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

A. O. Al Adawi · I. Barnes · I. A. Khan · A. M. Al Subhi · A. A. Al Jahwari · M. L. Deadman · B. D. Wingfield · M. J. Wingfield

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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 these native, leguminous hosts include vascular discolouration and partial or complete wilt of affected trees. A species of *Ceratocystis* was consistently isolated from symptomatic material. Morphological comparisons and analyses of DNA sequence data of the ITS, β-tubulin, and EF 1-α gene regions showed that the *Ceratocystis* isolates obtained from both tree species represent *C. manginecans*. This is the same pathogen that is causing the devastating mango sudden decline disease in Oman and Pakistan. This is also the same pathogen that has been reported causing a wilting disease on *Acacia mangium* in Indonesia. Cross inoculation with *C. manginecans* isolates from *P. cineraria*, *D. sissoo* and mango showed that the fungus can cause disease on all three trees.

A. O. Al Adawi · M. J. Wingfield
Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

I. Barnes (✉) · B. D. Wingfield
Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
e-mail: irene.barnes@fabi.up.ac.za

I. A. Khan
University of Agriculture, Faisalabad 38040, Pakistan

A. M. Al Subhi · M. L. Deadman
Department of Crop Sciences, Sultan Qaboos University, P.O. Box 34, Al Khod 123, Sultanate of Oman

A. O. Al Adawi · A. A. Al Jahwari
Ghadafan Agriculture Research Station, Ministry of Agriculture and Fisheries, P. O. Box 204, Sohar 311, Sultanate of Oman

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Introduction

Ceratocystis fimbriata sensu lato Ellis & Halst represents a well recognized group of cryptic fungal species that cause serious diseases on various agricultural and tree crops (Kile 1993; Roux and Wingfield 2009). The pathogen was first described from sweet potato (*Ipomoea batatas*) causing tuber black rot (Halsted and Fairchild 1891) and species in this complex have subsequently been reported from many agricultural crops worldwide, causing vascular discolouration, wilt, canker and root disease. For example, species in the *C. fimbriata* s. l. complex have been reported as serious pathogens 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; Roux and Wingfield 2009).

Ceratocystis fimbriata s. l. represents a complex of cryptic species that are increasingly being differentiated as additional molecular techniques, and especially DNA sequence comparisons, are applied to the group (Wingfield et al. 1996; Barnes et al. 2003; Engelbrecht and 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). For example, wilt disease of *Acacia mearnsii* (black wattle) for which the causal agent was first reported as *C. fimbriata* (Morris et al. 1993) was later recognised as a 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* s. l. complex including *C. pirilliformis* Barnes & M. J. Wingfield isolated from

Eucalyptus (Barnes et al. 2003), *C. cacaofunesta* Engelbrecht & Harrington infecting cacao and *C. platani* (J. M. Walter) Engelbrecht & Harrington on plane and sycamore trees (Engelbrecht and Harrington 2005), *C. neglecta* M. van Wyk, J. 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 in the Botryosphaeriaceae (Al Adawi 2002; Al Adawi et al. 2003) but it was later shown to be caused by a species in the *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 a new species, *Ceratocystis manginecans* M. van Wyk, A. Al Adawi & M. J. Wingf. (Van Wyk et al. 2007).

Ceratocystis manginecans has killed many thousands of mango 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; Al Adawi et al. 2013). *Ceratocystis manginecans* has also recently been found in Indonesia where, together with *C. acaciivora* 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* (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*. *Prosopis cineraria* (Leguminosae) is an important desert tree 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* (locally known as Shisham) trees since 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 multi-purpose tree that has a valuable timber and it is grown in plantations, alongside canals and roadsides, and to define field boundaries on private land (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 native trees using morphological and DNA sequence comparisons. Furthermore, host specificity and possible host jumps were considered by conducting reciprocal inoculations on mango (*Mangifera indica*), *P. cineraria* and *D. sissoo* trees.

Material and methods

Sample collection and fungal isolation

During December 2004, samples were collected from *P. cineraria* trees showing symptoms of wilt at Wilayat Sohar in Oman. Symptoms included single branches exhibiting wilt symptoms, dark grey-brown vascular discolouration of affected branches or tree trunks and often wilt of entire trees (Fig. 1a and b). Samples were collected by removing the bark and cutting longitudinal strips (approx. 50 mm) from freshly infected xylem with stain. Additional samples were taken during the following two years from different areas all 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 trees included black-grey staining of the xylem tissues, leaves first turning brown from the tops to the bottoms of trees, and death of the entire trees (Fig. 1c and d). After removing the bark from trees that had recently wilted, longitudinal strips of discoloured (streaked) vascular tissue were removed for isolation. For both *P. cineraria* and *D. sissoo*, wood samples were stored in plastic bags and preserved in a refrigerator prior to isolations being made.

Because the vascular discolouration 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 causing these symptoms. For this reason, carrot baiting (Moller and De Vay 1968a) was used where discoloured wood was placed between two slices of carrot that had first been treated with streptomycin sulphate (100 mg/l) 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 °C) for 7–10 days to induce sporulation directly on infected tissue.

Single ascospore mass 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 °C.

Morphological characterization

The morphological characteristics of the *Ceratocystis* sp. isolated from *P. cineraria* and *D. sissoo* trees were

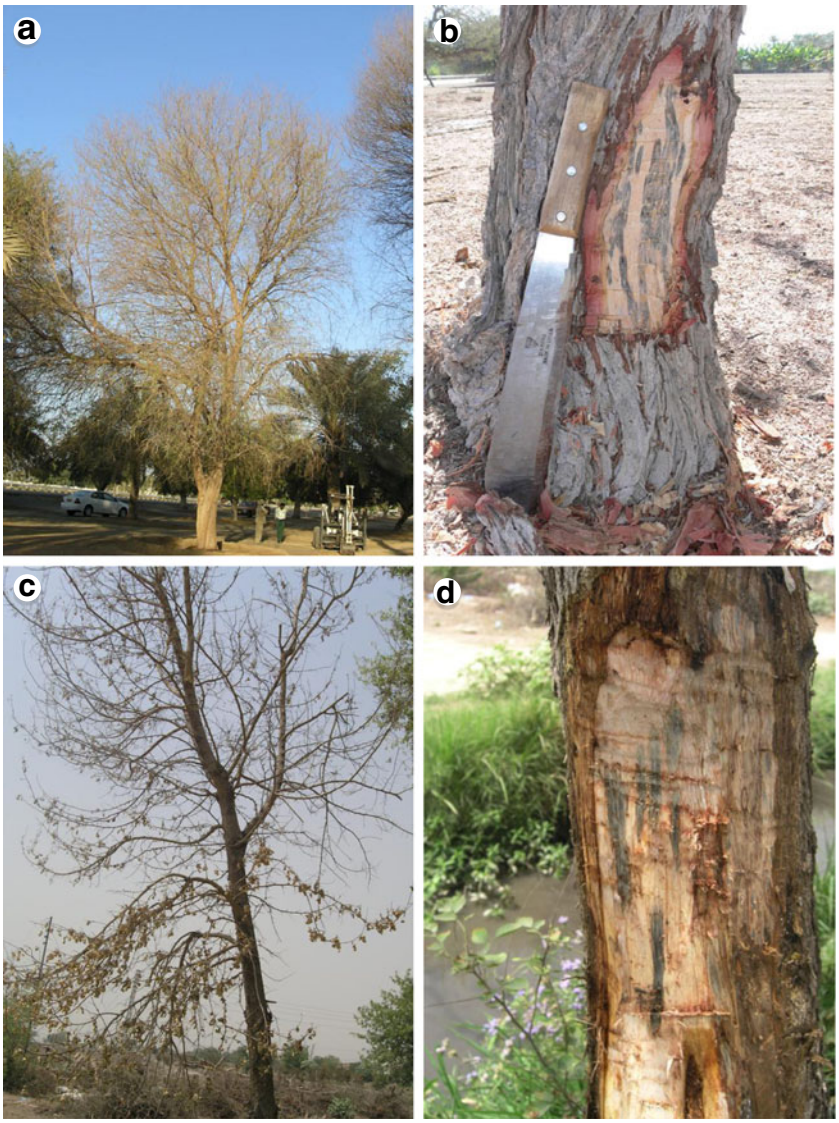


Fig. 1 Symptoms of wilt and die-back on *P. cineraria* and *D. sissoo* **a** wilted *P. cineraria*, **b** dark staining of the xylem tissues in *P. cineraria*, **c** dying *D. sissoo* tree, **d** vascular discolouration of infected *D. sissoo*

compared with *C. manginecans* 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 and CMW23641), two isolates of *Ceratocystis* sp. from *P. cineraria* (CMW17568 and CMW17570) and two from *D. sissoo* (CMW23623 and CMW23625) were selected for morphological comparisons. Morphological observations of the *Ceratocystis* isolates were made using fungal structures produced on 2 % MEA plates incubated for 10 days at 25 °C. Samples were prepared by mounting fungal structures on glass slides in lactic acid and observing these 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 primary and secondary conidia and chlamydospores.

Rates of growth were measured for each representative isolate grown on MEA and incubated in the dark at different temperatures ranging from 5 to 35 °C at 5 °C intervals. Diameters of cultures were measured across two perpendicular axes after 7 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 SAS version 8 (SAS institute, Cary, NC).

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 24 h and then ground into a fine powder using a Geno Grinder (Glenmills). DNA extraction was performed using a phenol: chloroform (1:1) extraction protocol as described by Barnes et al. (2001). 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

Table 1 *Ceratocystis* isolates used in the morphological comparisons, phylogenetic analyses and inoculation trials

Identity	Culture no.[a]	Host	Geographic origin	GenBank accession no[b].
C. manginecans	**CMW17225**[c]	*Prosopis cineraria*	Sohar, Oman	Identical to AY953383
				Identical to EF433308
				Identical to EU588652
	CMW17568 [d, e]	*P. cineraria*	Sohar, Oman	Identical to AY953383 (group 1) and identical to EU588657 (group 2)
				Identical to EF433308
				Identical to EU588650
	CMW17570 [d, e]	*P. cineraria*	Sohar, Oman	Identical to EU588657
				Identical to EF433308
				Identical to EU588650
	CMW23623 [e]	*Dalbergia sissoo*	Faisalabad, Pakistan	Identical to EU588657
				Identical to EF433308
				Identical to EU588650
	CMW23624	*D. sissoo*	Faisalabad, Pakistan	Identical to EU588657
				Identical to EF433308
				Identical to EU588650
	CMW23625 [d]	*D. sissoo*	Shorkot, Pakistan	Identical to EU588657
				Identical to EF433308
				Identical to EU588650
	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
	CMW21125	*Acacia crassicarpa*	Indonesia	EU588663
				EU588642
				EU588652
	CMW21127	*A. crassicarpa*	Indonesia	EU588664
				EU588643
				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

Table 1 (continued)

Identity	Culture no.[a]	Host	Geographic origin	GenBank accession no[b].
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
	CMW6579	*E. nitens*	Australia	AF427105
				DQ371653
				AY528983

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

[b] GenBank accession number for the ITS, β-tubulin 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

10 µl of RNase A (10 mg/ml, Roche Diagnostics, South Africa) and incubated at 37 °C 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 5.8 s 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 EF1-728 F 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 °C followed by ten cycles of 20 s at 94 °C, 40 s at 55 °C and 45 s at 72 °C. The last three temperature intervals were repeated for another 30 cycles with a 5 s increase per cycle for the annealing step at 55 °C, followed by a final elongation step for 10 min at 72 °C. 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 °C for10 s; 50 °C for 4 s; 60 °C 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 (Table 1).

Sequences were aligned using the programme MEGA version 5 (Tamura et al. 2011) and manually adjusted where necessary. Phylogenetic analyses 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, metrics 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 PAUP v. 4.0b10 and MrModeltest 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. Burn-in 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).

Inoculation trials

The first of two inoculation trials 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 and 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. The *P. cineraria* plants 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 *P. cineraria* plants were inoculated with each *Ceratocystis* isolate and the same number of seedlings was inoculated with a sterile plug of MEA to serve as controls. The plants were arranged in a randomized block design. All inoculated wounds were covered with moistened sterile cotton pads and sealed with Parafilm.

The inoculated plants 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 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 plants (Pairi cultivar), one-year-old plants of *P. cineraria* propagated from local Omani seed and ten-month-old *D. sissoo* plants propagated from seed, were grown in the same potting media as described for the first trial. All plants were kept under shade house conditions and irrigated twice per week. Two weeks prior to inoculation, the plants were transferred to a laboratory with a temperature of 24–26 °C and irrigated twice per week. The height and diameter of each plant was measured at the time of inoculation.

The inoculation was conducted using two isolates (CMW13851 and CMW13854) of *C. manginecans* from mango, two isolates (CMW17225 and CMW17568) of the *Ceratocystis* sp. from *P. cineraria* and two isolates (CMW23623 and CMW23625) of the *Ceratocystis* sp. from *D. sissoo*. Inoculum of each isolate was grown on MEA for 2 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 plants. 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 plants per host and three plants 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 plants were measured after 30 days.

Statistical analyses of the results were carried out for the 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, Fisher's least significant difference (LSD) 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).

Results

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

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

Host	Area	Year	Recovery of *C. manginecans*	
P. cineraria	Sohar	2004		1/4 (25 %)
		2005		3/6 (50 %)
		2006		3/5 (60 %)
			Total	7/15 (46.7 %)
D. sissoo	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		$p=0.085$	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 °C		0	0	**0**	0	0	**0**	0	0	**0**
	10 °C		0	0	**0**	0	0	**0**	0	0	**0**
	15 °C	$p<0.0001$	11b	9.3c	**10.15c**	13.9a	12.8a	**13.35a**	11.1b	12.5ab	**11.8b**
	20 °C	$p<0.0001$	20.7ab	22.9a	**22.1a**	20.7bc	19.3c	**20b**	19.6bc	19.8bc	**19.7b**
	25 °C	$p=0.0022$	30.2ab	32a	**31.1a**	31.9a	29.8ab	**30.85a**	27.4b	30.2ab	**28.8b**
	30 °C	$p<0.0001$	31a	30.2a	**30.6a**	26.4b	30.7a	**28.55b**	31.2a	30.8a	**31a**
	35 °C		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

Forestry and Agriculture Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

Morphological characterization

Isolates on MEA from *P. cineraria* and *D. sissoo* clearly represented a member of the *C. fimbriata* s. l. complex based on morphological characteristics of both the sexual and asexual structures (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. cineraria* and *D. sissoo* produced greyish olive cultures and the shape and colour of the ascomatal bases, 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.*

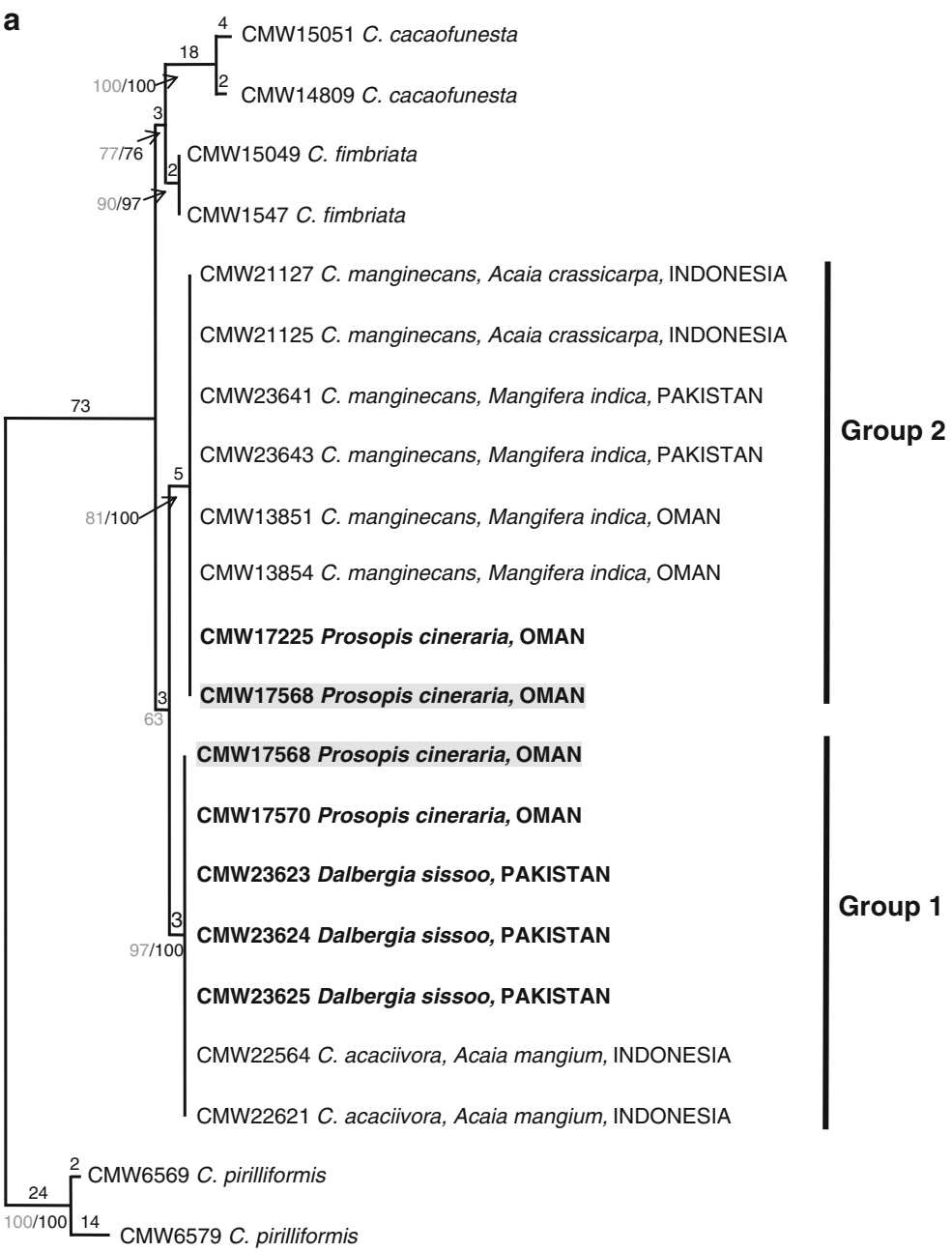
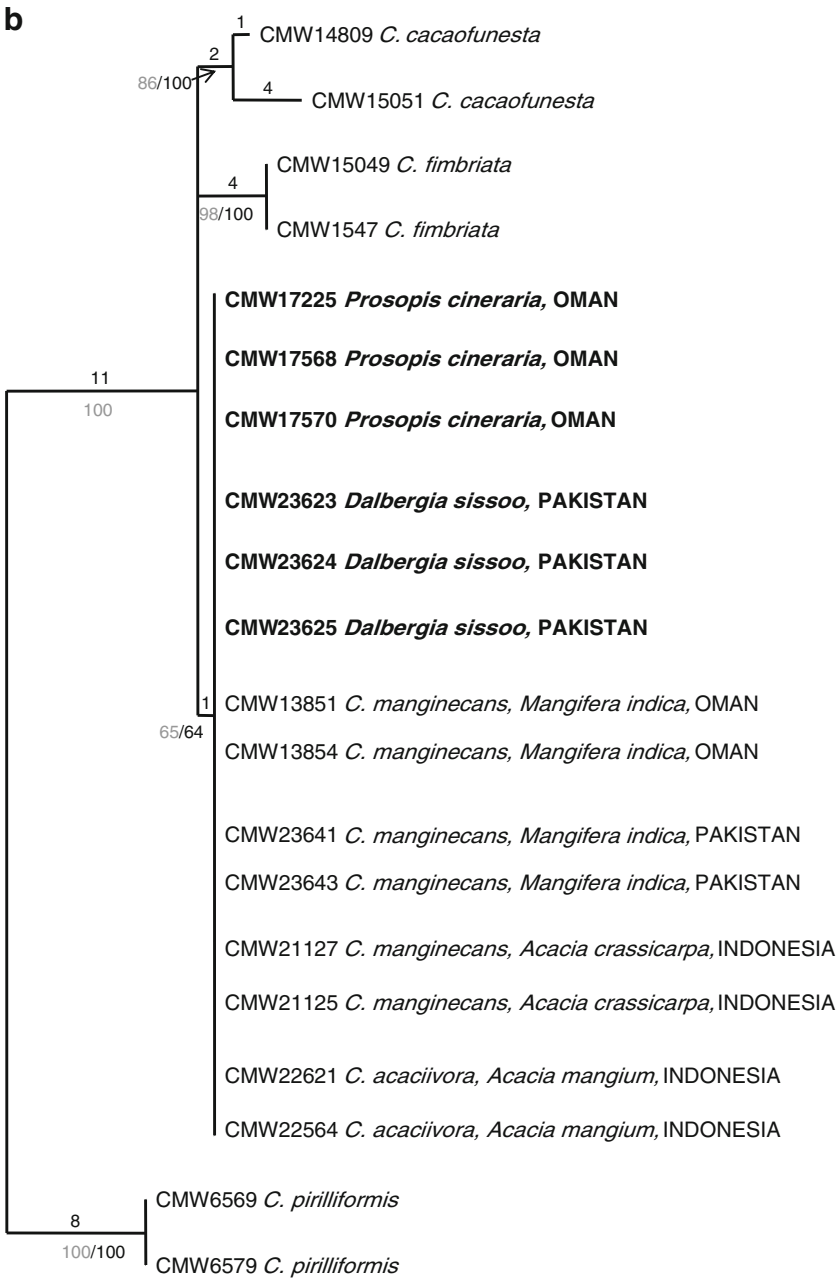


Fig. 2 Maximum parsimony trees resulting from the analysis of sequence data of the (**a**) ITS (**b**) β–tubulin, (**c**) TEF-1α gene regions. Bootstrap values and posterior probability values (*in bold*) are indicated below the branches. Branch lengths are represented above the branches for all trees. Isolates in bold are those sequenced in this study. The isolates reside in two groups in the ITS phylogeny; Group 1 includes *C. acaciivora* isolates and Group 2 includes *C. manginecans* isolates. ITS sequences obtained from isolate CMW17568 fall into both groups showing that this isolate has different ITS types and does not reflect different species. The β–tubulin and TEF-1α phylogenies do not differentiate between isolates of *C. acaciivora* and *C. manginecans*

Fig. 2 (continued)



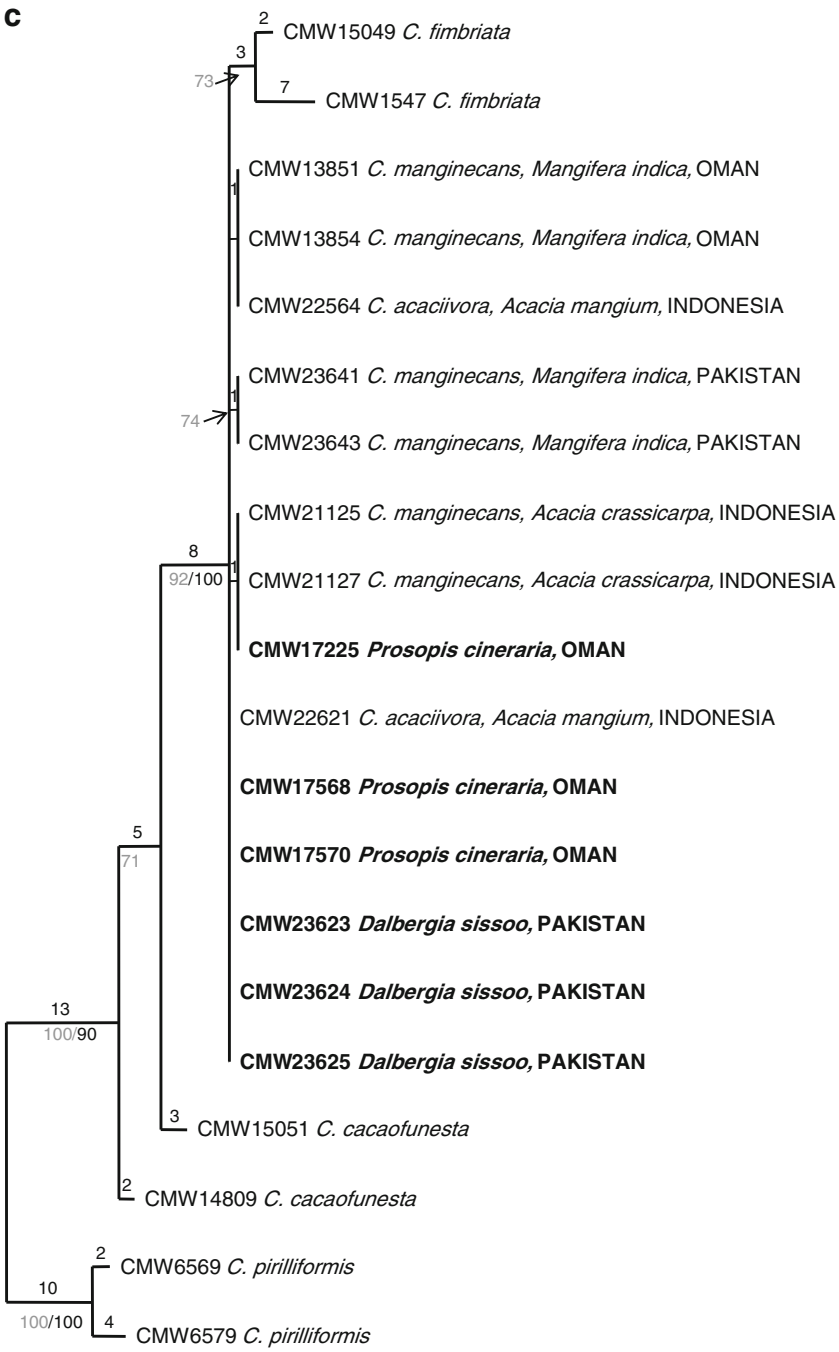
cineraria, *D. sissoo* and *C. manginecans* for the ascomatal bases, ascospores and chlamydospores. Variation was observed in the lengths of the ascomatal necks for isolates from all hosts, but they were still within the ranges reported for *C. manginecans* (Van Wyk et al. 2007). The lengths and widths 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* isolates from mango and the isolates from *P. cineraria* and *D. sissoo*. No growth occurred in any of the isolates at 5 °C, 10 °C and 35 °C. Optimum growth for all the *Ceratocystis* isolates occurred between 25 °C and 30 °C.

DNA extraction, amplification, sequencing and phylogenetic analyses

The total number of characters in the ITS dataset after alignment and inclusion of gaps was 560 bp. 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 the ITS gene region divided all the isolates from mango, *P. cineraria* and *D. sissoo* from Pakistan and Oman into two, well supported clades. Two

Fig. 2 (continued)



isolates from *P. cineraria* (CMW17225 and CMW17568) grouped together with the isolates of *C. manginecans* from mango and *A. crassicarpa* designated as Group 2 in the study of Tarigan et al. (2011) (Fig. 2a). All the *D. sissoo* isolates and two *P. cineraria* isolates (CMW17568 and CMW17570) resided in Group 1 with isolates of *C. acaciivora* from *A. mangium* (posterior probability value (PP)= 100 %, bootstrap value (BP)=96 %).

As a consequence of the placement of the *P. cineraria* isolates from Oman into two different *Ceratocystis* groups (Fig. 2a) based on ITS sequences with *C. manginecans* residing in Group 2 and *C. acaciivora* residing in Group 1, all sequencing of the ITS region were repeated. Repeated sequencing of the isolates revealed that a single isolate (CMW17568) from *P. cineraria* contained both of the ITS sequences that define Groups 1 and Group 2 as different species. Sequence variability between these two groups was represented by a 7 bp sequence difference in the ITS1 region that included 5 single-base indels and 2 base substitutions, and a single indel in the ITS2 region (Table 4).

Table 4 Sequence variation in the two ITS types (Group 1 and Group 2) found in *Ceratocystis manginecans* isolates from *P. cineraria*, *D. sissoo* and mango

Host	Isolate no.[a]	112[b]	118	121	127	132	140	181	472	
Shisham	**CMW23623**	**T**	**T**	**G**	**G**	-	**T**	**A**	**C**	GROUP 1
(*Dalbergia sissoo*)	**CMW23624**	**T**	**T**	**G**	**G**	-	**T**	**A**	**C**	
	CMW23625	**T**	**T**	**G**	**G**	-	**T**	**A**	**C**	
Ghaf	**CMW17570**	**T**	**T**	**G**	**G**	-	**T**	**A**	**C**	
(*Prosopis cineraria*)	**CMW17568**	**T**	**T**	**G**	**G**	**-**	**T**	**A**	**C**	
	CMW17568	**-**	**-**	**A**	**A**	**A**	**-**	**-**	**-**	
	CMW17225	-	-	**A**	**A**	**A**	-	-	-	
Mango	CMW13851	-	-	A	A	A	-	-	-	GROUP 2
(*Mangifera indica*)	CMW13854	-	-	A	A	A	-	-	-	
	CMW23641	-	-	A	A	A	-	-	-	
	CMW23643	-	-	A	A	A	-	-	-	
Acacia crassicarpa	CMW21125	-	-	A	A	A	-	-	-	
	CMW21127	-	-	A	A	A	-	-	-	

[a] The ITS region of all the isolates represented in bold were sequenced twice, using the same single spore culture to validate the results. The highlighted isolate contains both ITS types that was used to designate isolates into Group 1 and Group 2 in Tarigan et al. (2011) and Fig. 2a

[b] Nucleotide position after alignment

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 more than 100 trees using 39 informative characters. Tree length was 52 steps and the CI and RI values 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).

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 beginning to die at the end of the experiment. Analysis of variance for lesion length in *P. cineraria* showed 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. Furthermore, statistical analyses showed significant differences in lesion length between treated *P. cineraria* and the control seedlings (6.3 mm) (Fig. 3). Re-isolation from inoculated seedlings yielded a *Ceratocystis* sp. and the fungus was not isolated from the control seedlings.

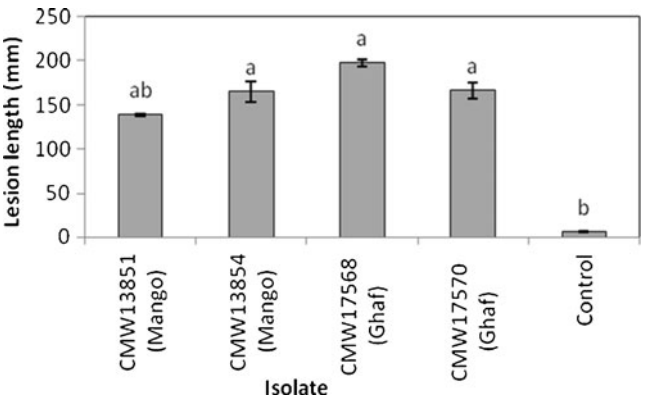


Fig. 3 Lesion lengths arising from inoculations on *P. cineraria* (Ghaf) seedlings using *Ceratocystis manginecans* isolated from mango (CMW13851 and CMW13854) and *Ceratocystis* isolates from *P. cineraria* (CMW17568 and CMW17570). Analysis of variance was calculated using a GLM model. Columns represent 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 $P < 0.05$ using Fisher's (LSD) test

All mango seedlings inoculated with *C. manginecans* and *Ceratocystis* isolates from *P. cineraria* and *D. sissoo* 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 the *Ceratocystis* isolates used in the inoculations. Average lesion lengths produced by the *C. manginecans* isolates (CMW13851 and CMW13854) from mango, *Ceratocystis* isolates from *P. cineraria* (CMW17225 and CMW17568) and from *D. sissoo* (CMW23623 and CMW23625) inoculated into mango seedlings were 406.7, 300, 266.7, 300, 300 and 263.3 mm respectively (Fig. 4a). The average lesion length on the control seedlings (58.3 mm) was significantly smaller than those for all the test plants.

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 no significant differences between the isolates tested (Fig. 4b). 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 from those for the controls that had an average lesion length of 5 mm.

D. sissoo seedlings inoculated with different *Ceratocystis* isolates showed mortality in four 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 (Fig. 4c). Although longer lesions were produced on *D. sissoo* seedlings inoculated with the different *Ceratocystis* isolates (ranging from 63.3 to 120 mm) there were no significant differences in lesion lengths of the majority of the *Ceratocystis* isolates compared to those for the control inoculations where the average lesion length was 5 mm.

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

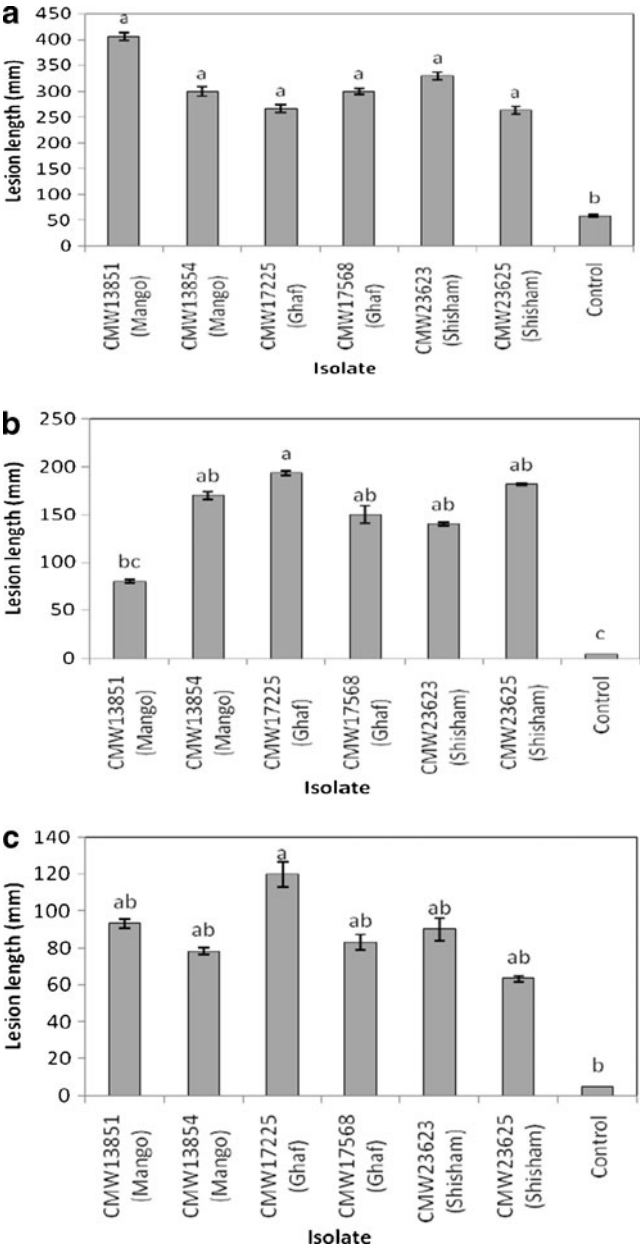


Fig. 4 Cross inoculation experiments on mango (**a**) *P. cineraria* (Ghaf) (**b**) and *D. sissoo* (Shisham) seedlings (**c**) using *Ceratocystis manginecans* isolated from mango (CMW13851 and CMW13854), *Ceratocystis* isolates from *P. cineraria* (CMW17225 and CMW17568) and from *D. sissoo* (CMW23623 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 $P < 0.05$ using Fisher's (LSD) test

Discussion

In this study, *P. cineraria* and *D. sissoo*, were found to represent two new leguminous hosts 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; the same was true in Pakistan for *D. sissoo*. The fact that *C. manginecans*, 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), is infecting native trees in these countries is serious and 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. In addition, the pathogenicity of *C. manginecans* to both these hosts was demonstrated in inoculation trials and it is clear 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.

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. acaciivora*, killing *A. mangium* in Indonesia (Tarigan et al. 2011). This is particularly interesting because *A. mangium*, like *P. cineraria* and *D. sissoo*, is a leguminous tree. This supports the view that *C. manginecans* has a host range beyond mango and in time it might be found to infect other trees.

Ceratocystis manginecans forms part of the *C. fimbriata* s. l. complex, which is typified by *C. fimbriata* sensu stricto that causes black rot of sweet potato (Engelbrecht and 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 and Harrington 2005; Johnson et al. 2005; Van Wyk et al. 2007; Rodas et al. 2008; Van Wyk et al. 2009; 2010; 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.

The ITS region has been used as the primary gene region with which to distinguish species in *Ceratocystis* and in fungi as a whole (Lieckfeldt and Seifert 2000; Van Wyk et al. 2009; 2010; 2011). It has been identified as a barcoding gene (Seifert 2009; Schoch et al. 2012) and just a few base changes in this sequence can define closely related, yet distinct species. The discovery of two ITS types in one isolate in *C. manginecans* in this study is not unusual (O'Donnell and Cigelnik 1997; Ko and Jung 2002; Pannecoucque and Höfte 2009) but suggests caution be applied when using this gene region to infer support for the delineation of species in the *Ceratocystis* species complex. This is especially in light of the fact that one of the ITS types in *C. manginecans* has exactly the same sequence that defines *C. acaciivora*. Although the combined phylogeny of three gene regions strongly supported the separation of *C. manginecans* and *C. acaciivora* (Tarigan et al. 2011), when analyzed separately, no resolution between these two species was evident in either the β-tubulin or EF 1-α phylogenies. This implies that most of the phylogenetic signal arose from the ITS region. The result of the present study questions the validity of *C. acaciivora* and further work is on-going to resolve this question.

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; Al Adawi et al. 2012). 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 and Peck 1994; Pena and 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 wood boring insects vector the pathogen; alternatively it may be carried to wounds on trees by opportunistic insects such as nitidulid beetles that are well-recognised as vectors of *Ceratocystis* spp. in the *C. fimbriata* s. l. complex (Moller and De Vay 1968b; Heath et al. 2009; Roux and 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; Wingfield et al. 2010). In this regard, the wilt disease of *P. cineraria* and *D. sissoo* has 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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