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Triggering dieback in an invasive plant: endophyte diversity and pathogenicity

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Abstract Dieback causes a progressive reduction in plant population health, resulting in the death of plant parts and often plant death. It is prevalent in many invasive woody weeds in Australia and has been suggested as a potential mechanism for biocontrol of these species. *Parkinsonia aculeata* one such invasive tree in northern Australia. It has naturalised across a wide range of climatic zones and some populations have been heavily reduced by dieback occurrence. The cause(s) of dieback in parkinsonia remain elusive, although fungal endophytes have been previously implicated. In this study, we characterised the culturable fungal endophyte community of healthy and dieback-affected parkinsonia using culture-based techniques, and identified cultured isolates via amplicon sequencing of the internal transcribed spacer (ITS) of the rDNA operon. Eight isolates, identified as pathogens, were selected for a 10-week pathogenicity trial, including water stress treatments, on parkinsonia seedlings. We isolated a taxonomically diverse fungal community from parkinsonia, representing 54 unique species from 25 families. Communities were similar across healthy and dieback-affected plants, but differed by plant tissue. Of the eight putative pathogenic isolates tested in the pathogenicity trial, inoculation with *Lasiodiplodia pseudotheobromae*, *Botryosphaeria dothidea* and *Pestalotiopsis mangiferae* resulted in the largest lesions, but systemic infection or dieback-like symptoms were not observed in any treatment despite plant stress being induced by drought or inundation. We concluded that inoculation of parkinsonia with the tested putative fungal pathogens is unlikely to result in dieback, which has implications for future work in biocontrol of parkinsonia.

Keywords *Parkinsonia aculeata* · Fungal community · Inoculation trial · Weeds · Biological control

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Introduction

Parkinsonia aculeata (parkinsonia, family: Fabaceae) is a spiny, leguminous, thicket-forming tree, native to the Americas, but a serious invader in northern Australia (Thorp and Lynch 2000). The management of parkinsonia is expensive and labour-intensive and usually involves the use of herbicides followed by manual removal of dead trees (Deveze et al. 2004). Since the most recent estimates of population extent exceeds 3 million ha (van Klinken et al. 2009), more efficient and autonomous control methods are sought. The most promising mechanism for parkinsonia control has been the occurrence of dieback in some populations (van Klinken et al. 2009). We define dieback as a progressive reduction in plant health, resulting in the death of plant parts, often followed by outright tree death that may result in local population decline, either as a gradual or sudden occurrence (Mueller-Dombois 1987). Parkinsonia dieback begins with defoliation, followed by browning of the stems starting at the stem tips, and usually resulting in whole tree mortality (Diplock 2016).

Dieback has been observed in a number of Australian Weeds of National Significance (WONS), but has not been observed in locally-occurring native species (Raghavendra et al. 2016; van Klinken et al. 2009; Wilson and Pitkethley 1992) and there is no evidence that dieback occurs in these WONS' native ranges. If the cause of parkinsonia dieback is identified there is potential for its use as a self-sustaining biological control agent to be used alongside other control methods.

Plants host a diverse community of fungal species, the vast majority of which are mutualistic or benign endophytes but some may be pathogenic or saprophytic (Hawksworth 2001). In previous work, endophyte communities (archaea, bacteria and fungi) were analysed for correlation with dieback occurrence in parkinsonia using terminal restriction fragment length polymorphism (T-RFLP) analysis (Steinrucken et al. 2016). Bacterial community composition was not significantly correlated to parkinsonia dieback, and although significant correlations with archaeal OTUs and dieback were observed, little is known about archaeal endophytes and few archaea have ever been cultured (Schleper et al. 2005). With regard to endophytic fungal communities, in their previous work Steinrucken et al. (2016) also found a significant correlation between fungal community composition and dieback occurrence, suggesting the involvement of multiple fungal endophytic species which differ in composition across plant parts. Although this work demonstrated the potential involvement of fungal endophytes in parkinsonia dieback, the method of community fingerprinting with T-RFLP did not allow assignment of taxonomy or ecological roles.

Diplock (2016) and Toh (2009) isolated, identified and tested a number of endophytic fungal pathogens reported to be involved in parkinsonia dieback. In their studies one species stood out as a potential causal agent: *Lasiodiplodia pseudotheobromae*. This species has been implicated in dieback of other non-native tree species globally including multiple Australian leguminous and woody WONS (Diplock 2016; Haque 2015; Sacdalan 2015; Toh 2009), *Prunus* spp. in South Africa (Damm et al. 2007), mango in Egypt (Ismail et al. 2012) and *Acacia* spp. in Australia (Adair et al. 2009). In testing the pathogenicity of *L. pseudotheobromae* on parkinsonia, Toh (2009) inoculated sterile vermiculite substrate with colonised millet seed before transplanting parkinsonia seedlings into the mixture one week post-emergence. This study showed that *L. pseudotheobromae* (isolate NT039; Genbank Accession no. KX893409) was the most virulent of 83 tested, including other Botryosphaeriaceae. A concurrent four-year field trial on adult parkinsonia trees involved inserting colonised millet seed into holes drilled into the base of the trees (Diplock 2016). On some sites, the treatment resulted in lesion formation by *L. pseudotheobromae*, but was unable to recreate dieback symptoms or tree mortality. The study was further complicated by wounding reactions, bacterial contamination and adverse environmental conditions including a flood and fire (Diplock 2016).

Although it has been implicated in disease and dieback of woody hosts, *L. pseudotheobromae* has also been associated with healthy hosts as a non-pathogenic endophyte (Jami et al. 2013; Slippers and Wingfield 2007). A number of other Botryosphaeriaceae species have both pathogenic and endophytic associations with their host and many can be triggered to become pathogenic in the presence of abiotic factors such as water stress (Mehl et al. 2013; Schulz et al. 1998). These species are termed 'latent pathogens': microorganisms that remain benign or mutualistic until triggered to be pathogenic by an external factor such as environmental stress to the host, or co-infection by a more virulent pathogen (Slippers and Wingfield 2007). It is therefore difficult to predict whether endophytic fungi could be pathogenic under certain circumstances or if they are simply opportunistic, becoming pathogenic or saprophytic when the plant is stressed.

The interaction between the host, its environment and pathogens plays an integral part in the occurrence of disease (Agrios 2005). Conceptual models described by Houston (1992); Manion (1991) and Whyte et al. (2016) attempt to characterise the interactions between these inciting and contributing factors and how they relate to dieback occurrence. This complexity means it is unclear whether symptoms of dieback in parkinsonia are the primary cause of dieback or are the results of secondary infections by opportunistic pathogens, triggered by other biotic or abiotic factors. Parkinsonia and many other dieback-affected WONS are spread across regions of northern Australia that are subject to long-term drought and intermittent flooding, so it is possible that dieback is partly triggered by water availability. This has been observed in the decline of black alder (*Alnus glutinosa*) by the pathogen *Phytophthora alni*, whose virulence is associated with flooding episodes (Webber et al. 2004). Similarly, in drought-stressed oak trees, a number of ascomycete pathogens such as *Biscogniauxia mediterranea* take advantage of weakened host tissues and become more virulent, causing decline in several species (La Porta et al. 2008).

In this study we describe the culturable fungal endophyte community in healthy and dieback-affected parkinsonia from regions previously shown to have dieback/endophyte community correlations, and we identify putative pathogens to test against parkinsonia seedlings exposed to excessive, limiting, or optimal water treatments in a glasshouse inoculation study. We consequently address the following question: Can we induce systemic infection and dieback-like symptoms in parkinsonia, by inoculating plants with the selected putative fungal pathogens, and will water stress enhance this effect?

Materials and methods

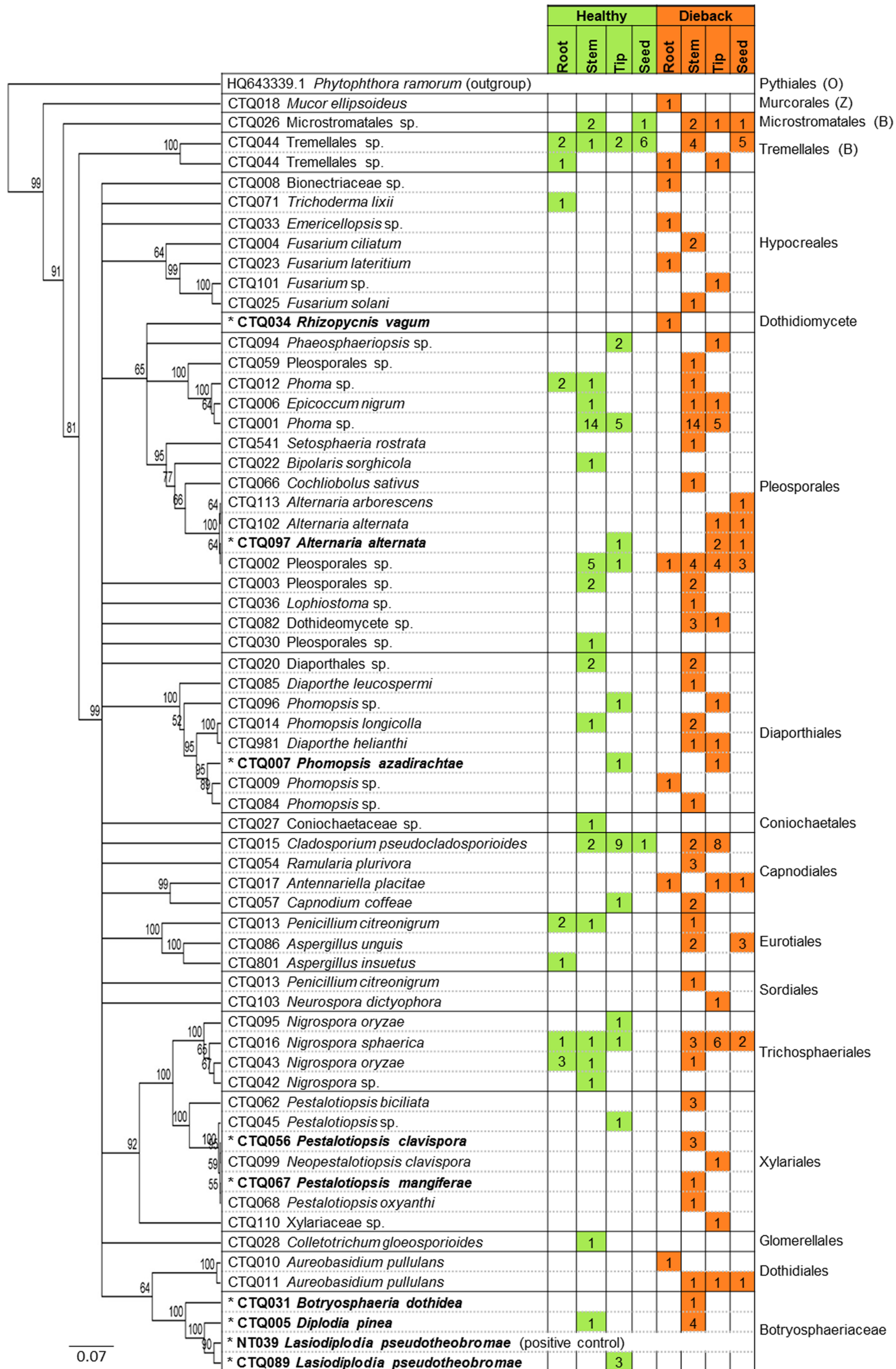
Sampling, identification and analysis of the fungal endophyte community

We sampled sub-dermal tissue from three roots, three secondary stems and three stem tips and seeds (when available) of five trees in each of three healthy and three dieback-affected parkinsonia populations near Charters Towers, Queensland. Endophyte communities have been previously shown to be structured by plant part (Steinrucken et al. 2016; Rudgers and Orr 2009). These plant parts were chosen to ensure any stratification of endophyte communities across an individual plant was accounted for, and since leaves and seeds were not always available, stem tips were collected. Sampling was conducted in March 2013 and repeated on the same trees in May 2013 in order to ensure sampled healthy trees did not develop dieback-like symptoms between sampling periods (they did not) and to avoid isolating a community of endophytes representative of only one point in time. Between sampling of different trees and plant parts, all tools were sterilized using 50% NaClO and then rinsed with sterile water. Samples from different plant parts and trees were stored in separate paper bags at 5 °C for up to 48 h until processing. Plant parts were vigorously pre-washed in distilled H_2O for 20 s. An ethanol (70%) and UV-sterilized laminar flow cabinet was used for subsequent steps. For stems, stem tips and seeds a three-stage ethanol-bleach-ethanol surface sterilization method was used as recommended by Bills (1996). Seeds were then imbibed in 95 °C sterile, distilled H_2O for 12 h. Roots were washed for 30 s in sterile, distilled H_2O containing 0.1% Tween-20™ (Sigma-Aldrich, St Louis, MO, USA) since harsher sterilization techniques are not recommended for roots (Thorn et al. 2007). All samples were blotted dry with sterile filter paper and surface sterilization was checked by sliding tissue over the surface of 50% Potato Dextrose Agar amended with streptomycin (sPDA; 35 mg L^{-1}) and incubating at 30 °C for seven days (Bacon and Hinton 2007). The bark of stem tips and stems, the seed coat of seeds and a small portion of root cortex was then removed using a sterile scalpel. Three tissue plugs (3–5 mm^2) from each sample were placed on sPDA media and were maintained at room temperature in the dark for seven days. Isolates were subcultured daily, or when mycelial growth was observed.

Once pure fungal isolates were obtained, genomic DNA was isolated using a MO BIO Powersoil® DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), and identified via sequencing of amplified ITS rDNA amplicons. PCR reactions were undertaken in a total volume of 20 µl and consisted of 0.2 U BIOTAQ™ DNA polymerase (Bioline, London, UK), 10× NH_4 Buffer (2 uL per reaction), $MgCl_2$ (60 mM per reaction), dNTPs (50 mM each per reaction), ITS1 (5′-TCCG TAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCT CCGCTTATTGATATGC-3′) primers (4 mM each per reaction; Gardes and Bruns 1993), and 2 uL extracted DNA per reaction. PCR reactions were run at 94 °C for 3 min; 34 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a final extension step of 72 °C for 10 min. PCR products were purified using the Wizard®SV Gel and PCR Clean-Up System (Promega Madison, WI, USA), and sequenced by Sanger sequencing using the same forward primer (ITS1), in one direction, at the Hawkesbury Institute for the Environment using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's instructions. Sequence chromatograms were analysed in Geneious® V6.1.6 (Biomatters Ltd., Auckland, New Zealand) and underwent BLASTn searches on the National Center for Biotechnology Information (NCBI) nucleotide database on 6th May 2016. Closest match was determined by comparing maximum sequence length and lowest e-values. The cut-off point for assigning species names to closest match on the database was 97–100% identity; genus names were 94–97% identity; family name 90–94% identity; and sequences with lower identity with members of several families were identified only at the ordinal level (Vega et al. 2010). Sequences sharing less than 85% identity with closest match sequences or sharing higher identity to unidentified sequences in GenBank, were identified only to class or phylum (Vega et al. 2010). All taxonomic classifications required >95% query coverage and sequences with the same % ID for different organisms were identified to the closest common taxonomic level. We aligned unique sequences using MUSCLE Alignment (Edgar 2004) with eight iterations over 456 bases as implemented in Geneious® v8.1 and constructed a neighbour-joining tree based on the UPGMA model with a *Phytophthora ramorum* voucher sequence as the outgroup (Fig. 1).

Glass house pathogenicity trial

One month old parkinsonia seedlings grown from seed and collected from healthy populations in Charters Towers (QLD) were re-potted in 0.8 L free-draining square plastic pots in media consisting of 8 parts fine/medium pit sand, 1 part Mikskaar White Peat and 1 part Mikskaar Professional® substrate 250 (pH 5.2–6; Mikskaar AS, Tallinn, Estonia) and amended with 2.8 g/L Basacote® Plus Prilled slow release fertilizer, 1.5 g/L Osmoform® slow release fertiliser (Everris International B.V., Geldermalsen, The Netherlands) and 0.2 g/L SierraForm GT® Anti Stress (Everris International B.V). Plants were grown in an evaporatively-cooled glasshouse (21–27 °C) watered every second day, fertilised monthly with All Purpose Soluble Fertilizer (Hortico®, Padstow NSW, Australia), and treated for mites, thrips, scale and powdery mildew with Crown® SureGrow (Everris International B.V)


Healthy
Dieback
Root
Stem
Tip
Seed
HQ643339.1 Phytophthora ramorum (outgroup)
CTQ018 Mucor ellipsoideus
CTQ026 Microstromatales sp.
CTQ044 Tremellales sp.
CTQ044 Tremellales sp.
CTQ008 Bionectriaceae sp.
CTQ071 Trichoderma lixii
CTQ033 Emericellopsis sp.
CTQ004 Fusarium ciliatum
CTQ023 Fusarium lateritium
CTQ101 Fusarium sp.
CTQ025 Fusarium solani
* CTQ034 Rhizopycnis vagum
CTQ094 Phaeosphaeriopsis sp.
CTQ059 Pleosporales sp.
CTQ012 Phoma sp.
CTQ006 Epicoccum nigrum
CTQ001 Phoma sp.
CTQ541 Setosphaeria rostrata
CTQ022 Bipolaris sorghicola
CTQ066 Cochliobolus sativus
CTQ113 Alternaria arborescens
CTQ102 Alternaria alternata
* CTQ097 Alternaria alternata
CTQ002 Pleosporales sp.
CTQ003 Pleosporales sp.
CTQ036 Lophiostoma sp.
CTQ082 Dothideomycete sp.
CTQ030 Pleosporales sp.
CTQ020 Diaporthales sp.
CTQ085 Diaporthe leucospermi
CTQ096 Phomopsis sp.
CTQ014 Phomopsis longicolla
CTQ981 Diaporthe helianthi
* CTQ007 Phomopsis azadirachtae
CTQ009 Phomopsis sp.
CTQ084 Phomopsis sp.
CTQ027 Coniochaetaceae sp.
CTQ015 Cladosporium pseudocladosporioides
CTQ054 Ramularia plurivora
CTQ017 Antennariella placitae
CTQ057 Capnodium coffeae
CTQ013 Penicillium citreonigrum
CTQ086 Aspergillus unguis
CTQ801 Aspergillus insuetus
CTQ013 Penicillium citreonigrum
CTQ103 Neurospora dictyophora
CTQ095 Nigrospora oryzae
CTQ016 Nigrospora sphaerica
CTQ043 Nigrospora oryzae
CTQ042 Nigrospora sp.
CTQ062 Pestalotiopsis biciliata
CTQ045 Pestalotiopsis sp.
* CTQ056 Pestalotiopsis clavispora
CTQ099 Neopestalotiopsis clavispora
* CTQ067 Pestalotiopsis mangiferae
CTQ068 Pestalotiopsis oxyanthi
CTQ110 Xylariaceae sp.
CTQ028 Colletotrichum gloeosporioides
CTQ010 Aureobasidium pullulans
CTQ011 Aureobasidium pullulans
* CTQ031 Botryosphaeria dothidea
* CTQ005 Diplodia pinea
* NT039 Lasiodiplodia pseudotheobromae (positive control)
* CTQ089 Lasiodiplodia pseudotheobromae
Pythiales (O)
Murcorales (Z)
Microstromatales (B)
Tremellales (B)
Hypocreales
Dothidiomycete
Pleosporales
Diaporthiales
Coniochaetales
Capnodiales
Eurotiales
Sordiales
Trichosphaeriales
Xylariales
Glomerellales
Dothidiales
Botryosphaeriaceae
0.07

◀ **Fig. 1** Neighbour-joining tree based (TreeBASE submission no. 20057) on the UPGMA Model constructed using Geneious® v8.1 on a 458 bp length MUSCLE alignment of ITS1-ITS4 sequences from representative endophytic fungal taxa (Table 2) including the number of those isolates isolated from each plant tissue type. Bootstrap values ($n = 1000$ replicates) are shown on the intercepts. Outgroup is a *Phytophthora ramorum* (HQ643339.1). Isolates used in the pathogenicity trial are in bold and indicated with *. Ordinal groups indicated on right. All isolates are Ascomycetes apart from those in orders marked with (B) Basidiomycete, (Z) Zygomycete and (O) Oomycete

at 2.5 mL/L at 3 and 6 months, and weekly with predatory mites. After ten months, 258 healthy plants were selected for this trial, and randomly arranged in a temperature-controlled glasshouse. Plants were not fertilized after this point and, during the trial, glasshouse pests were controlled only using predatory mites (*Neoseiulus californicus;* Bugs for Bugs, Mundubbera, QLD, Australia).

Eighty-six plants (the control group) were watered as before with 100 mL water every second day; 86 were placed in white plastic trays with the water level maintained at 5 cm depth, inundating the roots and the third group of 86 plants were drip-fed 8–10 mL water twice a week to simulate drought conditions. Glasshouse conditions were set at 28 °C during the day, 21 °C at night and 60% constant humidity for one week before inoculation and then a further ten weeks from January to March 2015 at the Ecoscience Precinct, Brisbane, Australia.

Eight fungal isolates were chosen from the identified endophytic species, identified via sequencing as species previously reported to be pathogenic and cause dieback in their host (Table 1). Representing five families (Table 1), all but one (CTQ089 *L. pseudotheobromae*) were isolated from dieback-affected plants. For a positive control we also included a *L. pseudotheobromae* isolate (NT039), obtained from the University of Queensland culture collection, which had been isolated and tested in previous dieback studies, and shown to be pathogenic on parkinsonia (Diplock 2016; Toh 2009). All nine isolates tested were ascomycetes. We tested the effect of three water stress treatments (drought, inundation and 'normal') on the pathogenicity of the selected fungal isolates and the growth of twelve month-old parkinsonia seedlings.

The nine isolates were passaged through Granny Smith apples to ensure they had not lost their pathogenicity due to prolonged subculturing (Erwin and Ribeiro 1996), and then re-isolated on PDA without streptomycin for use in subsequent inoculations. After seven days, underbark inoculation was carried out on surface-sterilised stems at approximately 7 cm above soil surface. Incisions of 8–10 mm long were made with a sterile scalpel blade. A 5 mm^2 mycelial plug was fully inserted into the wound and the stem was bound with Parafilm® (Bemis, Oshkosh, WI, USA) to facilitate healing. The negative control (five of the 86 plants in each water treatment) consisted of a sterile PDA plug. Plants were arranged randomly within a split-plot design, with each fungal inoculant (subplot) occurring once nested within each water

Table 1 Fungal species information for identified isolates used in the pathogenicity trial

Isolate	GenBank accession	Host[a]	Species	Family	Previous implications in host dieback
CTQE056	KT699873	D: S	*Pestalotiopsis clavispora*	Amphisaeriaceae	Mango (Ismail et al. 2013), blueberry (González et al. 2012)
CTQE067	KT699874	D: S	*Pestalotiopsis mangiferae*	Amphisaeriaceae	Mango (Johnson et al. 1992), Chinese Bayberry (Chen et al. 2013)
CTQE005	KT699869	D: S, H: S	*Diplodia pinea*	Botryosphaeriaceae	*Pinus* spp. (de Wet et al. 2000)
CTQE089	KT699875	H: T	*Lasiodiplodia pseudotheobromae*	Botryosphaeriaceae	Invasive trees in Australia (Haque 2015; Sacdalan 2015; Toh 2009)
CTQE031	KT699871	D: S	*Botryosphaeria dothidea*	Botryosphaeriaceae	Fruit and nut trees (Slippers and Wingfield 2007)
NT039 [b]	KX893409	D:S	*Lasiodiplodia pseudotheobromae*	Botryosphaeriaceae	Isolated from dieback-affected *Parkinsonia aculeata* (Diplock 2016)
CTQE034	KT699872	D: R	*Rhizopycnis vagum*	Morosphaeriaceae	Musk-melon and *Medicago sativa* (Armengol et al. 2003)
CTQE097	KT699876	D: TE, H: T	*Alternaria alternata*	Pleosporaceae	Kiwi (Tsahouridou and Thanassoulopoulos 2000), *Fraxinus excelsior* (Bakys et al. 2009), grape (Ferreira et al. 1989).
CTQE007	KT699870	D: T, H: T	*Phomopsis azadirachtae*	Valsaceae	*Azadirachta indica* (Zwolinski et al. 1990)

[a] Disease status (D = dieback, H = healthy): plant part (E = seed, T = stem tip, S = stem, R = root) of *Parkinsonia aculeata* tree from which this species was isolated

[b] Obtained from the University of Queensland culture collection

Table 2 Endophytes isolated in this study and identified by ITS sequencing to closest match (CM) in the NCBI nucleotide database. When the sequences of two or more isolates were identical, a representative isolate was chosen and taxonomic identities were assigned† (GenBank Accessions KX893353-KX893409; KT699869-KT699876)

Isolate[a]	CM Order	CM Family	CM Species	CM Accession	% Similarity	% Coverage	Rep. isolate	Rep. isolate identification†	Assigned accession
CTQE031[b]	Botryosphaeriales	Botryosphaeriaceae	*Botryosphaeria dothidea*	LC120711	100	100	CTQE031	*Botryosphaeria dothidea*	KT699871
CTQE005[b]			*Diplodia pinea*	KU319042	98	100	CTQE005	*Diplodia pinea*	KT699869
CTQE064			*Diplodia pinea*	KU319042	97.8	100	CTQE005	*Diplodia pinea*	KT699869
CTQE065			*Diplodia pinea*	KU319042	97.7	100	CTQE005	*Diplodia pinea*	KT699869
CTQE089[b]			*Lasiodiplodia pseudotheobromae*	KT075144	99.8	100	CTQE089	*Lasiodiplodia pseudotheobromae*	KT699875
CTQE090			*Lasiodiplodia pseudotheobromae*	KT075144	100	100	CTQE089	*Lasiodiplodia pseudotheobromae*	KT699875
CTQE092			*Lasiodiplodia pseudotheobromae*	KT075144	100	100	CTQE089	*Lasiodiplodia pseudotheobromae*	KT699875
CTQE017	Capnodiales	Antennulariellaceae	*Antennariella placitae*	JN116688	97.6	100	CTQE017	*Antennariella placitae*	KX893367
CTQE024			*Antennariella placitae*	JN116688	97.6	100	CTQE017	*Antennariella placitae*	KX893367
CTQE057	Capnodiales	Capnodiaceae	*Capnodium coffeae*	DQ491515	99.2	96.08	CTQE057	*Capnodium coffeae*	KX893384
CTQE091			*Capnodium coffeae*	DQ491515	99.2	96.06	CTQE057	*Capnodium coffeae*	KX893384
CTQE015	Capnodiales	Mycosphaerellaceae	*Cladosporium pseudocladosporioides*	KT877407	100	100	CTQE015	*Cladosporium pseudocladosporioides*	KX893365
CTQE054			*Ramularia plurivora*	KJ504782	100	100	CTQE054	*Ramularia plurivora*	KX893383
CTQE027	Coniochaetales	Coniochaetaceae	*Lecythophora hoffmannii*	JN942898	92.7	98.68	CTQE027	Coniochaetaceae sp.	KX893374
CTQE981	Diaporthales	Valsaceae	*Diaporthe helianthi*	KM979834	99.2	100	CTQE981	*Diaporthe helianthi*	KX893408
CTQE085			*Diaporthe leucospermi*	KT323120	100	100	CTQE085	*Diaporthe leucospermi*	KX893394
CTQE020			*Diatractium cordianum*	EU541488	87.6	98.21	CTQE020	Diaporthales sp.	KX893369
CTQE007[b]			*Phomopsis azadirachtae*	KJ427811	100	99.07	CTQE007	*Phomopsis azadirachtae*	KT699870
CTQE014			*Phomopsis longicolla*	FJ462759	99.3	100	CTQE014	*Phomopsis longicolla*	KX893364
CTQE009			*Phomopsis* sp.	JQ341094	95.8	100	CTQE009	*Phomopsis* sp.	KX893359
CTQE084			*Phomopsis* sp.	KP006360	99.8	100	CTQE084	*Phomopsis* sp.	KX893393
CTQE096			*Phomopsis* sp.	DQ780461	98	100	CTQE096	*Phomopsis* sp.	KX893398
CTQE010	Dothideales	Dothioraceae	*Aureobasidium pullulans*	FJ744598	99.8	100	CTQE010	*Aureobasidium pullulans*	KX893360
CTQE011			*Aureobasidium pullulans*	JQ235065	100	100	CTQE011	*Aureobasidium pullulans*	KX893361
CTQE034[b]	Dothidiomycete	Dothidiomycete	*Rhizopycnis vagum*	KF494167	99.9	100	CTQE034	*Rhizopycnis vagum*	KT699872
CTQE801	Eurotiales	Trichocomaceae	*Aspergillus insuetus*	NR_131292	100	100	CTQE801	*Aspergillus insuetus*	KX893407
CTQE086			*Aspergillus unguis*	KC478524	99.2	100	CTQE086	*Aspergillus unguis*	KX893395
CTQE013			*Penicillium citreonigrum*	KT316706	99.6	100	CTQE013	*Penicillium citreonigrum*	KX893363
CTQE076			*Penicillium citreonigrum*	KT316706	100	100	CTQE013	*Penicillium citreonigrum*	KX893363
CTQE079			*Penicillium citreonigrum*	KT316706	100	100	CTQE013	*Penicillium citreonigrum*	KX893363
CTQE111			*Penicillium citreonigrum*	KT316706	98.2	100	CTQE013	*Penicillium citreonigrum*	KX893363
CTQE028	Glomerellales	Glomerellaceae	*Colletotrichum gloeosporioides*	KU820630	100	100	CTQE028	*Colletotrichum gloeosporioides*	KX893375
CTQE008	Hypocreales	Bionectriaceae	*Clonostachys rosea*	KR093840	91.8	99.81	CTQE008	Bionectriaceae sp.	KX893358
CTQE071		Hypocreaceae	*Trichoderma lixii*	KU934235	100	100	CTQE071	*Trichoderma lixii*	KX893390
CTQE033		Hypocreales	*Emericellopsis* sp.	KF1915990	100	100	CTQE033	*Emericellopsis* sp.	KX893377
CTQE004		Nectriaceae	*Fusarium ciliatum*	HQ897818	100	100	CTQE004	*Fusarium ciliatum*	KX893356
CTQE060			*Fusarium ciliatum*	HQ897818	100	100	CTQE004	*Fusarium ciliatum*	KX893356
CTQE023			*Fusarium lateritium*	JN198452	100	10	CTQE023	*Fusarium lateritium*	KX893371
CTQE025			*Fusarium solani*	KF918565	100	100	CTQE025	*Fusarium solani*	KX893372
CTQE101			*Fusarium* sp.	KU881904	95.5	100	CTQE101	*Fusarium* sp.	KX893401

Table 2 (continued)

Isolate[a]	CM Order	CM Family	CM Species	CM Accession	% Similarity	% Coverage	Rep. isolate	Rep. isolate identification[†]	Assigned accession
CTQE026	Microstromatales	Microstromataceae	*Microstromatales* sp.	EF060728	96	99.67	CTQE026	Microstromatales sp.	KX893373
CTQE018	Mucorales	Mucoraceae	*Mucor ellipsoideus*	NR_111683	99.6	100	CTQE018	*Mucor ellipsoideus*	KX893368
CTQE082	Pleosporales	Didymellaceae	*Leptosphaeria senegalensis*	KJ439197	83.8	89.81	CTQE082	Dothideomycete sp.	KX893392
CTQE083			*Leptosphaeria senegalensis*	KJ439197	85	87.29	CTQE082	Dothideomycete sp.	KX893392
CTQE036	Pleosporales	Lophiostomataceae	*Lophiostoma* sp.	GQ254683	99.4	100	CTQE036	*Lophiostoma* sp.	KX893378
CTQE030			*Massarina armatispora*	AF383955	88.8	98.61	CTQE030	Pleosporales sp.	KX893376
CTQE002		Pleosporaceae	*Alternaria alternata*	KU041713	100	100	CTQE002	*Alternaria alternata*	KX893354
CTQE097[b]			*Alternaria alternata*	KU041713	99	100	CTQE097	*Alternaria alternata*	KT699876
CTQE102			*Alternaria alternata*	KT192219	99.9	100	CTQE102	*Alternaria alternata*	KX893402
CTQE113			*Alternaria arborescens*	KP942903	98.3	100	CTQE113	*Alternaria arborescens*	KX893405
CTQE022			*Bipolaris sorghicola*	KU232899	100	100	CTQE022	*Bipolaris sorghicola*	KX893370
CTQE066			*Cochliobolus sativus*	JQ753975	100	100	CTQE066	*Cochliobolus sativus*	KX893387
CTQE541			*Setosphaeria rostrata*	KT933715	100	100	CTQE541	*Setosphaeria rostrata*	KX893406
CTQE006		Pleosporales *incertae sedis*	*Epicoccum nigrum*	KR095197	100	100	CTQE006	*Epicoccum nigrum*	KX893357
CTQE059			*Peyronellaea glomerata*	JN850981	90	100	CTQE059	Pleosporales sp.	KX893385
CTQE094			*Phaeosphaeriopsis* sp.	KP230840	99.2	100	CTQE094	*Phaeosphaeriopsis* sp.	KX893396
CTQE001			*Phoma* sp.	FJ985695	99.8	100	CTQE001	*Phoma* sp.	KX893353
CTQE039			*Phoma* sp.	FJ985695	99.8	100	CTQE001	*Phoma* sp.	KX893353
CTQE088			*Phoma* sp.	FJ985695	99.8	100	CTQE001	*Phoma* sp.	KX893353
CTQE961			*Phoma* sp.	FJ985695	99.8	100	CTQE001	*Phoma* sp.	KX893353
CTQE012			*Phoma* sp.	KM259932	100	100	CTQE012	*Phoma* sp.	KX893362
CTQE080			*Phoma* sp.	KM259932	100	100	CTQE012	*Phoma* sp.	KX893362
CTQE003			*Pleosporales* sp.	KR909157	99.4	100	CTQE003	Pleosporales sp.	KX893355
CTQE098	Sordariales	Chaetomiaceae	*Chaetomium* sp.	KM520348	100	100	CTQE098	*Chaetomium* sp.	KX893399
CTQE103		Sordariaceae	*Neurospora dictyophora*	AY681181	98.9	100	CTQE103	*Neurospora dictyophora*	KX893403
CTQE044	Tremellales	Tremellaceae	*Cryptococcus* sp.	HQ623585	88.9	90.41	CTQE044	Tremellales sp.	KX893381
CTQE073			*Cryptococcus* sp.	HQ623585	88.9	88.49	CTQE044	Tremellales sp.	KX893381
CTQE075			*Cryptococcus* sp.	HQ623585	88.7	90.41	CTQE044	Tremellales sp.	KX893381
CTQE077			*Cryptococcus* sp.	HQ623585	88.6	88.49	CTQE044	Tremellales sp.	KX893381
CTQE078			*Cryptococcus* sp.	HQ623585	88.7	88.68	CTQE044	Tremellales sp.	KX893381
CTQE087			*Cryptococcus* sp.	HQ623584	88.7	88.49	CTQE077	Tremellales sp.	KX893391
CTQE043	Trichosphaeriales	Trichosphaeriaceae	*Nigrospora oryzae*	KC771471	100	100	CTQE043	*Nigrospora oryzae*	KX893380
CTQE072			*Nigrospora oryzae*	KC771471	99	100	CTQE043	*Nigrospora oryzae*	KX893380
CTQE095			*Nigrospora oryzae*	KF23404	98.4	100	CTQE095	*Nigrospora oryzae*	KX893397
CTQE042			*Nigrospora* sp.	KF128783	99.8	99.61	CTQE042	*Nigrospora* sp.	KX893379
CTQE016			*Nigrospora sphaerica*	KU878079	100	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE041			*Nigrospora sphaerica*	KU878079	100	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE055			*Nigrospora sphaerica*	KU878079	95.9	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE070			*Nigrospora sphaerica*	KU878079	100	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE100			*Nigrospora sphaerica*	KU878079	99	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE093			*Nigrospora sphaerica*	KU878079	99.8	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE099	Xylariales	Amphisphaeriaceae	*Neopestalotiopsis clavispora*	KP075005	100	100	CTQE099	*Neopestalotiopsis clavispora*	KX893400
CTQE062			*Pestalotiopsis biciliata*	KM199305	99.6	100	CTQE062	*Pestalotiopsis biciliata*	KX893386
CTQE063			*Pestalotiopsis biciliata*	KM199305	99.6	100	CTQE062	*Pestalotiopsis biciliata*	KX893386
CTQE056[b]			*Pestalotiopsis clavispora*	JX045815	100	100	CTQE056	*Pestalotiopsis clavispora*	KT699873

Table 2 (continued)

Isolate[a]	CM Order	CM Family	CM Species	CM Accession	% Similarity	% Coverage	Rep. isolate	Rep. isolate identification[†]	Assigned accession
CTQE067[b]			*Pestalotiopsis mangiferae*	KM510410	100	100	CTQE067	*Pestalotiopsis mangiferae*	KT699874
CTQE068			*Pestalotiopsis oxyanthi*	KT716303	100	100	CTQE068	*Pestalotiopsis oxyanthi*	KX893388
CTQE045			*Pestalotiopsis* sp.	GU592002	100	100	CTQE045	*Pestalotiopsis* sp.	KX893382
CTQE110		Xylariaceae	*Xylariaceae* sp.	AB741586	99.2	100	CTQE110	Xylariaceae sp.	KX893404

[a] Isolates CTQ001-CTQ036 (n = 31) were recovered from samples collected in March 2013, all others were isolated from samples collected in May 2013 (n = 58)

[b] Isolates tested in the pathogenicity trial (Table 1)

[†] Assigned identities: Species name approximately 97–100% sequence identity with closest match; genus ~94–97%; family ~90–94%; order 85–90%; class or phylum <85% (Vega et al. 2010)

treatment (main plot), each with nine replicates (therefore, 9 isolates × 9 replicates =81, + 5 negative controls =86 plants × 3 water treatments =258 plants in total).

Immediately prior to inoculation and at the end of the trial plant growth by height (cm) from the soil surface and stem girth (mm) at the site of inoculation was measured. We also monitored any damage by mites (% foliage damage). At the conclusion of the trial (10 weeks following inoculation) plants were harvested at the root collar. After harvest, lesions were bisected with a sterile blade. Underbark lesion size, identified by discolouration from the site of inoculation, and any scarring was measured. To confirm that lesions were associated with the inoculated pathogen and to look for any systemic infection by the inoculated pathogen, a small amount of tissue was sampled from the lesion or cut site of three plants in each replicate group; 1 cm above and below the lesion; and 10 cm above the lesion. Tissue samples were plated on sPDA and incubated for 1 week at room temperature in the dark, and isolates were identified via ITS sequencing as above. Roots were freed from soil by carefully running them under water, being careful not to wash away fine roots and as with the above-ground parts, were placed in paper bags and dried in an oven at 60 °C for 14 days. We recorded the dry weight of above and below-ground parts.

We tested the effects of water treatment and inoculated isolate on lesion length and three measures of plant health: the change in height and stem circumference over the ten-week inoculation trial and post-harvest dry mass at the conclusion of the trial. Data were treated as a split-plot design (Schwarz 2015) in R (R Core Team 2016) using the 'lme4' (Bates et al. 2015) and 'lmerTest' (Kuznetsova et al. 2016) packages for analysis of variance (ANOVA) with Kenward-Roger approximation for degrees of freedom. ANOVAs were followed by post-hoc testing using Tukey HSD (Tukey 1949) using the 'multcomp' (Hothorn et al. 2008) package.

Results

Fungal endophytes in healthy and dieback-affected *Parkinsonia aculeata*

We cultured a total of 213 fungal isolates from multiple plant parts in healthy and dieback-affected parkinsonia and identified 54 unique operational taxonomic units (OTUs) through DNA sequencing of the ITS rDNA region (Table 2). The identified isolates (GenBank Accessions KT699870-KT699873; KX893353-KX893409) represented 16 fungal orders and 25 families. The majority (90%) were Ascomycetes while seven Basiodiomycetes and one Zygomycete were also isolated. Fungi from the order Pleosporales had the greatest number of representative isolates (Fig. 2) with a total of 85 isolates, but only 5 unique OTUs. The Xylariales were well represented (12 isolates, 9 unique

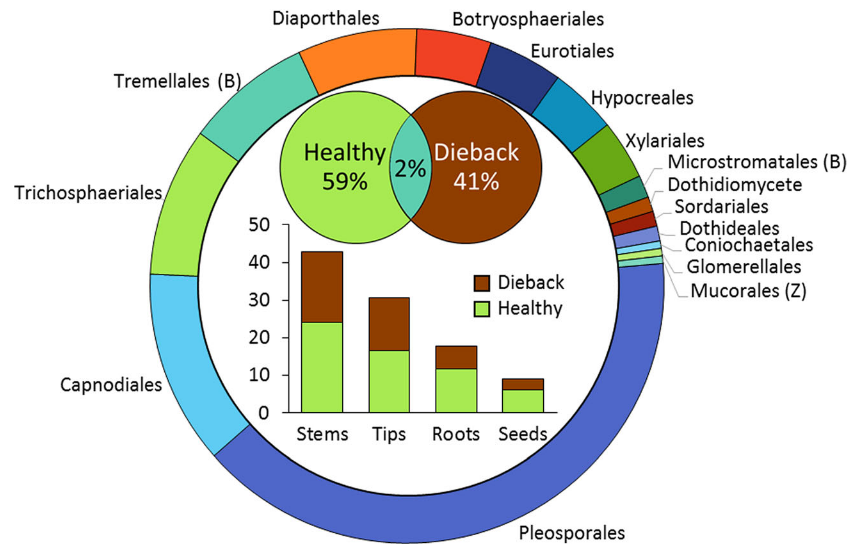


Fig. 2 Fungal community overview showing the proportion of taxonomic orders represented where (B) are Basidiomycetes, (Z) is a Zygomycete and all others are Ascomycetes; the proportion of isolates isolated by tissue type and host disease status; and the percentage of isolates found in healthy, dieback-affected parkinsonia, or both

species), as were the Hypocreales (10 isolates, 8 unique species) and Eurotiales (9 isolates, 5 unique species). We recovered 31 isolates from samples collected in March 2013 and 58 isolates from samples collected in May 2013. We isolated the greatest number of endophytes from parkinsonia stems with 28% of isolates from dieback trees and 11% from healthy trees (Fig. 2). This was followed by the branch tips (17% from dieback, 12% from healthy), the seeds (9% dieback, 4% healthy) and roots (4% dieback, 5% healthy). We isolated four species of Botryosphaeriaceae (8 isolates), and although four of these isolates were *L. pseudotheobromae*, this species was only isolated from healthy parkinsonia (Table 2). Overall, we isolated more endophytes from dieback-affected parkinsonia (61%) compared to healthy parkinsonia (39%) trees. Of the isolated taxa, 32 were previously shown to be pathogenic according to the literature, 27 had history as dieback pathogens, but only nine were involved in dieback of trees. Three of these were *Pestalotiopsis* (*P. clavispora*, *P. mangiferae* and *P. visimae*). Due to the number of available plants and our desire to maximise statistical power and therefore the number of replicates, we decided to exclude *P. visimae* from this trial.

Pathogenicity testing of isolates with a water stress interaction

There was a statistically significant difference in lesion length, explained by inoculated isolate (F = 2.347, $p = 0.01$), but no effect by water treatment or the interaction between these factors ($p > 0.05$; Table 3). Universally, lesion size was greater underbark than on the surface. The length of incision in the negative controls (sterile ½ PDA plug) was consistent with the length of the underbark "lesion", which we assume was a result of scarring, so we concluded that no lesion was formed for the negative control treatment. The positive control (*L. pseudotheobromae*, NT039) consistently formed larger lesions than any other pathogen tested (40.57 ± 0.51 mm; Fig. 3) and contributed significantly to variation in lesion length in post-hoc testing (Table 4). *Pestalotiopsis mangiferae* (CTQE067), *L. pseudotheobromae* (CTQE089), *Botryosphaeria dothidea* (CTQE031) and *Pestalotiopsis clavispora* (CTQE056) caused similar sized underbark lesions (23.94 ± 0.47 mm; Fig. 3). *Diplodia pinea* (CTQE005) and *Phomopsis azadirachtae* (CTQE007) resulted in the smallest lesions, with *P. azadirachtae* (12.83 ± 0.15 mm) only just

Table 3 ANOVA testing the effects of water treatment and inoculated isolate on lesion length and three measures of *Parkinsonia aculeata* plant health, where 'growth' is the change in height or stem circumference over the ten-week inoculation trial

		Lesion length		Growth (Height)		Growth (Circumference)		Post-harvest biomass	
Source of variation	**df**	**F**	***P*-value**	**F**	***P*-value**	**F**	***P*-value**	**F**	***P*-value**
Isolate	8	2.35	0.02	0.20	0.99	0.07	1.00	0.18	0.99
Water treatment	2	0.36	0.70	14.08	<0.001	10.82	<0.001	8.38	<0.001
Interaction	16	0.12	0.99	0.22	1.00	0.05	1.00	0.18	1.0

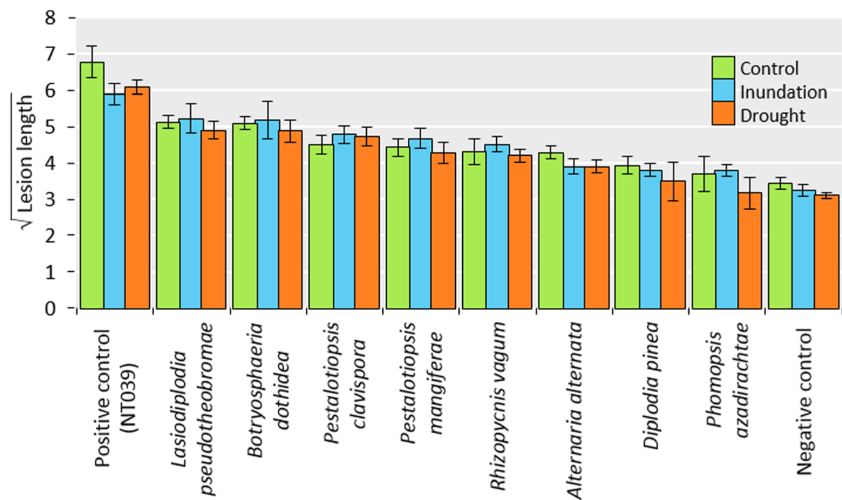


Fig. 3 Average underbark lesion length on one-year-old *Parkinsonia aculeata* seedlings by isolate and water treatment, at the conclusion of this 10 week glasshouse pathogenicity trial

exceeding the inoculation site scar length (10.67 ± 0.02 mm) but was greater than the negative control (11.77 ± 0.33 mm).

There was a statistically significant difference in plant health measurements by water treatment. Water treatment affected plant growth by height (F = 14.08, $p < 0.001$) stem circumference (F = 10.82, $p < 0.001$), and post-harvest biomass (F = 8.38, $p < 0.001$) but we found no significant effect of inoculated isolate or the interaction between isolate and water treatment on any recorded plant heath measurements ($p > 0.05$). Drought-affected plants showed the greatest levels of water stress (Table 5), which was reflected in their relatively slower growth rate over 10 weeks during the trial with smaller changes in stem circumference and height (Fig. 4a, b); lower post-harvest dry-mass (Fig. 4c); and their increased susceptibility to pests (Fig. 5). They also had a lower above-ground: below-ground plant dry mass ratio (1.43 ± 0.09) than inundated plants (2.08 ± 0.08) or control plants (2.13 ± 0.10). Plants that were inundated had reduced height compared to the control treatment (Fig. 4a); however, stem girth and post-harvest dry mass were similar between inundated plants and those in the control water regime (Table 5).

Despite confirming local infection by the inoculated pathogen via re-isolation from lesions, we were unable to re-isolate the pathogen more than 2 cm away from the lesion in any of the plants post-harvest. Isolates from post-harvest, healthy, plant tissue were identified as *Myrothecium verrucaria*, a ubiquitous contaminant and plant pathogen; *Phoma sp*. and *Chaetomium globosum* – both endophytes; and *Fusarium oxysporum* and *Penicillium verruculosum*, which are common saprotrophs and endophytes (Nguyen et al. 2016). We did not observe any dieback-like symptoms in the plants such as loss of foliage, internal staining or death of plant parts, other than lesions at the inoculation site. Any loss of foliage in drought-affected plants was consistent across inoculated pathogens, so was presumed to be due to water availability or mite damage, not infection by a pathogen. We therefore found no evidence of systemic infection by any inoculated pathogen, regardless of lesion size or level of stress.

Table 4 Tukey Q statistics (where $P < 0.05$) for post-hoc pairwise analysis of the effect of inoculated isolate[a] on underbark lesion length in *Parkinsonia aculeata*

	CTQE005	CTQE007	CTQE031	CTQE034	CTQE056	CTQE067	CTQE089	CTQE097
CTQE007	-							
CTQE031	5.11	6.80						
CTQE034	-	-	-					
CTQE056	-	5.65	-	-				
CTQE067	5.44	7.14	-	-	-			
CTQE089	5.58	7.28	-	-	-	-		
CTQE097	-	-	-	-	-	4.62	4.76	
NT039	14.48	16.33	9.59	12.25	10.73	9.26	9.12	13.66

[a] CTQE005 *Diplodia pinea;* CTQE007 *Phomopsis azadirachtae;* CTQE031 *Botryosphaeria dothidea;* CTQE034 *Rhizopycnis vagum;* CTQE056 *Pestalotiopsis clavispora;* CTQE067 *Pestalotiopsis mangiferae;* CTQE089 *Lasiodiplodia pseudotheobromae;* CTQE097 *Alternaria alternata;* NT039 Positive control *L. pseudotheobromae*

- Not significant where $P > 0.05$

Table 5 Tukey Q statistics (where $P < 0.05$) for post-hoc pairwise analysis of the effect of water treatment on three measurements of plant health at the end of the 10 week *Parkinsonia aculeata* inoculation trial

Water treatments	Growth (Height)	Growth (Circumference)	Post-harvest biomass
Control vs inundate	2.73	-	-
Control vs drought	5.31	4.00	3.85
Inundate vs drought	2.58	4.01	3.14

- Not significant where $P > 0.05$

Discussion

Despite significant levels of water stress, and a decrease in the health of stressed plants, underbark inoculation by any of the chosen fungal isolates did not cause systemic infection or dieback-like symptoms in parkinsonia. Four of the isolates tested in this trial were members of the Botryosphaeriaceae, many of which are known to persist as latent pathogens within their host (Jami et al. 2013; Mehl et al. 2013; Slippers and Wingfield 2007). This family of pathogens grow both intracellularly and intercellularly and after infection, are known to move via the mesophyll and vascular bundle (Mehl et al. 2013). Host response involving the formation of a new periderm can also lead to infection of the xylem and phloem tissue (Rayachhetry et al. 1996) leading to systemic infection within eight weeks. *Lasiodiplodia pseudotheobromae* (CTQ089) was only isolated from healthy, symptomless parkinsonia during field sampling, yet formed a significantly larger lesion in the pathogenicity trial than some of the other isolates that were isolated from dieback-affected parkinsonia. This supports the idea that at least this strain of *L. pseudotheobromae* is a latent pathogen (Jami et al. 2013). Lesions formed by *Diplodia pinea* were relatively small, and this may be because it is potentially more pathogenic to other tree species such as *Pinus* sp. (de Wet et al. 2000). *Botryosphaeria dothidea* has been observed to cause girdling and death in defoliated downy birch (*Betula pubescens*) stems after just four weeks, suggesting that defoliation stress might be essential for increased *B. dothidea* virulence (Crist and Schoeneweiss 1974).

Pestalotiopsis spp. are responsible for a number of plant diseases, mostly in the tropics (Chen et al. 2013; Espinoza et al. 2008; Ismail et al. 2013; Keith et al. 2006) and are commonly isolated as saprobes, although some are likely to have both endophytic and pathogenic stages in their lifecycles (Maharachchikumbura et al. 2011). Endophytes from this group are ubiquitous and not associated with geographic limits, but their host colonisation rates are lower in monsoon seasons than in the dryer winter season (Tejesvi et al. 2005). This indicates that they may be limited by drought-like conditions, and take advantage of their host in sustained wet weather. In our study, we did not observe significant variation in lesion length between inundated and drought-affected plants by either *P. clavispora* or *P. mangiferae*. This might be because parkinsonia is relatively healthy in inundated conditions compared to drought-affected conditions (Fig. 4), thereby not presenting with the stress required by the two *Pestalotiopsis* spp. for increased colonisation or pathogenicity. There are no records of dieback occurrence in parkinsonia in relation to rainfall conditions in the field.

Out of the other three isolates used in this study, only *Alternaria alternata* and *Rhizopycnis vagum* caused underbark lesions that were significantly greater than the negative control. *A. alternata* is known to produce host-specific phytotoxins which may cause defoliation (Babu et al. 2003). This species may therefore require a susceptible host for it to be more virulent. *Rhizopycnis vagum* is most frequently a root-colonizing endophyte (Knapp et al. 2012), although some

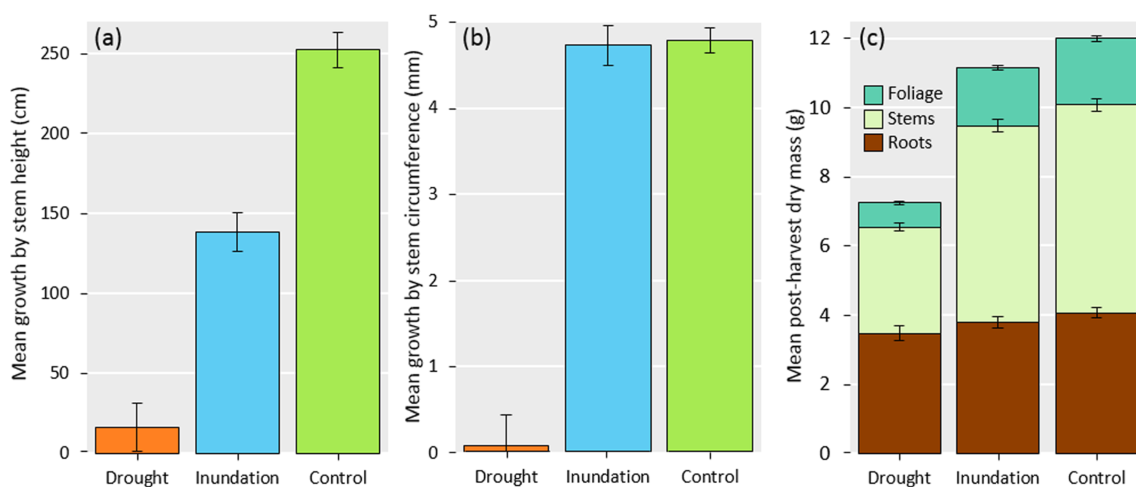


Fig. 4 The effects of water treatment on the average change in *Parkinsonia aculeata* plant height **a** and stem circumference **b** from the start to the end of the 10 week pathogenicity trial, and average post-harvest dry mass of roots, stems and foliage **c** at the conclusion of the trial

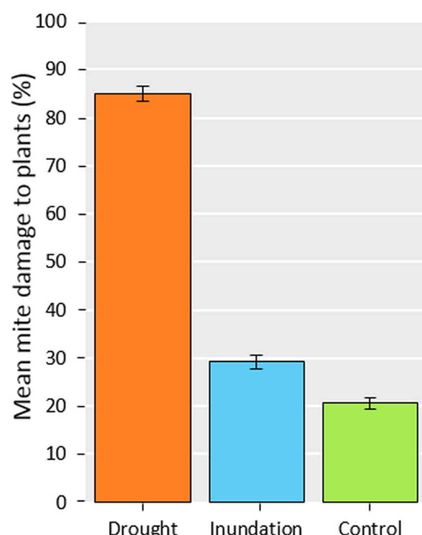


Fig. 5 Mite damage to *Parkinsonia aculeata* plants by water treatment at the conclusion of this glasshouse pathogenicity trial

studies have shown it to be pathogenic to musk-melon roots (Armengol et al. 2003) and involved in mature watermelon vine decline (Westphal et al. 2011). We isolated it from the roots of dieback-affected parkinsonia, but it too, only resulted in small localised lesions when inoculated. Westphal et al. (2011) suggests *Rhizopycnis vagum* may require other factors to increase disease severity, such as soil inoculation.

We attempted to ensure that each inoculated isolate was triggered into pathogenicity by first passaging the isolate through an apple. Although we achieved local infection in the plant, we did not observe systemic infection, despite first ensuring that the host was under water stress. It is possible that extending the length of the trial past 10 weeks may have resulted in eventual mortality. Incubation times vary between studies (Ismail et al. 2013; Pitt et al. 2013) but many report significant results within 10 days of inoculation (e.g., Armengol et al. 2003; Stukely and Crane 1994). Additionally, any response observed in an inoculation trial may be different to that observed in the field, even under similar conditions. The age of the plant tissue may affect the plant's response to inoculation, and the endophyte community hosted by plants in the field may be different to those hosted by glasshouse plants grown from seed. A latent pathogen may only be triggered into pathogenicity by a combination of these factors which may also explain the lack of dieback symptoms observed in this glasshouse trial. Plants are complex organisms, playing host to multiple taxonomic and trophic groups, with environmental responses ranging from inherent to symbiotically-assisted. It is therefore difficult to predict or monitor infection from inoculation with one organism, without distinctive symptoms. Future pathogenicity work in this system, as demonstrated in Toh (2009) using seedlings, should be assessed histologically during and after the trial.

We isolated a taxonomically diverse range of fungal endophytes from multiple plant parts of healthy and dieback parkinsonia, including some reportedly pathogenic species, with a total of 54 unique taxa from 204 isolates as identified by ITS amplicon sequencing. These species came mostly from dieback-affected plants, and the greatest number were isolated from stems. The fungal endophyte community of other invasive plants is similar in regards to culturable endophyte species found in this study. Diplock (2016) only isolated fungi from stems and identified 20 unique fungal endophyte species of 48 isolates associated with dieback-affected parkinsonia. We identified 31 taxa from dieback stems collected in the same region. Twenty-four fungal endophyte taxa (out of 1352 isolates) were recovered from healthy and dieback-affected *Mimosa pigra* stems by Sacdalan (2015), and 23 taxa from 166 isolates from healthy and dieback-affected *Vachellia nilotica* subsp. *indica* stems and roots (Haque 2015). Overall, the number of taxa recovered from dieback-affected plants in this study was greater than from healthy plants, which is expected if additional dieback-causing pathogens are present, or as the host is colonised by incoming saprophytes during cell death brought on by dieback. The composition of endophyte communities between individual hosts and host species may also differ due to local environmental conditions, distance decay (i.e. increasing dissimilarity between communities with increasing spatial distance; Peršoh 2015) and mode of endophyte transmission (i.e. vertical vs. horizontal). However, there is a high chance that the isolates recovered in this study are dominant and/or fast growing members of the parkinsonia endophyte community, since these species are more likely to be isolated. Conversely, this also implies that slower-growing or more benign species may not have been recovered and that unculturable taxa were missed. Steinrucken et al. (2016) recovered over 150 unique OTUs from dieback-affected plant parts and over 70 from healthy plant parts, which is more than double those isolated in this study. The availability of molecular techniques and the decreasing price of high-throughput sequencing technology has consistently demonstrated that the diversity of fungi is grossly underestimated by culture-based studies (Peay et al. 2016).

Our observations support the idea that some of the fungal endophytes isolated from parkinsonia, particularly the Botryosphaeriaceae, exist commonly as endophytes and may act as latent pathogens but in order to cause disease in their host some external environmental trigger is required. Despite the formation of localised lesions, no dieback-like symptoms were observed via underbark inoculation of parkinsonia with the eight chosen isolates in this study. Under the right conditions (e.g., a specific environmental stress or the presence of other microorganisms) however, underbark inoculation may still be an appropriate method for testing other potential putative pathogens. In the future, other factors such as salinity, heat, and defoliation stress could be used during pathogenicity

screening, and may provide insight into host susceptibility to dieback-associated pathogens. More thorough reporting of dieback occurrence in the field, and any associated environmental conditions, would also aid greatly in determining which stress factors are important for disease expression. Any potential dieback-causing agent(s) identified should be systematically tested for host-specificity (see Wapshere 1974; Evans 2000) – particularly against locally occurring native plant species – prior to release and widespread use as a biocontrol agent(s). Dieback syndromes adversely affect many desired tree species globally, but with the right combination of effective and specific dieback-causing pathogens, efficient inoculation techniques and conducive conditions, dieback may become an alternative tool for use in large scale weed management.

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