Investigating the Presence of Biotic Agents Associated with Mundulla Yellows

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ABSTRACT

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The role of biotic agents in the dieback syndrome Mundulla Yellows (MY) was investigated by analysis of 40 *Eucalyptus camaldulensis*, *E. leucoxylon*, or *E. cladocalyx* trees and soil samples from South Australia and Victoria, Australia. No pathogenic fungi, bacteria, phytoplasmas, or insect pests or vectors were found to be associated with MY. However, nematode analysis identified *Merlinius* spp. to be associated with soil, but not roots, from symptomatic trees. Interveneal chlorosis symptoms were not transmissible by seed, mechanical inoculation, or grafting using plant material derived from symptomatic trees. Virus-like particles were detected at a single symptomatic study site using transmission electron microscopy. MY symptoms were induced in *E. camaldulensis* seedlings by sowing seed from asymptomatic trees into sterilized and unsterilized soil collected from underneath symptomatic trees. Significantly, sterilized soil induced more severe symptoms in seedlings than unsterilized soil. Soil collected from under asymptomatic trees did not induce MY symptoms. This preliminary investigation indicates that, with the exception of *Merlinius* spp., pathogenic organisms and pests were not consistently associated with MY symptoms.

Mundulla Yellows (MY) is a progressive dieback syndrome of *Eucalyptus* and other native species, first reported in the 1970s in *Eucalyptus camaldulensis*. It first was observed near Mundulla, South Australia (SA) (9) but has been reported as occurring in most states of Australia and, more recently, in Spain (8).

The first symptom of MY is leaf interveneal chlorosis, which can occur on trees of any age (Fig. 1A). Yellowing is observed initially in immature leaves on a single branch and spreads to mature leaves, leading to defoliation (Fig. 1B). Any resulting epicormic growth is stunted and chlorotic. MY symptoms gradually spread throughout the tree, causing the whole canopy to die back over several years or decades (Fig. 1C), resulting in tree death.

The symptoms predominantly occur in disturbed vegetation, particularly near roadsides and paths. At least 87 species and 29 genera have been documented as expressing MY-like symptoms in South Australia (17).

Recently, Hanold and Randles observed transmission of MY symptoms in grafting experiments and hypothesized that MY is

likely to be associated with a contagious, biotic agent (9). RNA species (200 to 600 nucleotides) of unknown identity were detected in MY-affected *Eucalyptus* spp. in Australia (7) and in Southern Spain and on the island of Ibiza, Spain (8). However, the RNA also was found in a proportion of healthy trees (7). It was hypothesized that asymptomatic trees containing the MY-associated RNA may have been latently infected with an RNA-based pathogen.

Because of the proximity to roads, pathways, and disturbed vegetation, it also has been hypothesized that root-adsorbed herbicides, such as atrazine and simazine, may contribute to MY symptoms (15).

Despite being observed in Australia for at least 30 years, the cause of MY has not been determined. The following study represents a systematic analysis and com-

parison of symptomatic and asymptomatic trees for the presence and association of a biotic agent or agents in the development of MY symptoms. A separate article will address the role of abiotic factors in MY.

MATERIALS AND METHODS

Study sites. Ten study sites (approximately 150 by 100 m) were selected in Mundulla, South Australia and Lara, Victoria, Australia. Five sites contained trees that were affected with typical MY symptoms and five sites contained trees where MY symptoms could not be found. All sites were geographically separated from each other by at least 2 km. All sites contained trees of (predominantly) a single *Eucalyptus* spp., either *E. camaldulensis*, *E. leucoxylon*, or *E. cladocalyx* (Table 1). Two additional sites were used to collect seed from MY-affected *E. arenacea* and *Corymbia ficifolia* for seed transmission experiments (Table 1). Four trees from each of the 10 study sites were selected for investigation. The trees ranged in height and chest height diameter from 3 to 38 m and 8 to 185 cm, respectively. Each tree was tested for the presence of fungi, nematodes, bacteria, phytoplasma, viruses, and viroids. Insects were collected from tree foliage and understory vegetation to investigate the presence of pests and disease vectors at each site. To determine the possible cause of MY, the results of biotic testing were compared between the 20 symptomatic and 20 asymptomatic trees selected for this study.

Graft transmission. Branch pieces (approximately 1 cm in diameter) were collected from asymptomatic and symptomatic *E. camaldulensis* at Victorian and

Table 1. Study sites selected in Victoria (Vic) and South Australia (SA), Australia, investigating the cause of Mundulla Yellows (MY)

MY symptoms	Site no.	Site name and location	Species
Symptomatic	3	Carew, SA	<i>Eucalyptus leucoxylon</i>
Symptomatic	4	Buckingham Road, Mundulla, SA	<i>E. camaldulensis</i>
Symptomatic	5	Lara Lake Wetlands, Vic	<i>E. leucoxylon</i>
Symptomatic	6	Chirnside Park, Vic	<i>E. leucoxylon</i>
Symptomatic	8	West Geelong, Vic	<i>E. cladocalyx</i>
Symptomatic	11	Desert Camp Conservation Park, SA	<i>E. arenacea</i>
Symptomatic	12	Geelong, Vic	<i>Corymbia ficifolia</i>
Asymptomatic	1	Lowe's, Mundulla, SA	<i>E. leucoxylon</i>
Asymptomatic	2	Poocher Swamp, Mundulla, SA	<i>E. camaldulensis</i>
Asymptomatic	9	You Yangs Regional Park, Vic	<i>E. camaldulensis</i>
Asymptomatic	10	Anakie, Vic	<i>E. leucoxylon</i>
Asymptomatic	7	Main St., Lara, Vic	<i>E. cladocalyx</i>

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South Australian study sites (Victoria: October and December 2003 and March 2004; SA: November 2004). All leaves were removed from 10-cm-long scions and then grafted using either a “tongue-and-groove” method (17 scions, Victorian samples) or “whip-graft” method (12 scions, South Australian samples) onto 12-month-old unaffected *E. camaldulensis* rootstock using the

Fig. 1. A, Close-up of interveinal chlorosis in *Eucalyptus camaldulensis* foliage, Mundulla, South Australia. B, Mundulla Yellows (MY)-affected *E. camaldulensis*, Lara, Victoria. C, Advanced MY symptoms on *E. camaldulensis*, Mundulla, South Australia.

Fig. 2. Severely affected *Eucalyptus camaldulensis* scions were grafted onto unaffected rootstock. The new growth above and below the graft union was nonsymptomatic.

Fig. 3. A, Mundulla Yellows-affected *Corymbia ficifolia*, Geelong, from which seed were collected and planted in potting mix. B, The seedlings grown from this seed were healthy and green.

method of Toogood (23). Grafted plants were grown in Nu-Erth regular grade potting mix containing composted organic material, sand, and all the necessary trace elements required for healthy plant establishment conforming to the Australian Standard for potting mixes (AS 3743-1996).

All grafted trees were maintained in the glasshouse at temperatures between 15 and 25°C and fertilized every 6 months with a slow-release fertilizer (5 g of Osmocote-Outdoor and Indoor Plant Food).

Seed transmission. Capsules containing seed were collected from severely affected MY *C. ficifolia* (Geelong, Victoria) and *E. arenacea* (Desert Camp Conservation Park, SA). Capsules were incubated at 25°C for 1 week and the resultant released seed was sown into sterilized vermiculite in March 2003 and April 2003, respectively. Seed germinated within 3 weeks of sowing and seedlings were transplanted into 10-cm pots containing sterilized potting mixture and maintained in the glasshouse at 18 to 25°C. After 2 weeks, the seedlings were fertilized with a slow-release fertilizer (Osmocote Outdoor and Indoor Plant Food).

Soil sterilization experiments. To investigate the possible presence of soil-

Fig. 4. A, *Eucalyptus camaldulensis* seed collected from unaffected trees grown in soil from unaffected sites. B, seed collected from unaffected trees grown in unsterilized soil from MY-affected sites, and C, seed collected from unaffected trees grown in sterilized soil from MY-affected sites.

borne pathogens, *E. camaldulensis* seed was collected from asymptomatic trees located in Barmah State Forest, Victoria, Australia (MY has not been reported within this state forest). The seed (0.1 g) was sown into small plastic trays containing 200 g of sterilized (using a microwave to maintain soil temperature at approximately 80°C for 30 min) or nonsterilized soil from each of the four South Australian study sites (two symptomatic and two asymptomatic sites). All soil was collected from a depth of 0 to 20 cm. In all, eight sterilized and eight unsterilized replicates were included. The trays were monitored daily and watered approximately twice weekly to maintain soil moisture at field capacity. The seedlings were assessed visually for MY-like symptoms (interveinal chlorosis) and growth rate monitored for 3 months.

Virus and viroid. Herbaceous indexing. Symptomatic and asymptomatic leaves from all trees from all Victorian and South Australian study sites were collected for herbaceous indexing for virus. The leaves were ground in 10 ml of 0.05 M, phosphate buffer (1 M K_2HPO_4 /1 M KH_2PO_4 stock solutions), pH 7, with a mortar and pestle. Herbaceous indicators (*Nicotiana tabacum*, *N. glutinosa*, *N. rustica*, and *Chenopodium amaranticolor*) were dusted with carborundum powder and rub inoculated with leaf sap in March 2004 (Victorian study sites) and November 2004 (South Australian study sites). All indicators inoculated with Victorian material were monitored for the onset of symptoms for 5 months. Foliage samples collected for herbaceous indexing from South Australian sites were rub inoculated (as per Victorian samples) onto indicators in November 2004.

Transmission electron microscopy. Leaves from all 40 trees across the 10 study sites were collected during spring or summer and were examined for the presence of viruses. Approximately 5 mm² of leaf tissue was excised and macerated in a drop of sterile distilled water (sdH₂O). A carbon-coated formvar grid was placed onto the macerated drop to adsorb sample for 1 min. The grid was inverted and a drop of 2% aqueous ammonium molybdate was placed directly onto the grid for 30 s. Excess stain was blotted off the grid with a piece of filter paper and the grid left to air dry for 5 min. The grid was examined at 100 kV with a Jeol 200CX transmission electron microscope.

RNA analysis. Total RNA was extracted from tissue of all 40 symptomatic and asymptomatic trees using the modified method of Wan Chow Wah and Symons (25). RNA samples were digested with DNase (Promega Corporation) according to the manufacturer's instructions prior to two-dimensional (2D) reverse polyacrylamide gel electrophoresis (PAGE) (5% bis:acrylamide, 0.12% temed, and 0.07%

ammonium persulfate [APS] in 1× Tris-borate EDTA) analysis. Total RNA from pear infected with pear blister canker viroid (PBCVd) was included as a positive control. In addition, RNA also was extracted from healthy pear and used as a negative control. Return-PAGE gels were run and bands were visualized following the method of Singh and Boucher (21).

Fungi. Ten leaves from the 20 symptomatic and 20 asymptomatic trees were removed and washed in sdH₂O. Approximately 40 g of leaf tissue was excised and ground in 5 ml of sdH₂O. Serial dilutions of sap (100 µl) were plated onto potato dextrose agar containing achromycin at 100 µg/ml (PDA+A) and incubated at 22°C for 28 days. Plates were scored for fungal species and numbers of colonies.

A 10- to 20-cm twig or branch from each tree was sterilized in 70% ethanol. The branch was split lengthwise and small pieces of heartwood were excised (approximately 10 cm in length) and incubated on PDA+A for 28 days. Fungal species were identified and their number was recorded. Split branches and leaves were placed in plastic containers on moist tissue paper and incubated at room temperature for 28 days. Samples were monitored weekly and the tissue paper remoistened when necessary.

Five 200-g subsamples of soil (including small root pieces) were collected from a depth of 15 cm beneath the 20 symptomatic and 20 asymptomatic trees. The samples from each tree were pooled and *Phytophthora* baiting was conducted using the eucalyptus cotyledon method (14) followed by plating onto P₅ARPH media (11).

Bacteria. Ten leaves from each of the 20 symptomatic and 20 asymptomatic trees were surface sterilized and excised across the midrib in 1-by-2-cm² sections, homogenized gently with a mortar and pestle, and serially diluted using sdH₂O. Aliquots (100 µl) of each dilution were plated on to King's B medium (KB) and incubated at 25°C for 7 days. Several different colony types from each sample were subcultured and identified using fatty acid methyl analysis (FAME) as described by Weller et al. (27) by New South Wales Agriculture. General biochemical testing was done on each colony type, including gram stains, catalase and oxidase tests, and fluorescence under UV. Polymerase chain reaction (PCR) was used to test for *Xylella fastidiosa* using the method of Minsavage et al. (16) and *Liberobacter asiaticum* using the method of Jagoueix et al. (10).

In addition to fatty acid analysis, some colonies were identified using PCR and DNA sequencing. A loopful of pure, 1-day-old culture, growing on KB, was transferred to 200 µl of sdH₂O in a sterile Eppendorf tube. The suspension was homogenized and boiled for 5 min, then chilled on ice for 5 min. An aliquot (1 µl)

of the boiled suspension was used as template in the PCR reaction. A fragment of 16S rDNA was amplified using the primer pair fD2 and rp1, using the conditions described by Weiseberg et al. (26). The PCR products were purified using the QIAGEN Qiaquick PCR purification kit as per the manufacturer's instructions. Direct DNA sequencing was completed using Big Dye sequencing (BigDye Terminator v3.1, Cycle Sequencing Kits; Applied Biosystems) chemistry by Micromon DNA Sequencing Service (Monash University, Australia).

Phytoplasma. DNA was extracted from 1 g of leaf midrib from the 20 symptomatic and 20 asymptomatic trees as described previously (13). A DNA extract of tomato tissue containing Tomato Big Bud (TBB) phytoplasma was used as the positive control for PCR reactions. A nested PCR procedure was carried out with two sets of primers; P1 (2) and P7 (19) were used in the first PCR reaction, and fU5 (12) and m23sr (18) were used in the second reaction. The PCR procedure was described previously (5).

Nematodes. Soil and root material was collected from four points 1 m from the base of each tree at a depth of 30 to 40 cm. The four samples from each tree were pooled and mixed thoroughly and a subsample of 200 g was collected. Roots were separated from the soil. Nematodes were extracted from soil using the method of Whitehead and Hemming (28). Nematodes were identified and counted using a dissecting microscope at ×20 or ×40 magnification.

Root material (10 g) was collected from each soil sample and washed to remove soil and debris. Nematodes were extracted from roots based on the method of Fallis (4). Roots were blotted dry on paper towel, weighed, cut into 1-cm pieces, and macerated in 100 ml of water with an electric blender for 5 s at high speed. The macerate was poured onto tissue paper supported on wire mesh in a tray. Water was added to cover the root pieces, which were incubated at room temperature for 3 days. The root pieces were discarded and the suspension containing nematodes was collected in a beaker and left to settle for 2 h at room temperature. The supernatant was siphoned off and the nematodes were identified as above.

The Mann-Whitney (nonparametric) test was used to analyze the significance of the nematode numbers (22).

Insects. Sweep netting of understory grasses of large trees and foliage from smaller trees was conducted to investigate the presence of possible vectors of pathogens or insect pests that may be associated with MY symptoms. A yellow sticky trap was deployed in each tree canopy at all sites. At South Australian sites, insects were collected from foliage and branches that were removed with extendable loppers

or a tree saw. A subsample of the branch was transferred to a plastic bag and kept at 4°C until examined. Insects were pinned and identified to at least order or suborder level, with some identified to genus and species levels.

RESULTS

Graft transmission. Initial attempts at root or scion grafting of 5 asymptomatic and 12 symptomatic *E. camaldulensis* scions (from Victorian sites) onto asymptomatic *E. camaldulensis* rootstocks achieved only 5 successful grafts (29%); 3 from asymptomatic and 2 from symptomatic trees. Repeated grafting experiments of 12 scions from symptomatic trees (from South Australian sites) using a whip-graft technique resulted in 7 successful graft unions (58%).

All successful graft unions resulted in healthy new growth. After monitoring for more than 16 months (Victorian samples) and 5 months (South Australian samples), MY symptoms had not developed on either the scions or the rootstocks (Fig. 2). Some general yellowing of foliage was observed in both grafted asymptomatic and symptomatic plants during the growing period which was corrected using Aquasol and Osmocote. Powdery mildew also affected some plants during their growth. The grafted trees will continue to be monitored over the next 3 years to observe any delayed onset of symptoms.

Seed transmission. No MY symptoms developed in any of the *C. ficifolia* (Fig. 3A and B) or *E. arenacea* seedlings over the past 2 years. Although these preliminary results suggest that MY is not transmissible by seed, the trees will be continually monitored to observe any delayed onset of symptoms.

Soil sterilization experiments. Seed collected from healthy *E. camaldulensis* and grown in bulk subsoils from two independent sites with asymptomatic trees emerged as healthy and green seedlings, regardless of whether the soil was sterilized or unsterilized (Fig. 4A). Seed grown in soils from two sites containing sympto-

matic trees emerged as chlorotic seedlings with purple or red pigmentation of their vascular tissue (Fig. 4B). Surprisingly, seedlings grown in sterilized soil collected from sites with symptomatic trees had more severe symptoms and stunted growth (Fig. 4C) compared with seedlings grown in unsterilized soils from the same symptomatic sites (Fig. 4B).

Virus and viroid. *Herbaceous indexing.* Viral symptoms did not develop on any inoculated indicator plants using material from Victorian sites, despite monitoring for 5 months, whereas the positive control, *Potato virus Y* (PVY), produced typical PVY symptoms within 3 weeks of inoculation. No interveinal chlorosis or virus-like particles were observed in the inoculated indicator samples with transmission electron microscopy (TEM). Sap inoculation with material from SA has resulted in a single indicator plant (*N. tabacum*) with virus-like symptoms. This plant shows leaf blistering and curling and currently is being grown for subinoculation onto more indicators and further characterization by molecular methods.

TEM. Virus-like particles were detected in 4 of the 40 samples, and all 4 samples were collected from symptomatic *E. camaldulensis* trees at a single study site,

at Buckingham Road, SA. Virus-like particles were rigid and ranged in size from approximately 18 nm in diameter to 191 to 900 nm in length. Molecular characterization of these particles is in progress. No virus-like particles were detected at any other sites.

RNA analysis. 2D-PAGE analysis of total RNA revealed nucleic acid profiles that were almost identical between all 40 *Eucalyptus* spp. samples. Five faint bands were observed per sample, and no bands were found exclusively in symptomatic trees. The bands detected in the *Eucalyptus* spp. samples were mostly larger than the control band, and none were equivalent in size to the PBCVd control band. A band of 315 nucleotides was detected for the positive control (PBCVd).

Fungi. No known species of pathogenic fungi were found to be associated with MY-symptomatic trees. Many of the fungal colonies could not be identified but there were no colony types consistently associated with symptomatic or asymptomatic tissue. Both symptomatic and asymptomatic samples contained common species of fungi and yeast (Tables 2 to 4). Many unidentified sterile fungi were identified in split branches and leaves from both symptomatic and asymptomatic trees. *My-*

Table 3. Fungi isolated from tree heartwood, as part of the investigation into the cause of Mundulla Yellow^a

Site ^b	Symp ^c	<i>Eucalyptus</i> spp.	Fungi identified			
			<i>Chaetomium</i>	<i>Phaeoacremonium</i>	<i>Cladosporium</i>	<i>Penicillium</i>
1	A	<i>leucoxydon</i>	+	–	+	+
2	A	<i>camaldulensis</i>	–	–	–	–
7	A	<i>cladocalyx</i>	–	–	–	–
9	A	<i>camaldulensis</i>	–	–	–	+
10	A	<i>leucoxydon</i>	+	–	–	–
3	S	<i>leucoxydon</i>	–	+	–	–
4	S	<i>camaldulensis</i>	–	–	–	+
5	S	<i>leucoxydon</i>	–	–	+	+
6	S	<i>leucoxydon</i>	+	+	–	–
8	S	<i>cladocalyx</i>	–	–	–	–

^a Samples were pooled from four trees per site; + = presence and – = absence of fungi.

^b Sites are as follows: 1 = Lowe's, 2 = Poocher Swamp, 3 = Carew, 4 = Buckingham Road, 5 = Lara Lake Wetlands, 6 = Chirnside Park, 7 = Main St., Lara, 8 = West Geelong, 9 = You Yangs, and 10 = Anakie.

^c Asymptomatic (A) or symptomatic (S).

Table 2. Fungi isolated from leaf sap, as part of the investigation into the cause of Mundulla Yellow^a

Site ^b	Symptoms ^c	<i>Eucalyptus</i> spp.	Fungi identified					
			<i>Microsphaeropsis</i>	<i>Cladosporium</i>	<i>Mycosphaerella</i>	<i>Sporothrix</i>	<i>Penicillium</i>	<i>Oidiodendron</i>
1	A	<i>leucoxydon</i>	+	+	+	–	+	+
2	A	<i>camaldulensis</i>	+	–	+	+	–	–
7	A	<i>cladocalyx</i>	+	+	+	–	+	–
9	A	<i>camaldulensis</i>	+	+	+	–	–	–
10	A	<i>leucoxydon</i>	+	+	+	+	+	+
3	S	<i>leucoxydon</i>	+	+	+	+	–	–
4	S	<i>camaldulensis</i>	+	+	+	–	–	+
5	S	<i>leucoxydon</i>	+	+	+	+	+	–
6	S	<i>leucoxydon</i>	+	+	+	+	–	–
8	S	<i>cladocalyx</i>	+	+	+	–	–	+

^a Samples were pooled from four trees per site; + = presence and – = absence of fungi.

^b Sites are as follows: 1 = Lowe's, 2 = Poocher Swamp, 3 = Carew, 4 = Buckingham Road, 5 = Lara Lake Wetlands, 6 = Chirnside Park, 7 = Main St., Lara, 8 = West Geelong, 9 = You Yangs, and 10 = Anakie.

^c Asymptomatic (A) or symptomatic (S).

cosphaerella spp. (6) were identified in all leaf sap samples (Table 2). An unidentified *Phaeoacremonium* spp. (3) was isolated from heartwood from two symptomatic sites only (Table 3). Many unidentified sterile fungi were isolated in split branches and leaves from both asymptomatic and symptomatic trees, as well as the species listed (Table 4). There was no difference in frequency of any of the colony types between asymptomatic and symptomatic trees. No *Phytophthora* spp. were identified in symptomatic or asymptomatic trees by baiting soils.

Bacteria. No bacterial ooze was observed when pieces of symptomatic leaves were examined under the microscope. In isolations onto KB, there were a higher number of colonies found in trees from asymptomatic sites compared with the symptomatic trees. The most common species isolated were identified by fatty acid analysis as *Sphingomonas* spp., *Flavobacterium* spp., and *Pseudomonas putida*. No known bacterial pathogens were detected except in leaves from trees in one asymptomatic site, where rDNA from one isolate had a 90% sequence similarity to *Curtobacterium flaccumfaciens*

pv. *poinsettiae*. *Xylella fastidiosa* and *Liberobacter asiaticum* were not detected by PCR in any of the 40 trees examined.

Phytoplasma. Phytoplasmas were not detected in any of the 40 trees. PCR detection for phytoplasmas produced a 1,447-bp band in 4 of 20 asymptomatic trees and in 0 of 20 symptomatic trees. DNA sequencing indicated a high sequence similarity with the 16S rRNA gene from the bacterium *Streptococcus* spp.

Nematodes. The total number of nematodes present in soil samples (120,825) was greater than in roots (48,956), with *Tylenchus* spp. (20) being the most ubiquitous nematodes extracted from both symptomatic and asymptomatic sites from soils and roots. (Table 5 and 6). Use of the Mann-Whitney test (22) to determine whether there was any significance in nematode numbers between symptomatic and asymptomatic sites revealed that only *Merlinius* spp. numbers were significantly higher in soil samples collected from sites having symptomatic trees compared with sites having asymptomatic trees ($P < 0.01$). *Merlinius* spp. are ectoparasitic (20), accounting for their absence from all root samples (Table 6).

Insects. Insect sampling over two seasons did not identify any pests or vectors that were associated exclusively with symptomatic trees (Table 7). Families of insects that are known to contain vectors of plant pathogens were identified at both symptomatic and asymptomatic sites (e.g., leafhoppers [Cicadellidae], psyllids [Psylloidea], and aphids). The vegetable leafhopper and the Australian grass leafhopper, *Nesocluha pallida*, (Deltocephalinae) were identified in understory grass sweeps. Psyllids were identified at all study sites. Aphids were detected in samples at two asymptomatic sites and one symptomatic site. Xylem-feeding spittlebugs were identified at one site in the late spring sampling.

DISCUSSION

The most revealing outcome of this work relates to the soil sterilization experiments. Healthy *E. camaldulensis* seed sown in sterilized and unsterilized soils from MY-symptomatic sites produced seedlings with distinctive MY chlorotic symptoms, with the symptoms being more severe in sterilized soils from symptomatic sites. Conversely, planting of healthy *E.*

Table 4. Fungi isolated from leaves and split branches, as part of the investigation into the cause of Mundulla Yellow^a

Site ^b	Symptoms ^c	<i>Eucalyptus</i> spp.	Fungi identified from leaves			Fungi identified from split branches		
			<i>Trichoderma</i>	<i>Cladosporium</i>	<i>Penicillium</i>	<i>Trichoderma</i>	<i>Cladosporium</i>	<i>Rhizopus</i>
1	A	<i>leucoxydon</i>	+	+	+	–	–	–
2	A	<i>camaldulensis</i>	+	–	+	–	–	+
7	A	<i>cladocalyx</i>	–	–	–	+	–	–
9	A	<i>camaldulensis</i>	+	+	–	+	+	–
10	A	<i>leucoxydon</i>	+	–	+	–	–	–
3	S	<i>leucoxydon</i>	–	–	–	–	–	–
4	S	<i>camaldulensis</i>	+	–	–	–	–	–
5	S	<i>leucoxydon</i>	–	–	–	+	–	+
6	S	<i>leucoxydon</i>	–	+	–	–	–	–
8	S	<i>cladocalyx</i>	–	–	–	–	–	+

^a Samples were pooled from four trees per site; + = presence and – = absence of fungi.

^b Sites are as follows: 1 = Lowe's, 2 = Poocher Swamp, 3 = Carew, 4 = Buckingham Road, 5 = Lara Lake Wetlands, 6 = Chirnside Park, 7 = Main St., Lara, 8 = West Geelong, 9 = You Yangs, and 10 = Anakie.

^c Asymptomatic (A) or symptomatic (S).

Table 5. Nematode species and numbers identified from soils, as part of the investigation into the cause of Mundulla Yellow^a

Site ^b	Sym ^c	<i>Eucalyptus</i> spp.												Total no.
			Praty	Ty	Hemi	Moru	Paraty	Het	Hemicr	Paratric	Helico	Paralo	Mer	
1	A	<i>leucoxydon</i>	396	672	12	84	396	108	0	0	0	0	0	1,668
2	A	<i>camaldulensis</i>	0	2,658	0	1,080	361	0	156	48	0	0	0	4,303
7	A	<i>cladocalyx</i>	6	174	0	0	400	0	0	0	0	0	6	586
9	A	<i>camaldulensis</i>	0	54,378	0	0	1,944	0	0	0	0	6	0	56,328
10	A	<i>leucoxydon</i>	0	240	666	0	6	0	0	0	0	0	0	912
3	S	<i>leucoxydon</i>	6	2,497	0	0	618	0	12	0	30	6	231	3,400
4	S	<i>camaldulensis</i>	132	810	0	18	8,358	0	0	0	0	102	84	9,504
5	S	<i>leucoxydon</i>	222	1,458	0	0	102	0	0	0	0	0	78	1,860
6	S	<i>leucoxydon</i>	66	39,474	0	0	1,122	0	0	0	0	0	72	40,734
8	S	<i>cladocalyx</i>	792	312	84	0	...	0	48	0	0	0	246	1,482
P value	...	...	0.10	0.69	0.72	0.44	0.84	...	1.00	...	...	0.72	0.01	...
Total no.	...	...	1,620	102,673	762	1,182	13,307	108	216	48	30	114	717	...

^a Nematode abbreviations are as follows: Ty (*Tylenchus* spp.), Hemi (*Hemicylophora* spp.), Moru (*Morulanius* spp.), Paraty (*Paratylenchus* spp.), Het (*Heterodera* spp.), Hemicr (*Hemicriconemoides* spp.), Paratric (*Paratrichodorus* spp.), Paralo (*Paralongidorus* spp.), Mer (*Merlinius* spp.), and Helico (*Helicotylenchus* spp.).

^b Sites are as follows: 1 = Lowe's, 2 = Poocher Swamp, 3 = Carew, 4 = Buckingham Road, 5 = Lara Lake Wetlands, 6 = Chirnside Park, 7 = Main St., Lara, 8 = West Geelong, 9 = You Yangs, and 10 = Anakie.

^c Asymptomatic (A) or symptomatic (S).

camaldulensis seedlings into sterilized and unsterilized soils from asymptomatic sites did not produce MY symptoms. All resulting leaves were asymptomatic. These findings suggest that soil abiotic factors contribute to the development of MY symptoms. The heating of symptomatic soils during the sterilization process markedly increased the severity of symptoms in seedlings. To explain this phenomenon and to identify which abiotic factors are involved in MY symptom development, extensive sampling of soil and foliage from symptomatic and asymptomatic trees has been undertaken and will be published elsewhere (B. Czerniakowski, R. Crnov, I. W. Smith, and J. E. Luck, *unpublished*).

No bacterial pathogens were associated with MY-affected trees and, in this study, no phytoplasmas were detected in either asymptomatic or symptomatic trees. In other studies, phytoplasmas have been identified in *Eucalyptus* spp. but no correlation was found with MY symptoms (5). Fungal analysis did not detect any known fungal pathogens exclusively associated with MY-affected trees. No known insect pests or vectors of plant pathogens were identified as being associated with MY-affected trees. No biotic agent was transmitted in grafting experiments, onto herbaceous indicators, or through seed. The grafting data reported here does not support the hypothesis that MY is a graft-transmissible agent (9).

Virus-like particles were detected at one South Australian symptomatic study site, using TEM. The absence of these particles in symptomatic trees from all other sites suggests that it is unlikely that a virus is the causal agent of MY. A possible scenario is that the yellow leaves on trees at this site (symptomatic of MY) may have attracted insects vectoring a virus. The virus may be contributing to MY tree symptoms or may be cryptic within the host. Grafting experiments and herbaceous indexing experiments of these virus-like particles onto indicators, using material from this single site, has not produced interveinal chlorosis in foliage. Experiments are in progress to identify the virus-

Table 6. Nematode species and numbers identified from roots, as part of the investigation into the cause of Mundulla Yellows^a

Site ^b	Symp ^c	<i>Eucalyptus</i> spp.	Praty	Ty	Hemi	Moru	Paraty	Het	Hemicr	Paratric	Helico	Paralo	Mer	Total no.
1	A	<i>leucoxyton</i>	0	341	0	0	0	2,803	0	0	0	0	0	3,144
2	A	<i>camaldulensis</i>	0	3,075	0	0	58	0	0	0	0	0	0	3,144
7	A	<i>cladocalyx</i>	0	0	0	0	0	0	0	0	0	0	0	0
9	A	<i>camaldulensis</i>	0	23,302	0	0	81	0	0	0	0	0	0	23,383
10	A	<i>leucoxyton</i>	0	328	0	0	0	0	0	0	0	0	0	328
3	S	<i>leucoxyton</i>	212	183	0	0	0	0	0	0	0	0	0	395
4	S	<i>camaldulensis</i>	6,959	373	0	0	98	0	0	0	0	0	0	7,430
5	S	<i>leucoxyton</i>	10,571	0	0	0	0	0	0	0	0	0	0	10,571
6	S	<i>leucoxyton</i>	0	517	0	0	0	0	0	0	0	0	0	517
8	S	<i>cladocalyx</i>	0	49	0	0	0	0	0	0	6	0	0	55
P value	...	...	0.17	0.46	...	...	1.00	...	...	...	...	...	...	...
Total no.	...	...	17,742	28,168	0	0	237	2,803	0	0	6	0	0	...

^a Nematode abbreviations are as follows: Ty (*Tylenchus* spp.), Hemi (*Hemicylophora* spp.), Moru (*Morulum* spp.), Paraty (*Paratylenchus* spp.), Het (*Heterodera* spp.), Hemicr (*Hemicriconemoides* spp.), Paratric (*Paratrichodorus* spp.), Paralo (*Paralongidorus* spp.), Mer (*Merlinius* spp.) and Helico (*Helicotylenchus* spp.).

^b Sites are as follows: 1 = Lowe's, 2 = Poocher Swamp, 3 = Carew, 4 = Buckingham Road, 5 = Lara Lake Wetlands, 6 = Chirnside Park, 7 = Main St., Lara, 8 = West Geelong, 9 = You Yangs, and 10 = Anakie.

^c Asymptomatic (A) or symptomatic (S).

Table 7. Examples of insects collected during spring 2004 from South Australian symptomatic and asymptomatic sites, as part of the investigation into the cause of Mundulla Yellows

Site, class (if not Insecta), order or suborder	Superfamily or family	Species name	Common name
Buckingham Road, symptomatic			
Diptera	Undetermined	Undetermined	Flies
Hemiptera; Heteroptera	Tingidae	Undetermined	Lace bugs
Hemiptera; Homoptera	Cicadellidae; Typhlocybinae	<i>Zygina zealandica</i>	Leafhoppers
Hemiptera; Homoptera	Cicadellidae; Deltocephalinae	<i>Arawa</i> spp., <i>Balclutha</i> spp.	Leafhoppers
Hemiptera; Homoptera	Delphacidae	<i>Toya</i> spp.	Delphacid planthoppers
Hemiptera; Homoptera	Psylloidea	Undetermined	Psyllids
Hymenoptera	Undetermined	Undetermined	Wasps
Hymenoptera	Formicidae; Dolichoderinae	<i>Iridomyrmex</i> spp.	(Meat ants)
Psocoptera	Undetermined	Undetermined	Booklice/psocids
Thysanoptera	Undetermined	Undetermined	Thrips
Lowe's, asymptomatic			
Arachnida; Acarina	Undetermined	Undetermined	Mites
Arachnida; Araneae	Undetermined	Undetermined	Spiders
Coleoptera	Lathriididae	Undetermined	(Minute mould beetles)
Coleoptera	(other families)	Undetermined	Beetles
Diptera; Nematocera	Undetermined	Undetermined	Flies
Diptera; Brachycera	Undetermined	Undetermined	Flies
Hemiptera; Heteroptera	Miridae	Undetermined	Mirid bugs
Hemiptera; Homoptera	Aphididae	Undetermined	Aphids
Hemiptera; Homoptera	Cicadellidae; Typhlocybinae	<i>Zygina zealandica</i>	Leafhoppers
Hemiptera; Homoptera	Cicadellidae; Deltocephalinae	<i>Arawa</i> spp., <i>Nesoclutha pallida</i>	Leafhoppers
Hemiptera; Homoptera	Cicadellidae; Ulopinae; Cephalinae	<i>Cephalelini</i> spp.	Seed-mimicking leafhoppers
Hemiptera; Homoptera	Psylloidea	Undetermined	Psyllids
Hymenoptera	Undetermined	Undetermined	Wasps
Hymenoptera	Formicidae; Dolichoderinae	<i>Iridomyrmex</i> spp.	(Meat ants)
Hymenoptera	Formicidae; Dolichoderinae	<i>Ochetellus</i> spp.	Spinifex ant
Hymenoptera	Formicidae; Myrmicinae	<i>Podomyrma</i> spp.	Muscleman tree-ants
Orthoptera	Undetermined	Undetermined	Grasshoppers

like particles. The complete data set, including TEM photos of particles, will be reported subsequently. Spherical virus-like particles were isolated from diseased *E. macrorhyncha* by Brzostowski and Grace in 1974, but particles were never purified or characterized (1).

Multiple faint RNA bands were identified in both symptomatic and asymptomatic trees using 2-D reverse PAGE analysis for the detection of viroids. Importantly, no bands were associated exclusively with symptomatic trees. The full characterization of the multiple RNA bands will determine whether the RNA is associated with a pathogen or is derived from the host. Triki et. al. (24) reported the role of Mn deficiency in a yellowing disorder of date palms and described the association of a double-stranded RNA with symptomatic trees. Its subsequent characterization revealed that the RNA was chloroplast derived and not a pathogenic agent. To address the possibility of asymptomatic trees being latently infected with a virus or "virus-like" agent, sampling methods specifically included the geographical separation of asymptomatic sites from symptomatic sites. Grafting, inoculation, and TEM results do not support the association of a virus-like organism with MY symptoms.

The association of *Merlinius* spp. with symptomatic trees is interesting. It is not known what role this nematode, commonly referred to as the stunt nematode, has in MY etiology. There is little data available on the pathogenicity of this organism, particularly in association with eucalypts (20). A primary pathogenic role is unlikely, given that typical MY symptoms were induced with sterilized soil. It is more likely that *Merlinius* spp. play a secondary role in symptom development or that they are associated with grasses surrounding the trees. Inoculation studies will determine whether the presence of *Merlinius* spp. contributes to MY symptoms.

This study did not demonstrate the involvement of pests or pathogens in the etiology of MY. Data indicating that soil factors play a role in the development of MY symptoms will be presented in a subsequent article (B. Czerniakowski, R. Crnov, I. W. Smith, and J. E. Luck, *unpublished*).

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