980).

The fact that there was some inconsistency in symptom development on seedlings exposed to *H. mangiferae* is not surprising. It would be most unlikely that individual insects would carry consistent loads of inoculum and efficacy of transmission would also not be expected to be consistent from plant to plant. Furthermore, plants propagated from seed such as in this study would have had different genetic backgrounds, thus influencing susceptibility.

The exact manner in which *H. mangiferae* vectors *C. manginecans* is not known. In other insect-vectored pathogens, spores can adhere to the outer surface of insects, can be carried in the insects' gut or can be transferred via specialized mycangia (Francke-Grossman 1967, Malloch & Blackwell 1993). Further studies are required to resolve mode of transport of the pathogen, but the fact that beetles consistently carry *C. manginecans* and that they can transfer the pathogen to healthy plants, is unequivocal. Furthermore, results of this study, together with previous finding of a high isolation rate (13-83%) of *C. manginecans* from *H.*

mangiferae associated with the disease (Al Adawi *et al*. 2006) fulfill Leach's principles regarding insect vectorship (Leach 1940). Confirmation of *H. mangiferae* as the primary vector for the mango wilt pathogen is important for an integrated disease management program. Fungicides and insecticides, alone and in combination, have been tested but their efficacy, at least in Oman, remains equivocal (Ministry of Agriculture and Fisheris, *unpublished data*). *H. mangiferae* produces several generations per year and it may be possible to manage emerging adults with insecticide applications but adults and larvae within galleries are unlikely to be controlled (S. Al Khatri, *personal communication*). Thus, a more comprehensive understanding of the biology of *H. mangiferae* is now required.

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Table 1. Association of bark beetle (*H. mangiferae*) infestation with mango wilt disease conducted in two areas in the Al Batinah region, Sultanate of Oman.

		Trees with wilt disease symptoms (%)			Trees infested by *H. mangiferae*	
Treatments		**Healthy**	**Diseased**	**Dead**	**Asymptomatic**	**Infested**
Area	*P* value	0.889	0.013	0.325	0.580	0.079
Liwa		38.1a[a]	49.5a	12.4a	31.7a	55.9a
Sohar		36.9a	33.9b	19.4a	27.1a	43.7a
Tree type	*P* value	0.094	0.006	0.184	0.017	0.001
Local (Seed propagated)		30.1a	48.9a	21.0a	18.6b	60.3a
Exotic (Grafted)		44.7a	31.7b	11.9a	39.3a	37.1b

[a] Column means followed by the same letter are not significantly different according to Tukey's test ($P \leq 0.05$).

Table 2. Correlation between irrigation, water salinity and percentage of mango trees infested with bark beetles (*H. mangiferae*) in surveyed mango farms conducted in two areas of the Al Batinah region.

		R	***P* value**
All trees	*Healthy*	-0.014	0.173
	Diseased	0.013	0.062
	Dead	0.000	0.951
	Free of bark beetle	-0.010	0.280
	Infested	0.010	0.134
Local (Seed propagated)	*Healthy*	0.001	0.905
	Diseased	0.005	0.597
	Dead	-0.005	0.559
	Free of bark beetle	0.006	0.466
	Infested	0.000	0.971
Exotic (Grafted)	*Healthy*	-0.032	0.012
	Diseased	0.005	0.583
	Dead	0.010	0.273
	Free of bark beetle	-0.029	0.027
	Infested	0.002	0.866

Table 3. Percent beetle colonization and rate of disease development after exposing mango plants to *H. mangiferae*.

Seedling	Type	Seedling height (cm)	Seedling diameter (mm)[a]	% Bark beetle colonization[b]	Gummosis appearance (days after exposure)[b]	Wilt appearance (days after exposure)[b]
1	+ *H. mangiferae*	115	24	100 (20/20)	1	-
2	+ *H. mangiferae*	82	16	50 (10/20)	26	64
3	+ *H. mangiferae*	110	20	55 (11/20)	4	42
4	+ *H. mangiferae*	100	23	50 (10/20)	11	64
5	+ *H. mangiferae*	102	20	100 (20/20)	5	-
6	+ *H. mangiferae*	93	16	40 (8/20)	22	42
7	+ *H. mangiferae*	126	23	100 (20/20)	22	-
8	+ *H. mangiferae*	123	27	100 (20/20)	22	-
9	+ *H. mangiferae*	74	16	100 (20/20)	-	-
10	+ *H. mangiferae*	86	16	100 (20/20)	-	-
	Mean	**101.1**	**20.1**			
11	- *H. mangiferae*	69	19	-	-	-
12	- *H. mangiferae*	88	20	-	-	-
13	- *H. mangiferae*	95	16	-	-	-
14	- *H. mangiferae*	112	19	-	-	-
15	- *H. mangiferae*	70	13	-	-	-
	Mean	**86.8**	**17.4**			

[a] Measured at 10 cm above soil level.

[b] "-" indicates no colonization/gummosis/wilt.

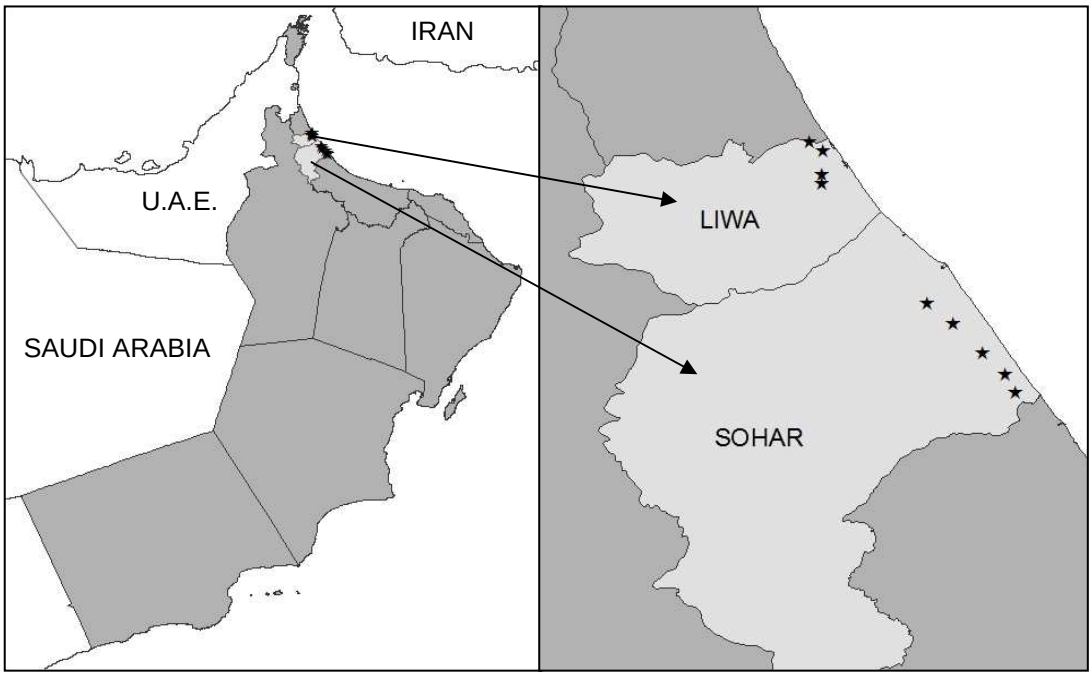

Fig. 1. Distribution of the farms in Sohar and Liwa in the Sultanate of Oman that were surveyed for mango wilt disease incidence and association with *H. mangifera*e infestation.

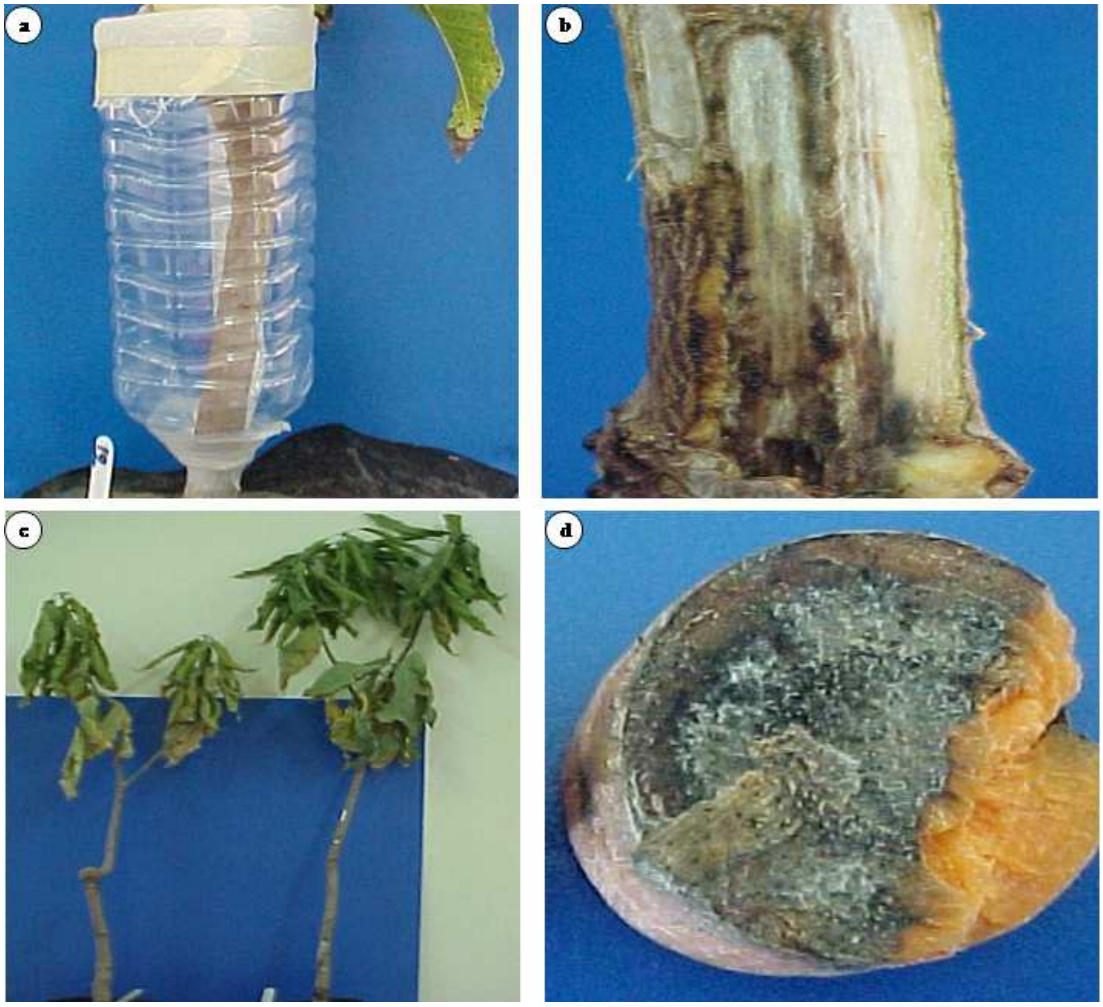

Fig. 2. Inoculation procedure and disease development in mango seedlings exposed to *Hypocryphalus mangiferae*; (a) plastic cage surrounding mango stem, (b) gummosis and vascular discolouration, (c) wilted mango seedlings, (d) *Ceratocystis manginecans* isolated on carrot discs.

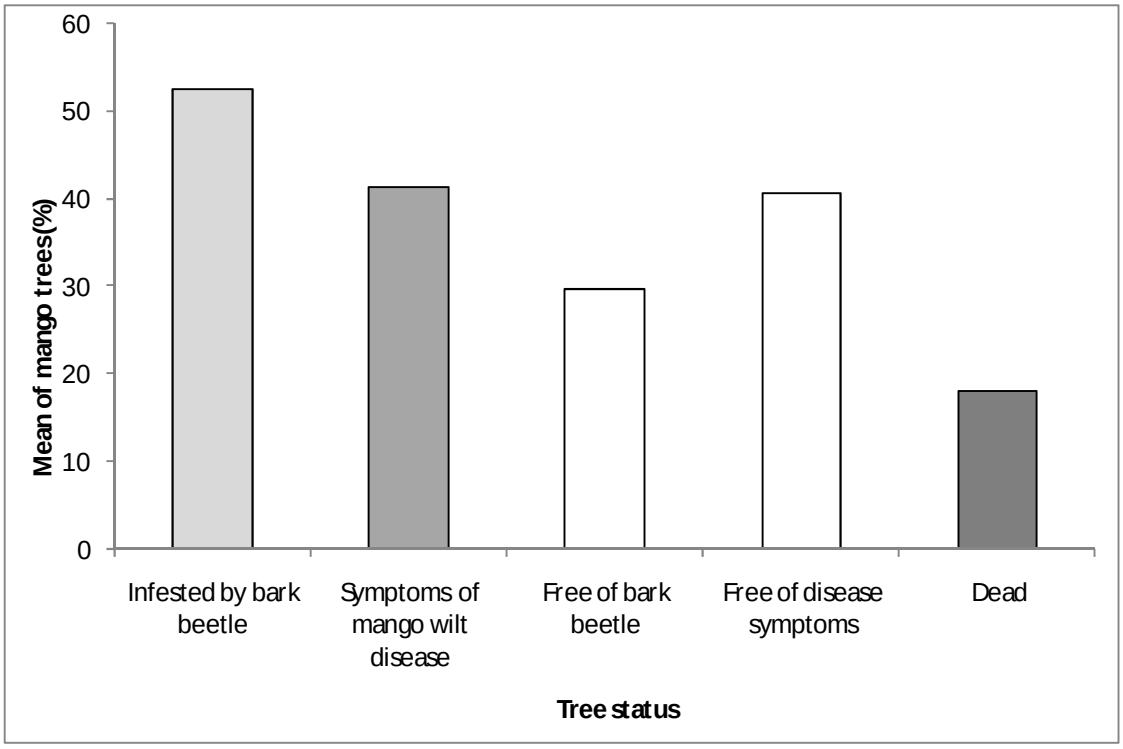

Fig. 3. Observations of infestation by bark beetle (*Hypocryphalus mangiferae*) and symptoms on mango trees surveyed on the farms in the Sohar and Liwa areas in the Al Batinah region.

CHAPTER 4

Clonal structures of *Ceratocystis manginecans* populations from mango wilt disease in Oman and Pakistan

ABSTRACT

Ceratocystis manginecans has recently been described from Oman and Pakistan where the fungus causes a serious wilt disease of mango. In both countries, the disease has moved rapidly throughout mango producing areas leading to the mortality of thousands of mango trees. The disease is associated with the infestation of the wood-boring beetle *Hypocryphalus mangiferae* that consistently carries *C. manginecans*. The aim of this study was to consider the population structure of *C. manginecans* isolated from Oman and Pakistan by using microsatellite markers and amplified fragment length polymorphisms (AFLP's). Population genetic analysis of *C. manginecans* isolates from diseased mango tissue or bark beetles associated with the disease in Oman and Pakistan, showed no genetic diversity. The apparently clonal nature of the population suggests strongly that *C. manginecans* was introduced into these countries as a single event or from another clonal source.

4.1. INTRODUCTION

Ceratocystis manginecans M. Van Wyk, A Al Adawi, & M.J. Wingf was recently described as a serious pathogen causing wilt of mango trees (*Mangifera indica* L.) in Oman and Pakistan (Al Adawi *et al*. 2006; Van Wyk *et al*. 2007). Although mango has been planted in Oman and Pakistan for several centuries, the disease was first observed as recently as 1998 in both countries. In Oman, mango wilt disease was first reported on a small number of trees in Barka, southern Al Batinah. The disease subsequently spread to all mango growing areas killing thousands of trees (Al Adawi *et al*. 2003; Al Adawi *et al*. 2006). Although the mango industry in Oman is relatively small (8600t per annum), it accounts for 30% of mango consumption in the country (Ministry of Agriculture and Fisheries [MAF], 2009b; Royel Oman Police [ROP] 2002; 2008). In contrast, Pakistan is a major mango producer and exporter, with production exceeding 1.7 mt per annum and net profits from mango exports exceeding 20 million $US 2007 (FAOSTAT 2007). The recent establishment and spread of mango wilt disease has resulted in substantial loss and threatens mango production in both countries (Kazmi *et al*. 2005; Al Adawi *et al*. 2006).

Disease symptoms in Oman and Pakistan include vascular discoloration, gummosis and wilt of part, or the whole of the infected trees. The disease is usually accompanied by visible infestation by the wood boring beetle *Hypocryphalus mangiferae* (Coleoptera: Scolytinae) (Al Adawi *et al*. 2006; Van Wyk *et al*. 2007). The first record of *H. mangiferae* attacking mango trees in Oman and Pakistan was concurrent with the first report of mango wilt disease. Furthermore, the pathogen responsible for the disease, *C. manginecans* (Al Adawi *et al*. 2006), was recovered from *H. mangiferae* isolated from diseased mango trees in both countries (Al Adawi *et al*. 2006; Van Wyk *et al*. 2007). In Oman, the high levels of recovery (13- 83%) of *C. manginecans* from *H. mangiferae* associated with the disease and

the random distribution and rapid progress of mango wilt across northern Oman, has suggested the involvement of the bark beetle as vector for the pathogen (Al Adawi *et al*. 2006).

Ceratocystis manginecans is a recently described species in the *C. fimbriata sensu lato* complex (Van Wyk *et al*. 2007). This complex includes several newly described and numerous other unresolved species isolated from a wide range of hosts in many parts of the world (Kile 1993; Roux *et al*. 2000; Webster and Butler 1967a, b; Barnes *et al*. 2001; Baker *et al*. 2003). Such species include *C. albifundus* M.J. Wingf., De Beer & Morris, the cause of a serious wilt disease of *Acacia mearnsii* (Wingfield *et al.* 1996); *C. pirilliformis* Barnes & M.J. Wingf. from *Eucalyptus* (Barnes *et al*. 2003); *C. cacaofunsta* Engelbrecht & Harrington. from *Theobroma cacao* and *C. platani* Engelbrecht & Harrington from *Platanus* sp. (Engelbrecht & Harrington 2005).

Mango wilt disease has been known in Brazil since the 1930's (Viegas 1960) and has similar symptoms to those observed in Oman and Pakistan. The causal agent of the disease in Brazil resides in the *C. fimbriata* complex that is believed to include a number of distinct taxa (Van Wyk *et al*. 2007; Van Wyk *et al*. 2011) or *C. fimbriata sensu stricto* (Harrington *et al*. 2011) but the relatedness of these to *C. manginecans* remains to be fully resolved. The purported vector of *C. manginecans* in Oman, *H. mangiferae* is also associated with mango wilt in Brazil (Castro 1960; Rossetto *et al*. 1980) where it is considered an invasive alien species.

In Sao Paulo State, Brazil, two strains of *C. fimbriata s.l* were found to be predominant. Mango varieties evaluated for resistance showed differential responses. The varieties Coração Boi, Espada, Ubá and Vitória were resistant to one strain but susceptible to the second (Ribeiro 1993; Ribeiro *et al*. 1995). Given the fact that the pathogen in Brazil may

encompass a number of discrete taxa (Van Wyk *et al*. 2011) or is one having considerable genetic variability, information on population genetic structure will be important for the development of effective management strategies for mango wilt disease in Oman, Pakistan and elsewhere (McDonald 1997; McDonald & Linde 2002; Milgroom & Peever 2003).

The origin of *C. manginecans* in Pakistan and Oman is unknown but field evidence suggests that it is an introduced pathogen. It is known that there have been large consignments of mango fruit imported into Oman from Pakistan (ROP 2000; 2002; 2004; 2006; 2008) and the proximity of the two countries encourages regular unauthorised introductions of mango planting material into Oman from Pakistan (Al Adawi, *personal observation*). The health status of this material is difficult to determine but illicit trade represents a clear means by which pathogens could enter Oman.

The aim of this study was to gain an understanding of the population structure of *C. manginecans* isolates from both diseased mango trees and bark beetles associated with mango wilt in Oman and Pakistan by using two sets of microsatellite markers previously developed for *C. fimbriata* (Barnes *et al.* 2001, Steimel *et al.* 2004) and an AFLP (Vos *et al.* 1995) protocols. In this way it was anticipated that it might be possible to better understand the origin of the pathogen in Oman and to inform programmes focussed on developing disease-tolerant planting stock.

4.2. MATERIALS AND METHODS

4.2.1. Sampling and fungal isolations

Isolates of *C. manginecans* were collected from stems of diseased mango trees and from *H. mangiferae* beetles in Oman and Pakistan. Samples from Oman were collected

between November 2003 and June 2004 from five areas (Shinas, Liwa, Sohar, Rustaq and Quriayat) where disease incidence was high. Liwa and Shinas are within 50 km of Sohar while Rustaq and Quriayat are 110 and 250 km distant from each other respectively (Fig 1). Between them, these areas represent 41.8% of the total mango production in Oman (Ministry of Agriculture and Fisheries [MAF] 2009a). Diseased mango samples from Pakistan were collected during May 2006 from Faisalabad and Multan (230 Km apart) in the province of Punjab (Fig 2). In addition, thirty adult *H. mangiferae* were collected from mango trees from Faisalabad and Multan using an aspirator. Punjab represents 52% of the total area under mango cultivation in Pakistan with an annual production of over 700,000 tons (67%) (Collins *et al*. 2006).

Samples were made by cutting pieces of woody tissue from the leading edges of lesions on the stems of trees. These samples were washed with tap water; surface disinfested in 1% NaOCl for 1 min, rinsed in sterile distilled water and blotted dry on sterile filter paper. To bait for *C. manginecans* and induce fungal sporulation, infected wood pieces were incubated in moist chambers and/or placed between two slices of carrot pre-treated with streptomycin (100 mg/l) (Moller & De Vay 1968).

Single adult *H. mangiferae* beetles were placed in a cavity made on the inner surface of a pair of carrot discs pre-treated by soaking in a streptomycin solution. Carrot discs inoculated with either diseased wood pieces or beetles were incubated for 7-10 days at room temperature (22-25^{o}C) under conditions of high humidity. Once ascomata had developed on the carrot discs, single ascospore masses were transferred onto 2% malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin (100 mg/l).

To confirm pathogen identity, fungal structures from randomly selected ten-day-old cultures of isolates were transferred to glass slides, mounted in lactic acid and examined

under a compound microscope. *C. manginecans* isolates were identified based on morphological characteristics such as colour of colonies, ascomatal shape, presence of hat shaped ascospores and production of cylindrical conidia (Van Wyk *et al.* 2007). All isolates used were preserved in the culture collection (CMW) at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

4.2.2. DNA extraction

All isolates were grown on 2% MEA and incubated for two weeks at 25 ^{o}C. Mycelium, including ascomata and ascospores was scraped from the agar surface using a sterile spatula and transferred to 1.5 ml Eppendorf tubes. These were freeze dried, the mycelium crushed to a fine powder using a glass rod and the DNA was extracted following the method described by Barnes *et al*. (2001). Extracted DNA was run on 1.5% agarose gels stained with ethidium bromide. The presence and intensity of the extracted DNA for each isolate was examined under ultraviolet (UV) illumination. DNA concentrations were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, Delaware) and DNA dilutions (30-60 ng/µl) made with sterile distilled water.

4.2.3. SSR-PCR and genescan analysis

Cross-species amplification of the available 27 microsatellite markers developed for *C. fimbriata* (Barnes *et al*. 2001; Steimel *et al.* 2004) were tested on three isolates of *C. manginecans*. PCR's were performed using 25 µl reaction mixtures consisting of 2 ng DNA, 2.5 µl of Expand HF buffer containing 1.5 mM $MgCl_2$, 300 nM of the forward and reverse microsatellite primers, 200 µM of dNTP and 0.35 U of *Taq* polymerase. Amplification of microsatellite primers was carried out using an Eppendorf thermocycler programmed as described previously (Barnes *et al*. 2001; Steimel *et al.* 2004). PCR products were separated

by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

Primers that successfully amplified *C. manginecans* were used on the isolates obtained from Oman and Pakistan following the same methods described above. Primers were fluorescently labelled with PET, VIC, FAM or NED dyes using the G5 labelling kit (Applied Biosystems, Warrington, UK).

Microsatellite products were multiplexed according to the fluorescent dye and the amplicon size. This was to enable the simultaneous analysis of the maximum possible loci in a single gel run. The fluorescently labelled SSR-PCR amplicons (0.5 µl containing approx. 1.5 ng DNA) were mixed with 0.4 µl GeneScan-500 Liz internal size standard (Applied Biosystems, Foster City, California) and 10 µl formamide. The majority of samples were run on an ABI Prism 377 DNA sequencer (Applied Biosystems) and the remaining samples were separated using an ABI Prism 3100 DNA sequencer. Due to allele shifts between instruments, three reference samples were analyzed on both instruments. GENOMAPPER version 3.0 (Applied Biosystems, Foster City, California) was used to analyze gels and determine allele sizes. All gel runs contained the same reference samples to ensure reproducibility.

All alleles that were only one base pair different from each other at a locus were sequenced to confirm the allele scoring. The PCR reactions for three isolates (CMW15337, 23629, 23637) of *C. manginecans* were repeated using same conditions described above but with non-fluorescent primers. The PCR products were purified using 6% Sephadex G-50 columns (1g sephadex in 15 ml sterile water, Sigma-Aldrich, Steinheim, Germany). Sequencing reactions were prepared using 5-10 ng of cleaned PCR product, 1µl primer, 2 µl 5x sequencing buffer, 2 µl of ABI Prism Big Dye Terminator mix, v. 3. 1 (Applied

Biosystems Inc., Foster City, California) in a total volume of 10 µl. Sequencing PCR consisted of 25 cycles at 96 ºC for 10 s; 50 ºC for 4 s; 60 ºC for 4 min. Sequencing reactions were cleaned using Sephadex G-50. Sequences were determined using an ABI PRISM 3100 Autosequencer (Applied BioSystems, Foster City, Califronia, USA) and aligned using MEGA 4.0 (Kumar *et al*. 2004).

Selected isolates from the *C. fimbriata s.l* clade (Harrington 2009) and *C. platani* were included in this study (Table 1) to test the robustness of the microsatellite locus amplification and for comparison with *C. manginecans* isolates.

4.2.4. AFLP analysis

Twenty one isolates of *C. manginecans* (nine from Oman and 12 from Pakistan) and three isolates of *C. platani* were screened using the AFLP protocol (Vos *et al.* 1995) modified by Myburg *et al.* (2001; Table 1). DNA was digested using restriction endonucleases *Eco*RI and *Mse*I and the restricted fragments were ligated to the corresponding adapters. The pre-amplification reaction was performed with two pre-selective primers (M E00 and M M00) using the following PCR mixture: 5 µl Restriction/ Ligation (R/L) mixtures; 1x PCR buffer; 0.2 mM dNTPs and 1.5 units *Taq* polymerase. The PCR conditions for pre-amplification of the R/L DNA fragments involved: 30 s at 72 ºC, 25 cycles of 30 s at 94 ºC, 30 s at 56 ºC, and 1 min at 72 ºC with an increment of 1 s per cycle, followed by 2 min at 72 ºC. The PCR mixture for selective amplification was carried out as above using one primer combinations (Mo4 + E-AC) and the *Eco*RI primer was labelled with the infrared dye, IRDye 700 (LI-COR, Lincoln, NE). The final PCR amplification consisted of 13 cycles of 10 s at 94 ºC, 30 s at 65 ºC with a decrement of 0.7 ºC per cycle, 1 min at 72 ºC; followed by 23 cycles of 10 s at 94 ºC, 30 s at 56 ºC, 1 min at 72 ºC with an increment of 1 s per cycle; and a final extension step at 72 ºC for 1 min. Formamide loading buffer was added to the products resulting from

the final amplification and these were run on a polyacrylamide gel using automated DNA sequencer 4200 LI-COR (Myburg *et al.* 2001). AFLP gel images generated from LI-COR automated sequences were visually evaluated and banding patterns of *C. manginecans* isolated from mango and *H. mangiferae* from Oman and Pakistan were compared to the *C. platani* isolates.

4.3. RESULTS

4.3.1. Sampling and isolations

Seventy nine isolates of *C. manginecans* were isolated from diseased mango trees and from bark beetles (*H. mangiferae*) from five areas of Oman (Table 1, Fig 1). Isolations from Faisalabad, Pakistan, yielded 17 *C. manginecans* isolates, nine of which were from bark beetles (Table 1, Fig 2). In total, 96 isolates of *C. manginecans*, three isolates of *C. platani* and six isolates of species from the *C. fimbriata s.l* clade representing those from different hosts and locations were used for the SSR studies (Table 1).

4.3.2. SSR-PCR and Genescan analysis

From the 27 loci amplified with the microsatellite markers, four loci (AG17/18, CF21/22, CAG900 and GACA60) were excluded from the analysis as they produced monomorphic alleles in all isolates, including those from the *C. fimbriata s.l* group. Loci AG1/2, AG7/8, AG17/18, CF11/12, CF15/16, CF17/18, CF23/24, CAT1200 and GACA60 amplified alleles with a one base pair polymorphism. After sequencing, 100% sequence similarity was found between the alleles that had one base pair difference between them. They were thus scored as a single allele with following sizes; 264, 283, 304, 219, 474, 270, 251, 158, 373 and 186.

Sizes of the SSR-PCR products in the remaining 23 loci ranged from 123 to 481 bp. In the *C. manginecans* populations from Oman and Pakistan, twenty six alleles were obtained from the amplification of twenty three polymorphic SSR loci. Two alleles amplified in loci CF11/12 and AAG9 but in both cases, one of the alleles had a low allele frequency value of 0.01 and 0.03 respectively. The remaining loci yielded monomorphic alleles in all isolates of *C. manginecans* from Oman and Pakistan. Therefore, in almost all loci examined, the allelic frequency values were one and gene diversity values were zero for the *C. manginecans* isolates from Oman and Pakistan (Table 2). All isolates of *C. manginecans* from either diseased mango or bark beetles represented a single multilocus genotype for the above mentioned loci. Furthermore, all the *C. manginecans* isolates had the same alleles as the two *C. fimbriata s.l* isolates from mango in Brazil at ten of the loci (43%) and they shared the same allele with the Brazilian fig and Eucalyptus isolates at six loci (26%). *C. platani* isolates shared the same alleles with the *C. manginecans* populations in three microsatellite loci (13%).

For the *C. fimbriata s.l* isolates, polymorphisms were observed despite the limited number of isolates used in this study. In the Brazilian mango isolates, six loci were polymorphic and each isolate had a different multilocus genotype.

Three loci (AAG9, CAT3K and CAT9X) amplified more than one allele per locus within the Oman and Pakistan *C. manginecans* population. Locus AAG9, amplified multiple alleles in one isolate of *C. manginecans* from Oman. One of these alleles was the same as that in the Brazilian mango and *Eucalyptus* isolates of *C. fimbriata s.l.* In addition, locus CAT3K amplified double alleles (309/321) for *C. manginecans* isolated from Oman and Pakistan and shared one of the alleles (309) with all the Brazilian *C. fimbriata s.l* isolates, irrespective of their host origin (Table 3).

4.3.3. AFLP profiles

The AFLP profiles of 18 loci for all isolates of *C. manginecans* isolated from diseased mango trees and bark beetles associated with mango wilt disease in Oman and Pakistan were identical. The three isolates of *C. platani* from *Platanus* that were used as controls and shared 14/18 (78%) bands with the *C. manginecans* isolates in Mo4 + E-AC primer combination. In addition, 10 unique AFLP bands were present in the profiles of the *C. platani* isolates and these bands were not present in the *C. manginecans* profiles.

4.4. DISCUSSION

Microsatellite and AFLP analyses this study showed a complete lack of variation amongst isolates of *C. manginecans*. Furthermore, there were no intra or inter geographical differences between *C. manginecans* isolates from Oman and Pakistan or between isolates from mango trees or bark beetles. The results, therefore, suggest a recent founder event with *C. manginecans* having been recently introduced and established in the region.

Results of this study affirm the efficacy of the microsatellite markers developed previously for *C. fimbriata s.l.* for studying populations of isolates of *C. manginecans* as well as *C. albifundus*, *C. platani*, *C. cacaofunesta* and recently *C. pirilliformis* (Engelbrecht *et al*. 2004; Barnes *et al*. 2005; Engelbrecht *et al*. 2007; Kamgan *et al*. 2009). This might indicate that flanking regions surrounding these markers are highly conserved across taxa, allowing cross species amplification of those markers (Selkoe & Toonen 2006).

The overall genetic analysis of *C. manginecans* isolates from Oman and Pakistan has shown that they represent a single clone of the pathogen. This supports the view that mango wilt disease in Oman and Pakistan emerged from single introduction of a single haplotype

and the population has expanded clonally to infect mango trees in many parts of both countries. However, the source of *C. manginecans* in Oman and Pakistan is unknown. Tracking records of customs and plant quarantine offices in Oman has revealed that there have been regular importations of mango fruit and germplasm from India and Pakistan (ROP Foreign trade statistics 2002; 2008). In addition, there is evidence of illegal introductions of mango seedlings into Oman from Pakistan that is facilitated by the proximity of the two countries (Al Adawi, *personal observation*). It is, therefore, possible that Pakistan represents the source of infection in Oman.

There are possible origins of *C. manginecans* in Oman other than Pakistan. For example there are recent but inconclusive observations that mango wilt disease in India and Bangladesh (Ploetz & Freeman 2009; Mosharraf Hossain, *personal communication*) and this might be the source of the infections in both Pakistan and Oman. Results of this study, however, provide no clues as to the origin of the pathogen, which may have come from South America that appears to be an area of substantial genetic variation for *C. fimbriata s.l.* including the many cryptic species encompassed by this group encompasses (Barnes *et al*. 2001; Baker *et al*. 2003; Engelbrecht & Harrington 2005; Rodas *et al*. 2008; Van Wyk *et al*. 2009; Van Wyk *et al*. 2010).

Prevention of further introductions of *C. manginecans* into Oman and Pakistan will be important as this will preclude an expansion of the genetic diversity of the pathogen in these countries. *C. manginecans* reproduces asexually via conidia and by ascospores arising from unidirectional mating type switching (Webster 1967; Webster & Butler 1967a, b; Harrington & McNew 1997; Witthuhn *et al*. 2000). The fungus is able to outcross and there is emerging evidence of hybrids between species in the *C. fimbriata s.l.* complex (B. Wingfield, *unpublished*). A greater genetic diversity in *C. manginecans*, emerging from sexual

outcrossing or hybridisation will complicate efforts to manage mango wilt disease in Oman and neighbouring countries. Clearly very effort should be made to prevent the introduction of strains of *C. manginecans* or other species in the *C. fimbriata s.l.* complex.

The lack of genetic variation between isolates of *C. manginecans* from *H. mangiferae* and mango in Oman and Pakistan suggests the direct involvement of *H. mangiferae* as a vector for the spread of the fungus in these countries (Rossetto *et al*. 1980; Al Adawi *et al*. 2006; Van Wyk *et al*. 2007; Masood *et al*. 2008). *H. mangiferae* is host specific on mango and is indigenous to southern Asia where mango originated. Other than in Brazil, Oman and Pakistan where the insect is found in association with *C. manginecans*, *H. mangiferae* also occurs in many parts of the world including India, Malaysia, Indonesia and southern Florida where mango wilt disease is not known (Wood 1982; Butani 1993; Atkinson & Peck 1994). However, *C. manginecans* was recently reported in Indonesia associated with wilt and die-back disease on *Acacia* spp (Tarigan *et al*. 2011) and *C. fimbriata s.l* has been reported to cause disease on various crops including coffee (*Coffea arabica* L.), taro (*Colocasia esculenta*), pomegranate (*Punica granatum*) and rubber (*Hevea brasiliensis*) in the same area (Ploetz & Prakash 1997; Somasekhara 1999). Yet there has been no connection between these diseases and *H. mangiferae*, suggesting that the *C. manginecans* has not co-evolved with the insect (Ploetz & Freeman 2009).

The management of mango wilt disease involves using systematic fungicides and resistance mango cultivars (Rossetto *et al*. 1996; Ploetz & Freeman 2009). The development of genetic material resistant to the mango wilt pathogen, *C. manginecans*, is considered an important element in disease management. The Ministry of Agriculture in Oman, in collaboration with FAO, has begun a programme to introduce new mango germplasm from Brazil and Australia and to evaluate this for to infection by *C. manginecans* under local

conditions. The results of the present study showing clonality of the pathogen in Oman suggests that selection and breeding for resistance will be simpler than it would have been if *C. manginecans* were represented a diverse population of isolates.

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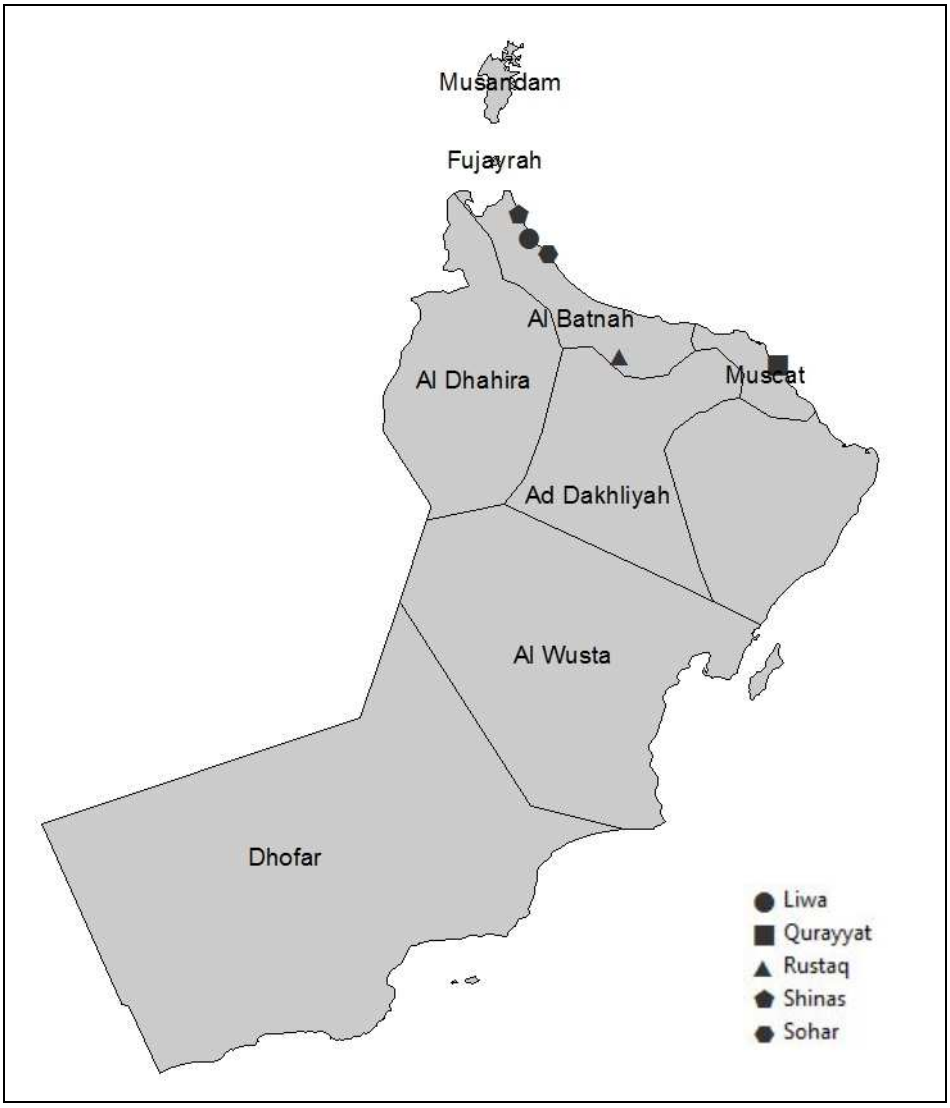

Fig. 1. Map of Oman showing locations for *C. manginecans* collection.

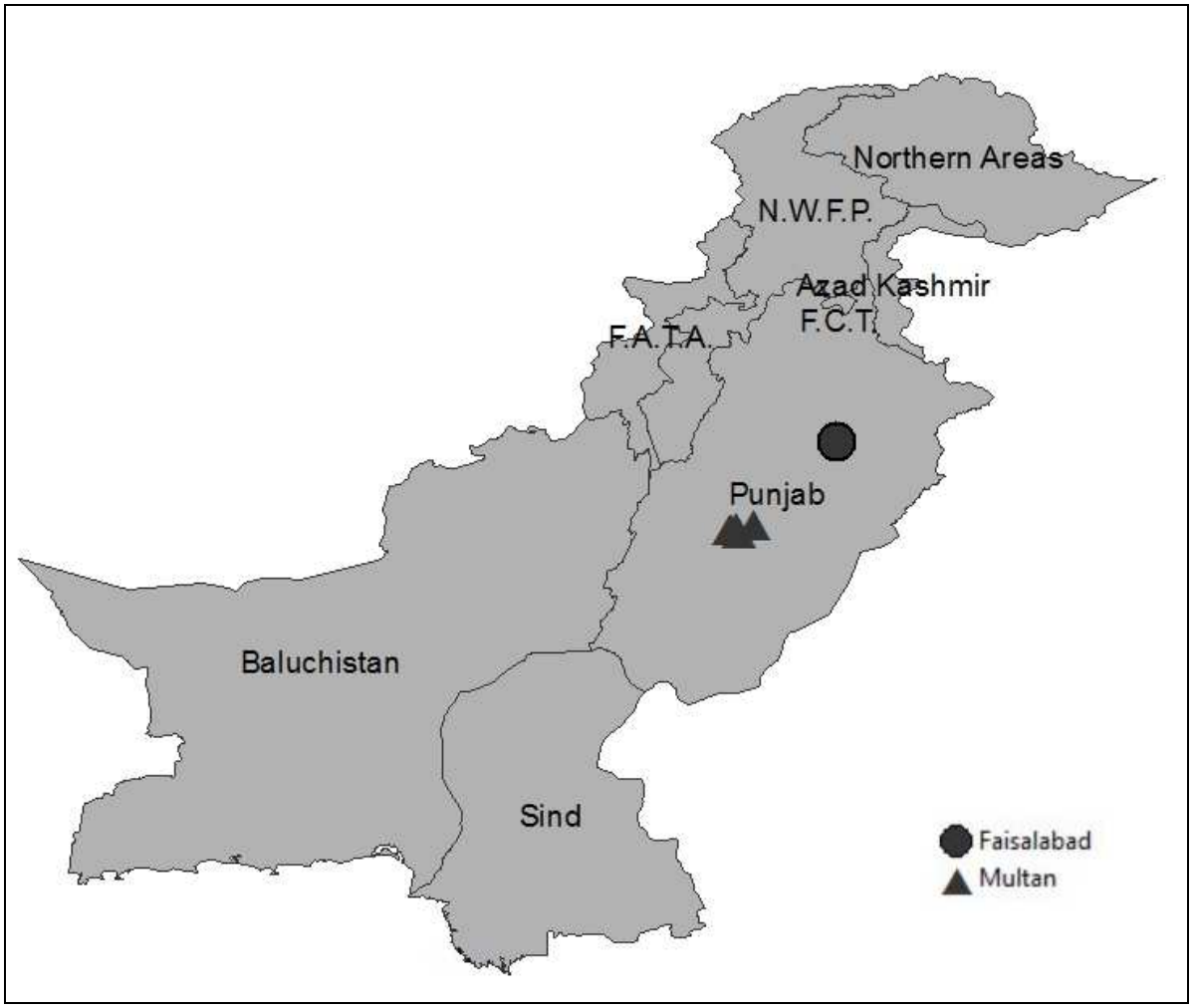

Fig. 2. Map of Pakistan showing locations for *C. manginecans* collection.

Table 1. Isolates of *Ceratocystis manginecans* used in this study. *C. fimbriata s.l* and *C. platani* isolates were used as controls.

***Ceratocystis* spp.**	**CMW isolates[a]**	**Source**	**Area**	**Country**	**Sample size**
C. manginecans	**15337[b]** – 15353	*Mangifera indica*	Shinas	Oman	16(2)[c]
	15366, 15369, 15370, 15385 - 15389, 15391	*M. indica*	Liwa	”	9(3)
	15313, **15314,** 15315 - **15316**, 15367, 15390	*M. indica*	Sohar	”	6(3)
	15371 – 15377	*M. indica*	Quariyat	”	7(1)
				Total	**38(9)**
C. manginecans	**15317** – 15336	*Hypocryphalus mangiferae*	Sohar	Oman	20(1)
	15354 - 15365, **15384**	*H. mangiferae*	Liwa	”	13(3)
	15368	*H. mangiferae*	Rustaq	”	1(1)
	15378 – **15381, 15382,** 15383, 15392	*H. mangiferae*	Quariyat	”	7(1)
				Total	**41(6)**
C. manginecans	17567	*M. indica*	Faisalabad	Pakistan	1(1)
	23637- 23642, 23643	*M. indica*	Multan	”	7(3)
				Total	**8(4)**
	23628, 23629, **23630-23634,** 23635, 23636	*H. mangiferae*	Faisalabad	Pakistan	**9(1)**
C. fimbriata s.l	14797, 15052	*M. indica*	-	Brazil	2
	14806	*Ficus* sp.	-	Brazil	1
	4903	*Eucalyptus* sp.	-	Brazil	1
C. platani	**1894**	*Platanus* sp.	-	Switzerland	1
	2219	*Platanus* sp.	-	France	1
	2242	*Platanus* sp.	-	Italy	1

[a] All the *C. manginecans* isolates were collected by A. O. Al Adawi and are maintained in the culture collection (CMW) of the Forestry and Agriculture Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b] Isolate number in bold were used in the AFLP analysis.
[c] Number in parenthesis indicates the number of sites where the samples were collected.

Table 2. Summary statistics for *Ceratocystis manginecans* isolates from Pakistan and Oman using 23 microsatellite markers.

	No. of isolates		No. of alleles		Allele frequency				H[a]	
Loci	**Oman**	**Pakistan**	**Oman**	**Pakistan**	**Oman**		**Pakistan**		**Oman**	**Pakistan**
					A	**B**	**A**	**B**		
AG1/2	79	7	1	1	1.00		1.00		0.00	0.00
AG7/8	79	17	1	1	1.00		1.00		0.00	0.00
AG15/16	79	17	1	1	1.00		1.00		0.00	0.00
CF5/6	79	17	1	1	1.00		1.00		0.00	0.00
CF11/12	79	17	2	1	0.97	0.03	1.00		0.05	0.00
CF13/14	79	17	1	1	1.00		1.00		0.00	0.00
CF15/16	79	16	1	1	1.00		1.00		0.00	0.00
CF17/18	79	17	1	1	1.00		1.00		0.00	0.00
CF23/24	79	17	1	1	1.00		1.00		0.00	0.00
AAG8	79	13	1	1	1.00		1.00		0.00	0.00
AAG9	79	17	2	1	0.99	0.01	1.00		0.03	0.00
CAA9	78	17	1	1	1.00		1.00		0.00	0.00
CAA10	77	17	1	1	1.00		1.00		0.00	0.00
CAA15	78	17	1	1	1.00		1.00		0.00	0.00
CAA38	73	16	1	1	1.00		1.00		0.00	0.00
CAA80	78	17	1	1	1.00		1.00		0.00	0.00
CAT1	76	17	1	1	1.00		1.00		0.00	0.00
CAT3K	79	17	1	1	1.00		1.00		0.00	0.00
CAT9X	79	17	1	1	1.00		1.00		0.00	0.00
CAT1200	79	17	1	1	1.00		1.00		0.00	0.00
CAG5	77	17	1	1	1.00		1.00		0.00	0.00
CAG15	79	16	1	1	1.00		1.00		0.00	0.00
GACA650	79	17	1	1	1.00		1.00		0.00	0.00

[a] H = Nei's (1973) gene diversity

CHAPTER 5

Ceratocystis manginecans associated with a serious wilt disease of two native legume trees in Oman and Pakistan

ABSTRACT

A serious wilt disease has recently been found on *Prosopis cineraria* (Ghaf) in Oman and on *Dalbergia sissoo* (Shisham) in Pakistan. Disease symptoms on both hosts include vascular discolouration and partial or complete wilt of affected trees. A species of *Ceratocystis* was consistently isolated from discoloured, recently infected wood of dying *P. cineraria* and *D. sissoo* exhibiting wilt symptoms. Morphological comparisons and analyses of DNA sequence data showed that the *Ceratocystis* isolates obtained from both tree species were the same. Furthermore, that they represented *C. manginecans*, a serious wilt pathogen of mango in Oman and Pakistan. This represents first report of *C. manginecans* infecting native trees, both belonging to the leguminosae. Cross inoculation of *C. manginecans* isolates from *P. cineraria*, *D. sissoo* and mango showed that the fungus can cause disease on all three host plants.

5.1. INTRODUCTION

Ceratocystis fimbriata sensu lato Ellis & Halst is a well recognized group of fungi that cause serious diseases on various agricultural crops (Kile 1993). This pathogen was first described from sweet potato (*Ipomoea batatas*) causing a black rot (Halsted & Fairchild, 1891). Since then, these fungi have been reported from many agricultural crops worldwide, causing vascular discolouration, wilt, cankers and root rot (Kile 1993). *C. fimbriata s. l.* has been reported as a serious pathogen causing substantial economic losses in hosts such as *Annona squamosa*, *Citrus* spp., *Coffea arabica*, *Colocasia esculenta*, *Crotalaria juncea*, *Ficus carica*, *Punica granatum*, *Mangifera indica*, *Eucalyptus* spp. and *Acacia* spp. (Kile 1993; CAB International 2001).

Ceratocystis fimbriata s. l. represents a complex of cryptic species that are increasingly being recognised as additional molecular techniques and especially DNA sequence comparisons are applied to this group of fungi (Wingfield *et al*. 1996; Barnes *et al*. 2003; Engelbrecht & Harrington 2005; Johnson *et al*. 2005; Van Wyk *et al*. 2007; Rodas *et al*. 2008; Van Wyk *et al*. 2009; Van Wyk *et al*. 2010). An example is that of a wilt disease of *Acacia mearnsii* (black wattle) for which the causal agent was first reported as *C. fimbriata* (Morris *et al*. 1993) and later described as new species, *C. albifundus* De Beer, Wingfield & Morris, based on morphological and DNA sequence comparisons (Wingfield *et al*. 1996). Subsequently, many additional species have been described in the *C. fimbriata* complex including *C. pirilliformis* Barnes & M. J. Wingfield isolated from *Eucalyptus* (Barnes *et al*. 2003), *C. cacaofunesta* Engelbrecht & Harrington infecting cacao and *C. platani* Engelbrecht & Harrington on plane and sycamore trees (Engelbrecht & Harrington 2005), *C. neglecta* M. van Wyk, Jol. Roux & C. Rodas and *C. fimbriatomima* M. van Wyk & M.J. Wingf. isolated from *Eucalyptus* sp. (Rodas *et al*. 2008; Van Wyk *et al*. 2009), *C. colombiana* M. van Wyk &

M.J. Wingf. and *C. papillata* M. van Wyk & M.J. Wingf. isolated from coffee, cacao and citrus (Van Wyk *et al*. 2010).

A serious disease of Mango (*Mangifera indica*) similar to a disease of this tree known as 'seca" (Viegas 1960; Ploetz 2003) was reported in Oman and Pakistan in 1998 (Malik *et al*. 2005; Al Adawi *et al*. 2006). For some years the disease in Oman was thought to be caused by a species of *Botryosphaeriaceae* (Al Adawi 2002; Al Adawi *et al*. 2003) but it was later shown to be caused by a species member of *C. fimbriata s. l.* complex (Al Adawi *et al*. 2003; Al Adawi *et al*. 2006). After the application of DNA sequence and morphological comparisons, the fungus was described as the new species, *Ceratocystis manginecans* M. Van Wyk, A. Al Adawi & M.J. Wingf. sp. nov. (Van Wyk *et al*. 2007).

Ceratocystis manginecans has killed many thousands of trees in Oman and Pakistan (Kazmi *et al*. 2005; Al Adawi *et al*. 2006; Van Wyk *et al*. 2007). The fungus is closely associated with a wood boring beetle *Hypocryphalus mangiferae* (Curculionidae: Scoltyinae) that has been shown to carry it to healthy trees (Al Adawi *et al*. 2006; Chapter 3). *Ceratocystis manginecans* has also recently been found in Indonesia where together with *C. acaciavora* Tarigan & M. van Wyk it is closely associated with the rapid wilt and death of the leguminous plantation tree, *Acacia mangium* (Tarigan *et al*. 2011).

Recently, native *Prosopis cineraria* L. Druce (locally known as Ghaf) trees in Oman have begun to show symptoms of wilt similar to those seen on mango trees infected with *C. manginecans*. *P. cineraria* (Leguminosae) is an important desert species and is one of the few trees capable of surviving without irrigation in harsh and arid conditions (Brown 1991; 1992). Similarly, in Pakistan, a dramatic wilt disease has been observed on native *Dalbergia sissoo*, Roxb. (locally known as Shisham) trees in 1995 (Kazmi *et al*. 2005). *Dalbergia sissoo*, like *P. cineraria* is a legume and is indigenous to Haryana and other parts of India, Pakistan,

Nepal and Bangladesh (Tantau *et al*. 2005). *Dalbergia sissoo* is a multipurpose tree that has a valuable timber and it is grown in plantations, beside canals and roadsides, and as field boundaries in private lands (Khan *et al*. 2004).

A *Ceratocystis* sp. was recently reported associated with *P. cinerea* and *D. sissoo* in Oman and Pakistan (Al Adawi *et al*. 2009, Poussio *et al*. 2010). The main objective of this study was to identify the species of *Ceratocystis* responsible for the disease associated with these trees by using morphological comparisons and DNA sequencing. Furthermore, host specificity and possible host jumps were investigated through cross inoculation in mango, *P. cineraria* and *D. sissoo* trees.

5.2. MATERIALS AND METHODS

5.2.1. Sample collection and fungal isolation

During December 2004, samples from *P. cineraria* trees showing symptoms of wilt were collected from Wilayat Sohar in Oman. Symptoms included single branches exhibiting wilt symptoms, dark grey-brown vascular discolouration of the affected branches or tree trunks and finally, wilt of the entire trees (Figs 1a & b). Samples were collected by removing the bark and cutting longitudinal strips of wood tissue (approx. 50 mm) from freshly infected xylem with stain symptoms. Additional samples were made during the following two years from different areas but in the vicinity of Sohar.

In May 2006, plantations of *D. sissoo* in Fasilabad, Shorkot, Chenab Negar and Multan, Pakistan were visited to examine disease symptoms in these areas. Symptoms on the tree included black-grey staining of the xylem tissues; leaves first turned brown from the tops to the bottoms of trees, followed by death of the entire trees (Fig 1 c & d). After removing the

bark from trees that had recently wilted, it was possible to cut longitudinal strips of discoloured (streaked) vascular tissue for isolations. In both the cases of *P. cineraria* and *D. sissoo*, wood samples for investigation were stored in plastic bags and preserved in a refrigerator prior to undertaking isolations in a laboratory.

Because the vascular discoloration on both *P. cineraria* and *D. sissoo* was very similar to that observed on mango trees dying as result of infection by *C. manginecans*, it was suspected that a *Ceratocystis* sp. might be involved. For this reason, carrot baiting (Moller & De Vay 1968a) was carried out where discoloured wood was placed between two slices of carrot that had first been treated with streptomycin sulphate (100 mg/l) (Moller & De Vay 1968a) and incubated at room temperature to induce fungal sporulation on the carrot slices. In addition, pieces of discoloured wood were placed in moist chambers at room temperature (25 ^{o}C) for 7-10 days to induce sporulation directly on infected tissue.

Ascospore masses that developed at the apices of ascomata on infected wood or carrot slices were transferred to 2% malt extract agar (MEA, 20 g/l malt, 20 g/l agar) (Biolab, Midrand, South Africa) in Petri dishes. These cultures were incubated at 25 ºC.

5.2.2. Morphological characterization

The morphological characteristics of the *Ceratocystis* sp. isolated from *P. cineraria* and *D. sissoo* trees were compared to those of *C. manginecans* isolates collected from mango trees in Oman and Pakistan (Van Wyk *et al*. 2007) (Table 1). Two isolates of *C. manginecans* from mango in Oman and Pakistan (CMW13854 CMW23641), two isolates of *Ceratocystis* sp. from *P. cineraria* (CMW17568 & CMW17570) and two from *D. sissoo* (CMW23623 & CMW23625) were selected for morphological comparisons. Morphological observations of the *Ceratocystis* isolates was made from fungal structures produced on 2%

MEA plates incubated for 10 days at 25 ºC. Samples were prepared by mounting fungal structures on glass slides in lactic acid and observed under a light microscope. For each isolate, 25 measurements were taken for the lengths and widths of the ascomatal bases, necks ascospores as well as the primary and secondary conidia and chlamydospores.

The rates of growth were measured for each representative isolate grown in MEA and incubated in dark at different temperatures ranging from 5 to 35 ºC at 5 ºC increments. The diameters of cultures were measured across two perpendicular axes after seven days with five replicate plates at each temperature for each isolate. All the morphological data were analyzed by analysis of variance (ANOVA) and means were compared using Tukey's test. All statistical analyses were performed using the SAS version 8 (SAS institute, Cary, NC).

5.2.3. DNA extraction, amplification, sequencing and phylogenetic analyses

Mycelium from 12-day-old cultures grown on 2% MEA plates was scraped from the surface of cultures, freeze dried for 24h and then ground into a fine powder using Geno Grinder (Glenmills). DNA extraction was performed using the phenol: chloroform (1:1) extraction protocol as described by Barnes *et al*. (2001a). Isolated DNA was cleaned by washing with 70% ethanol and dried under a vacuum. The isolated DNA was re-suspended in 50 µl sterile SABAX water with 10 µl of RNase A (10 mg/ml, Roche Diagnostics, South Africa) and incubated at 37 ºC for approximately 2 h to digest any residual RNA. The concentration and purity of the DNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, Delaware, U.S.A.). DNA was diluted with deionised sterile water to concentrations of 5-20 ng/µl.

Amplification of the ITS1 and ITS2 regions flanking the 5.8s ribosomal RNA gene was carried out with universal primers ITS1 and ITS4 (White *et al*., 1990). Part of the β-

tubulin (BT) gene region was amplified using primers βt2a and βt2b (Glass and Donaldson 1995) and part of the translation elongation factor (TEF-1α) gene was amplified using the EF1-728F and EF1-986R primer set (Jacobs *et al*. 2004). PCR reactions were prepared in a total volume of 25 µl that included 1.5 µL of diluted genomic DNA, 1 U of *Taq* polymerase (Roche Molecular Biochemicals), 2.5 µL of 10 x PCR buffer containing 2.0 mM $MgCl_2$, 0.5 µL of 10 mM of each primer and 2.5 µL of 10 mM of dNTPs. Amplifications were performed in a Mastercycler gradient thermal cycler (Eppendorf, Germany) using the following parameters: a 2-min step at 96 ºC followed by ten cycles of 20s at 94 ºC, 40s at 55 ºC and 45s at 72 ºC. The last three temperature intervals were repeated for another 30 cycles with a 5s increase per cycle for the annealing step at 55 ºC, followed by a final elongation step for 10 min at 72 ºC. PCR amplicons were visualized under UV light on 1% agarose gels (Roche Diagnostics, Mannheim). PCR amplification products were purified using 6% Sephadex G-50 columns (1 g sephadex in 15 ml sterile water, Sigma-Aldrich, Steinheim, Germany). Sequencing reactions were prepared in 10 µL total volumes containing 2 µL purified PCR product, 1 µL of 10 mM of the same primers used for the first PCR amplification and 2 µL 5x dilution buffer and ABI Prism Big Dye Terminator mix, v.3.1 supplied by the manufacturer (Applied Biosystems Inc., Foster City, California). Sequencing PCR cycles consisted of 25 repetitions at 96 ºC for10s; 50 ºC for 4s; 60 ºC for 4 min. Sequencing reactions were cleaned using Sephadex G-50. Sequences were determined using an ABI PRISM 3100 Autosequencer (Applied BioSystems, Foster City, California, USA). Sequences available in Genbank that were the most similar to the DNA sequences produced in this study and those of recently described species in the *C. fimbriata s. l* complex were used in the data sets for alignments.

Sequences were aligned using the programme MEGA version 5 (Tamura *et al*. 2011) and manually adjusted where necessary. Phylogenetic analysis and most parsimonious trees (MP) for each data set were generated in PAUP v. 4.0b10 (Swofford 2002). All characters were assigned equal weight and gaps were treated as a fifth character (new state). The heuristic search with 100 random stepwise additions and tree bisection reconnections (TBR) was employed as the swapping algorithm. Branch support for nodes was obtained by performing 1000 bootstrap replicates of the aligned sequences. For parsimony analysis, measures that were calculated included tree length (TL), retention index (RI) and consistency index (CI). *C. pirilliformis* was used as the outgroup taxon and the in-group was considered to be monophyletic.

The DNA substitution model for data sets representing each gene region was determined by Akaike Information Criterion (AIC) using PAUP4.0b10 and MrModeltest programme version 2.3 (Nylander 2004). These models were incorporated in Bayesian analyses using the programme MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Four chains of Markov Chain Monte Carlo (MCMC) were concurrently run from a random start for 1 million generations and the trees were sampled every 100th generation. Burnin values were calculated for each data set using Tracer programme version 1.4 and these values were incorporated in MrBayes to exclude all trees sampled before the stationary phase was reached. Posterior probabilities were calculated from a 50% majority rule consensus tree constructed from the remaining trees (Rannala and Yang 1996).

5.2.4. Inoculation trials

A first trial was designed to evaluate the pathogenicity of the *Ceratocystis* sp. from *P. cineraria* and *C. manginecans* on *P. cineraria* seedlings. This trial was conducted using two isolates of *C. manginecans* from mango (CMW13851 and CMW13854) and two isolates

(CMW17568 & CMW17570) of the *Ceratocystis* sp. from *P. cineraria* (Table 1). One-year-old *P. cineraria* plants grown from locally collected seeds in 13 cm diameter pots containing loamy soil mixed with peat moss were used in this trial. Seedlings of *P. cineraria* were wounded with a sterile scalpel by making an l-shaped incision (10 mm long) on the stems of the seedlings, approximately 20 cm above the soil level and inserting a mycelial plug of agar (4 mm diam) into each wound site. Seven seedlings of *P. cineraria* were inoculated with each *Ceratocystis* isolate and the same number of seedlings was inoculated with a sterile plug of MEA to serve as controls. These seedlings were arranged in a randomized block design. All inoculated wounds were covered with moistened sterile cotton pads and sealed with Parafilm.

All seedlings were kept in a shade house and watered twice a week. After 60 days, the bark tissue of the inoculated seedlings was removed above and below the inoculation site and the lengths of the lesions were measured. To re-isolate the inoculated pathogen, wood samples were taken from the margins of the lesions and plated out onto MEA plates or placed between two carrot slices.

In a second inoculation trial, host specificity on mango, *P. cineraria* and *D. sissoo* was tested. Nine-month-old mango seedlings (Pairi cultivar), one-year-old seedlings of *P. cineraria* propagated from local Omani seed and ten-month-old *D. sissoo* plants propagated from seeds, were grown in the same potting media as described for the first trial. All seedlings were kept under shade house conditions and irrigated twice per week. Two weeks prior to inoculation, all seedlings were transferred to a laboratory with a temperature of 24-26^{o}C and irrigated twice per week. The height and diameter of each seedling was measured at the time of inoculation. The inoculation was conducted using two isolates (CMW13851 & CMW13854) of *C. manginecans* from mango, two isolates (CMW17225 & CMW17568) of the *Ceratocystis* sp. from *P. cineraria* and two isolates (CMW23623 & CMW23625) of the

Ceratocystis sp. from *D. sissoo*. Inoculum of each isolate was grown on MEA for two weeks and 4 mm diam. mycelial plugs made using a cork borer were inserted into wounds of equivalent size made to remove the bark and expose the cambium on the stems of the seedlings. Points of inoculation were 10 cm above the soil level in the case of *P. cineraria* and *D. sissoo* and 10 cm above the graft union in the case of the mango plants. Each isolate was inoculated into three seedlings per host and three seedlings of each host were used as controls, inoculating them in an identical manner with a 4 mm sterile MEA plug. Lesion lengths on the inoculated seedlings were measured after 30 days.

Statistical analyses of the results were carried out for two trials using ANOVA in order to compare lesion lengths between isolates and host types compared in the second inoculation trial. In cases with significant ($P < 0.05$) variation among isolates, Tukey's test was used to compare means. Statistical analyses of the data were performed using SAS statistical software (version 8.2, SAS Institute, Cary, NC, USA).

5.3. RESULTS

5.3.1. Sample collection and fungal isolation

Isolation from symptomatic xylem tissue on *P. cineraria* and *D. sissoo* using both carrot baiting and moist chambers yielded 14 isolates of a *Ceratocystis* species (Table 2). The overall percentage of isolation from *P. cineraria* and *D. sissoo* samples from different areas was 46.7% and 38.9% respectively. Cultures of the isolates obtained in this study are preserved in the culture collection (CMW) of the Forestry and Agriculture Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

5.3.2. Morphological characterization

Isolates on MEA from *P. cineraria* and *D. sissoo* clearly represented a species member of the *C. fimbriata s. l.* complex based on the ascomatal morphology and size, ascospore shape as well as conidiophore and conidial morphology (Upadhyay 1981; Van Wyk *et al*. 2007). Ascomata were black with globose bases and long necks. Hat shaped and hyaline ascospores exuded from the apices of the ascomata and globose to oval and olive brown chlamydospores were present. Tubular conidiophores giving rise to hyaline and cylindrical conidia were present (Table 3).

All of the morphological characteristics of the isolates examined in this study were similar to those in the descriptions given for *C. manginecans* isolated from diseased mango trees (Van Wyk *et al*. 2007). Isolates from *P. cinerarea* and *D. sissoo* produced greyish olive cultures and the shape and colour of the ascomatal base, primary conidia and secondary condia were similar to those of *C. manginecans* isolated from mango from Pakistan and Oman (Van Wyk *et al*. 2007) (Table 3). There were no significant differences in the dimensions of the structures in the isolates from *P. cineraria* and *D. sissoo* and *C. manginecans* for the ascomatal bases, ascospores and chlamydospores. Variation was observed in the lengths of the ascomatal necks for isolates from all hosts, but they were still within the ranges reported for *C. manginecans* (Van Wyk *et al*. 2007). The lengths and widths of the primary and secondary conidia overlapped between the three groups of isolates (Table 3).

There were no observable differences in the growth for *C. manginecans* from mango and those from *P. cineraria* and *D. sissoo*. No growth occurred in any of the isolates at 5 ºC, 10 ºC and 35 ºC. Optimum growth for all the *Ceratocystis* isolates occurred at 25 ºC to 30 ºC.

5.3.3. DNA extraction, amplification, sequencing and phylogenetic analyses

The total number of characters in the ITS dataset after alignment and inclusion of gaps was 560bp. The parsimony analysis for the ITS gene region was based on 117 parsimony informative characters, which resulted in six most parsimonious trees with a tree length of 131 steps, consistency index (CI) of 0.970 and a retention index (RI) of 0.979. The phylogenetic analysis for ITS gene region divided all the isolates from mango, *P. cineraria* and *D. sissoo* from Pakistan and Oman into two well supported clades. Two isolates from *P. cineraria* (CMW17225 and CMW17568) grouped together with the isolates of *C. manginecans* from mango and *A. crassicarpa* designated as Group 1 (Fig 2a). All the *D. sissoo* isolates and two *P. cineraria* isolates (CMW17568 and CMW17570) fell into the group with isolates of *C. acaciivora* isolated from *Acacia mangium* (posterior probability value (PP) = 100%, bootstrap value (BP) = 96%). These two groups (Fig 2a) were closely related to isolates of *C. fimbriata senso stricto* from sweet potato and *C. cacaofunesta* from cacao (Engelbrecht & Harrington 2005).

Due to the placement of the *P. cineraria* isolates from Oman into two different *Ceratocystis* species based on ITS sequences (*C. manginecans* – designated as Group 1, and *C. acaciivora*, designated as Group 2 in Fig 2a), all ITS sequences in this study were repeated. The repeated sequencing of the isolates revealed that isolate CMW17568 from *P. cineraria* contained both of the ITS sequences that were defining Groups 1 and Group 2 as different species. Sequence variability between these two groups (and species) comprises a seven bp sequence difference in the exon regions that includes 5, single-base indels and 2 bases that are substitutions (Table 4).

The total number of characters after alignment and inclusion of gaps for the BT sequence data was 551 bp. The maximum parsimony analysis was based on 26 informative

characters and resulted in one tree with a tree length of 26 steps and a CI and RI value of 1. The BT sequences for *P. cineraria* and *D. sissoo* were 100% identical to those of *C. manginecans* from mango in Oman and Pakistan. In addition, there was no resolution in the sequence alignments between the *Ceratocystis* isolates in this study and those of the species *C. manginecans* and *C. acaciivora.* All the BT sequences from Oman, Pakistan and Indonesia used in this study were identical and formed a weakly supported monophyletic clade (PP=64%, BP=63%) (Fig 2b).

The total number of characters after alignment and inclusion of gaps for the TEF-1α sequence data was 756 bp. The maximum parsimony analysis yielded over 100 trees using 39 informative characters. Tree length was 52 steps and the CI and RI were 0.788 and 0.814 respectively. Sequence alignment for the TEF-1α revealed no significant phylogenetic difference between the isolates from *P. cineraria* and *D. sissoo* with those of *C. manginecans* and *C. acaciivora*, which all clustered together (PP=100%, BP=93%) (Fig 2c). All sequences obtained in this study for the ITS, BT and were deposited into GenBank with accession numbers XXXX to XXXX (Table 1).

5.3.4. Inoculation trials

All *P. cineraria* seedlings inoculated in the first trial with isolates from *P. cineraria* and *C. manginecans* exhibited vascular discolouration with 59% (19/32) of inoculated seedlings died at the end of the experiment. Analysis of variance for lesion length in *P. cineraria* indicated no significant differences in lesion lengths among all the isolates inoculated onto this host. Both isolates of *C. manginecans* from mango (CMW13851 and CMW13854) and the *Ceratocystis* isolates from *P. cineraria* (CMW17568 and CMW17570) gave rise to long lesions in *P. cineraria* seedlings ranging from 138.8 mm to 197.5 mm long. Furthermore, statistical analyses showed significant differences in lesion length between

treated *P. cineraria* and the control seedlings (6.3 mm) (Fig 2). Re-isolation from inoculated seedlings yielded a *Ceratocystis sp.* and the fungus was not isolated from the control seedlings.

All mango seedlings inoculated with *C. manginecans* and *Ceratocystis* isolates from *P. cineraria* and *D. sissoo* in second inoculation trail showed typical symptoms of mango wilt disease. The symptoms included extensive vascular discolouration in all inoculated seedlings and wilt was recorded in over 66% (12/18) of the inoculated seedlings. There were no significant differences in lesion length produced by all the *Ceratocystis* isolates used in the inoculation. Average lesion lengths produced by the *C. manginecans* isolates (CMW13851 & CMW13854) from mango, *Ceratocystis* isolates from *P. cineraria* (CMW17225 & CMW17568) and from *D. sissoo* (CMW23623 & CMW23625) inoculated into mango seedlings were 406.7, 300, 266.7, 300, 300 and 263.3 mm respectively. The average lesion lengths on the control seedlings (58.3 mm) were significantly different from those for all the test plants (Fig 3a).

P. cineraria seedlings inoculated with different isolates of *Ceratocystis* showed wilt symptoms in 72% (13/18) of the inoculated seedlings. Analysis of variance for lesion length in *P. cineraria* seedlings revealed there was no significant difference between the isolates tested. The lesion lengths produced on the *P. cineraria* seedlings inoculated with the *Ceratocystis* isolates ranged from 80 to 193.3 mm, which was significantly different to those for the controls that had an average lesion length of 5 mm (Fig 3b).

D. sissoo seedlings inoculated with different *Ceratocystis* isolates showed mortality in four out of 18 (22%) seedlings. Isolates from *P. cineraria*, *D. sissoo* and *C. manginecans* did not differ significantly in their ability to cause lesions on *D. sissoo* plants. *D. sissoo* seedlings inoculated with different *Ceratocystis* isolates produced longer lesions (ranging from 63.3 to

120 mm) but there were no significant difference in lesion lengths produced by the majority of the *Ceratocystis* isolates compared to those for the control inoculations where the average lesion length as 5 mm (Fig 3c).

Re-isolation from selected inoculated seedlings of the three hosts yielded cultures of a *Ceratocystis* sp. No *Ceratocystis* isolates were retrieved the control seedlings for any of the three hosts.

5.4. DISCUSSION

In this study, two novel leguminous hosts, *P. cineraria* and *D. sissoo,* were found for *C. manginecans*. In Oman, where the pathogen was first discovered (Al Adawi *et al*. 2006; Van Wyk *et al*. 2007), *P. cineraria* trees showed typical symptoms of infection by the fungus and the same was true in Pakistan for *D. sissoo*. The fact that *C. manginecans*, which is a serious wilt pathogen of mango in Oman (Al Adawi *et al*. 2006; Van Wyk *et al*. 2007) and Pakistan (Kazmi *et al*. 2005; Van Wyk *et al*. 2007), has apparently undergone a host jump to infect native trees in these countries is serious and it could potentially lead to the devastation of important components of the natural biodiversity of Oman and Pakistan.

The identity of *C. manginecans* as the fungus associated with the wilt disease of *P. cineraria* and *D. sissoo* was determined based on morphological characteristics as well as DNA sequence comparisons. The results were unequivocal that the fungus is the same as that infecting mango in both Oman and Pakistan. Furthermore, pathogenicity of *C. manginecans* to both *P. cineraria* and *D. sissoo* was demonstrated by inoculation trials and there seems little doubt that the fungus is responsible for the widespread death of these trees. It was also possible to show that isolates of the pathogen from these trees are able to infect and kill

mango plants. This supports the view that *C. manginecans* has a wider host range than only mango and in time it might be found to infect other trees.

Ceratocystis manginecans is best known due to the severe damage that it has caused on mango in Oman and Pakistan (Kazmi *et al*. 2005; Al Adawi *et al*. 2006; Van Wyk *et al*. 2007). But it has recently also been found, together with *C. acaciavora,* killing *Acacia mangium* in Indonesia (Tarigan *et al*. 2011). This is particularly interesting because *A. mangium*, like *P. cineraria* and *D. sissoo*, is a legume. However, mango is distantly related to these trees and this might simply be a coincidence.

Al Adawi *et al.* (Chapter 4), have speculated on the likely origin of *C. manginecans* and have suggested that the pathogen might have been introduced into Oman and Pakistan from South America. This view emerged from the fact that a mango disease, very similar to that found in Oman, is found on these trees in Brazil (Viegas 1960; Ploetz 2003). While *C. manginecans* has not been found in Brazil associated with mango wilt, a number of species complex closely related to it (Van Wyk *et al*. 2007) and residing in the *C. fimbriata s.l.* cause the disease there.

Ceratocystis manginecans forms part of the *C. fimbriata s.l.* complex, which is typified by *C. fimbriata s.s.* which causes black rot of sweet potato (Engelbrecht & Harrington 2005). Phylogenetic inference based on DNA sequence comparisons has made it possible to distinguish many different lineages in the complex and some of these have been treated as distinct species (Wingfield *et al*. 1996; Barnes *et al*. 2003; Engelbrecht & Harrington 2005; Johnson *et al*. 2005; Van Wyk *et al*. 2007; Rodas *et al*. 2008; Van Wyk *et al*. 2009; Van Wyk *et al*. 2010; Van Wyk *et al*. 2011). Most of these species can also be distinguished based on morphological characters although these are subtle and experience with all the members of the group is needed to clearly recognise them. There is some

disagreement as to the species boundaries amongst isolates of *C. fimbriata s.l.* but our view is that there is more than sufficient evidence to justify the recognition of *C. manginecans*. Furthermore, recognition of this species as distinct makes it possible to select trees with resistance to it and equally importantly, to institute actions to prevent its movement outside its current area of occurrence.

The mango disease caused by *C. manginecans* in Oman and Pakistan is closely associated with the wood boring insect *H. mangiferae* (Al Adawi *et al*. 2006; Van Wyk *et al*. 2007). This insect is native to south East Asia including the native range of mango and it is apparently specific to these trees (Castro 1960; Butani 1993; Atkinson & Peck 1994; Pena & Mohyuddin 1997). No insects were found associated with the wilt disease of *P. cineraria* and *D. sissoo* reported in this study. However, *Ceratocystis* spp. require wounds to infect trees and it is possible that a wood boring insect vectors the pathogen, or alternatively it is carried to wounds on trees by opportunistic insects such as nitidulid beetles that are well-recognised as vectors of *Ceratocystis* spp. (Moller & De Vay 1968b; Heath *et al*. 2009; Roux & Wingfield 2009).

The wilt disease of *P. cineraria* and *D. sissoo* appears to be serious and it is clearly a new host tree/pathogen association (Roy 2001) that has apparently occurred due to a host shift (Slippers *et al*. 2005). This category of diseases is increasing in importance and they have the capacity to devastate native trees that have not previously encountered them (Roy 2001; Anderson *et al*. 2004; Slippers *et al*. 2005; Woolhouse *et al*. 2005; Desprez-Loustau *et al*. 2007). In this regard, the wilt disease of *P. cineraria* and *D. sissoo* have the capacity to impact seriously on the natural diversity of Oman and Pakistan and studies should be instituted to understand them better.

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Table1. *Ceratocystis* isolates used in the morphological comparisons, phylogenetic study and inoculation trials.

Identity	Culture no.[a]	Host	Geographic origin	GenBank accession no[b].
C. manginecans	**CMW17225**[c]	*Prosopis cineraria*	Sohar, Oman	Xxxxx
				Xxxxx
				Xxxxx
	CMW17568[d, e]	*P. cineraria*	Sohar, Oman	Xxxxx
				Xxxxx
				Xxxxx
	CMW17570[d, e]	*P. cineraria*	Sohar, Oman	Xxxxx
				Xxxxx
				Xxxxx
	CMW23623[e]	*Dalbergia sissoo*	Faisalabad, Pakistan	Xxxxx
				Xxxxx
				Xxxxx
	CMW23624	*D. sissoo*	Faisalabad, Pakistan	Xxxxx
				Xxxxx
				Xxxxx
	CMW23625[d]	*D. sissoo*	Shorkot, Pakistan	Xxxxx
				Xxxxx
				Xxxxx
C. manginecans	CMW13851[d]	*Mangifera indica*	Sohar, Oman	AY953383
				EF433308
				EF433317
	CMW13854[d, e]	*M. indica*	Shinas, Oman	AY953385
				EF433310
				EF433319
	CMW23641[e]	*M. indica*	Multan, Pakistan	EF433305
				EF433314
				EF433323
	CMW23643	*M. indica*	Multan, Pakistan	EF433304
				EF433313
				EF433322
	CMW21127	*Acacia crassicarpa*	Indonesia	EU588663
				EU588642
				EU588652
	CMW21127	*A. crassicarpa*	Indonesia	EU588664
				EU588643

Identity	Culture no.[a]	Host	Geographic origin	GenBank accession no[b].
				EU588653
C. acaciivora	CMW22564	*A. mangium*	Indonesia	EU588657
				EU588637
				EU588647
	CMW22621	*A. mangium*	Indonesia	EU588661
				EU588640
				EU588650
C. fimbriata s. s	CMW15049	*Ipomaea batatas*	U.S.A	DQ520629
				EF070442
				EF070394
	CMW1547	*I. batatas*	Papua New Guinea	AF264904
				EF070443
				EF070395
C. cacaofunesta	CMW15051	*Theobroma cacao*	Costa Rica	DQ520636
				EF070427
				EF070398
C. cacaofunesta	CMW14809	*T. cacao*	Ecuador	DQ520637
				EF070428
				EF070399
C. pirilliformis	CMW6569	*Eucalyptus nitens*	Australia	AF427104
				DQ371652
				AY528982
C. pirilliformis	CMW6579	*E. nitens*	Australia	AF427105
				DQ371653
				AY528983

[a] Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

[b] GenBank accession no. for the ITS, Beta-tublin and EF sequences respectively, for each isolate.

[c] Isolates in bold were sequenced during this study.

[d] Isolates used in the inoculation trial.

[e] Isolates used in the morphological characterizations.

Table 2. Recovery of *Ceratocystis manginecans* from carrot baiting and moist chambers from samples collected from dying *P. cineraria* and *D. sissoo* trees in Oman and Pakistan.

Host	Area	Year	Recovery of *Ceratocystis manginecans*
Ghaf	**Sohar**	**2004**	1/4 (25%)
		2005	3/6 (50%)
		2006	3/5 (60%)
		Total	**7/15 (46.7%)**
Shisham	**Faisalabad**	**2006**	3/6 (50%)
	Shorkot		2/7 (28.6%)
	Chenab Nagar		2/4 (50%)
	Multan		0/1 (0 %)
		Total	**7/18 (38.9 %)**

Table 3. Morphology of selected *Ceratocystis manginecans* isolates from mango, *P. cineraria* and *D. sissoo*.

Isolates/ Morphological characters[a]		*P* value	Mango			*P. cinerea*			*D. sissoo*		
			CMW13854	CMW23641	Mean	CMW17568	CMW17570	Mean	CMW23623	CMW23625	Mean
Neck (l)		*p*<0.0001	543.24b[c]	518.27b	**530.75c**	525.51b	620.67a	**620.67a**	620.67a	620.67a	**620.67a**
Neck (w) top		*p*<0.0001	20a	17.36b	**18.68a**	17.56b	17.74b	**17.65b**	18.79ab	19.01ab	**18.91a**
Neck (w) bottom		*p*=0.1805	38.22a	29.96a	**34.09a**	33.79a	31.03a	**32.41a**	31.65a	31.94a	**31.79a**
Ascomatal base (w)		*p*<0.0018	198.18ab	178.31b	**188.25a**	206.85a	186.94ab	**196.90a**	186.74ab	206.15a	**196.44a**
Ascomatal base (l)		*p*<0.0024	190.32ab	185.66ab	**187.99a**	210.37a	176.56b	**193.47a**	181.17b	200.54ab	**190.85a**
Ostiolar hyphae (l)		*p*<0.0003	53.89b	57.94ab	**55.92b**	57.3ab	61.24ab	**59.27b**	64.29a	63.89a	**64.09a**
Hat-shaped ascospores (l)		*p*=0.4358	3.47a	3.32a	**3.39a**	3.42a	3.3a	**3.36a**	3.33a	3.4a	**3.63a**
Ascospores (w) without sheath		*p*=0085	4.9b	5.01ab	**4.95a**	5.16ab	4.9b	**5.03a**	5.34a	4.83b	**5.09a**
Ascospores (w) with sheath		*p*=0.002	6.03b	6.28ab	**6.16a**	6.08ab	5.98	**6.03a**	6.5a	5.89b	**6.21a**
Primary conidia (l)		*p*=0.075	22.72ab	19.26b	**20.99a**	21.34ab	21.98ab	**21.66a**	22.2ab	23.37a	**22.79a**
Primary conidia (w)		*p*<0.0001	4.24b	4.32b	**4.28b**	5.52a	4.48b	**5a**	4.3b	4.19b	**4.243b**
Secondary Conidia (l)		*p*<0.0001	9.79a	8.53b	**9.16a**	10.22a	8.66b	**9.44a**	8.17b	9.98a	**9.08a**
Secondary Conidia (w)		*p*<0.0001	6.8a	6.2b	**6.5a**	5.06c	6.74a	**5.90b**	5.97b	6.98a	**6.48a**
Chlamydospores (l)		*p*=0.0361	13.58a	12.99a	**13.29a**	14.06a	13.18a	**13.62a**	13.9a	13.92a	**13.91a**
Chlamydospores (w)		*p*<0.0020	10.49b	11.13ab	**13.29a**	11.43a	10.22b	**13.62a**	10.78ab	11.13ab	**10.95a**
Culture[b] growth rate at	**5 ºC**		0	0	**0**	0	0	**0**	0	0	**0**
	10ºC		0	0	**0**	0	0	**0**	0	0	**0**
	15ºC	*p*<0.0001	11b	9.3c	**10.15c**	13.9a	12.8a	**13.35a**	11.1b	12.5ab	**11.8b**
	20ºC	*p*<0.0001	20.7ab	22.9a	**22.1a**	20.7bc	19.3c	**20b**	19.6bc	19.8bc	**19.7b**
	25ºC	*p*=0.0022	30.2ab	32a	**31.1a**	31.9a	29.8ab	**30.85a**	27.4b	30.2ab	**28.8b**
	30ºC	*p*<0.0001	31a	30.2a	**30.6a**	26.4b	30.7a	**28.55b**	31.2a	30.8a	**31a**
	35ºC		0	0	**0**	0	0	**0**	0	0	**0**

[a] All morphological characters represent an average for 25 measurements for each morphological structure measured in µm.

[b] Growth rate measurements represent an average of diameters of cultures measured in mm at each temperature after seven days.

[c] Means of isolates with the same letter are not significantly different according to Tukey test.

Table 4. Polymorphic sites in the sequence of the ITS gene region for *Ceratocystis manginecans* isolates from *P. cineraria*, *D. sissoo* and mango.

Host	Isolate no.[a]	148[b]	163	166	172	177	184	547
Ghaf	**CMW17225[c]**	**-**	**-**	**A**	**A**	**A**	**-**	**C**
(*Prosopis cineraria*)	**CMW17568**	**-**	**-**	**A**	**A**	**A**	**-**	**C**
	CMW17568	**T**	**T**	**G**	**G**	**-**	**T**	**-**
	CMW17570	**T**	**T**	**G**	**G**	**-**	**T**	**-**
Shisham	**CMW23623**	**T**	**T**	**G**	**G**	**-**	**T**	**-**
(*Dalbergia sissoo*)	**CMW23624**	**T**	**T**	**G**	**G**	**-**	**T**	**-**
	CMW23625	**T**	**T**	**G**	**G**	**-**	**T**	**-**
Mango	CMW13851	-	-	A	A	A	-	C
(*Mangifera indica*)	CMW13854	-	-	A	A	A	-	C
	CMW23641	-	-	A	A	A	-	C
	CMW23643	-	-	A	A	A	-	C
Acacia crassicarpa	CMW21125	-	-	A	A	A	-	C
	CMW21127	-	-	A	A	A	-	C

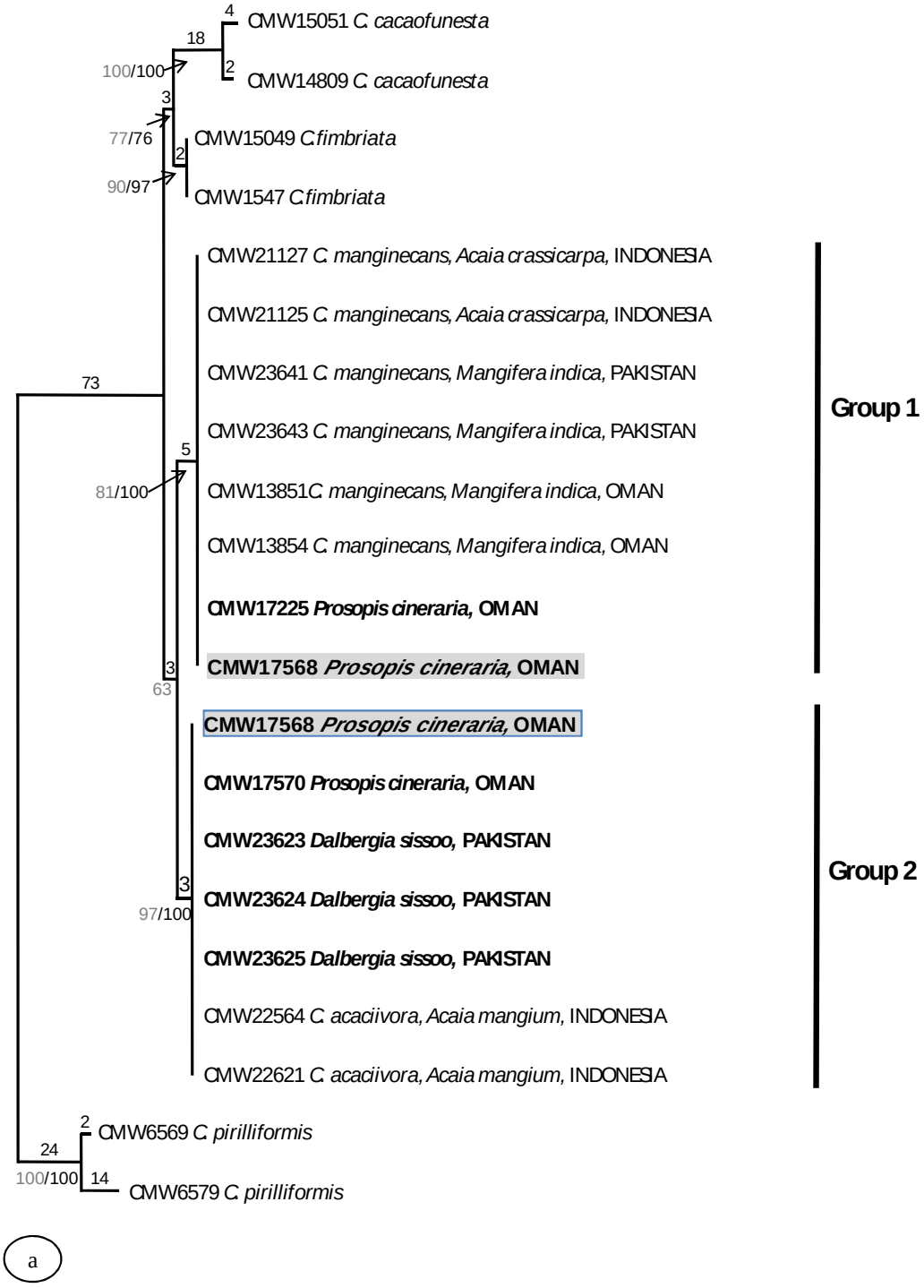
[a] The ITS region of all the isolates represented were sequenced twice to validate the results.

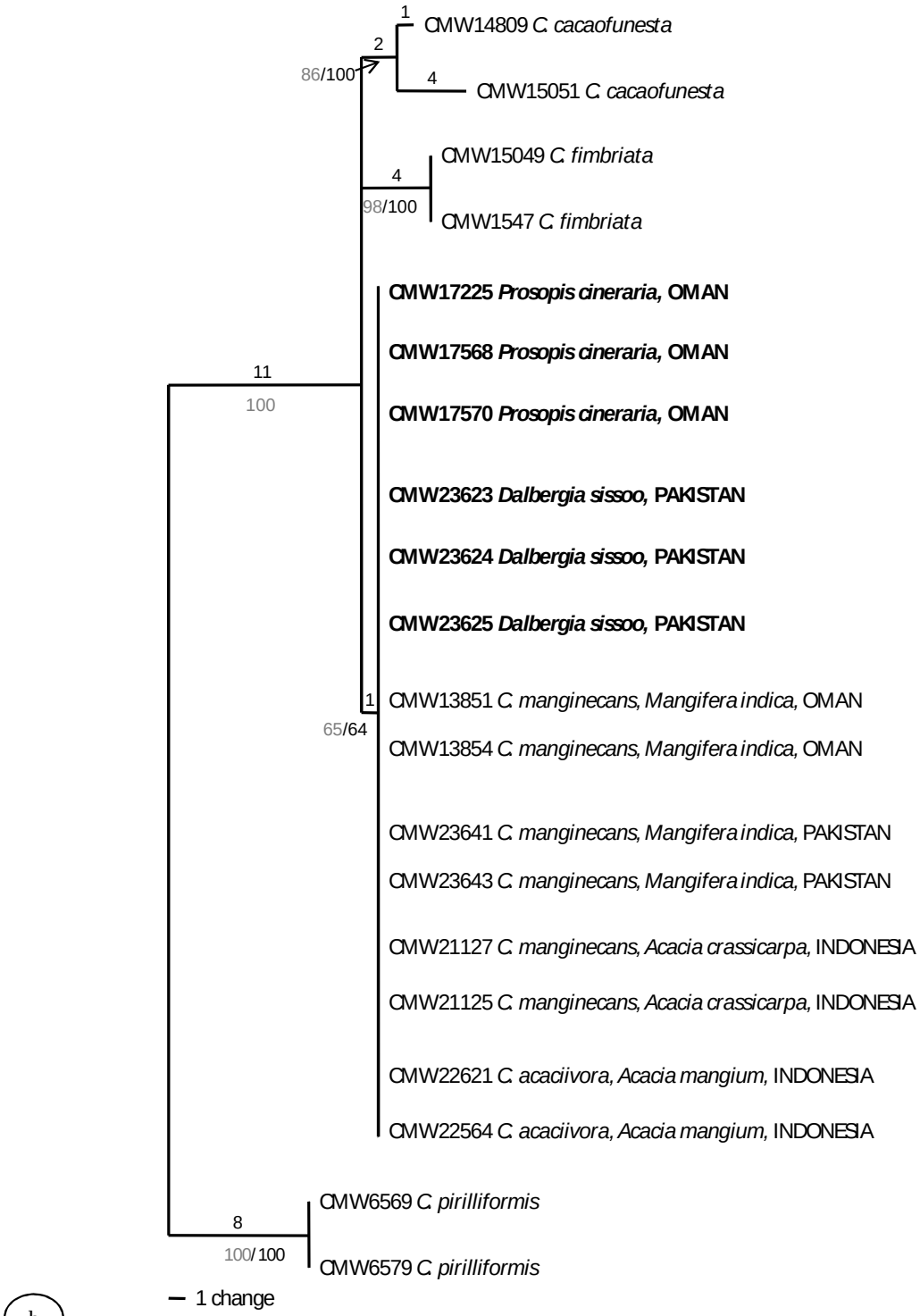
[b] Nucleotide position after alignment.

[c] Isolate in bold was sequenced two times using the same single spored culture.

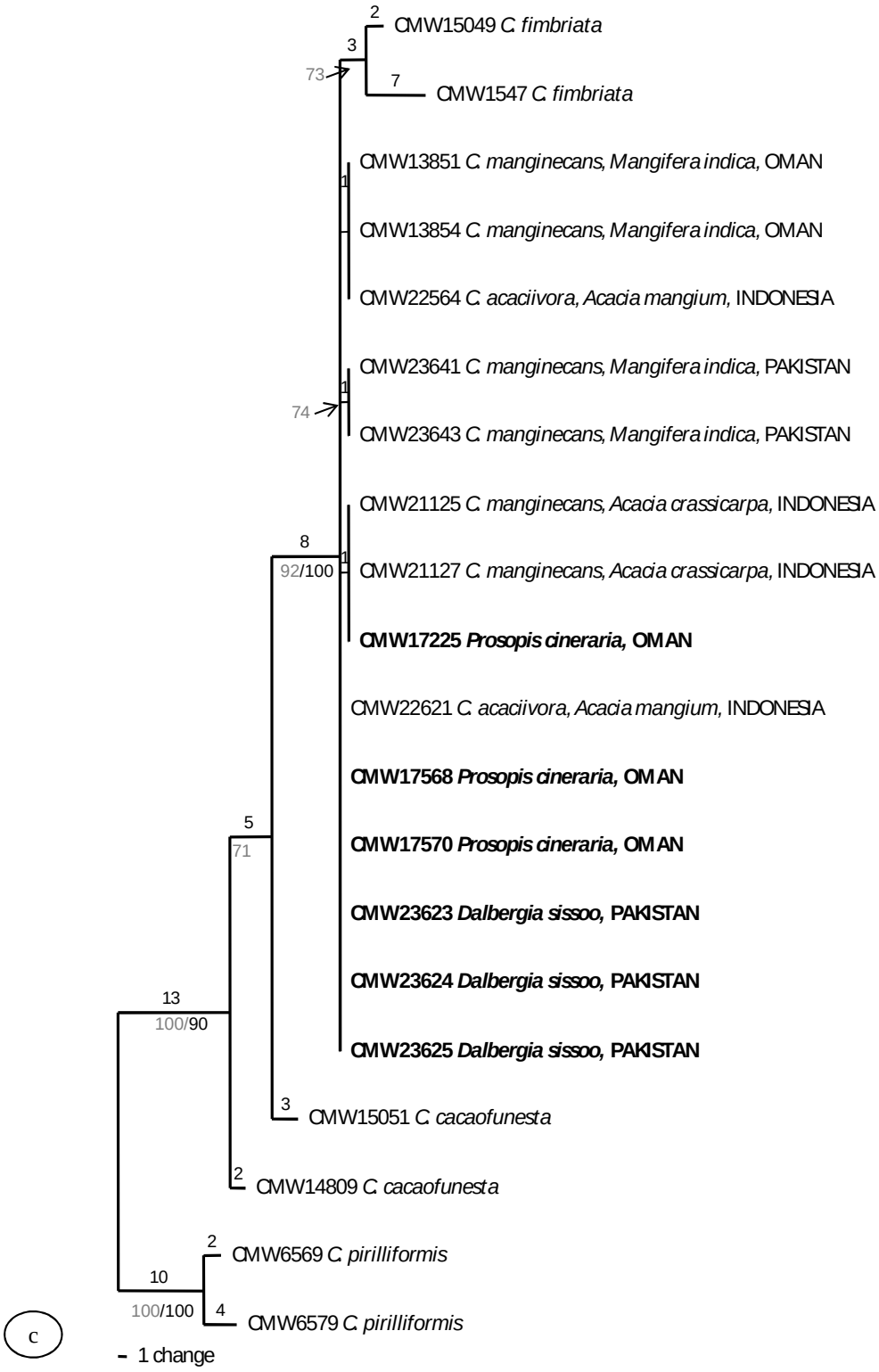
Fig. 1. Symptoms of wilt and die-back on *P. cineraria* and *D. sissoo;* (a) wilted *P. cineraria*, (b) dark staining of the xylem tissues in *P. cineraria*, (c) Dying *D. sissoo* tree, (d) vascular discolouration of infected *D. sissoo*.

Fig. 2. Maximum parsimony trees resulting from the analysis of sequence data of the (a) ITS, (b) β–tubulin, (c) TEF-1α gene regions. Bootstrap values and posterior probability values (in bold) are indicated below the branches. Branch lengths are represented above the branches for all trees.





b



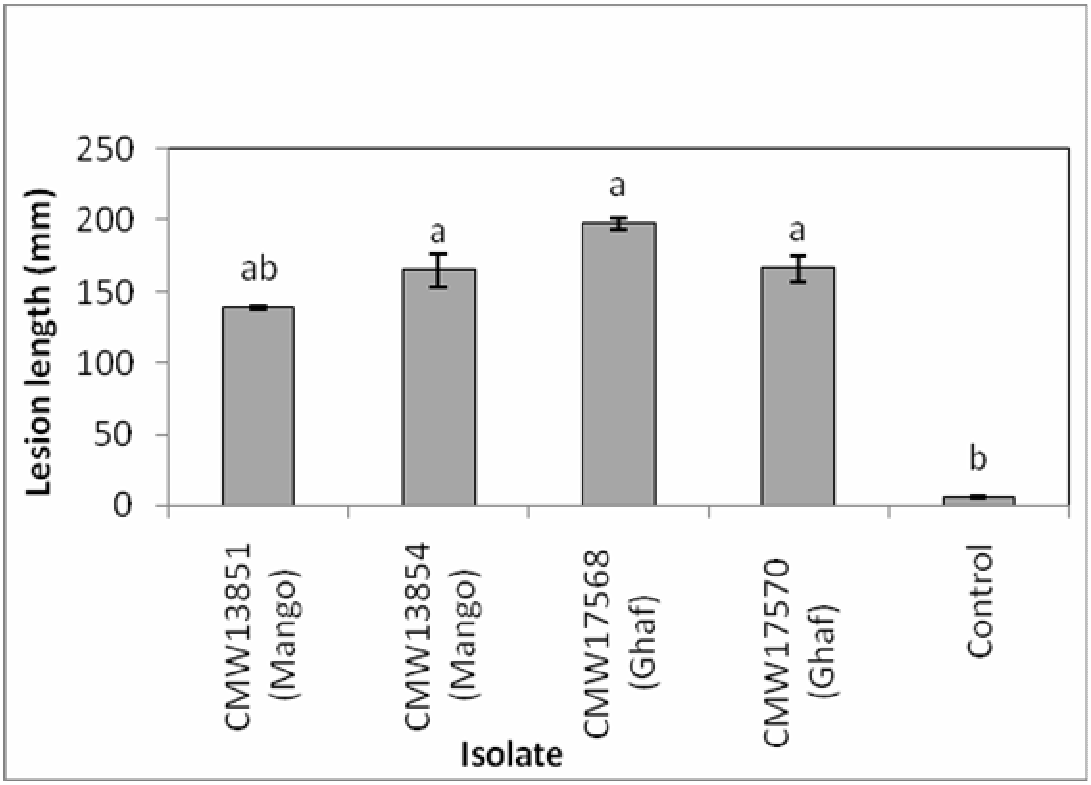
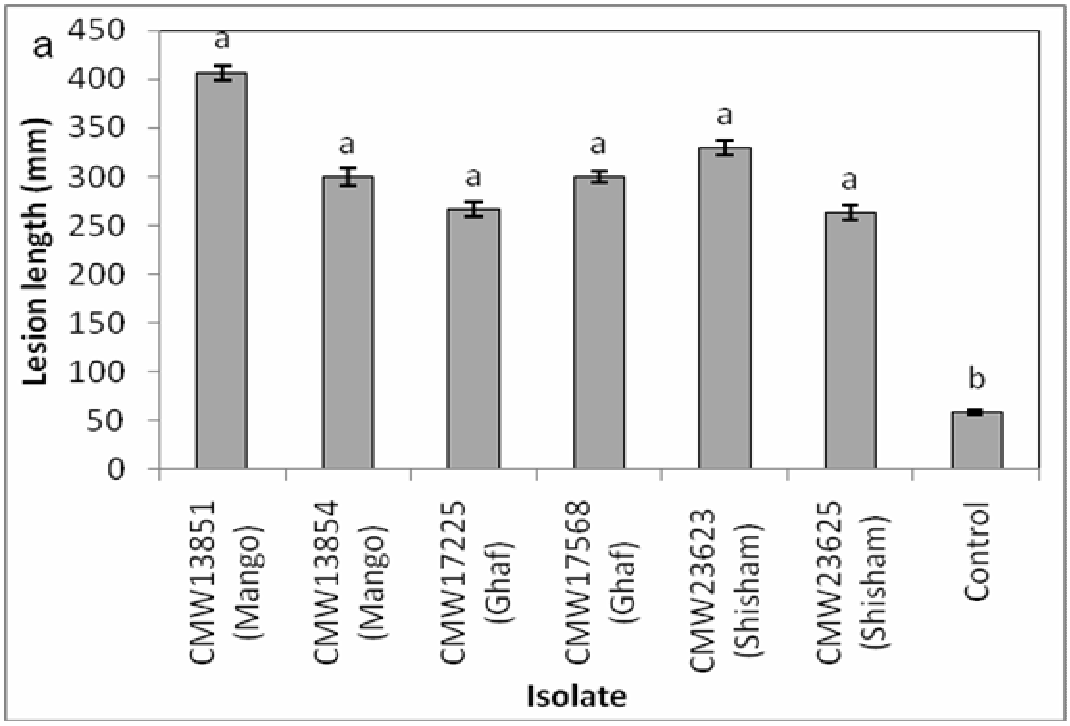
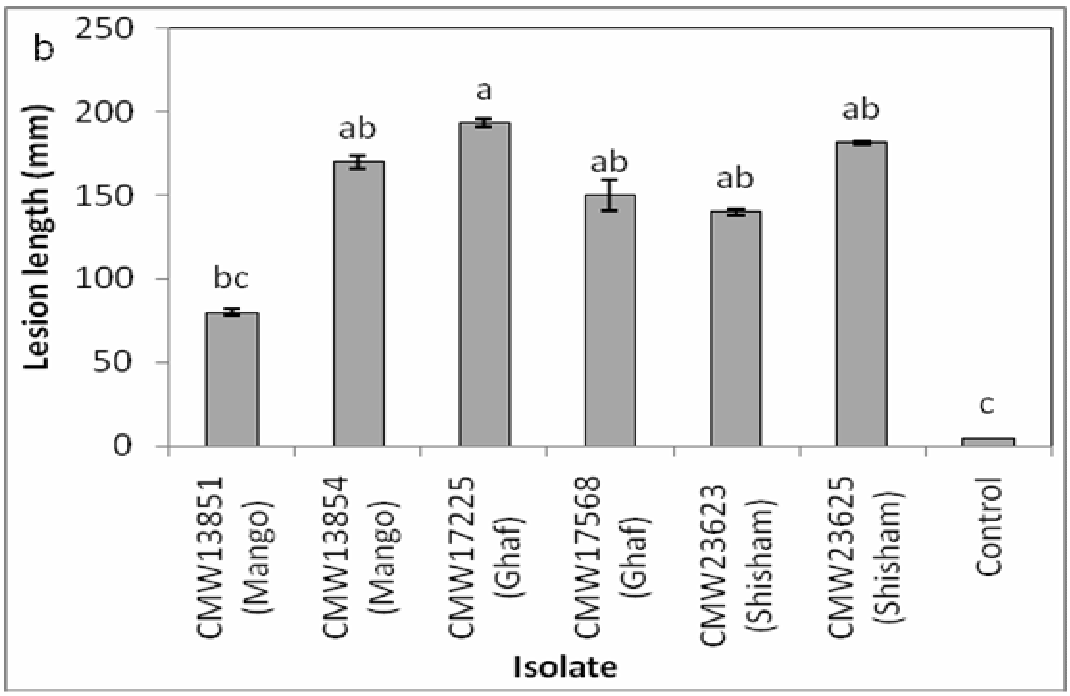


Fig. 3. Inoculation experiments on *P. cineraria* (Ghaf) seedlings using *Ceratocystis manginecans* isolated from mango (CMW13851 and CMW13854) and *Ceratocystis* isolates isolated from *P. cineraria* (CMW17568 and CMW17570). Analysis of variance was calculated using a GLM model. Columns represent means of lesion lengths produced by each isolate. Lesion lengths of the isolates marked with same letter were not significantly different from each other at $P < 0.05$ according to Tukey's test.





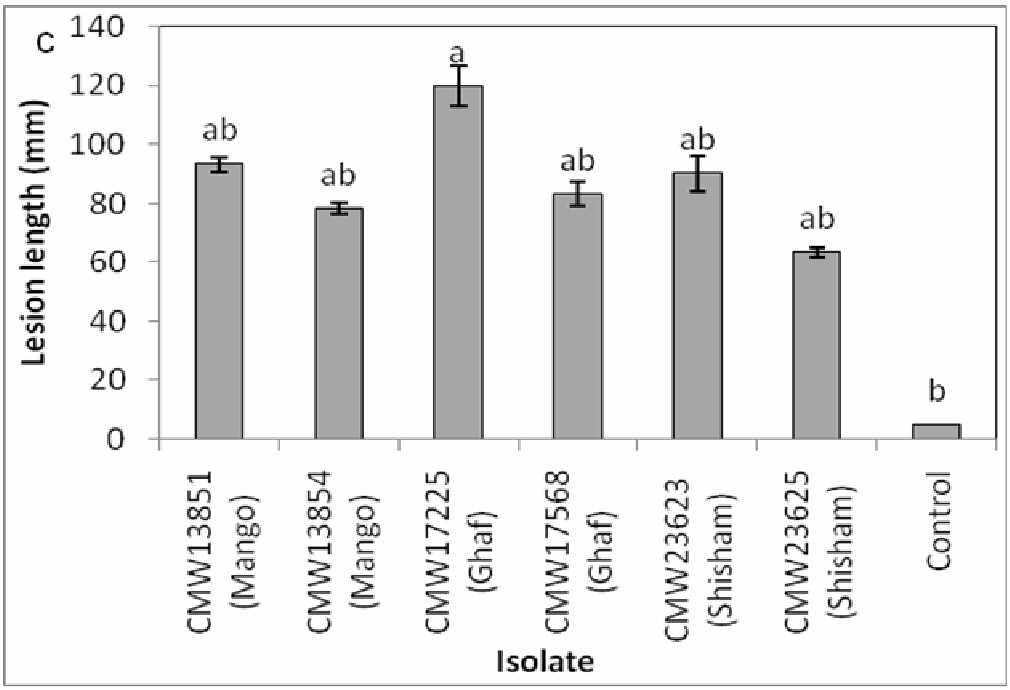


Fig. 4. Cross inoculation experiments on mango (a) *P. cineraria* (Ghaf) (b) and *D. sissoo* (Shisham) seedlings (c) using *Ceratocystis manginecans* isolated from mango (CMW13851 and CMW13854), *Ceratocystis* isolates from *P. cineraria* (CMW17225 and CMW17568) and from *D. sissoo* (CMW23623 and CMW23625). Analysis of variance was calculated using a GLM model. Columns represent means of lesion length for each isolate. Isolates marked with the same letter were not significantly different from each other at $P < 0.05$ according to Tukey's test.

CHAPTER 6

Evaluation of mango cultivars for resistance to infection by *Ceratocystis manginecans*

ABSTRACT

Ceratocystis manginecans has been reported to cause a serious wilt disease of mango in Oman and Pakistan. To identify plants resistant to this disease, thirty mango cultivars were artificially inoculated with two isolates of *C. manginecans* in three trials. Statistical analysis revealed significant differences in lesion lengths among mango cultivars. Similarly, there were significant differences in the aggressiveness of the isolates used for inoculations. However, in trials where more than one isolate was used, there was no significant isolate x cultivar interaction suggesting that isolates do not affect the ranking of cultivars as susceptible or resistant. Cultivar Pairi and local mango cultivars had the longest lesions and were ranked as highly susceptible. In contrast, cultivars Hindi Besennara, Sherokerzam, Mulgoa, Baneshan, Rose and Alumpur Baneshan, had the smallest lesions and are considered as relatively resistant against *C. manginecans*. The inoculation results are concurrent with the incidence of wilt of these cultivars under field conditions.

6.1. INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most important perennial fruit crops in the Sultanate of Oman (MoA 2009a; MoA 2009b). Mango wilt disease, caused by *Ceratocystis manginecans* M. Van Wyk, A. Al-Adawi, & M. J. Wingf (Al Adawi *et al*. 2006; Van Wyk *et al.* 2007), is the most serious threat to the production of mango in the country. Since it was first reported in 1998, the disease has led to the death of many thousands of productive mango trees in the country and has been accompanied by a loss of valuable germplasm (Al Adawi *et al*. 2006).

Mango wilt disease in Oman is closely associated with the wood-boring beetle, *Hypocryphalus mangiferae* (Coleoptera: Scolytinae) that infests healthy trees. The bark beetle carries *C. manginecans* and as it bores into the wood, it creates an open wound for infection by the pathogen. Trees infected with the pathogen exude gum from infected stems/branches and the wood displays brown to black vascular discoloration. As the disease advances, tree parts or entire trees wilt and subsequently die. The disease is most serious in trees propagated from local seed sources and on exotic cultivars that are grafted on rootstock of local Omani cultivars. Where trees are grafted onto susceptible rootstocks, that rootstock is preferentially infested by wood boring insects and *C. manginecans* and tree death is more rapid than in the case of trees propagated from local seed sources (Al Adawi *et al*. 2006).

A mango wilt disease very similar to that found in Oman has been reported from Pakistan. *Ceratocystis manginecans* and *H. mangiferae* have also been recovered from dying trees in that country (Malik *et al.* 2005; Van Wyk *et al*. 2007). Recent genetic analysis of *C. manginecans* populations from Oman and Pakistan using microsatellite markers has shown that the isolates from both countries collected from dying trees and from *H. mangiferae* are identical

(Al Adawi, *unpublished*). The population of *C. manginecans* collected from Oman and Pakistan represents a single genotype that was most probably introduced into these countries via mango propagation materials from a single source (Van Wyk *et al*. 2007; Al Adawi, *unpublished*).

In 2001, the Ministry of Agriculture and Fisheries in Oman initiated a mango wilt disease management programme. This programme involved eradication of severely infected and dead mango trees, pruning of dead branches and using systemic insecticides and fungicides applied to infected trees through spraying or soil drenching. Following these measures, more than 13% of the mango trees in the Al Batinah region were eradicated (Al Adawi *et al*. 2006). Despite this aggressive programme, mango wilt disease continues to progress and occurs in all areas where mango trees are propagated. Since its first appearance in Oman, more than 60% of mango trees have been affected by the disease in certain regions (Al Adawi *et al*. 2003).

Control of the mango wilt disease using fungicides and insecticides has not been effective. This is in part because *C. manginecans* infects trees internally and the bark beetle larvae penetrate the wood. Consequently, development of genetic material resistant to infection by *C. manginecans* is considered as the most appropriate means to reduce the impact of the disease (Ploetz & Freeman 2009). The aim of this study was to evaluate available local and exotic monoembryonic mango germplasm for resistance to infection by *C. manginecans*.

6.2. MATERIALS AND METHODS

6.2.1. Field evaluation of mango cultivars

The incidence of mango wilt disease was assessed in a farm in Barka (20 km west of Muscat) during April 2001. The mean incidence of mango wilt disease in Barka area was estimated at 13.6% during 2001 (Al Adawi *et al*. 2006). A plot size of 1.4 ha with trees of over

six-years-old, and a multiplicity of mango cultivars was selected for incidence assessment. All 13 mango cultivars were grafted on local rootstocks with the exception of local, seed propagated cultivars. The incidence of mango wilt disease was assessed as the number of wilted trees of each cultivars present in 2001 and was reassessed after three years.

Disease incidence was also assessed on mango genebank material at Waqaibah (24°20′42.79″N, 56°43′25.93″E) and Wadi Haibi (24°19′10.37″N, 56°43′00.62″E) close to Sohar in North East Oman during 2002 and 2003. The genebank consisted of 32 local mango accessions collected from various mango cultivation areas in Oman, in addition to the 23 exotic cultivars imported as part of a cultivar performance assessment programme. The age of all local mango accessions and six of the exotic cultivars (Arumanis, Hamlet, Golek, Oaies, Taimour and Zabidia) at time of the first disease incidence assessment was five-years-old with 3 trees per accession. Sixteen of the exotic mango cultivars grown at Waqaibah were 12 twelve-years-old with 16 trees per cultivar. At Wadi Haibi, five exotic mango cultivars (Baramasi, Dasheheri, Neelum, Rose and Tenneru) of three-year old trees were also included in the wilt incidence evaluation. The number of wilted trees of each cultivar was recorded monthly. An incidence disease scale was used where 0 – 20% indicated tolerant cultivars, 21 – 40% moderately tolerant cultivars, 41 – 60% moderately susceptible cultivars, 61 – 80% susceptible cultivars and 81 – 100% highly susceptible cultivars.

6.2.2. Inoculum and inoculations

Thirty mango cultivars from diverse sources were evaluated for resistance to *C. manginecans* (Table 1). Due to variable availability of plants, three separate inoculation trials were conducted in April 2005, February 2007 and November 2009. Each of the mango cultivars

was grafted onto root-stocks of undefined local origin and grown in 13 cm diameter pots containing loamy soil mixed with peat moss (1:1 v:v) and kept under shade-house conditions.

Inoculum of *C. manginecans* was grown on malt extract agar (MEA) for two weeks at 25^{o}C. Mango plants were inoculated by making a 1cm, I-shaped incision 20cm above the graft union using a sterile scalpel and following the methods described by Al Adawi *et al*. (2006).

6.2.3. Inoculation trials

In the first trial, six monoembryonic mango cultivars including Baramasi, Dasheheri, Pairi, Rose, Tenneru and Zafran were artificially inoculated with two isolates (CMW15351 and CMW13854) of *C. manginecans* shown to be pathogenic in previous pathogencity tests (Al Adawi *et al*. 2006). Twenty six-month-old seedlings with an average scion diameter of 14.2 mm (at inoculation position) were used. Seven plants of each cultivar were inoculated with each isolate by inserting mycelial discs (2mm diameter) into the wounds on the stems. Five plants of each mango cultivar were also inoculated with sterile MEA as controls. All wounds were covered with moistened, sterile cotton pads and sealed with Parafilm to maintain a humid environment. After 60 days of inoculation, the bark was removed to expose the discolored xylem and the lesion length was measured.

The second trial included thirteen mango cultivars, eight Indian monoembryonic cultivars (Baramasi, Dasheheri, Imampasand, Langra, Pairi, Rose, Tenneru and Zafran), one Egyptian polyembryonic cultivar (Hinidi Besennara), and four locally developed cultivars (Ishbiah, Mantkah Al thour, Muscati and Sohar 2005). Nine, eighteen-month-old mango plants, with an average scion diameter of 11.3 mm, were inoculated as above using *C. manginecans* isolate CMW13854 and a control of sterile MEA. Lesion length was evaluated 60 days after inoculation.

In the third trial, twenty eight mango cultivars were used including local and exotic monoembryonic and polyembryonic mango cultivars (Table 5). Twenty two-month-old seedlings with an average scion diameter of 12.7 mm, were inoculated as above using two isolates (CMW13851 and CMW13854) of *C. manginecans*. Each isolate was inoculated on four plants of each cultivar and an equal number of control plants were inoculated with sterile MEA. Lesion lengths were measured after 36 days.

6.2.4. Data analysis

Statistical analyses were performed using SAS (SAS Institute, Cary, NC, USA). Analysis of variance according to the general linear model procedure (GLM) was used to compare lesion lengths between isolates and mango cultivars. Where there were significant differences, Duncan's multiple range tests was used to separate means. The interaction between isolate and cultivar in the first and last trials were examined and the data of the three trails were combined after determining that there was no significant interaction. Where cultivars had not been evaluated in one or two trials, they were treated as missing data in the combined data set. The differences in lesion lengths for combined data and among isolates, years and cultivars were tested using analysis of variance and significant differences between means were separated by Duncan's multiple range test. Because isolate CMW13854 was used in all three trials, the data were reanalyzed using inoculation data for only this isolate to evaluate possible differences between trails.

6.3. RESULTS

6.3.1. Field evaluation of mango cultivars

The assessment of wilt incidence in Barka during the period 2001 - 2004 revealed that almost half of the mango trees present in 2001 (101/208 = 48.6%) had been killed by 2004. The number of dead trees was higher in local germplasm (43/69 = 62.3%). The proportion of killed trees was also high in Tenneru (3/4 = 75%), Alphonso (11/19 = 57.9%), Langra (3/6 = 50%), Imampasand (1/2 =50%), Neelum (9/19 = 47.4%) and Pairi (9/20 = 45%). The cultivars Rose (2/8 = 25%), Baramasi (1/4 = 25%), Bangalora (0/4 =0%) and Baneshan (0/1 = 0%) were least affected.

During 2002 - 2003, the overall incidence of tree death due to mango wilt was 53.2% (194/365) in the mango germplasm trial at Sohar. Local mango germplasm showed high incidence, with 26 of the 32 accessions (81.3%) showing tree losses between 66.7 and 100%. Ten of the 23 (43.5%) exotic cultivars had tree losses between 50 and 100%. Local germplasm Al Arash and Oad Al Roob both showed 100% wilt incidence in the germplasm trial. The highest incidence among exotic cultivars was recorded in Bangalora (81.3%), Neelum (66.7%), Golek 68.8%), Alphonso (62.5%), Pairi (56.3%), Imampasand (56.3%) and Sherokerzam (50.0%). Lowest disease incidence among exotic cultivars was recorded in Langra (37.5%), Baramasi (37.5%), Baneshan (31.3%), Zafran (31.3%), Rose (30.8%), Hindi Besennara (25.0%), Mulgoa (25.0%), Tenneru (24.0%) and Dasheheri (18.8%) (Table 6).

6.3.2. Artificial inoculation of mango cultivars

Statistical analyses of the results of the inoculation trial conducted in 2005 revealed significant differences ($P < 0.0001$) in lesion length between mango cultivars (Table 2). Significantly longer lesions ($P = 0.005$) were produced by isolates CMW13854 (X =26.12 cm)

and CMW15351 (X =19.20 cm) compared to control inoculations (X =1.12 cm). Mean lesion length in cultivar Pairi (40.3 cm) was significantly greater than that on cultivar Rose, which had the smallest (X =11.1 cm) lesions of all cultivars tested (Table 3).

For the trial conducted in 2007, there were significant differences ($P < 0.0001$) in the response of different cultivars to inoculation with *C. manginecans* (Table 2). Results of this trial also showed that cultivar Pairi and the four local mango cultivars tested were highly susceptible to infection, having the most extensive lesion development (X =42.11 cm). Cultivar Rose (X =18.1 cm), Hindi Besennara (X =22.4 cm) and Baramasi (X =22.7 cm) had the smallest lesions. Insignificant lesions (X =1.3 cm) were associated with the control inoculations (Table 4).

In the trial conducted in 2009, there were significant ($P < 0.0001$) differences in lesion length among cultivars (Table 5). Cultivar Pairi was highly susceptible to infection, having the most extensive lesion development (X = 60.8 cm). The six local mango accessions Al Batikah, Al Arash, Ishbiah, Oad Al Roob and Sohar 2005 also had extensive lesion development. Some of this material died during the trial (Table 5). Cultivars with the most limited lesion development included Baneshan, Rose and Alumpur Baneshan. Among the Egyptian polyembryonic cultivars tested in this trail, only Golek was highly susceptible to infection. In contrast, the Taimor and Hindi Besennara were less susceptible to infection and their mean lesion lengths (22.8 cm and 18.9 cm respectively) were similar to those of the least susceptible Indian cultivars. The lesions associated with control inoculations were small and significantly different from those where *C. manginecans* was used as inoculum ($P < 0.0001$).

Analysis of variance for lesion length where the data for three trails were combined revealed significant differences ($P < 0.0001$) among cultivars and between isolates ($P = 0.0046$) tested in different trials. There was no significant difference between trial years ($P = 0.3893$), the

isolate x cultivar interaction was not significant ($P = 0.7073$) but there was a significant year x cultivar interaction ($P < 0.0001$). Inoculations using isolate CMW13854 were repeated in three trails and this isolate also consistently produced the most extensive lesion development compared with other isolates (CMW13851 and CMW15351) tested in 2005 and 2009.

Analysis of variance of lesion length for pooled data for the three trails but including only data for isolate CMW13854 revealed significant differences between cultivars ($P < 0.0001$), no significant difference ($P = 0.4466$) between trail years, and a significant ($P = 0.0006$) year x cultivar interaction. Pairi and local mango cultivars were consistently highly susceptible to infection by isolate CMW13854. Likewise, the cultivars Rose, Baneshan and Alumpur Baneshan consistently displayed the lowest levels of infection by this isolate (Table 6).

6.4. DISCUSSION

Thirty mango cultivars were evaluated for resistance to infection by *C. manginecans* in three consecutive inoculation trails. Although the isolates did not have the same level of aggressiveness, there was no significant isolate x cultivar interaction. Cultivars did, however, differ in their susceptibility to infection and results were consistent with field observations where local germplasm was generally more susceptible than that from outside Oman.

Inoculation trials showed that none of the cultivars tested were immune, or showed high levels of resistance to infection by *C. manginecans*. Ranking of mango cultivars based on lesion length following artificial inoculation generally correlated with field assessments of the disease. Cultivar Pairi was highly susceptible having the longest lesions after inoculation. Similarly, most of the local mango accessions included in the third inoculation such as Al Batikah, Ishbiah and Al Arash had the longest lesions and the highest levels of mortality at 88, 37 and 25%

respectively. This is consistent with mango wilt disease incidence evaluation in the mango genebank at Sohar where Pairi and local cultivars such as Al Batikah, Al Arash, and Oad Al Roob were severely affected by the disease with mortality incidences of 56.3, 33.3, 100 and 100% respectively. In the cases where local mango accessions were used as the scion, the disease symptoms were first expressed on the scion part of infected trees which represents the more susceptible portion of the trees. Furthermore, higher levels of disease were found in local mango cultivars compared to exotic cultivars. Disease severity assessments at a farm in the Barka area showed consistently higher levels of disease in local mango cultivars other than a limited number of exotic cultivars such as Pairi (Al Adawi 2002; Al Adawi *et al*. 2006). Therefore, high incidence of wilt in some exotic cultivars might be attributed to the death of local rootstock material due to *C. manginecans* infection, rather than susceptibility of exotic cultivars.

Results of this study showed that there are some reasonable levels of resistance to infection by *C. manginecans* in some mango cultivars. In the largest inoculation trial conducted in 2009 and taking into consideration inoculations with isolate CMW13854 in all three trials, cultivars Hindi Besennara, Mulgoa, Baneshan, Rose and Alumpur Baneshan are the most resistant. Some of these cultivars have been assessed for disease incidence and severity at the mango genebank at Sohar and at Barka and they have also been shown to be relatively resistant to infection under natural conditions (Al Adawi 2002). Thus, the mango wilt management program should consider excluding the highly susceptible mango cultivars and to rather use these cultivars for future propagation.

In all trials, control plants were randomly placed between those that were inoculated. A small number of control plants in the 2009 trial developed vascular discoloration similar to that in the inoculated plants, probably due to insects or mites carrying inoculum to wounds made on

the control plants. *Ceratocystis manginecans* produces a very strong banana odour and inoculum would have been attractive to insects that could move between plants (Kile 1993; Van wyk *et al.* 2007). While the control plants were not required for comparisons of response between different mango cultivars, they were included to provide a basis of comparison in the inoculations.

There was a significant year x cultivar interaction in the inoculation trials. Scion diameter could have contributed to this interaction. Scion diameter reflects tissue age and this varied significantly ($P < 0.0001$) among inoculated cultivars within and between trials. Mango trees undergo phenological changes including annual vegetative and reproductive growth and consequently, physiological changes (Davie & van Vuuren 1998; Davenport 2009) that are influenced by the environment. Host physiological status such as tissue age and phenological change are well-known factors that can affect pathogen progress within inoculated tissue (Eversmeyer *et al.* 1980; Tomerlin *et al.* 1983; Turechek & Stevenson 1998; Prell & Day 2001; Pariaud *et al*. 2009). The possible effect of physiological status during cultivar evaluation for resistance against *C. manginecans* needs further investigation.

In the 2005 and 2009 trials, isolates of *C. manginecans* showed significant differences in aggressiveness to mango cultivars. However, all of these isolates of *C. manginecans* were pathogenic and produced vascular discoloration in all the tested mango cultivars. The isolate CMW13854 was shown to be most aggressive in all inoculation trials but none of the isolates interacted differentially with mango cultivars in the 2005 and 2009 trails. Van der Plank (1984) suggested that a non-significant isolate x cultivar interaction would indicate horizontal resistance. Horizontal resistance is known to be durable since it is determined polygenically by the combined action of many minor genes. Most of the mango cultivars grown in Oman are derived from Indian monoembryonic cultivars that have a single zygotic embryo and produce

hybrid seedlings (Iyer & Schnell 2009). Hence, seed propagation from those cultivars will produce plants with substantial genotypic variation and give rise to diverse genotypes. Therefore, the levels of resistance in mango cultivars to infection by *C. manginecans* (and other desirable characteristics) could be enhanced through an extensive breeding program (Prell & Day 2001).

Breeding for resistance to *C. manginecans* would probably need to include hybridization between resistant cultivars in order to produce highly resistant cultivars. Furthermore, hybridization between resistant mango cultivars and those with lower levels of resistance would potentially yield resistance cultivars with more suitable horticultural criteria. Remnant mango trees left after the serious mango wilt epidemic represent a potential source of horizontal resistance and should be selected and evaluated for their resistance to infection. For example, breeding programs in Brazil have yielded mango cultivars resistant to infection by *Ceratocystis fimbriata s.l.* such as IAC 101 Coquinho, IAC 102 Touro, IAC 104 Dura and IAC 106 Jasmin that are descendents of susceptible cultivars including Coquinho and Jasmin (Ribeiro *et al*. 1995; Rossetto *et al*. 1996).

In order to replace current susceptible local monoembryonic rootstock cultivars, resistant polyembryonic cultivars as potential future rootstocks represent a high priority in the breeding program. Currently, Taimour and Hindi Besennara represent a suitable option to serve as rootstocks until further highly resistant cultivars can be found. Therefore, the current breeding program in Oman includes a selection of remnant mango trees and introduction of monoembryonic and polyembryonic mango germplasm from various part of the world for evaluation of resistance against *C. manginecans*.

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Table 1. Embryo type and origin of 30 mango cultivars used in resistance evaluations against *Ceratocystis manginecans* during three inoculation trails.

No.	Cultivar	Embryo type[a]	Origin	No.	Cultivar	Embryo type	Origin
1	Amrapali[b]	M	India	16	Langra	M	India
2	Alumpur Baneshan	M	India	17	Mantkah Al thour	M	Local accession
3	Al Arash	M	Local accession	18	Mulgoa	M	India
4	Al Batikah	M	Local accession	19	Muscati	M	Local accession
5	Alphonso	M	India	20	Neelum	M	India
6	Bangalora	M	India	21	Oad Al Roob	M	Local accession
7	Baneshan	M	India	22	Pairi	M	India
8	Baramasi	M	India	23	Rose	M	India
9	Dasheheri	M	India	24	Sherokerzam	M	India
10	Golek	P	Egypt	25	Sindhu[c]	M	India
11	Hajeb	M	Local accession	26	Sohar 2005	M	Local accession
12	Hindi Besennara	P	Egypt	27	Sournka	M	India
13	Imampasand	M	India	28	Taimour	P	Egypt
14	Ishbiah	M	Local accession	29	Tenneru	M	India
15	Khoh	M	Local accession	30	Zafran	M	India

[a] M= Monoembryonic, P= Polyembryonic
[b] Amrapali = Dasheheri x Neelum
[c] Sindhu = Ratna (Neelum x Alphonso) x Alphonso

Table 2. Analysis of variance of lesion length caused by isolates of *Ceratocystis manginecans* inoculated on different mango cultivars during infection trails.

Year	Source of variation	df	Sum Square	Mean square	*F* value	*P* > *F*
2005	Isolate	1	1004.65	1004.65	8.41	0.0050
	Cultivar	5	6644.46	1328.89	11.12	0.0001
	Isolate * Cultivar	5	608.76	121.75	1.02	0.4130
2007	Cultivar	12	4186.55	348.88	5.24	0.0001
2009	Isolate	1	643.84	643.84	4.25	0.0407
	Cultivar	27	28931.78	1071.55	7.08	0.0001
	Isolate * Cultivar	27	2701.29	100.05	0.66	0.8971
All years	Isolate	2	1345.36	672.68	5.49	0.0046
	Cultivar	29	30574.84	1054.30	8.60	0.0001
	Year	2	232.09	116.05	0.95	0.3893
	Isolate * Cultivar	31	3210.73	103.57	0.84	0.7073
	Year * Cultivar	14	5577.23	398.37	3.25	0.0001
All years with isolate CMW13854 only	Cultivar	29	26639	918.6	6.9	0.0001
	Year	2	215.4	107.7	0.81	0.4466
	Year * Cultivar	15	5599	373.3	2.8	0.0006

Table 3. Mean Lesion length in six mango cultivars inoculated with two isolates of *Ceratocystis manginecans* in evaluation trail conducted during April 2005.

Cultivar	Scion diameter (mm)	Lesion length (cm)			
		CMW13854	**CMW15351**	**Mean**	**Control**
Pairi	16 ± 2.4	48.9 ± 15.1 a[a]	31.6 ± 8.9 a	40.3a	0.5 ± 0.6
Baramasi	16.3 ± 4.3	28.4 ± 19.3 b	20.4 ± 5.4 abc	24.4b	1.1 ± 0.9
Zafran	13.3 ± 2.4	22.5 ± 14.5 b	22.6 ± 11.8 ab	22.5bc	0.3 ± 0.5
Tenneru	13.4 ± 2.3	21.8 ± 8.1 b	16.2 ± 10.2 bc	19bc	1.5 ± 1.6
Dasheheri	15 ± 2.3	20.1 ± 6.9 b	17.1 ± 10.7 abc	18.6bc	2.3 ± 1.9
Rose	11.1 ± 4.1	15.1 ± 2.9 b	7.2 ± 6.3 c	11.1c	0.8 ± 0.4
Mean[b]	14.2 ± 3.5	26.1a	19.2b		1.1c

[a] Values within same column followed by the same latter are not significantly different at $P < 0.05$ using Duncan's multiple range test.
[b] Values within mean row followed by the same latter are not significantly different at $P < 0.05$ using Duncan's multiple range test.

Table 4. Mean Lesion length on 13 mango cultivars inoculated with one isolate of *Ceratocystis manginecans* in evaluation trail conducted during February 2007.

Cultivar	Scion diameter (mm)	Lesion length (cm)		Mortality (%)
		CMW13854	Control	
Pairi	9.4 ± 1.2	42.1 ± 8.2 a[a]	1	10
Tenneru	10.9 ± 1.5	35.4 ± 13 ab	1	0
Imampasand	12.3 ± 1.2	33.7 ± 8.8 abc	2	0
Mantkah Al thour	10 ± 1.8	33.5 ± 6.7 abc	4	0
Sohar 2005	16.8 ± 1.4	32.7 ± 12.7 abc	2	0
Dasheheri	13.3 ± 1.2	32.7 ± 7.5 abc	1	0
Muscati	13.2 ± 1.9	32 ± 9.1 bcd	2	0
Ishbiah	7.5 ± 1.1	31.6 ± 9.8 bcde	1	100
Zafran	10.9 ± 0.7	27.6 ± 4 bcde	1	0
Langra	13.1 ± 1.3	24.6 ± 6.1 cdef	1	0
Baramasi	8.7 ± 1.8	22.7 ± 3.5 def	1	11.1
Hindi Besennara	12.9 ± 2.4	22.4 ± 5.2 ef	1	0
Rose	8.7 ± 0.9	18.1 ± 5.4 f	1	0
Mean[b]	11.3 ± 2.9	30a	1.3b	

[a] Values within same column followed by the same latter are not significantly different at $P < 0.05$ using Duncan's multiple range test.
[b] Values within mean row followed by the same latter are not significantly different at $P < 0.05$ using Duncan's multiple range test.

Table 5. Mean lesion length of 28 mango cultivars inoculated with two isolates of *Ceratocystis manginecans* in infection trails conducted during December 2009.

Cultivar	Scion diameter	Lesion length (cm)				Mortality
	(mm)	CMW13851	CMW13854	Mean	Control	(%)
Pairi	17.5 ± 4.6	46.5 ± 29.6 ab[a]	75 ± 44.9 a	60.8a	21.3 ± 24.2	0
Golek	14.9 ± 5.7	53 ± 7.5 a	50.8 ± 9.7 b	51.9ab	25.8 ± 14.3	12
Al Batikah	10.6 ± 1.8	38.5 ± 2.4 abc	51 ± 10.6 b	44.8bc	1 ± 0	88
Ishbiah	13.2 ± 2.4	39.3 ± 9.8 abc	45 ± 16.3 bc	42.1bc	17.1 ± 11.1	37
Alphonso	13 ± 4.1	36 ± 12.3 bcde	36.3 ± 2.6 bcd	36.1cd	2.5 ± 2.4	0
Imampasand	14.3 ± 4.5	39.3 ± 10.9 abc	30.3 ± 14.4 bcd	34.8cde	0.5 ± 0	0
Al Arash	12.3 ± 3	31.5 ± 7.9 bcdef	37.8 ± 7.4 bcd	34.6cde	6.5 ± 5.7	25
Oad Al Roob	12.7 ± 2.3	28.5 ± 11.2 cdefg	35.3 ± 11.1 bcd	31.9cdef	6.1 ± 7.9	0
Khoh	12 ± 5.2	29.8 ± 14.7 bcdefg	33.5 ± 29.4 bcd	31.6cdef	32 ± 26.9	0
Sohar 2005	13.4 ± 2.1	25 ± 4.3 cdef	35.8 ± 11 bcd	30.4cdef	2.3 ± 2.5	0
Bangalora	17.5 ± 3.3	24 ± 12 cdefg	29.3 ± 19.9 bcd	26.6defg	1 ± 0	0
Langra	11.2 ± 3.4	28.3 ± 16.7 cdefg	18 ± 0 d	25.8defg	1 ± 0.6	0
Neelum	15.6 ± 5.1	21.8 ± 12.3 cdefg	29.3 ± 8.6 bcd	25.5defg	7.9 ± 8.3	0
Sournka	13 ± 7	19 ± 7.7 efg	28.3 ± 11 bcd	23.6defg	15.5 ± 20.5	0
Baramasi	6.9 ± 1	22.7 ± 5.5 cdefg	23.1 ± 10.8 cd	22.9defg	3.4 ± 5.5	0
Amrapali	12.8 ± 6.5	19 ± 9.4 efg	26.8 ± 13.7 bcd	22.9defg	6 ± 7.1	0
Taimour	13 ± 5.5	25.8 ± 14.9 cdef	19.8 ± 7.1 cd	22.8defg	3.4 ± 5.5	0
Sindhu	11.4 ± 4.6	19.5 ± 9.6 efg	24 ± 3.9 cd	21.8defg	3.4 ± 2.8	0
Dasheheri	12.7 ± 2.2	20.5 ± 7.2 defg	20.8 ± 8.8 cd	20.6efg	1 ± 0	0
Sherokerzam	12.1 ± 2.7	19.8 ± 3.4 efg	19.9 ± 6.9 cd	19.8fg	1 ± 0	0
Mulgoa	14.3 ± 3	18.5 ± 7.6 efg	20 ± 5.2 cd	19.3fg	1 ± 0	0
Hindi Besennara	14.8 ± 3	17.1 ± 4.3 fg	20.8 ± 5.3 cd	18.9fg	4.8 ± 5.7	0
Hajeb	10.9 ± 5.1	14.8 ± 5.4 fg	22.5 ± 3.4 cd	18.6fg	11.5 ± 0.7	0
Tenneru	14 ± 4.6	17.8 ± 9 efg	19 ± 3.7 d	18.4fg	1 ± 0	0
Zafran	10 ± 3.4	19.8 ± 2.5 efg	15.3 ± 4.9 d	17.5fg	1 ± 0	0
Baneshan	12.5 ± 4.5	11.9 ± 3.1 g	16.3 ± 9.1 d	14.1g	1 ± 0.3	0
Rose	6.7 ± 0.6	12.8 ± 6.4 g	15.3 ± 3.3 d	14g	6 ± 7.1	0
Alumpur Beneshan	11.6 ± 4.3	14 ± 3 fg	13.5 ± 7.8 d	13.8g	1.4 ± 0.5	0
Mean[b]	12.7 ± 4.6	25.5b	29.3a	'-	6.3c	'-

[a] Values within same column followed by the same latter are not significantly different at $P < 0.05$ using Duncan's multiple range test.
[b] Values within mean row followed by the same latter are not significantly different at $P < 0.05$ using Duncan's multiple range test.

Table 6. Combined data analysis of mean lesion length of 30 mango cultivars inoculated with isolates of *Ceratocystis manginecans* in three infection trails.

Cultivar	Trails	Mean Lesion length in three trails	Mean Lesion length in three trails using isolate CMW13854	Death incidence in Barka farm 2001 - 2004	Death incidence in Sohar genebank 2002 - 2003	
					Waqaibah	Wadi hebi
Golek	2009	51.9a[1]	50.8a	-	2/3 (66.7%)	-
Pairi	2005, 2007, 2009	46.1ab	51.1a	9/20 (45%)	9/16 (56.3%)	-
Al Batikah	2007, 2009	44.8abc	51a	-	1/3 (33.3%)	-
Ishbiah	2007, 2009	36.5bcd	35.7abc	-	-	-
Alphonso	2009	36.1bcd	36.3abc	11/19 (57.9%)	10/16 (62.5%)	-
Al Arash	2009	34.6cde	37.8ab	-	3/3 (100%)	-
Imampasand	2007, 2009	34.3cdef	32.5bcd	1/2 (50%)	9/16 (56.3%)	-
Mantkah Al thour	2007	33.5defg	33.5bc	-	-	-
Muscati	2007	32defgh	32bcd	0/1 (0%)	-	-
Oad Al Roob	2007, 2009	31.9defgh	35.3bc	-	3/3 (100%)	-
Khoh	2009	31.6defgh	33.5bc	-	-	-
Sohar 2005	2007, 2009	31.5defghi	33.8bc	-	-	-
Bangalora	2007, 2009	26.6defghij	29.3bcde	0/4 (0%)	13/16 (81.3%)	-
Neelum	2007, 2009	25.5defghijk	29.3bcde	9/19 (46.4%)	11/16 (68.8%)	9/22 (41%)
Langra	2007, 2009	25.defghijk	23.8bcde	3/6 (50%)	6/16 (37.5%)	-
Sournka	2009	23.6efghijk	28.3bcde	-	-	-
Baramasi	2005, 2007, 2009	23.6efghijk	25bcde	1/4 (25%)	6/16 (37.5%)	3/24(13%)

Cultivar	Trails	Mean lesion length in three trails	Mean lesion length in three trails using isolate CMW13854	Death incidence in Barka farm 2001 - 2004	Death incidence in Sohar genebank 2002 - 2003	
					Waqaibah	Wadi hebi
Amrapali[2]	2009	22.9fghijk	26.8bcde	0/1 (0%)	-	-
Tenneru	2005, 2007, 2009	22.8fghijk	26.5bcde	3/4 (75%)	-	6/25(24%)
Taimour	2009	22.8fghijk	19.8cde	-	1/3 (33.3%)	-
Dasheheri	2005, 2007, 2009	22.6fghijk	25.2bcde	4/10 (40%)	3/16 (18.8%)	1/22(5%)
Zafran	2005, 2007, 2009	22.3ghijk	22.9bcde	15/39 (38.5%)	5/16 (31.3%)	-
Sindhu[3]	2009	21.8ghijk	24bcde	-	-	-
Hindi Besennara	2007, 2009	20.6hijk	21.8bcde	-	4/16 (25%)	-
Sherokerzam	2009	19.8ijk	19.9cde	-	8/16 (50%)	-
Mulgoa	2009	19.3jk	20cde	-	4/16 (25%)	-
Hajeb	2009	18.6jk	22.5bcde	-	-	-
Baneshan	2009	14.1k	16.3de	0/1 (0%)	5/16 (31.3%)	-
Rose	2005, 2007, 2009	13.9k	16.5de	2/8 (25%)	-	8/26(31%)
Alumpur Beneshan	2009	13.8k	13.5e	-	5/16 (31.3%)	-

[1] Values within same column followed by the same latter are not significantly different at $P < 0.05$ using Duncan's multiple range test.

SUMMARY

Mango wilt disease represents one of destructive diseases threatening mango cultivation in areas where it has been reported. Studies in this thesis represent the first to be undertaken after it was recognized that the disease is caused by the vascular wilt and canker pathogen *Ceratocytis manginecans* in Oman and Pakistan. Studies in this thesis showed clearly that the pathogen in the area represents a single clonal entity, adding credence to the view that the pathogen was introduced into Oman and Pakistan. Furthermore, the pathogen was shown to be vectored by the bark beetle *Hypocryphalus mangiferae*, which is native to India and has apparently been introduced into Oman, Pakistan and various other parts of the world such as Brazil where mango wilt is a serious disease. Concurrent with the appearance of mango wilt, two leguminous trees *Prosopis cineraria* and *Dalbergia sissoo*, began to wilt and die in Oman and Pakistan respectively. In this study, it was possible to show that these trees are also dying as result of infection by *C. manginecans* and that the pathogen appears to have undergone a host shift to these native trees. Both these tree species were shown to be susceptible to be equally susceptible to infection by *C. manginecans* as is mango. A final part of this investigation considered opportunities to select mango cultivars resistant to mango wilt in Oman. Here, the local mango accessions other than Pairi were shown to be highly susceptible to infection. In contrast, several mango cultivars such as Hindi Besennara, Sherokerzam, Mulgoa, Baneshan, Rose and Alumpur Baneshan had small lesions after inoculation with *C. manginecans* and can be considered amongst the tolerant mango cultivars. These results were also consistent with field evaluations. Studies undertaken in this thesis have added substantial information concerning mango wilt disease in Oman and it is hoped that this will help to reduce the devastation due to *C. manginecans* in the future.