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The mango sudden decline pathogen, *Ceratocystis manginecans*, is vectored by *Hypocryphalus mangiferae* (Coleoptera: Scolytinae) in Oman

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Abstract In Oman, the bark beetle *Hypocryphalus mangiferae* is closely associated with trees affected by mango sudden decline 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 sudden decline disease symptoms and it was found on trees in the early stages of the disease. Healthy, 2-year-old mango seedlings were exposed to *H. mangiferae* collected from diseased mango trees. Seedlings were infested by the bark beetles and after 6 weeks, typical mango sudden decline disease symptoms were observed. *Ceratocystis manginecans* was isolated from the wilted mango seedlings while uncolonized control seedlings remained healthy. The results show that *H. mangiferae* vectors *C. manginecans* in Oman and is, therefore, an important factor in the epidemiology of this disease.

Keywords *Mangifera indica* · *Ceratocystis fimbriata* · Bark beetle · Dispersal · Disease incidence

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Introduction

A serious epidemic of a new mango disease, sudden decline, has led to the death of thousands of trees in Oman. Sudden decline was first noted during 1998 in the Barka area in the southern part of the Al Batinah region (Al Adawi et al. 2003). It was initially attributed to various factors, but is now known to be caused by the virulent fungal pathogen, *Ceratocystis manginecans* M. Van Wyk, A. Al Adawi and M.J. Wingf. (Al Adawi et al. 2006; Van Wyk et al. 2007).

When sudden decline 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 and 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 and 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 and Peck 1994; Butani 1993; Pena and Mohyuddin 1997).

Examination of mango trees suffering from sudden decline 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 wilt disease of mango with symptoms similar to those of sudden decline 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 wilt disease of mango, seca, has been known in Brazil since 1938 (Viegas 1960). In Brazil, *Ceratocystis fimbriata sensu lato* is consistently isolated from seca-affected mango trees and it has been confirmed as the causal agent (Viegas 1960; Ploetz 2003; Ferreira et al. 2010). *H. mangiferae* is also closely associated with seca in Brazil and *C. fimbriata s.l.* has been isolated from adults of the beetle (da Castro 1960; Rossetto et al. 1980; Ribeiro 1993).

During the past 12 years, sudden decline has spread rapidly in Oman from the south to the north of the Al Batinah region and to other regions including Muscat, Musandam, Dakhiliyah and Sharqiyah. Furthermore, the disease in Oman is randomly distributed within each area and the isolation rate for *C. manginecans* from *H. mangiferae* associated with the disease was over 80 % (Al Adawi et al. 2006). Isolates of *C. manginecans* from diseased mango trees and from *H. mangiferae* were pathogenic, and caused the vascular discolouration and wilt symptoms of sudden decline (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 confirm Leach's principles (Leach 1940) for the *H. mangiferae* × mango × *C. manginecans* interaction and sudden decline. Leach's principles state that the following roles must be demonstrated to prove insect transmission of a plant disease: 1) close association of the insect with diseased plants, 2) regular visits by insects to healthy plants under conditions suitable for the transmission of the disease, 3) presence of the pathogen in or on insects in nature or following visitation to diseased plants, and 4) the experimental reproduction of disease by insect visitation under controlled conditions (Leach 1940).

Materials and methods

Survey and isolations

During 2004, mango trees on 17 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 sudden decline 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 for seed-propagated (local unknown monoembryonic mango cultivars propagated directly through seeds) and grafted trees (mainly scion of different known Indian monoembryonic mango cultivars grafted over the rootstock of unknown local monoembryonic cultivars). Disease incidence was also calculated for trees with visible signs of disease such as gummosis, vascular discolouration under the bark and partial wilt. Data from the survey were statistically assessed by analysis of variance (ANOVA) using SAS software v. 8 (SAS Institute 2000). Mean *H. mangiferae* infestation levels and disease incidence in different areas, or where different propagation methods were applied, were separated by Tukey's test when ANOVA was significant ($P<0.05$). The effect of irrigation water salinity on *H. mangiferae* infestation levels and disease incidence in surveyed farms was investigated and data were analyzed through correlation analysis using SAS software v. 8 (SAS Institute 2000).

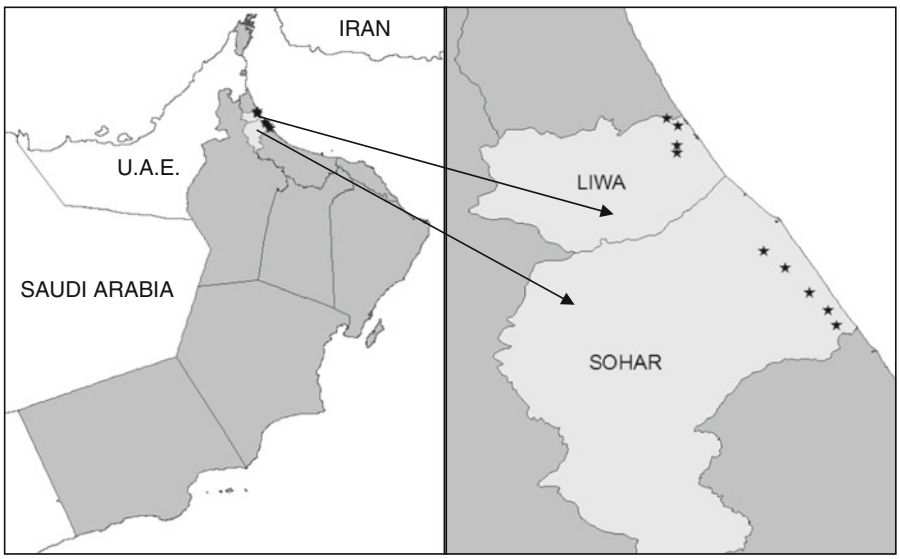

Fig. 1 Distribution of the farms in Sohar and Liwa in the Sultanate of Oman that were surveyed for mango wilt disease incidence and association with *H. mangiferae* infestation

Inoculation of mango seedlings with living *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, 10 cm above the soil level (Fig. 3a), and the top of the container was covered with nylon mesh to allow gas exchange but exclude unwanted infestations by *H. mangiferae*.

During March 2004, 225 individuals of *H. mangiferae* were collected from mango trees affected by sudden decline, using an aspirator. Twenty five of these insects were randomly selected and assayed for *C. manginecans* via carrot baiting (Moller and DeVay 1968). Twenty additional individuals were randomly selected and placed in the plastic containers surrounding each of the 10 plants. Five containers surrounding seedlings were left empty to serve as controls. All plants were maintained at 25±2 °C.

The number of individuals of *H. mangiferae* that infested plants was estimated by subtracting the number of dead beetles found at the base of each container from the total that was added to the container. Gummosis and wilt symptoms were recorded for each plant every 7 days. Vascular discolouration of the wood was recorded at the end of the experiment after 64 days when fungal isolations were conducted for all

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

Variables		Trees with decline disease symptoms (%)			Trees infested by *H. mangiferae*	
		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	19.3a	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 ($P \leq 0.05$)

plants. The exception was two seedlings that showed wilting symptoms after 42 days. The experiment ended for these two seedlings at 42 days after which vascular discolouration was measured and wood samples extracted.

Fungal isolations included removing the outer bark and then cutting out pieces of discoloured vascular tissue/wood. These wood samples were surface sterilized with 70 % ethanol and placed between fresh carrot slices (Moller and DeVay 1968). Carrot slices were checked after 7 days for the presence of *Ceratocystis* perithecia. Ascospore droplets from the tips of perithecial necks were transferred to 2 % Malt Extract Agar (MEA) (Oxoid, England) supplemented with streptomycin sulphate (500 mg/l) (Fluka, China) and incubated at 25±2 °C.

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 the *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 the 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 10× PCR buffer including 1.5 mM of $MgCl_2$, 2 μl of dNTPs (10 mM), 1 U of *Taq* polymerase (Roche Diagnostics, Germany), 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 5× 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 °C for 10 s; 50 °C for 4 s; 60 °C 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).

Results

Surveys and isolations

Approximately 1,090 mango trees in Sohar and Liwa were inspected for the presence of *H. mangiferae*. Statistical analysis revealed significant differences (F=12.4; df=4; P<0.0001) in the percentage of healthy trees, dead trees and those infested with *H. mangiferae*. Fifty-two percent of the trees examined were infested with *H. mangiferae* while 41 % showed mango sudden decline symptoms, and 18 % had died (Fig. 2). All trees with symptoms of sudden decline were infested with *H. mangiferae*, but an additional 11 % of the trees that were infested were free of gummosis and wilt symptoms indicating: possibly an early stage of disease development in healthy trees through introduction of *C. manginecans* inoculum by *H. mangiferae*; and an ability of *H. mangiferae* to infest healthy mango trees (Fig. 2). Survey data revealed non-significant but higher (F=3.3; df=1; P=0.079) *H. mangiferae* infestation levels in Liwa (55.9 %) compared to Sohar (43.7 %). Sudden decline incidence was significantly higher (F=7.03; df=1; P=0.013) in Liwa (49.5 %) than in Sohar (33.9 %). Differences in mortality levels between Sohar (19.3 %) and Liwa (12.4 %) were not significant (F=1.0; df=1; P=0.325) (Table 1).

H. mangiferae infestation (F=12.4; df=1; P=0.001) and disease incidence (F=8.82; df=1; P=0.006) in local seed-propagated mango trees was significantly higher (60.3 % and 48.9 % respectively) than in grafted trees (37.1 % and 31.7 %), but mortality was not significantly higher (F=1.85; df=1; P=0.184) in seed-propagated trees (21 %) than in grafted trees (11.9 %) (Table 1). The salinity levels of irrigation water ranged from 0.630 to 2.07 dS/m in Liwa and from 0.480 to 1.94 dS/m in Sohar. There was no

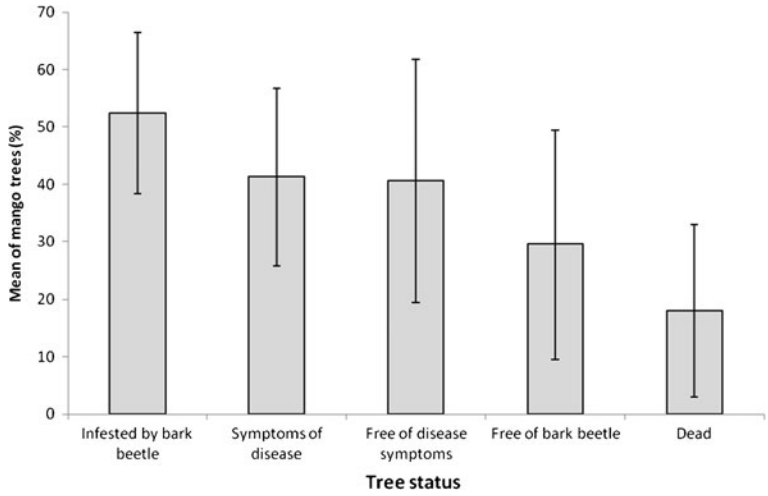

Fig. 2 Observations of infestation by bark beetle (*Hypocryphalus mangiferae*) and symptoms of sudden decline disease on mango trees surveyed on the 17 farms in the Sohar and Liwa areas in the North Al Batinah governate (Bars represent standard deviations)

correlation between water salinity and *H. mangiferae* infestation levels (r=0.01; P=0.134), disease incidence (r=0.013; P=0.062) or dead trees (r=0; P= 0.951) (Table 2).

Inoculation of mango seedlings with *H. mangiferae*

Ceratocystis manginecans was isolated from 12 % of the assayed individuals of *H. mangiferae.* In total, 80 % (159 of 200) of the *H. mangiferae* individuals entered the bark of challenged healthy plants, although infestation ranged from 40 to 100 % among the different plants (Table 3). Colonization by *H. mangiferae* was mainly initiated at the leaf scars (i.e. sites where leaves were removed from the stem to allow for the cage to be placed around the stem) 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 in colour, but later black-brown, was observed in 80 % of the exposed mango seedlings; no gummosis was observed on the control plants (Fig. 3b). The amount of gummosis varied among seedlings: four of the 10 tested seedlings developed gummosis within 2 weeks and four more showed gummosis after 3 weeks (Table 3). Wilt symptoms were first observed after 42 days (Table 3), starting with two of the seedlings. Four exposed seedlings developed wilt symptoms (Fig. 3c) and no wilt was observed in the control seedlings. Longitudinal sections through exposed stems revealed vascular discolouration (Fig. 3b) radiating from beetle entry points. Discolouration was not observed in the control seedlings.

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

Status of mango trees	r^a	P value
Infested	0.010	0.134
Diseased	0.013	0.062
Dead	0.000	0.951

[a] Value of linear correlation coefficient

Pathogen identification

Isolations from exposed seedlings (Fig. 3d) yielded *Ceratocystis*-like, grey-olive colonies on MEA. The fungus was isolated from the four wilted mango seedlings and from two that developed gummosis. No control seedlings yielded *Ceratocystis* isolates. 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 98 % similarity with sequences of *C. manginecans* from Oman (CMW13851 = AY953383.1, CMW13852 = AY953384.1, CMW13854 = AY953385.1).

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

Discussion

Results of this study provide clear evidence that *H. mangiferae* is a vector of the mango sudden decline pathogen, *C. manginecans*. Survey data demonstrated an intimate association between infestation by *H. mangiferae* and mango sudden decline disease, although *H. mangiferae* also infested healthy mango trees in the surveyed areas. There was no significant correlation between irrigation water salinity and level of *H. mangiferae* infestation or the incidence of sudden decline. Healthy mango plants exposed to *H. mangiferae* developed typical symptoms of sudden decline, including gummosis, vascular discolouration and wilt, and *C. manginecans* could be re-isolated from symptomatic tissue.

In 10 years, sudden decline disease has spread rapidly in Oman from the south to the north across the Al Batinah region (more than 250 km), as well as to other regions of the country. Active dispersal of *C. manginecans* by a bark beetle, such as *H. mangiferae*, would not be an unexpected explanation for such rapid spread (Webber and Gibbs 1989; Wainhouse et al. 1998; Storer et al. 1999).

Results of the survey in this study demonstrated a clear association between *H. mangiferae* and sudden decline in all affected mango trees. However, infestation by *H. mangiferae* was also found in 11 % of mango trees free of disease symptoms. That healthy mango trees were attacked by *H. mangiferae* supports the view that *H. mangiferae* is a primary vector that attacks non-stressed and healthy trees. In Brazil, *H. mangiferae* has been reported as common on mango trees without disease symptoms and on those on which symptoms have just begun to develop (da Castro 1960; Rossetto et al. 1980).

High levels of *H. mangiferae* infestation and sudden decline 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 indicate that non-grafted local mango varieties are highly susceptible to the disease compared to grafted plants of an exotic scion on a local rootstock. *H. mangiferae* was most commonly associated with local mango varieties and less so with exotic, grafted varieties. This is consistent with previous observations that sudden decline is significantly more common on local mango varieties

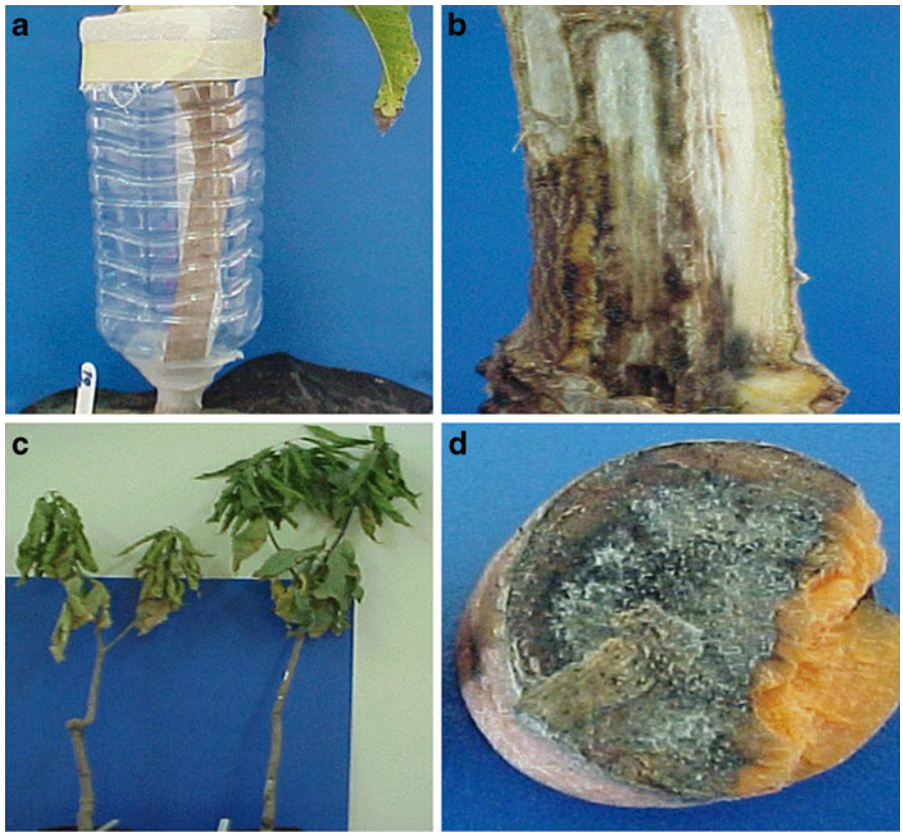

Fig. 3 Inoculation procedure and disease development in mango seedlings exposed to *Hypocryphalus mangiferae*; (**a**) plastic cage surrounding mango stem, (**b**) gummosis and vascular discolouration, (**c**) wilted mango seedlings, (**d**) *Ceratocystis manginecans* isolated on carrot discs

compared to those grafted with non-native scions (Al Adawi et al. 2006). The reason for the greater susceptibility of local varieties is unclear, but it is also consistent with the fact that large numbers of bark beetle tunnels were observed on the local rootstocks of grafted trees. This could be due to genetic differences between exotic and local varieties, expressed in differences in defence responses to bark beetle infestation and *C. manginecans* and this is consistent with similar studies (Al Sadi et al. 2010). Current research to select varieties resistant to sudden decline should also include investigations into susceptibility to infestation by *H. mangiferae*.

Although farms included in the survey varied in the salinity of irrigation water, only one farm out of the 17 surveyed had irrigation water salinity levels that were unsuitable (EC >2 dS/m) for mango cultivation (Meurant et al. 1999). This farm represented 7.4 % of all mango trees included in the survey. Although there was no correlation between irrigation water salinity and infestation by *H. mangiferae* or disease incidence, additional research on this topic is warranted considering the small number of plants that were exposed to high salinity in the present study.

The results of this study showed that *H. mangiferae* can colonize healthy mango seedlings and that the beetles, thereby, facilitate infection of mango by *C. manginecans*. The pathogen was isolated from plants exposed to insects subsequent to symptom development and sampled insects were shown to carry the fungus. Thus, *C. manginecans* can be spread to healthy plants by *H. mangiferae*. This is consistent with the seca scenario in Brazil in which *H. mangiferae* is a vector for *C. fimbriata s.l.* (da Castro 1960; Rossetto et al. 1980). In Brazil, only 1 % of *H. mangiferae* were contaminated with *C. fimbriata s.l* (Ribeiro and 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). 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. 2007; Masood et al. 2008). However, in Pakistan, 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).

The inconsistency in symptom development in the present study was not surprising. Individuals of *H. mangiferae* would carry inconsistent inoculum loads and efficacy of transmission would vary from plant to plant. The tested seedlings would also have non-uniform susceptibilities since the seedlings had heterogeneous genetic backgrounds.

The manner in which *H. mangiferae* disseminates *C. manginecans* is not known. Fungal pathogens can either adhere to the outer surface of insect vectors, be carried in their gut, or be transferred via specialized mycangia (Francke-Grosmann 1967; Malloch and Blackwell 1993). Further study is needed to determine how *C. manginecans* is associated with *H. mangiferae*. Furthermore, results of this study, together with the 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 (Leach 1940) regarding insect vector relationships presented in the introduction to this paper. This study demonstrated a close association between *H. mangiferae* and wilted mango trees (Role 1), the presence of 11 % *H. mangiferae* in healthy mango trees (Role 2), isolation of *C. manginecans* from 12 % of *H. mangiferae* (Role 3) and mango sudden decline disease symptoms were produced in healthy mango trees infested with living *H. mangiferae* (Role 4).

Confirmation of *H. mangiferae* as a vector for *C. manginecans* is important for an integrated disease management program. Fungicides and insecticides, alone and in combination, have been tested but with equivocal results (Ministry of Agriculture and Fisheries, 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 if its management is to play an important role in the management of sudden decline.

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