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ORIGINAL RESEARCH ARTICLE

First report of *Eutypella* species associated with dying cotton (*Gossypium hirsutum* L.)

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Abstract
A rapid increase in the number of *Gossypium hirsutum* L. plants suddenly wilting and dying in a commercial cotton field in Central Queensland initiated this study. The aim was to characterise fungal species recovered from discoloured vascular tissue of dead plants and determine if they contributed to their death. Isolations were consistently dominated by one fungus based on culture morphology. Identification was established on sequences of the internal transcribed spacer (ITS) region of ribosomal DNA and revealed that the isolates all belong to Diatrypaceae, with high homology to *Eutypella scoparia*. Further analyses showed that there were two distinct *Eutypella* species present in the isolates, which are quite different from *E. scoparia* from the sequence dissimilarity due to the presence of ITS1 and ITS2 insertions. In diseased root samples, community profiling showed two operational taxonomic units (OTUs) related to *E. scoparia* were the most abundant fungi accounting for 45 to 99% of all sequences. Pathogenicity tests showed that a *Eutypella* isolate when inoculated into the stem of healthy *G. hirsutum* using two inoculation methods caused cankerous growth and necrosis of vascular tissue, typical of trunk disease. The fungus caused a red—brown streaking of the vascular tissue like that observed in diseased field plants. This study shows that the fungal isolates, which form a distinct group within the *Eutypella*, are associated with the root and stem of dying *G. hirsutum* and were the dominant fungi of diseased roots. This is the first known case of *Eutypella* affecting cotton worldwide and is considered an expansion of this genus' host range.

Keywords Diatrypaceae · Tree diseases · Trunk canker · Cotton · Eutypella

Introduction

Cotton (*Gossypium hirsutum* L.) is a major commodity in Australian agriculture, representing 30—60% of the gross value of the total agricultural production in regions where it is grown (Cotton Australia 2014). Over a five-year average between 2013/14 and 2017/18, the Australian cotton crop was worth AUD $2 billion annually, underpinning the viability of 152 rural communities (Cotton Australia 2021). However, cotton diseases present a major constraint to cotton production, not only in Queensland, but all cotton growing regions in Australia. Cotton diseases impact profitability directly through crop losses (estimated at $27–75 million annually), as well as indirectly through the inability to plant in previously affected fields to cotton due to the risk of significant future yield losses (Sas 2020). In some cases, such as severe examples of Fusarium wilt, yield loss can be more than 80% when susceptible cultivars are grown (Stiller and Wilson 2014).

In the 2017/18 season, a single small patch of cotton plants (var. Sicot 714B3F) in an 18 ha field in Central Queensland were observed to suddenly wilt and die, which are the typical symptoms of a condition termed 'Sudden wilt'. This condition is potentially caused by *Fusarium* spp. and/or a physical effect of environmental conditions, as hot weather following an irrigation or rainfall favour development. Interestingly, Sudden wilt has not been observed to

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reoccur in the same location. However, unlike Sudden wilt, leaves remained attached, and the disease reoccurred in the same location in subsequent cotton plantings, significantly increasing in area affected with each planting of cotton. Hence, in the 2019/20 season the area of diseased cotton plants had increased to approximately 1 ha. Symptoms associated with the sudden decline and death of cotton include sudden wilting and plant death; bronzing of leaves and petioles; dead plants with blackened stems and leaves remaining on the plant; internal reddening of roots and root decay; in cross section the infected tissue may possess a wedge-shaped appearance and has a reddish-grey colour. Symptoms were observed to occur from squaring and at all stages of growth there on, occurring as either single plants or in patches. To distinguish from Sudden wilt, this disease is referred to as Reoccurring wilt. In the 2020/21 season, dead cotton plants exhibiting typical symptoms of Reoccurring wilt were observed on three additional farms in Central Queensland. In these fields only single plants scattered through the field tended to be affected, rather than patches of diseased plants. This pattern of disease spread suggests early development of the disease in these fields.

Preliminary investigations into the cause of plant death did not yield fungi known to be cotton pathogens, rather, a species of fungi belonging to the family Diatrypaceae was consistently isolated from symptomatic root and stem tissue. Diatrypaceous fungi are commonly found on dead or declining wood of angiosperms (Glawe and Rogers 1984). A few species in this family constitute important plant pathogens in native or cultivated plants, and among them, *Eutypa lata* has been the most studied as the cause of Eutypa dieback of grapevine (*Vitis vinifera*) (Carter 1957, 1991; Trouillas et al. 2011). In Australia *E. lata* is widespread across South Australian vineyards (Sosnowski et al. 2007; Pitt et al 2010). A taxonomic study of Diatrypaceous fungi associated with grapevines and other woody plants in Australia identified two new *Eutypella* species (Trouillas et al. 2011). Other species in this family which cause disease in woody plants include *E. parasitica*, causing Eutypella canker, a serious disease of sugar maple (*Acer saccharum* Marshall) in northern USA (Davidson and Lorenz 1938) and *E. scoparia*, which is responsible for canker of pecan (Gottwald 2001). The first record of *E. scoparia* in Australia was from dead twigs of two *Acacia* species from seed production areas in the Northern Territory (Yuan 1996). To our knowledge, there are no reports of Diatrypaceae fungi associated with dying cotton plants.

The aim of this study was to identify the species of Diatrypaceae isolated from dying cotton plants in Central Queensland. The composition of fungal communities associated with diseased cotton roots and associated soils was also determined, using Next-Generation Sequencing (NGS) methods, to determine which species were dominant. We also tested the pathogenicity of a representative isolate on healthy cotton plants to determine the potential involvement of *Eutypella* species in cotton plant death. Knowledge about the pathogen(s) involved in Reoccurring wilt of cotton will help design strategies to manage the disease in this crop.

Materials and methods

Collection of samples, isolation of fungi and morphological characteristics

Fifteen diseased cotton plants exhibiting typical symptoms of Reoccurring wilt were collected from commercial cotton fields in Central Queensland, Australia. A 5 cm length of tap root and/or lower stem with discoloured vascular tissue was cut, washed in deionized water, surface-sterilised by soaking in 70% ethanol for 3 min then rinsed in Millipore water. The bark was aseptically removed, and then small pieces of discoloured vascular tissue were cut out and placed on 90 mm Petri plates of ¼ strength Potato Dextrose Agar (Difco, Detroit, USA) amended with 50 mg/L Streptomycin Sulphate (Sigma, St Louis, USA) (¼PDAS) and incubated at laboratory light and room temperature conditions for 14 days. Plates were observed daily and hyphal tips from developing colonies were transferred to fresh ¼PDAS plates and incubated at laboratory conditions. Microscopic examination of conidial masses emerging out of pycnidia were observed in culture after 6 months incubation at room temperature. For microscopic examination of spores, pycnidia were placed on glass microscope slides and mounted in water. Fungal structures were observed using a Nikon CiL Phase Contrast microscope and photographs were taken with a Nikon 5MP colour digital camera, using Nikon application software. Spore measurements are based on 10 individual spores.

DNA extraction and polymerase chain reaction amplification of recovered isolates from dead cotton plants

Isolates were grown for 7—10 days on ¼PDAS, prior to DNA extraction. Mycelium was scraped from the surface of the cultures and total genomic DNA was extracted using the BioSprint® DNA Plant Kit (Qiagen) according to the manufacturer's instructions.

Molecular identification via ITS sequencing was performed on 13 of the isolates shown in Table 1. The ITS1, 5.8S, ITS2 regions and part of the D1 region of the LSU rDNA were amplified using the universal eukaryotic primers UN-UP18S42 (5'-CGTAACAAGGTTTCCGTAGGTGAA C-3') and UN-LO28S576B (5'-CTCCTTGGTCCGTGTTTC AAGACG-3') (Bakkeren et al. 2000). Each 30 µl reaction

Table 1 Molecular characterisation of *Eutypella* sp. in diseased cotton plants and roots through ITS sequencing examined in this study

Isolate code	Location	Herbarium number (BRIP)	Sequence labels	GenBank accessions
Eutypella isolates from diseased cotton plants				
CQ4-1	Theodore east	71492	CQ4-1	MZ918961
CQ4-2	Theodore east	71491	CQ4-2	MZ918956
CQ7-5	Giber Gunyah	71495	CQ7-5_RC	MZ918953
CQ7-15a	Giber Gunyah	72140a	CQ7-15a	MZ918957
CQ7-15c	Giber Gunyah	-	CQ7-15c	MZ918958
CQ7-15d	Giber Gunyah	-	CQ7-15d	MZ918959
CQ7-15e	Giber Gunyah	-	CQ7-15e	MZ918960
CQ7-15f	Giber Gunyah	70935a	CQ7-15f	MZ918964
CQ9-3a	Moura	70529a	CQ9-3a_RC	MZ918962
CQ9-3b	Moura	70529b	CQ9-3b	MZ918963
CQ9-3c	Moura	70529c	CQ9-3c_RC	MZ918954
CQ9-3d	Moura	-	-	
CQ10	Moura	71230a	CQ10_RC	MZ918955
QC9-3a	Moura	70529a	RM1	MZ918950
CQ9-3b	Moura	70529b	RM2	MZ918951
CQ9-3c	Moura	70529c	RM3	MZ918952
Community OTU amplicons associated with diseased cotton roots				
-	Moura	-	OTU_1	MZ920135
-	Moura	-	OTU_2	MZ920136
-	Moura	-	OTU_257	MZ920137
-	Moura	-	OTU_426	MZ920138

contained 0.3U Phusion® High-Fidelity DNA Polymerase (New England Biolabs), 1 × Phusion HF Buffer, 0.2 mM dNTPs, and 0.5 µM of each primer. PCR cycling conditions were as follows: initial denaturation at 98 °C for 30 s, then 30 cycles of 98 °C 15 s, 6 °C 15 s and 72 °C 30 s, and a final 5 min extension at 72 °C, which produced a 1 kb + amplicon. Unpurified PCR products were submitted to the Australian Genome Research Facility (AGRF) for dual direction sanger sequencing. Full sequence reads were trimmed and analysed using Genieous Prime Software v2019.0.4 (Biomatters Ltd.) and then compared to the GenBank database using BLASTn.

Living cultures of 10 of the isolates of *Eutypella* sp. were deposited in the Queensland Plant Pathology Herbarium (BRIP). Nine of these isolates were collected from diseased cotton in Central Queensland and one isolate, labelled CQ9-3d, was recovered from the cotyledon region of symptomatic cotton grown from seed in naturally infested field soil (Table 1).

Genetic analysis of fungal communities from root and soil samples

Roots from dead cotton plants exhibiting typical symptoms of Reoccurring wilt were collected from a commercial cotton field in Moura, Central Queensland for analyses of fungal community composition. This field was the location of the first detection of Reoccurring wilt, from which isolates CQ9-3a, CQ9-3b CQ9-3c, CQ9-3d originated. A total of four root samples with surface soil removed were analysed with minimal processing prior to DNA extraction. DNA from root samples was extracted using the SARDI RDTS laboratory procedure so that large sample size (1.75 g/sample) could be used to reduce field-based variation. Similarly, 250 g of surface soil samples collected from the root zone of the disease plants was used in the DNA extraction using the SARDI RDTS laboratory procedure (Ophel-Keller et al. 2008). Genetic composition of fungal communities associated with roots was determined using NG-amplicon (ITS-region) sequencing method, using the primers ITS1F (CTTGGTCATTTAGAG GAAGTAA) and 2R (GCTGCGTTCTTCATCGATGC), through the Australian Genome Research Facility (AGRF – www.agrf.org.au) sequencing service.

Full details for methods used for the bioinformatic analysis of the amplicon sequence data to determine the abundances of individual genera/species and the composition of total fungal community are similar to those described in Gupta et al. (2019). Representative sequences from each OTU were then classified by using the RDP

Naïve Bayesian Classifier with the Warcup training set (Deshpande et al. 2016).

Phylogenetic analyses and sequence alignment

The sequence data described above, obtained from the fungal isolates recovered from diseased cotton plants, were combined with the amplicon data from the community study for phylogenetic and sequence alignment studies. A set of 491 *Eutypella* reference sequences was downloaded from the GenBank nucleotide collection (searching for “eutypella internal transcribed spacer" on 13/5/2021). These sequences were reduced in number by clustering them at 97% identity, using usearch cluster_fast (Edgar 2010), and any sequences containing indeterminate bases (‘N’) were also discarded. This process left a set of 53 distinct ‘species-level’ sequences for comparing to the isolates. The isolates, amplicons and the various GenBank sequences had been produced using several different PCR primers, and subsequently differed in starting location, ending location and length. All the sequences were trimmed on the right at the end of ITS2/start of the LSU to remove any extraneous LSU sequence data. All the sequences started near the beginning of the ITS1 region, with the RM sequences starting slightly further into ITS1 than the others, and the OTU sequences slightly further back into the SSU. This ragged start to the sequences being compared led to small differences in the similarity metrics from otherwise identical sequences. This was corrected by trimming all the sequences to the start of the RM sequences, at location 1809 under the positioning scheme used in Toju et al (2012). This trimming at the start and tail produced a set of isolate and reference sequences that covered either almost all the ITS1 region, or the complete ITS1, 5.8S and ITS2 regions.

The complete set of amplicons, isolates, and clustered GenBank sequences was aligned using both Clustal Omega (Madeira et al. 2019) and Muscle (Edgar 2004). This initial alignment showed that the isolates were closest to, but distinct from, a group of *E. scoparia* sequences. The average identity of the isolates to this *E. scoparia* group was only 86% and was only 70% to the more distant Eutypella sequences. This ‘*E. scoparia*’ group of 12 distinct sequences was regarded as the nearest neighbours to the isolates and used in in the remaining comparisons. A full phylogenetic tree built from this initial set of Eutypella sequences can be found in Supplementary Fig. S1. Clustal Omega was also used to generate phylogenetic trees and identity matrices using the default parameters for alignment and tree construction (Madeira et al. 2019). Muscle was used to produce alignments, and these were then visualised using JalView (Waterhouse et al. 2009). The phylogenetic trees were plotted using FigTree (Rambaut 2018).

Pathogenicity assay

Pathogenicity of *Eutypella* species (CQ9-3a) was determined by inoculating cotton (*Gossypium hirsutum* variety Sicot 714 B3F) with the fungal isolate using two methods. For method 1, mycelial growth was scraped from a ¼PDAS plate into water. Using a hypodermic needle and syringe, the visually dense mycelial suspension was injected into vascular tissue of five-week-old plants between the cotyledons. For the control, sterile water was used. Plants were grown from seed in University of California (UC) potting mix consisting of 27% sand, 40% peat and 33% gravel plus nutrients (g/L: Blood and Bone 1.33, Dolomite 3.33, Super Phosphate Fine 1.33, Lime 1, Micromax 0.33, Potassium Nitrate 0.13, Potassium Sulphate Granular 0.13) in 10 cm diameter pots. Treatments were replicated five times ($n = 10$). The inoculated area was covered with Parafilm®. Plants were harvested 12 weeks after inoculation and examined for necrosis and vascular discolouration. Necrotic tissue developing outside the area of inoculation were cultured on growth medium (¼PDAS) and fungal isolates obtained from re-isolation were identified based on their morphology (colony shape, colour, growth rate) and by sequencing the ITS region as described earlier to fulfill Koch’s postulates. Healthy stem tissue was also plated on growth medium (¼PDAS).

For method 2, mycelial ¼PDAS plugs (5 mm) taken from the margin of a 14-day-old culture or sterile ¼PDAS plugs (control) were placed on one wound per stem using five mature plants per treatment ($n = 10$). Wounds were made into the core of the stem using a sterile scalpel blade. Inoculated wounds were wrapped in Parafilm®. Plants were grown from seed in UC potting mix in a controlled environmental cabinet for three months, cut above node two to remove growth. Once plants had new leaf growth, stems were inoculated. Using mature plants provided plants with stems that had a larger surface area for wounding and inoculation. Plants were harvested three weeks after inoculation and examined for necrosis and vascular discolouration. Recovery of inoculated fungus and identification was performed as described for Method 1 to fulfill Koch’s postulates.

Variety Sicot 714 B3F was chosen for pathogenicity assays as this was the variety from which the isolate was originally recovered in the field, and therefore known to be susceptible to this disease. The growth cabinet conditions were set to 28 °C, 16 h light/20 °C 8 h dark and 60% humidity. Pots were watered carefully to approximately field capacity, then watered as required.

Bioassay using naturally infested field soil

Field soil was collected from around the roots of cotton that had died from the disease Reoccurring wilt. The field was in Central Queensland where *Eutypella* sp. was first detected.

Five cotton plants (var. Sicot 714 B3F) were grown from seed in 15 cm pots in a controlled environmental cabinet for six months. Growth cabinet conditions were the same as described for pathogenicity assays. Plants were watered weekly until five months old. After this time watering was reduced to once a fortnight to provide water stress. At harvest, roots (tap and lateral) and stem vascular tissue from healthy and diseased plants were plated on ¼PDAS for pathogen recovery.

Results

Field symptoms

The initial symptoms of Reoccurring wilt observed in the field was a sudden wilting of plants, with the leaves and petioles developing a bronzed appearance. Death followed quickly, within one week of wilt commencement. Disease symptoms were observed to develop from squaring and throughout the growing season (Fig. 1a, b). The leaves remained attached on wilting and dying plants and stems of diseased plants become blackened (Fig. 1c, d). In cross-section, infected stem tissue had a wedge-shaped appearance and was reddish—grey in colour, and when bark was removed, reddening of the vascular tissue was observed (Fig. 1e-h).

Field observations of disease development over three seasons

Disease was first observed late in the season as a couple of dead plants in a single location. In the following season, plants succumbed to disease in the same location, however the area of diseased cotton plants had increased in size. In the third cotton crop, at the onset of disease symptoms, the

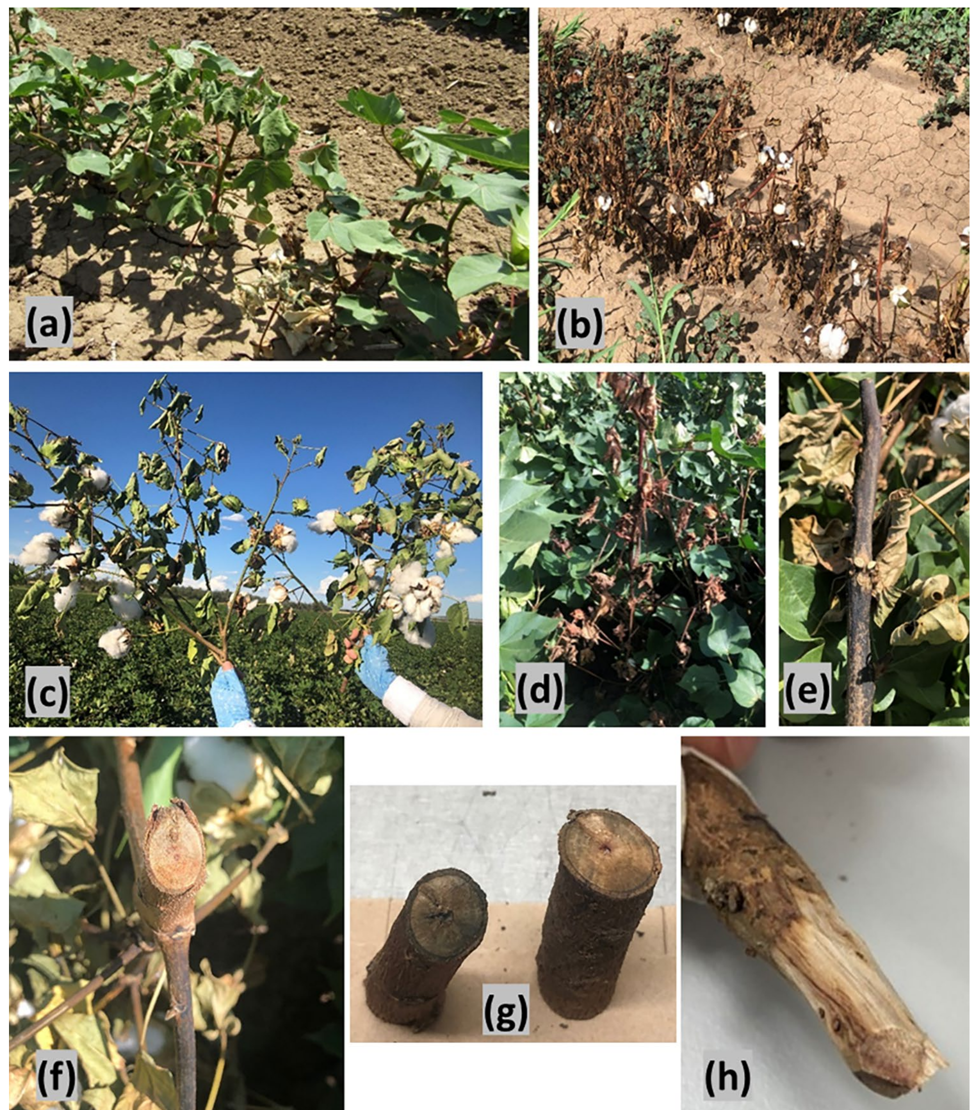

Fig. 1 Symptoms of Reoccurring wilt in field grown plants: Wilting and plant death early season (**a**) and significant plant death end of season (**b**) in Central Queensland. Wilting plant with leaves remaining attached (**c**), single, dead plant amongst healthy cotton (**d**), blackening of the stem (**e**), infected tissue may possess a wedge-shaped appearance and has a reddish—grey colour (**f**, **g**) and when bark is removed reddening of the vascular tissue may be evident (**h**) (Photos Linda Smith, DAF)

pattern of plant death was more closely monitored through the season. The initial location of diseased plants increased significantly, spreading across and down the rows. The pattern of spread down the row did not display movement like Fusarium wilt in furrow irrigated cropping, where the pathogen is carried down the row in irrigation water. The spread of the disease within the row was displayed as death of adjacent plants that continued through the season, in both directions, resulting over time in 20 or more dead plants in a row. The development of scattered single infected plants situated meters away from dead plants was common and occurred through the season. Over time the healthy plants adjacent to diseased plants at these new sites of infection also succumbed to disease. There was a consistent display of sudden wilting and dying of plants throughout the season.

Characteristics of fungal cultures

Diatrypaceae isolates were characterised by having white to white-cream cottony slow-growing low dense aerial mycelium on ¼PDAS which gradually darkened in the centre (Fig. 2a, b). The isolates reported in this work did not vary greatly in culture morphology. Spores were not readily produced on artificial media, with only one isolate forming small pycnidia (Fig. 2c). Conidia were filiform and mostly curved in shape measuring 11.31-19.34 µm long × 1.06-2.08 µm diam. (average $15.92 \pm 2.36 \times 1.80 \pm 0.30$, $n = 10$) (Fig. 2d). Pycnidia were only observed to develop in plate culture after at least four months at ambient laboratory light and temperature conditions. These morphological features correspond to descriptions of species in the Diatrypaceae family (Glawe and Rogers 1984). In all diseased plants sampled, *Eutypella* sp. was the predominant fungus recovered from the roots and blackened stem.

DNA extraction and polymerase chain reaction amplification of recovered isolates from dead cotton plants

The ITS PCRs amplified products larger than 1 kb for sequencing. Initial BLAST search results indicated the 13 isolates in this study had 96-97% homology with *E. scoparia*. All sequencing data have been deposited in the public database GenBank and is available with no restrictions (Table 1).

Genetic analysis of fungal communities from root and soil samples

A total of 279,347 (average 69837 ± 7277) of ITS-region quality filtered sequences were obtained from the four root and five soil samples samples which gave on average 54

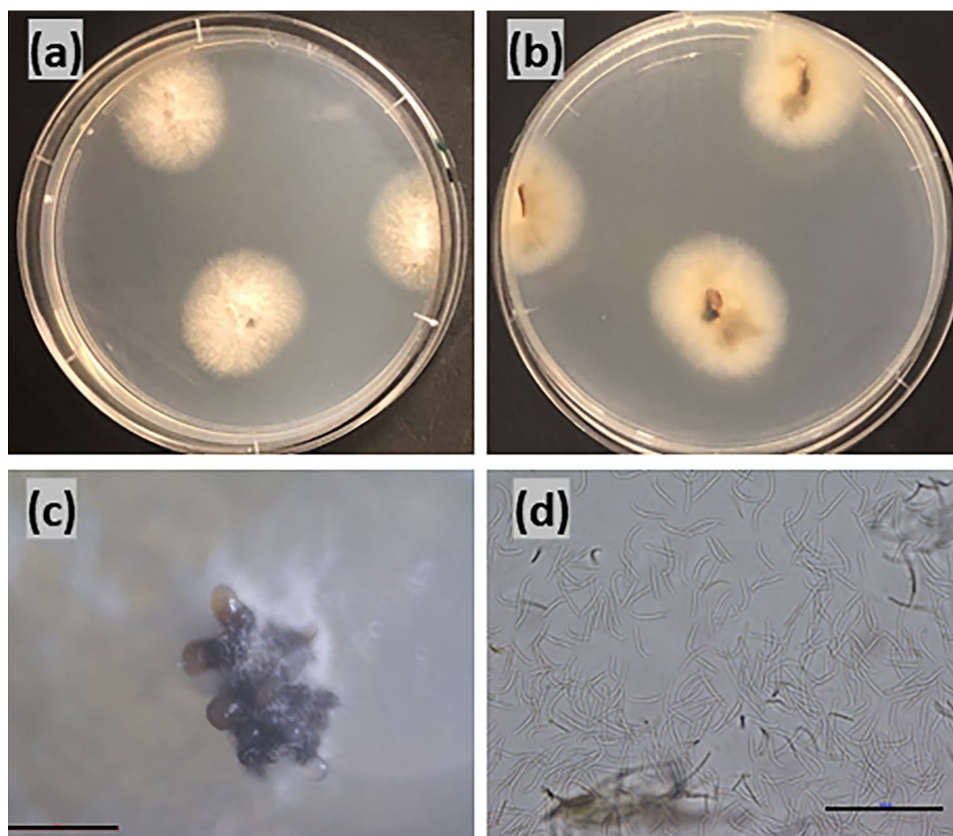

Fig. 2 Culture characteristic of *Eutypella* species on quarter strength potato dextrose agar amended with 50 mg/L of Streptomycin Sulphate after 10 days incubation at ambient laboratory light and temperature conditions (**a** = colony from above, **b** = colony from below). Conidial masses emerging out of the pycnidia (**c**) and hyaline, filiform conidia (**d**) after 6 months incubation. Scale bar is 500 µm (**c**) and 50 µm (**d**) (Photos Linda Smith, DAF)

fungal OTUs per root sample and 205 fungal OTUs per sample for soils. In each root sample, four to eight OTUs accounted for > 95% of sequences. In general, fungi belonging to the phylum Ascomycota accounted for > 95% of the fungal community and only 14 OTUs belonging to nine families contained > 1% of sequences. The genus *Eutypa* (represented by two OTUs of *Eutypella scoparia;* 81.7 to 89.7% sequence identity) were the most abundant fungi accounting for 45 to 99% of all sequences in the roots (Table 2). A total of 12 genera showed > 0.5% of total sequences. Unusually, no OTUs belonging to the phylum Glomeromycota, the group containing mycorrhizal fungi, were detected in the root samples.

Results for the composition of fungal community in the soils near the diseased roots indicated the presence of fungi belonging to five classes, 17 families with just 35 genera accounting for 99% of the total soil fungal community (Fig. S2). There was a total of 72 genera found in all the five samples with an average value of 58 ± 5 per sample. Members belonging to the families Dothideomycetes and Sordariomycetes in the phylum Ascomycota were the most abundant groups with an average value of 90 ± 5% per sample

Table 2 Genetic composition of fungal communities associated with diseased cotton plant roots, with classification as per Warcup reference dataset (Deshpande et al. 2016). The most abundant OTUs accounting for > 92.5% of total sequences are presented here, along with the identified pathogens. The full OTU table from the study can be found in Supplementary Table S1

OTU#	% of total sequences	Phylum	Class	Family	Genus	Species	% Identity
Sample #:Root 1							
OTU_2	44.6	Ascomycota	Sordariomycetres	Diatrypaceace	*Eutypa*	***Eutyphella scoparia***	89.7
OTU_3	38.1	Ascomycota	Dothideomycetes	Didymellaceace	*Atradidymella*	*Phoma fungicola*	98.9
OTU_5	5.5	Ascomycota	Sordariomycetres	Nectriaceace	*Gibberella*	*Fusarium equiseti*	100
OTU_10	5.2	Ascomycota	Dothideomycetes	Botryosphaerlaceace	*Botryosphaeria*	*Lasiodiploadia parva*	100
OTU_9	1.4	Ascomycota	Sordariomycetres	Nectriaceace	*Fusarium*	*Fusarium delphinoides*	100
OTU_4	1.2	Ascomycota	Dothideomycetes	Pleosporaceace	*Alternaria*	*Alternaria alternata*	100
Sample #:Root 2							
OTU_2	52.3	Ascomycota	Sordariomycetres	Diatrypaceace	*Eutypa*	***Eutyphella scoparia***	89.7
OTU_5	15.9	Ascomycota	Sordariomycetres	Nectriaceace	*Gibberella*	*Fusarium equiseti*	100
OTU_7	10.7	Ascomycota	Sordariomycetres	Nectriaceace	*Haematonectria*	*Fusarium falciforme*	100
OTU_4	5.5	Ascomycota	Dothideomycetes	Pleosporaceace	*Alternaria*	*Alternaria alternate*	100
OTU_13	3.1	Ascomycota	Eurotiomycetes	Trichocomaceace	*Petromyces*	*Aspergillus flavus*	100
OTU_15	2.2	Ascomycota	Dothideomycetes	Mycosphaerellaceace	*Davidiella*	*Cladosporium rectoides*	100
OTU_17	1.7	Ascomycota	Sordariomycetres	Plectosphaerellaceace	*Verticillium*	*Verticillium_ leptobactrum*	100
OTU_14	1.2	Ascomycota	Sordariomycetres	Hypocreales Incertaesedis	*Acremonium*	*Acremonium_ brachypenium*	90.5
Sample #:Root 3							
OTU_1	92	Ascomycota	Sordariomycetres	Diatrypaceace	*Eutypa*	***Eutyphella scoparia***	81.7
OTU_4	3.7	Ascomycota	Dothideomycetes	Pleosporaceace	*Alternaria*	*Alternaria alternata*	100
OTU_17	0.7	Ascomycota	Sordariomycetres	Plectosphaerellaceace	*Verticillium*	*Verticillium_ leptobactrum*	100
OTU_3	0.5	Ascomycota	Dothideomycetes	Didymellaceace	*Atradidymella*	*Phoma fungicola*	98.9
OTU_15	0.5	Ascomycota	Dothideomycetes	Mycosphaerellaceace	*Davidiella*	*Cladosporium rectoides*	100
OTU_44	0.4	Ascomycota	Dothideomycetes	Didymellaceace	*Didymella*	Stagonosporopsis_ dorenboschi	100
Sample #:Root 4							
OTU_1	99.4	Ascomycota	Sordariomycetres	Diatrypaceace	*Eutypa*	***Eutyphella scoparia***	81.7
OTU_257	0.2	Ascomycota	Sordariomycetres	Diatrypaceace	*Eutypa*	***Eutyphella scoparia***	86.7
OTU_75	0.1	Ascomycota	Sordariomycetres	Hypocreales Incertaesedis	*Sarocladium*	*Sarocladium strictum*	99.5
OTU_15	0.1	Ascomycota	Dothideomycetes	Mycosphaerellaceace	*Davidiella*	*Cladosporium rectoides*	100

(Fig. S2). Members from the phylum Basidiomycota were mainly found in the soil samples (2.8 to 28% of total OTUs) with just 0.2% of total sequences in the root samples. Average values for alpha-diversity measures such as Shannon index (H) and species richness (Margalef species richness, d) were 4.2 ± 0.1 and 57 ± 6, respectively.

Phylogenetic analyses and sequence alignment

The alignments generated using Muscle were used to infer the phylogenetic relationships of our isolates and other *Eutypella* species. The isolates and amplicons group into three clusters, two quite close together and one more distant, and all quite distinct from other published *E. scoparia* (Fig. 3). The relationships between these sequences becomes even clearer after looking at the actual sequence alignments. The first alignment (Fig. 4) shows just the ITS1 region which is present in all the isolates, amplicons, and reference sequences. The second alignment (Fig. 5) shows the ITS2 region which is found in only the isolates and reference sequences. The striking features in all these alignments are the large insertions in both the ITS1 and ITS2 regions. The isolates are not different from *E. scoparia sp.* just because of minor substitution differences, but because of these insertions. Most of the isolates have a 30 bp insertion in ITS1, with the OTU_2/RM3 group being the exception. A similar insert is only found in one of the GenBank sequences (MK59553 from US prairie soil). The OTU_2/RM3 group

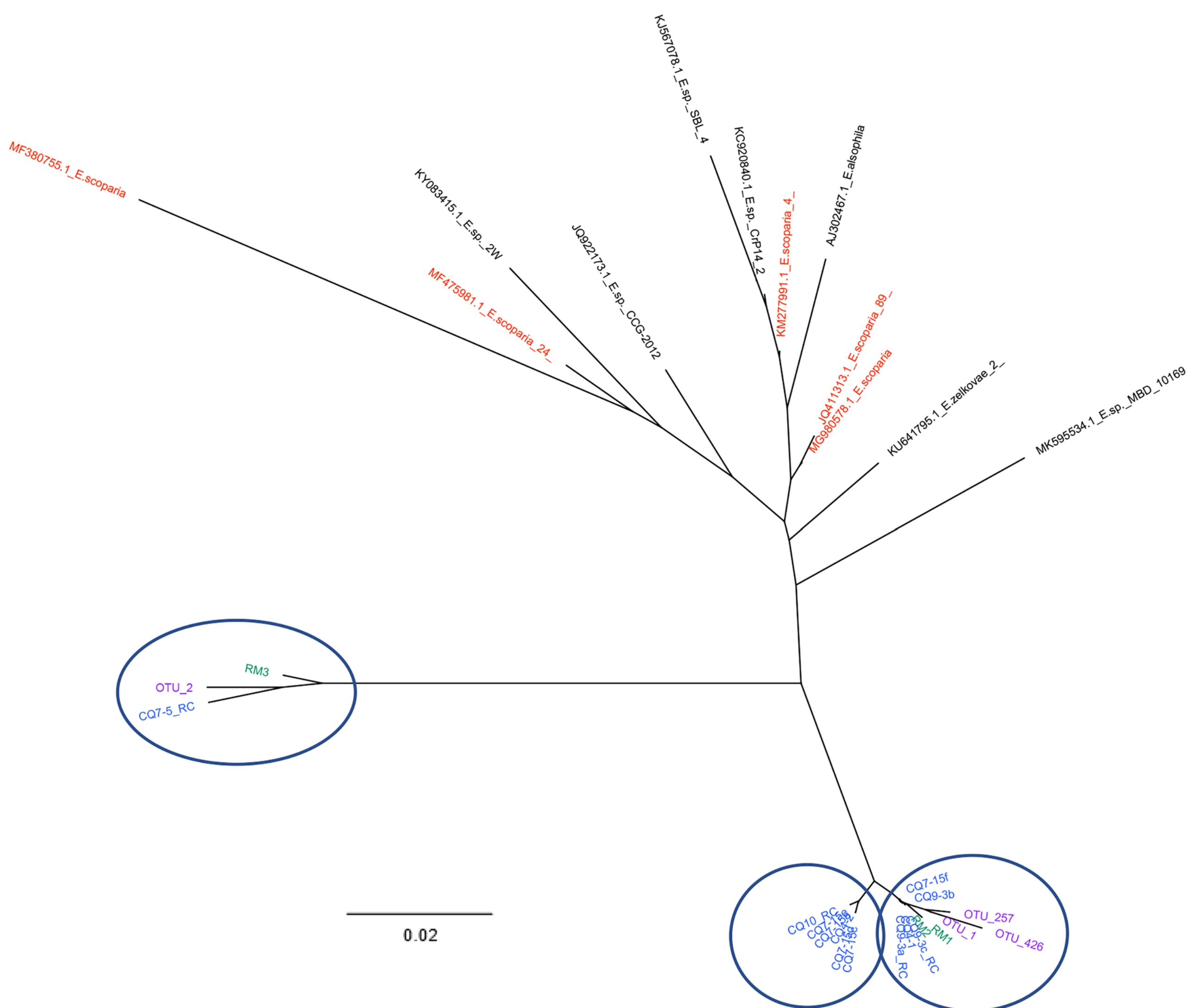

Fig. 3 Phylogenetic tree generated based on trimmed ITS1 (amplicons) and ITS1 + 5.8S + ITS2 (isolates) sequences. The specimens in this study are: *Eutypella* isolates from diseased cotton plants in blue, isolates from diseased cotton roots (RM3ex-type strains of *Eutypella scoparia)* in green, community OTU amplicons in purple) and other neigbouring *Eutypella* species from Genbank in black (and red for those identified as *Eutypella scoparia*)

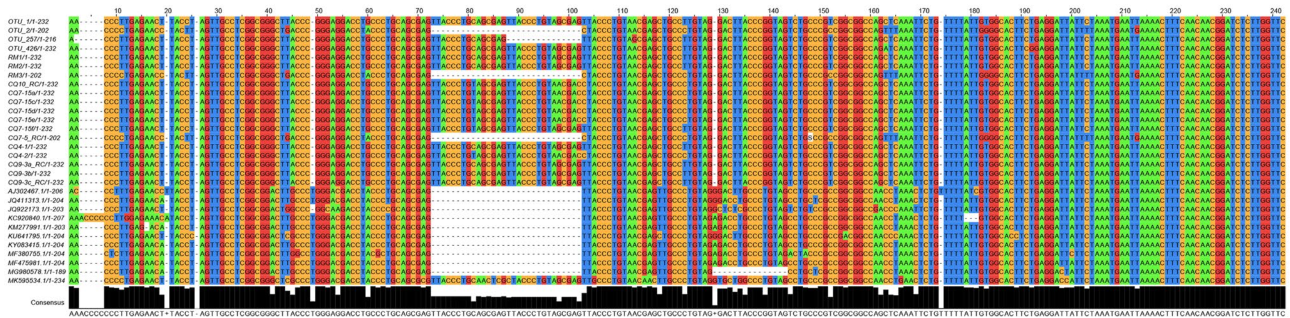

Fig. 4 Sequence alignments in the ITS1 region. The aligned sequences have all been trimmed to the start of the region found in the RM isolates, and finish at the start of the 5.8S region

does not have this ITS1 insert, and neither do the reference *E. scoparia sp.*, but this group is distinguishable from the other isolates by having two inserts in the ITS region (OTU_2 doesn't include the ITS2 region, but it is identical to RM3 in ITS1). Overall, these alignments and phylogenetic trees suggest that there are two species present in the isolates, and both of these are quite different from published *E. scoparia* and related *Eutypella* sp.

Pathogenicity assay

Method 1 needle inoculation

The inoculation point became swollen and necrotic in plants treated with *Eutypella* isolate (BRIP 70529a) whereas there was no reaction to water inoculation. Figure 6a and b shows the reaction eight days post inoculation.

Plants were harvested 12 weeks after inoculation. Plants inoculated with *Eutypella* sp. BRIP 70529a produced a swollen necrotic wound at the inoculation point (Fig. 6c). Beneath the bark the core of the stem was black extending from the inoculation point (Fig. 6d, e). There was no evidence of swelling, necrosis or blackening of stem tissue with water inoculation (Fig. 6f, g). No foliar symptoms were observed in the experiments conducted. Fungi resembling *Eutypella* were recovered from all fungal inoculated treatments. Results of sequencing confirmed that the same *Eutypella* species was re-isolated thereby fulfilling Koch's postulates. No fungi were recovered from the control treatment.

Method 2 mycelial plug inserted into wound

A wound was made in the mature stem to insert plug. Within three weeks a necrotic callous was observed in fungal inoculated plants (Fig. 7a). When the necrotic callous was cut away using a scalpel, the extent of discolouration was evident (Fig. 7b, c) and observed to extend 3 cm up the stem from the inoculation point and 1.5 cm below (Fig. 7d). In the control, stem wounding resulted in a mild stem browning (Fig. 7e, f). Fungi recovered from fungal inoculated plants were morphologically identical to *Eutypella* sp. Results of sequencing confirmed that the same *Eutypella* sp. was re-isolated thereby fulfilling Koch's postulates. No foliar symptoms were observed in the experiments conducted.

Bioassay using naturally infested field soil

After six months, of which the fifth month was under water stress, one out of five plants grown from seed in field soil suddenly wilted and died after four weeks of water stress. Other symptoms included leaves remaining

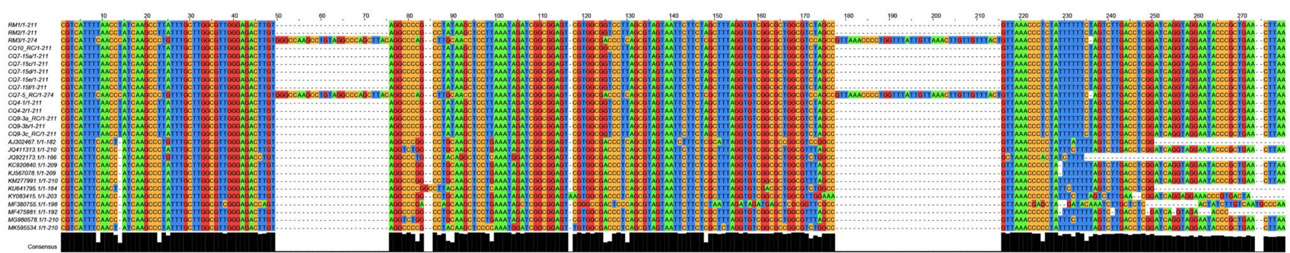

Fig. 5 Sequence alignments in ITS2 region. This alignment starts at the start of the ITS2 region (end of 5.8S) and finishes at the end of ITS2/start of LSU. Only the isolates and the reference sequences are included here as the primers used for the community profiling study only covered the ITS1 region

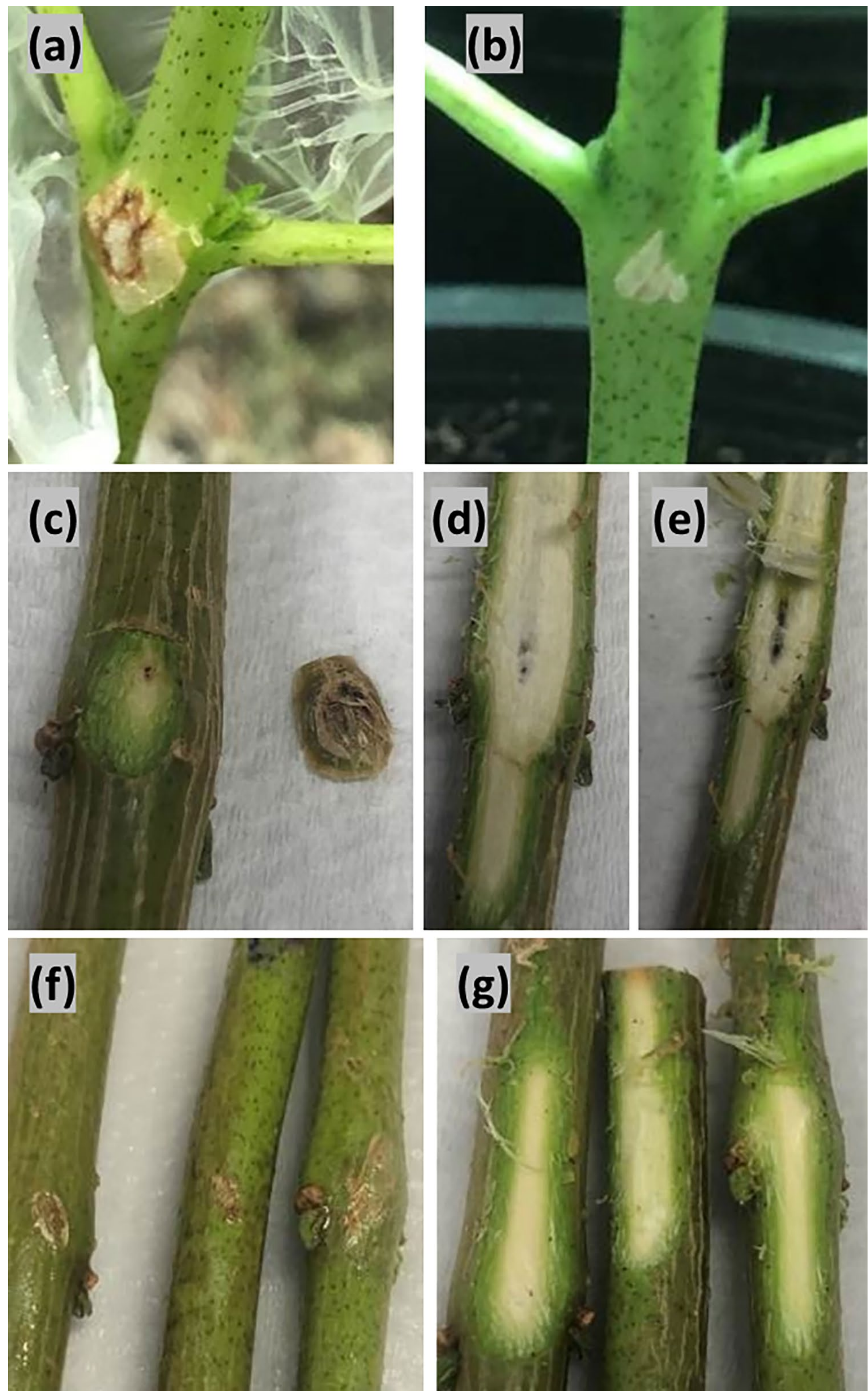

Fig. 6 Reaction eight days post needle inoculation with *Eutypella* isolate BRIP 70529a (**a**) and sterile water (**b**). After 12 weeks a swollen necrotic wound and internal stem blackening was observed when vascular tissue was removed using a scalpel blade (**c**, **d**, **e**). Plants inoculated with water as a control did not result in swelling, necrosis or blackening of stem tissue (**f**, **g**) (Photos Linda Smith, DAF)

attached, blackened stem, dry rot of roots and internal blackening of roots and stem (Fig. 8a-c). These symptoms were typical of those observed under field conditions for Reoccurring wilt. Numerous isolations from the tap root and blackened stem of this plant yielded *Eutypella* sp. In the tap root *Eutypella* sp. was predominately isolated, and from the stem, *Eutypella* sp. was the only fungus recovered, confirmed by morphology and molecular

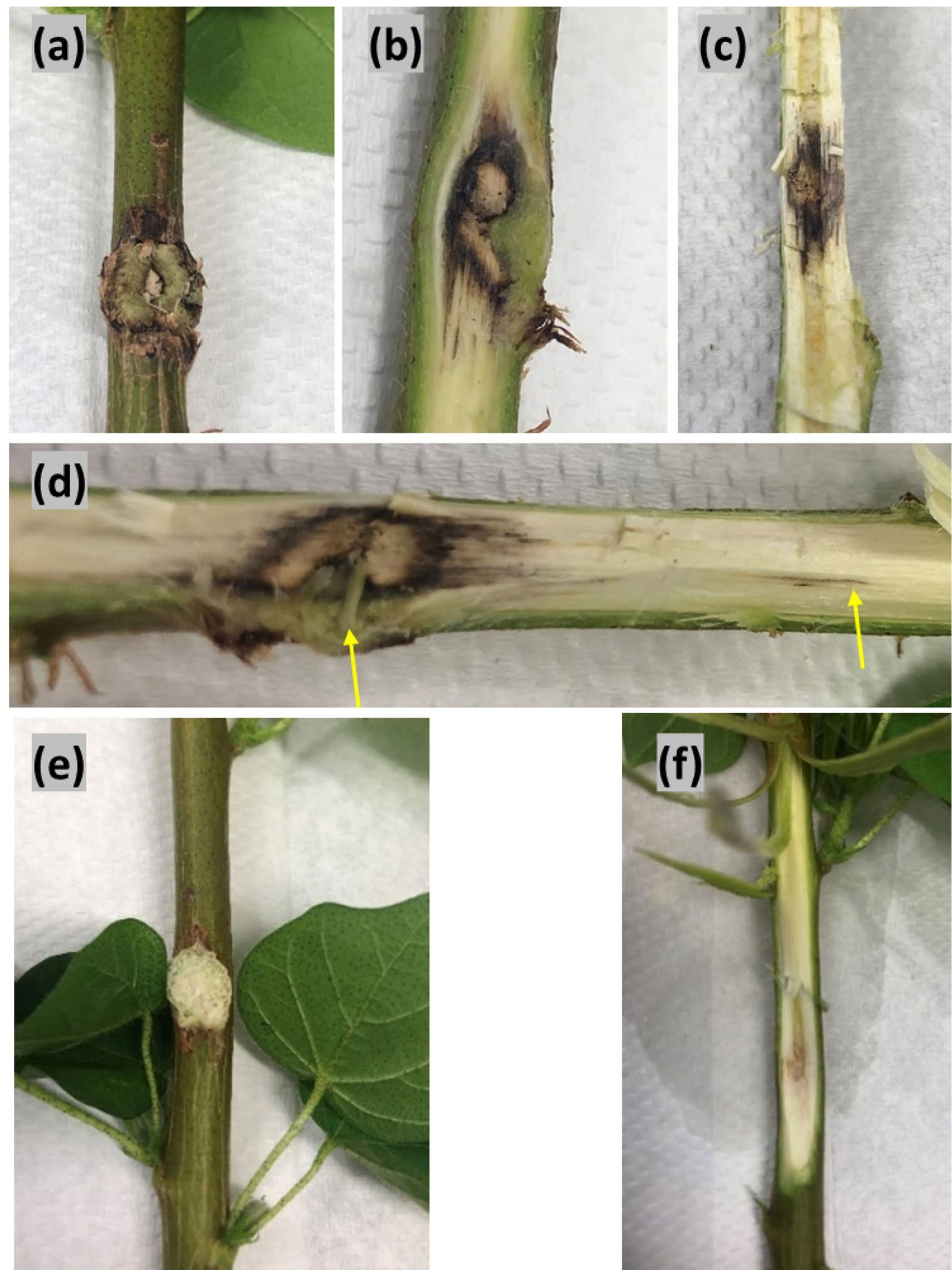

Fig. 7 Inoculation point (**a**) and blackening within stem core three weeks after inoculation with *Eutypella* isolate BRIP 70529a using the agar plug method (**b**, **c**). Vascular blackening extended both directions from inoculation point, with arrows indicating inoculation point and vascular discolouration 3 cm up the stem (**d**). Three weeks post inoculation with agar plug for control. Inoculation point (**e**) and slight browning internally at inoculation point (**f**) (Photos Linda Smith, DAF)

characterisation (Table 3). The four remaining plants appeared healthy based on above ground symptoms. The roots of these plants were larger with more lateral roots compared to the diseased plant. No dry rot was observed on roots of healthy plants. However, there was necrosis of some lateral roots (Fig. 8d). Plating of necrotic roots yielded the fungus *Rhizopycnis vagum*, which was confirmed by molecular characterisation (Table 3). No *Eutypella* sp. were recovered from healthy stem or root tissue.

Discussion

In recent years, there have been numerous studies conducted worldwide on branch and trunk canker diseases caused by species of Diatrypaceae on various woody plants (Carter 1957, 1991; Mayorquin et al. 2016; Moyo et al. 2018; Paolinelli-Alfonso et al. 2015; Pitt et al. 2013; Trouillas et al. 2011). However, to our knowledge, there have been no reports of species in this family associated

Table 3 Identification of fungi recovered from diseased cotton plants in bioassay studies to confirm pathogenicity of *Eutypella* isolate (BRIP70529a) and natural infection in field soil

Bioassay study	Inoculation method	Disease symptoms	Tissue sampled	Isolate No	Molecular identification[a]	Identification based on morphology
Inoculation with *Eutypella* sp. (BRIP70529a)	Wounded tissue, colonised agar plug	Swollen necrotic wound and internal blackening of stem	Surface canker/ lesion	1, 2	*Eutypella scoparia*	*Eutypella* sp.
			Black centre of stem	3, 4	*Eutypella scoparia*	*Eutypella* sp.
	Hypodermic needle, mycelial suspension	Swollen necrotic wound and internal blackening of stem	Blackened stem	5	*Eutypella scoparia*	*Eutypella* sp.
Naturally infested field soil	Natural infection	Sudden wilting and plant death, typical symptoms of Reoccurring wilt	Blackened tissue of tap root	6	*Eutypella scoparia*	*Eutypella* sp.
			Tap root, vascular tissue	7, 8	*Eutypella scoparia*	*Eutypella* sp.
			Rotten surface of tap root	9	*Eutypella scoparia*	*Eutypella* sp.
			Upper stem	10, 11	*Eutypella scoparia*	*Eutypella* sp.
		No symptoms of Reoccurring wilt, roots generally healthy, but with some necrosis	Necrotic roots	12, 13 (BRIP 72091a)	*Rhizopycnis vagum*	-

[a]Characterisation of ITS region; 96—97% homology within *Eutypella scoparia*

with diseased cotton plants. This study reports for the first time, species in the Diatrypaceae family associated with death of plants in commercial cotton fields in Australia. These species were associated with symptoms that include sudden wilting followed by rapid plant death occurring from the squaring stage and throughout the growing season to mature plants, external stem blackening, internal reddening of roots and root decay, and in cross section the infected tissue often possessed a wedge-shaped appearance and had a reddish—grey colour. Using morphological and molecular sequence data (ITS gene region), two distinct *Eutypella* species, both of which are undescribed, were identified from cotton stem and root samples showing necrosis and vascular discolouration. One of the *Eutypella* sp. was also confirmed as a pathogen of cotton in pot trials because it could produce stem canker, necrosis, and vascular discolouration of cotton stems, typical of trunk disease.

Under field conditions, foliar symptoms associated with Reoccurring wilt include sudden wilting, bronzing of leaves and petioles, and plant death, with leaves remaining on the plant. However, no foliar symptoms were observed in the two inoculation experiments under controlled environmental conditions. The conditions conducive to foliar symptom development are not yet understood and may not have been

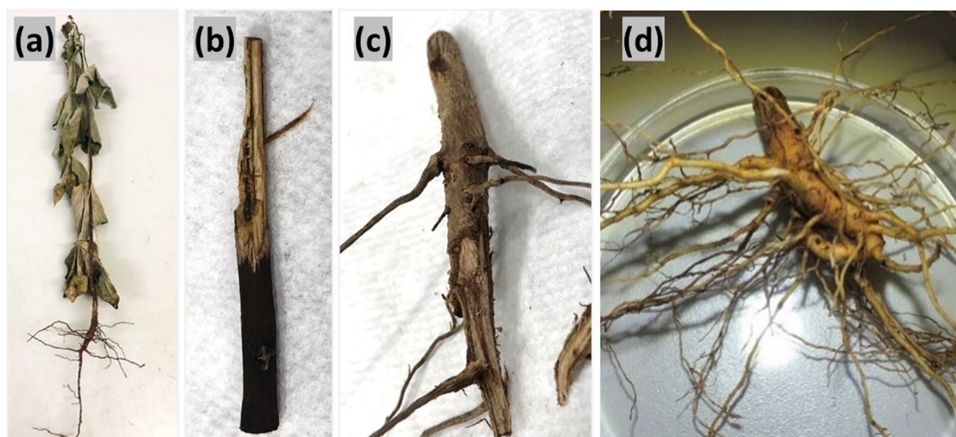

Fig. 8 Sudden wilting and death of cotton grown from seed in naturally infested field soil six months after planting. Whole plant (**a**); segment of blackened stem with internal blackening (**b**); and dry rot of roots with internal reddening and blackening (**c**). Unhealthy root system of cotton plant that appeared healthy above ground with no foliar symptoms yielded *Rhizopycnis vagum* from necrotic roots plated onto ¼PDAS (**d**) (Photos Linda Smith, DAF)

met in the pot experiments conducted, given that pathogenicity of *E. lata* in grapevine is triggered when host plants are under stress (Sosnowski et al. 2011). In pot trials, where cotton was grown for five months in soil collected from a pathogen infested field, followed by one month under water stress conditions, one plant out of five developed the full known range of symptoms typical of Reoccurring wilt. These results suggest that *Eutypella* pathogenicity to cotton may also be triggered when plants are under abiotic stress.

Diatrypaceae species are known to be associated with wedge-shaped necrosis on grapevine (Pearson and Burr 1981), with *E. lata* found to be the most dominant species with this symptom type in the study by Moyo et al. (2018). Cankers and dieback in grapevine were for a long time attributed to the diatrypaceous fungus *E. lata* (Moller and Kasimatis 1978). However more recently, different species within and between Botryosphaeriaceae and Diatrypaceae are frequently isolated together from the cankers, suggesting that grapevine trunk diseases are caused by complexes of several pathogens (Úrbez-Torres et al. 2009). A study by Pitt et al. (2013) showed that in addition to *E. lata*, seven species, including *Eutypella* were pathogenic on grapevine. In our study, fungal isolations from diseased cotton stems were dominated by the species *E. scoparia* (96—97% homology). To confirm that *Eutypella* species were the dominant coloniser of diseased cotton and not a biased result through the ability of the pathogen to be cultured on artificial media, the fungal species present in four diseased cotton roots were determined using amplicon (ITS1 region) sequencing of total fungal communities. Results concurred with isolation of culturable fungi, that the most abundant species was *E. scoparia*, which was represented by two closely related fungal OTUs, accounting for 45 to 99% of all sequences found in the diseased plant roots. Interestingly, OTU_1 accounted for 92 to 99% of all sequences in root samples 3 and 4, with no OTU_2 detected. In root samples 1 and 2, OTU_2 accounted for 44 to 52% of all sequences with no OTU_1 detected. Other fungal pathogens identified in diseased cotton roots that have been associated with stem and trunk canker and the decline of tree crops, include *Lasiodiplodia parva*, *Phoma fungicola* and *Fusarium falciforme* (Correia et al. 2013; Taieb et al. 2014; Crespo et al. 2019). However, these species made up a relatively smaller percentage of the total fungal communities associated with diseased cotton plant roots compared to *E. scoparia*, apart from *P. fungicola* accounting for 38% of all sequences in root sample 1. Whether Reoccurring wilt is caused by a complex of several pathogens like grapevine trunk diseases is not known and requires investigation.

The observation of a very low number of OTUs ($<0.4\%$ of total) representing the genus *Eutypa* in the soil samples (Fig. S2) suggests that it is not a dominant member of the soil fungal community even in diseased soils. Interestingly, no OTUs belonging to the phylum Glomeromycota, the group containing mycorrhizal fungi, were detected in the root samples and only one OTU in one soil sample. Mycorrhizal associations assist emerging cotton seedlings to access phosphorus and zinc more efficiently and are essential for normal cotton growth (Allen and Nehl 1999). In Australian cotton, arbuscular mycorrhizal fungi can colonize 50–70% of root length under field conditions (Eskandari et al. 2017). Mycorrhizal colonization in cotton plants assists with early season root and shoot development and increased growth rate and help plants to counter the stresses imposed by physico-chemical soil constraints (Eskandari et al. 2017). It would be beneficial to understand at what point in the cotton growing cycle that mycorrhizal colonisation of the roots was affected. In addition, no OTUs related to known beneficial fungal genera such as *Trichoderma* were found either in the roots or soil samples. In general, there was a less diverse fungal community in soil samples collected from near the diseased roots. For example, genetic diversity of fungal community indicated lower alpha-diversity measures such as Shannon index (H) and species richness (d) values, which were 4.2 ± 0.1 and 57 ± 6, respectively, compared to that generally observed in cotton fields, where H was 5.44 ± 0.04 and d was 188 ± 6 (Gupta et al. 2019; Cotton RDC Project Final Report unpublished). Our results clearly indicate that the root samples analysed represent diseased plant roots as healthy plant roots generally harbour a more diverse fungal community.

Members of the Diatrypaceae family are characterised by perithecia, which are small flask-shaped fruiting bodies that contains the sexually produced ascospores formed within an ascus (Glawe and Rogers 1984). In grapevines, perithecia of *E. lata* are formed in old, infected parts of vines (Trouillas and Gubler 2010). Under favourable environmental conditions, ascospores are discharged and spread by rain droplets and/or wind to short or long distances. (Rolshausen et al. 2015; Gramaje et al. 2018). Observations of disease spread in cotton fields suggest that the potential mechanism of dispersal could be via aerial spores rather than movement of inoculum in the soil. The pattern of spread down the rows did not appear to resemble the movement of soil-borne pathogens such as *Fusarium oxysporum* f. sp. *vasinfectum* in furrow-irrigated cotton, where the pathogen is carried down the row in irrigation water or in soil moved down the rows by farm implements (Davis et al. 2006). The spread of the disease Reoccurring wilt within a row was displayed as a plant developing symptoms and dying next to healthy plants, with this pattern of infection and death continuing through the season, in both directions along the row, as well as single infected plants developing at a distance within the row. Also, the development of disease across the rows and deeper in the field appeared initially as scattered single infected plants situated a metre to several meters away from dead plants,

which then expanded along the row, suggesting dispersal of the pathogen from a distance, potentially aerially.

The infection process for *E. lata* in grapevine is well understood. Ascospores land on susceptible exposed xylem tissues from pruning wounds or cuts, where they germinate and start new infections, colonising and killing the internal tissue as it progresses towards the base of the trunk (Rolshausen et al. 2015; Gramaje et al. 2018). When all woody tissue (xylem and phloem) is dead, transport of water and nutrients from the roots is stopped, and the vine dies. It is interesting that *E. lata* grows to the base of the trunk but not into the roots (Sosnowski 2018). For Reoccurring wilt, infection of the pathogen is not yet understood, however unlike *E. lata*, the cotton pathogen is capable of colonising both the above ground and below ground tissue, which is characterised by internal wedge-shaped lesions of dead tissue, confirmed by isolation of the pathogen. In addition, *Eutypella* sp. is also able to displace other root microbes such as mycorrhizae. Although *Eutypella* sp. was isolated from the roots of dead cotton plants and shown to cause disease typical of trunk canker when inoculated into the stem of cotton, an attempt to inoculate healthy cotton roots using mycelium to initiate disease was not successful. The pathogen was not able to be recovered from surface sterilised tap root or stem tissue on growth media collected from 12-week-old plants (unpublished data). Mycelium was used in pathogenicity tests because mycelium of *Eutypa* has been shown to be infectious, whereas conidia are not (Carter 1991). These results suggest that either the pathogen is not able to infect directly into the roots or the conditions were not conducive for infection and disease development. Further research is underway to determine mode of infection. In addition, it is understood that soil-borne pathogens are generally higher in root-zone soils that have diseased plants. Community analysis of the soil surrounding diseased roots showed that there was a low population of Diatrypaceae suggesting that this cotton pathogen may not be soil-borne, further supporting that the entry of the cotton pathogen may not be from the soil into the roots.

If the cotton pathogen is not soil-borne with infection initiated in the roots, the death of cotton grown in naturally infested field soil in pot assays must have been via another mechanism. In grapevines, although *E. lata* does not grow into the roots, it is able to colonise tissue from above ground infection and move down the vine to the base of the trunk (Sosnowski 2018). Dead wood with blackened surfaces is characteristic of infection by *E. lata* in grapevines, and when on the ground has been shown to be a source of inoculum (Sosnowski et al. 2006). Blackened cotton stems are also characteristic of infection by *Eutypella* sp., and the pathogen has only been able to be recovered from blackened stem tissue, and not from green stems, leaves or petioles. Soil collected from around diseased plants used in pot assays was not sieved prior to use and therefore may have contained dead plant material suitable as a source of inoculum. Overhead irrigation of 2 mm or more is known to trigger release of spores from fruiting structures on dead wood (Magarey and Carter 1986) and therefore the hand watering of pot bioassays could have initiated the release of ascospores if these were present.

Phylogenetic analyses of the combined ITS1 and ITS2 DNA sequence data of our *Eutypella* isolates identified three clusters, two quite close and one more distant. Comparison with reference sequences obtained from GenBank representing 491 *Eutypella* species, including *E. scoparia*, facilitated the proposal that our isolates consist of two novel species. The relationships between these sequences becomes even clearer after looking at the sequence alignments of the ITS1 and ITS2 regions. The striking features are the large insertions present in the isolates, meaning that these are not different from published *E. scoparia* and related species because of minor substitution differences, but because of these insertions as well. Most of the isolates have a 30 bp insertion in ITS1, with the OTU_2/RM3 group being the exception. The OTU_2/RM3 group does not have this insert, and neither do the *E. scoparia*, but this group is distinguishable by having two inserts (26 bp + 38 bp) in the ITS region (OTU_2 doesn't include the ITS2 region, but it is identical to RM3 in ITS1). Overall, the phylogenetic trees and these alignments would indicate there are two novel *Eutypella* species present in the isolates, both quite different from published *E. scoparia* and other members of the genus.

Summary

This study identified species of Diatrypaceae recovered from the stem and roots of dying and dead cotton plants from Central Queensland, that were distinct from, but closely related to, *E. scoparia*. The most abundant fungal species in diseased root tissue was identified as a relative of *E. scoparia* (< 89.7% match), which was represented by 2 OTUs. Pathogenicity of a representative isolate of *Eutypella* sp. on healthy cotton plants was confirmed, causing cankerous growth and necrosis of vascular tissue, typical of trunk disease. Phylogenetic analyses and sequence alignment of the combined ITS1 and ITS2 DNA sequence data determined there are two undescribed species of *Eutypella* present in the isolates, both quite different from *E. scoparia*. This is the first known case of *Eutypella* affecting cotton worldwide and is considered an expansion of this genus' host range. Knowledge of the pathogen(s) involved in Reoccurring wilt of cotton will help design strategies to manage the disease in this crop.

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Declarations

Conflicts of interest The authors declare that they have no conflict of interest.

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