

Alternaria infectoria and *Stemphylium herbarum*, two new pathogens of pyrethrum (*Tanacetum cinerariifolium*) in Australia

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Abstract Perithecia at the base of dead flower stems of pyrethrum (*Tanacetum cinerariifolium*) plants in yield-decline affected fields of northern Tasmania were identified as *Alternaria infectoria* and *Stemphylium herbarum*. Identification was based on morphological description of cultures established from single ascospores; and conidiospores; ascospore shape and septation; and multigene phylogenetic analyses using the internal transcribed spacer (ITS) region, translation elongation factor 1- α (*EF1*), polymerase II second largest subunit (*RPB2*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) genes. *Stemphylium herbarum* produced necrotic leaf lesions on both sides of the spray inoculated pyrethrum leaves which coalesced to encompass the entire leaf in in vivo and in vitro experiments. *Alternaria infectoria* produced small necrotic leaf lesions on both sides of the leaves two weeks after inoculation which did not expand and hence, was considered as a minor pathogen of pyrethrum. This is the first report of *A. infectoria* and *S. herbarum* as pathogens of pyrethrum in Australia. The role of *A. infectoria* and *S. herbarum* in pyrethrum yield-decline in northern Tasmania needs to be evaluated.

Keywords *Alternaria infectoria* · Multigene analyses · Perithecia · *Stemphylium herbarum* · *Tanacetum cinerariifolium*

Introduction

Pyrethrum, *Tanacetum cinerariifolium* is commercially used for production of natural pyrethrins (Pethybridge et al. 2008a). Pyrethrins are extracted from the achenes located within pyrethrum flower heads (Ambrizic et al. 2007). Approximately 3000 ha of pyrethrum, accounting for 70% of the world production of pyrethrins is grown in Australia each year (Hay et al. 2015). Pyrethrum seeds are sown in winter and the first harvest occurs in spring of the following year (15 to 17 months after planting). Pyrethrins yield is greatly affected by the foliar and soil-borne pathogens that cause reduced flower production. Ideally, pyrethrum plants can be harvested annually for three to four subsequent years, however, in fields severely affected by pathogens, growth is reduced after the first or the second harvesting season and the crops are then terminated (Hay et al. 2002).

During surveys conducted between April 2015 and September 2016 in northern Tasmania, necrotic leaf lesions, necrotic crown tissues and perithecia at the base of dead flower stems from the previous season's harvest were consistently observed on pyrethrum plants growing in yield-decline affected sites. Many foliar pathogens of pyrethrum such as *Stagonosporopsis tanacetii* causing ray blight disease of pyrethrum (Vaghefi et al. 2012), *Didymella tanacetii* the causal agent of pyrethrum tan spot (Pearce et al. 2015); *Colletotrichum tanacetii* causing anthracnose disease of pyrethrum (Barimani et al. 2013); *Sclerotinia sclerotiorum* and *S. minor* causing sclerotinia flower blight (Scott et al. 2014); winter blight caused by *Alternaria tenuissima* and pink spot caused by *Stemphylium botryosum* (Pethybridge et al. 2004); and Botrytis flower blight

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caused by *Botrytis cinerea* (Pethybridge et al. 2008a) have been reported on the affected plants.

Alternaria tenuissima was first reported as a pathogen of pyrethrum by Srinath and Sarwar (1965) with necrotic lesions on the apical part of the pyrethrum leaves. Later, winter blight disease of pyrethrum caused by *A. tenuissima* and pink spot disease caused by *Stemphylium botryosum* were reported as foliar pathogens with *A. tenuissima* producing necrotic leaf spots and *S. botryosum* brown water-soaked lesions with pink and brown margins on both sides of the leaves (Pethybridge et al. 2004; Pethybridge et al. 2008a). The teleomorph stage of *S. botryosum* was detected by Pethybridge et al. (2004) on dead plant material and the bases of old flower stems in pyrethrum fields, however, no further studies were undertaken to determine the contribution of this pathogen to pyrethrum yield reduction.

In a survey conducted in 2012 on foliar pathogens of pyrethrum causing necrotic leaf lesions, the isolation frequency of *Alternaria* spp. declined significantly from 92% of fields in winter to 40% in summer during three sampling times. Isolation frequency of *S. botryosum* was considerably lower (23% of fields in winter) (Hay et al. 2015). However, Hay et al. (2015) reported a flux in the population of *Alternaria* spp. and *S. botryosum* in different seasons of the year. Although these pathogens were mentioned as not being yield-limiting, the frequency of isolation from necrotic leaf lesions of pyrethrum was high (Hay et al. 2015). Pethybridge et al. (2003) frequently isolated *A. tenuissima* and *S. botryosum* from necrotic leaf spots of pyrethrum during October and November 2001, emphasizing that the population of these pathogens was higher in winter and spring compared to that in summer.

Alternaria are ubiquitous fungi with different lifestyles from saprophytic to phytopathogenic causing plant disease and post-harvest rots in wide variety of agricultural crops and ornamental plants. *Alternaria* species also cause human diseases resulting in problems in immunocompromised patients and are responsible for childhood asthma (Lawrence et al. 2013; Woudenberg et al. 2013). Taxonomy of *Alternaria* species has been changing since Nees first described it in 1816 based on production of multi-celled and melanised club-shaped spores in short or long chains (Woudenberg et al. 2013). Later, Wallroth in 1833 and Preuss in 1851 added two closely related genera of *Stemphylium* and *Ulocladium* to the *Alternaria* species complex based on production of phaeodictyospores = muriform spores (production of both longitudinal and transverse septa) and made the identification more complicated (Lawrence et al. 2013). However, Simmons in 2007 classified 275 species in the *Alternaria* complex according to morphological differences (Woudenberg et al. 2013). Currently, the *Alternaria* species complex possesses nine genera: *Alternaria*, *Stemphylium*, *Chalastospora*, *Crivellia*, *Embellisia*, *Nimbya*, *Ulocladium*, *Undifilum* and *Sinomyces*. The genus *Alternaria* includes eight sections with the isolates forming a sexual stage belonging to

A. infectoria (syn. *Lewia*) species group clading distinctly from other *Alternaria* sections due to morphological differences in conidiospores and chemical differences in toxin production (Inderbitzin et al. 2009; Lawrence et al. 2013; Woudenberg et al. 2013). *Alternaria* and *Stemphylium* are among the most common asexual morphs of the order *Pleosporales* (Zhang et al. 2012).

Stemphylium species are mostly saprophytic growing on dead plant materials, however, *S. botryosum*, *S. solani*, *S. vesicarium* and *S. loti* have been known as pathogens of agriculturally important crops and stone fruits (Wang et al. 2009). Simmons (1967) established criteria for morphological identification of various *Stemphylium* spp. and introduced *Pleospora herbarum* as teleomorph of *S. botryosum*. However, Simmons in 1985 subsequently reclassified *Stemphylium/Pleospora* holomorphs and reported *Pleaospora tarda* as the sexual stage of *S. botryosum* and *P. herbarum* the sexual stage of *S. herbarum* (Inderbitzin et al. 2009).

The aims of this study were to i) identify the species associated with the perithecia isolated from the base of the pyrethrum dead flower stems using multigene phylogenetic analyses and morphological identification ii) determine the pathogenicity of these species on pyrethrum.

Methods and materials

Sample collection

In a field survey in September 2016 from fields located in Table Cape and Burnie, northern Tasmania, seven plants showing perithecia at the base of dead flower stems were collected and transferred to the laboratory for isolate identification. Pieces (5–10 cm in length) of necrotic flower stem bases containing perithecia were cut from each plant, placed on moisturised filter papers with sterile water in Petri dishes and incubated at room temperature (23–25 °C) for 2 days. Fertile perithecia were then lifted from the surface of the flower stems using a sterilised needle; vortexed in sterilised water and cultured on WA for single sporing. Perithecia were also mounted on microscopic slides with clear lactic acid and gently tapped to release asci for morphological identification.

Five additional *Alternaria* spp. strains isolated from necrotic leaf lesions of pyrethrum in northern Tasmania were also collected. All other strains used in the study were reference strains derived from the national centre of biotechnology information (NCBI) database at <http://www.ncbi.nlm.nih.gov/>. A culture of each isolate was sent to the Queensland Plant Pathology Herbarium (BRIP), Brisbane, Australia to obtain culture collection reference numbers.

Taxonomy

Morphological descriptions were made of monosporic isolates grown on synthetic poor nutrient agar (SNA) with pieces of sterilised filter papers (double autoclaved for 20 min at 121 °C) on the surface of the agar as carbon source to induce sporulation (Crous et al. 2009). Cultures were incubated at 21–22 °C under cool white fluorescent light in a 8/16 h regime for 7 days (Woudenberg et al. 2013). Morphological descriptions of ascospores for the species that did not form perithecia on SNA, were made directly from the perithecia that were isolated from flower stems. Slides were prepared with clear lactic acid. Microscopic images were captured using a Leica DM2900 compound microscope with both bright field and differential interference contrast illuminations. Colony growth rate was measured in 2-day intervals for 7 days and pigmentation was rated according to Rayner et al. (1970) color chart. The size of 30 conidiospores and ascospores; and 10 samples of all other structures including asci and conidiophores were measured (Table 1).

Molecular identification

DNA extraction and PCR amplification

Fungal mycelia were scraped directly from 7-day-old single spored cultures on oatmeal agar (OA), DNA extracted using the DNeasy Plant Mini Kit (Qiagen Pty. Ltd., Australia) following the manufacturer’s instruction, and quantified by comparing the band density using Lambda DNA/HindIII Marker (Thermo Ficsher Scientific, Australia) after electrophoresis in 1.5% agarose gel. The ITS region (700 bp) was amplified using primer pairs V9G (de Hoog and Gerrits van den Ende 1998) and ITS4 (White et al. 1990). Approximately 420 bp of the *EF1* was amplified by PCR primers EF-728f (Carbone and Kohn 1999) and EF2 (O’Donnell et al. 1998); and 890 bp of the partial *RPB2* genes were amplified by PCR primers RPB2-5F2 (Sung et al. 2007) and fRPB2-7cR (Liu et al. 1999). The GAPDH region was amplified using primer pairs *gpd1* and *gpd2* designed by Berbee et al. (1999). PCR thermal cyclers for ITS were performed as described by White et al. (1990), for *RPB2* touchdown PCR, for *EF1* gradient PCR and for GAPDH were performed according to PCR conditions described in Woudenberg et al. (2013). DNA amplification was carried out using an Eppendorf thermal cycler (Pty. Ltd. Australia). PCR products were then purified using QIAquick PCR purification kit (Qiagen Pty. Ltd. Australia) and sequenced.

Multigene phylogenetic analysis

Purified amplicons were sequenced and sequence intensity was assessed using the Chromas Lite MFS computer package. Isolate sequences were tentatively identified after conducting

Table 1 Key morphological features of *A. infectoria* and *S. herbarum*, SD = standard deviation

Species	Growth rate after 7 d (mm)	Colony pigmentation	Conidiophores length	Conidiospores Length × Width (SD)	Asci	Ascospores Length × Width (SD)
<i>A. infectoria</i> (BRIP 65180)	35	Pale grey to silvery with white and fluffy aerial mycelia; reverse white thick margins with dark grey centre	11–21 (SD 7.11) µm	23.5–59.5 (SD 10.6) µm × 7–12 (SD 1.5) µm	122.5–137 (SD 10.4) µm × 16–16.5 (SD 0.40) µm	20–27.5 (SD 2.05) µm × 7.5–16 (SD 2.62) µm
<i>S. herbarum</i> (BRIP 65181)	30	Olive grey colonies with pale grey aerial mycelia, reverse black with thin white margins	17–57.5 (SD 21) µm	19.5–35.5 (SD 4.12) µm × 9–22 (SD 3) µm	143–188 (SD 14.5) µm × 27.5–33 (SD 2.1) µm	26–37 (SD 2.83) µm × 13–16 (SD 1.03) µm

BLAST searches of the database. Sequences were aligned manually using the sequence alignment editing program MEGA6 (Tamura et al. 2013). Consensus sequences were obtained from both forward and reverse sequences using DeNovo assembly in Geneious v 7.06 (Kearse et al. 2012). Consensus sequences were then pairwise aligned and annotated with the closely related reference sequences derived from the database within Geneious (Table 2). Maximum likelihood (ML) phylogenetic trees were constructed for each gene individually and in combination using MEGA6 software and PhyML (Guindon and Gascuel 2003) within Geneious.

Due to the lack of *RPB2* gene sequences for most *Stemphylium* species, two separate multigene phylogenetic trees including ML combined tree of ITS-*EF1*-GAPDH-*RPB2* for *Alternaria* and ITS-*EF1*-GAPDH for *Stemphylium* were generated. However, *Stemphylium* species were included in the combined phylogenetic tree constructed for *Alternaria*, where *RPB2* gene sequences were available. In the tree for *Alternaria*, species of closely related genera to *A. infectoria* group including species of sections *Chalastospora*, *Ulocladium* and *Stemphylium* were included. Species of section *Alternata* were also included to determine whether recovered strains isolated from the dead flower stem bases were associated with the sexual stages of *A. tenuissima* or *A. alternata*. Moreover, an individual *EF1* phylogenetic tree was constructed with all the aforementioned isolates plus the five isolates recovered from necrotic leaf lesions in 2016 to determine if they were anamorphs of *A. infectoria*. However, no further sequencing was carried out for these isolates.

In the combined tree for *Stemphylium* spp., species of different groups (Inderbitzin et al. 2009) were included to differentiate various groups in the *Stemphylium* species complex. The best substitution model was selected using MEGA6 with ML statistical analysis and complete deletion of the gaps. In PhyML analysis, the GTR substitution model was selected. To assess the relative stability of branches, bootstrap analysis with 1000 pseudoreplicates for ML analyses in MEGA6 and 500 pseudoreplicates in PhyML analyses was performed. Gaps were treated as missing data. Consensus sequences were deposited in the database and trees in TreeBASE at www.treebase.org. GenBank accession numbers for the reference markers are shown in Table 2.

Pathogenicity tests

Pathogenicity of *A. infectoria* and *S. herbarum* isolated from the dead pyrethrum flower stems was assessed in two trials. For each trial, the pyrethrum seedlings of cultivar Pyrate were germinated from steam sterilised seeds and raised in seedling mix in Tasmania (BRA Pty. Ltd.). Seedlings were transferred to 10 cm diam pots with potting mix, fertilised with 5 g of

Osmocote Plus (Scotts Australia Pty. Ltd.) per pot and grown in the glasshouse for two months.

Inoculum was prepared from 5-day-old single spored cultures on SNA by adding 10 mL of sterile water to each plate, gently scraping the colony surface with a glass spreader and then filtering the spore suspension using cheese cloth. The concentration of each spore suspension was quantified to 10^6 spore/mL using a haemocytometer. Two drops of 0.1% Tween-20 solution was added to each spore suspension (Pethybridge et al. 2008b).

In vivo experiment

To assess pathogenicity of each isolate to produce leaf lesions, three replicates of healthy Pyrate pyrethrum plants were each spray inoculated with 20 mL of 10^6 spore/mL spore suspension of each isolate of *A. infectoria* and *S. herbarum*. A hand sprayer was used to spray the leaves and inoculation was continued until just before runoff. Three control plants were sprayed with sterilised water. Each plant was then covered with a plastic bag to increase humidity after inoculation and induce germination of the spores. The plastic bags were removed after 24 h and the plants were then maintained in the glasshouse until necrotic lesions appeared on the leaves. Necrotic lesions were cultured on potato dextrose agar (PDA) for pathogen identification. Tissue surface sterilisation and culture were carried out by dipping the leaf sections into 80% ethanol for 30 s, 1% active ingredient (ai) sodium hypochlorite for 1 min and then rinsed two times in sterilised water for 1 min. They were then blotted on a sterilised paper towel and small 5 mm² sections of tissue were cultured onto WA and incubated according to the condition described above. Mycelium was subsequently subcultured onto PDA and incubated for a further 5 days at 23 °C.

In vitro experiment

An *in vitro* bioassay was undertaken with detached leaves of healthy pyrethrum plants inoculated by placing 10^6 spore/mL of spore suspension of each species onto the surface sterilised leaves. Controls were treated with sterilised water. Detached leaves were incubated in plastic containers, four leaves per container, with moistened filter papers at 21 °C for eight days and symptoms were assessed after eight days. A Leica DM205 FA stereomicroscope was used to assess the symptoms.

Results

Perithecia isolated from dead flower stems of all the seven plants in 2016 were associated with both *A. infectoria* and *S. herbarum* (Fig. 1).

Table 2 Accession numbers of *Alternaria* spp. and *Stemphylium* spp. used in this research

Isolates	Culture accession No.	Location	Isolate source	Accession No.			
				ITS	<i>EF1</i>	GAPDH	<i>RPB2</i>
<i>A. alternariae</i> ^a	CBS 126989	USA	<i>Daucus carota</i>	AF229485	KC584730	AY278815	KC584470
<i>A. alternata</i> ^a	CBS 916.96	India	<i>Arachis hypogaea</i>	AF347031	KC584634	AY278808	KC584375
<i>A. arboriscens</i> ^a	CBS 102607	USA	<i>Lycopersicon esculentum</i>	AF347033	KC584636	AY278810	KC584377
<i>A. armoraciae</i> ^a	CBS 118702	New Zealand	<i>Armoracia rusticana</i>	KC584182	KC584638	KC584099	KC584379
<i>A. breviformis</i> ^a	CBS 121331	Australia	<i>Triticum</i> sp.	FJ839608	KC584700	KC584148	KC584442
<i>A. capsici-annui</i> ^a	CBS 504.74	-	<i>Capsicum annuum</i>	KC584187	KC584644	KC584105	KC584385
<i>A. cerata</i> ^a	CBS 121340	Australia	<i>Elymus scabrus</i>	JN383482	KC584699	AY562398	KC584441
<i>A. conjuncta</i> ^a	CBS 196.86	Switzerland	<i>Pastinaca sativa</i>	FJ266475	KC584649	AY562401	KC584390
<i>A. ethzedia</i> ^a	CBS 197.86	Switzerland	<i>Brassica napus</i>	AF392987	KC584657	AY278795	KC584398
<i>A. gaisen</i> ^a	CBS 632.93	Japan	<i>Pyruca pyrifolia</i> cv. <i>Nijiseiki</i>	KC584197	KC584658	KC584116	KC584399
<i>A. infectoria</i> ^a	CBS 210.86	UK	<i>Triticum aestivum</i>	DQ323697	KC584662	AY278793	KC584404
<i>A. infectoria</i>	UMAi01- BRIP 65180	Australia	<i>Tanacetum cinerariifolium</i>	KY009902	KY009904	KY038017	KY009906
<i>A. obclavata</i> ^a	CBS 124120	USA	Air	KC584225	KC584701	KC584149	KC584443
<i>A. oudemansii</i> ^a	CBS 114.07	-	-	FJ266488	KC584746	KC584175	KC584486
<i>A. oregonensis</i> ^a	CBS 542.94	USA	<i>Triticum aestivum</i>	FJ266478	KC584674	FJ266491	KC584416
<i>A. tenuissima</i> ^a	CBS 918.96	UK	<i>Dianthus</i> sp.	AF347032	KC584693	AY278809	KC584435
<i>Alternaria</i> sp. ^a	CBS 198.67	USA	soil	AF229487	KC584737	KC584737	KC584477
<i>Curvularia australis</i> ^c	BRIP 12521	-	-	KJ415541	KJ415451	KJ415405	-
<i>Exerohilum pedicellatum</i> ^b	BMP0384	-	-	AF229478	JQ672389	AY278824	JQ646512
<i>S. alfalfae</i> ^c	CBS 192.86	USA	<i>Medicago sativa</i>	AF442775	AY324676	AY443874	-
<i>S. botryosum</i> ^c	CBS 714.68	Canada	<i>Medicago sativa</i>	KC584238	KC584729	AF443881	AF107804
<i>S. gracilariae</i> ^c	CBS 482.90	Israel	<i>Gracilaria</i> sp.	AY329217	AY324709	AY377021	-
<i>S. herbarum</i> ^c	CBS 191.86	India	<i>Medicago sativa</i>	KC584239	KC584731	AF443884	KC584471
<i>S. herbarum</i>	UMSh02- BRIP 65181	Australia	<i>Tanacetum cinerariifolium</i>	KY009903	KY009905	KY038018	KY009907
<i>S. lancipes</i> ^c	EGS 46–182	New Zealand	<i>Aguilegia</i> sp.	AY329203	AY324742	AF443886	-
<i>S. loti</i> ^c	NO 770	USA	<i>Lotus</i> sp.	AF442789	AY324775	AF443888	-
<i>S. lycopersici</i> ^c	Strain 05	China	<i>Lycopersicon esculentum</i>	KR911818	KR911808	KR911813	-
<i>S. majusculum</i> ^c	EGS 16–068	USA	<i>Latyrnus maritimus</i>	AY329228	AY324710	AY317032	-
<i>S. paludiscripti</i> ^c	EGS 31–016	USA	-	AY329231	AY324769	AY317035	-
<i>S. sarciniforme</i> ^c	EGS 29–188	USA	<i>Trifolium pratense</i>	AY329221	AY324746	AY317025	-
<i>S. sedicola</i> ^d	CBS 109843	Auckland	<i>Sedum spectabile</i>	AY329232	AY324713	AY317036	-
<i>S. solani</i> ^c	EGS 41–135	USA	<i>Lupinus</i> sp.	AY329214	AY324759	AY317018	-
<i>S. trifolii</i> ^c	EGS 12–142	USA	<i>Trifolium repens</i>	AY329218	AY324744	AY317022	-
<i>S. triglochinchicola</i> ^c	EGS 36–118	UK	<i>Triglochin maritima</i>	AY329175	AY324753	AF443901	-
<i>S. tomatonis</i> ^d	CBS 109844	USA	<i>Lycopersicon esculentum</i>	AY329229	AY324711	AY317033	-
<i>S. vesicarium</i> ^c	CNU094009	China	<i>Allium</i> sp.	JF331519	JF331628	JF331462	-
<i>S. xanthosomatis</i> ^c	EGS 17–137	New Caledonia	<i>Xanthosoma sagittifolium</i>	AY329206	AY324758	AF443903	-

^a (Woudenberg et al. 2013); ^b (Lawrence et al. 2013); ^c (Camara et al. 2002); ^d (Simmons 2001)

*BRIP, Queensland Plant Pathology herbarium; Brisbane, Australia; CBS, Centraalbureau voor Schimmelcultures, the Netherlands; BMP, BM Pryor, School of Plant Sciences, University of Arizona, Tuscon, Arizona 85,721; EGS, EG Simmons, Mycological Sreives, Crawfordsville, Indiana 47,933; NO, Nichole O’Nille, Culture Collection, Beltsville, MD; CNU, Chungnam National University, Yuseong, KungDong, Daejeon, Chungnam, Korea; UM, University of Melbourne; Australia

Taxonomy

Detailed taxonomic description of *A. infectoria* and *S. herbarum* are shown in Table 1.

Alternaria infectoria: Conidia obclavate in long or short chains predominantly without longitudinal septa and with short branched conidiophores growing in between the conidia. Ascospores muriform, 6–7 transverse septa with 3–4

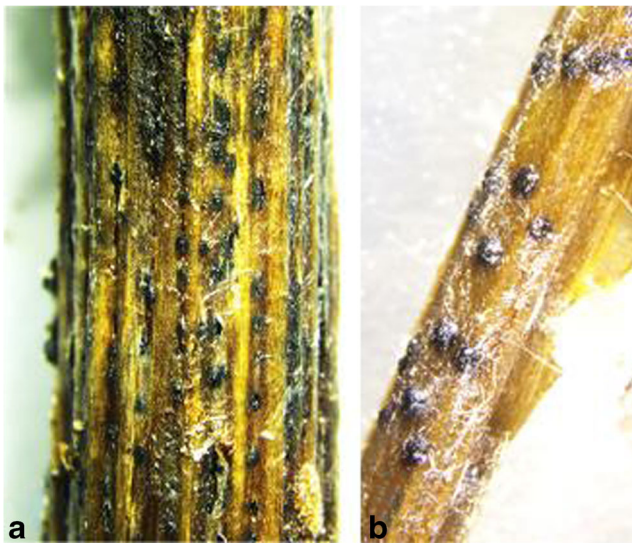


Fig. 1 **a** an old necrotic pyrethrum flower stem cut from a field grown plant with perithecia formed at the base; **b** perithecia of *S. herbarum* produced in vivo on a sterilised pyrethrum flower stem in WA, 10 days after inoculation

longitudinal septa in the central segments, end cells without septa. Cylindrical asci with 8 ascospores, straight or somewhat curved (Fig. 2). No perithecia formed on SNA with pieces of filter papers and WA with sterilised flower stems.

Stemphylium herbarum: Oblong-ovoid shaped conidia, light to dark brown with meristemic growth occasionally, multicelled with transverse and longitudinal septa. Conidiophores short and long, branched. Ascospores yellow, 7–9 transverse and many longitudinal septa in central and lateral segments. Cylindrical asci with 8 ascospores, curved or straight (Fig. 3). Perithecia produced on SNA and WA with pieces of sterilised flower stems (Fig. 1b).

Phylogeny

Multilocus combined phylogenetic analyses of ITS-*EF1-RPB2*-GAPDH; and ITS and *RPB2* individual trees (individual trees were not shown) confirmed that

Fig. 2 *Alternaria infectoria*; **a** colony morphology and pigmentation on OA; **b-e** conidia; **f** asci containing eight ascospores; **g** multi-celled ascospores. Scale bars **b-e** and **g** 20 μ m; **f** 50 μ m



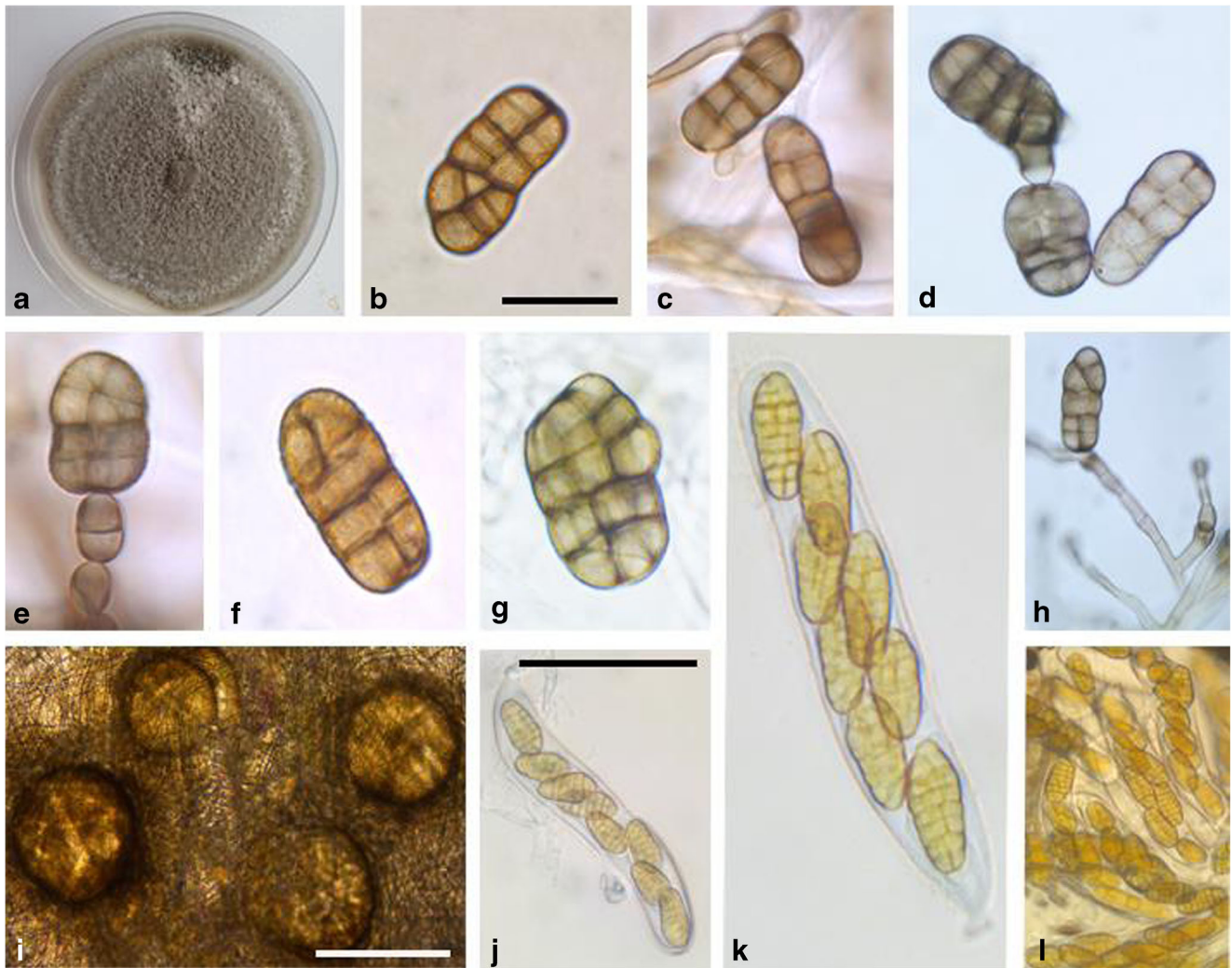


Fig. 3 *Stemphylium herbarum*; **a** colony morphology and pigmentation on OA; **b-g** conidia; **h** short and long conidiophores **i** perithecia on SNA; **j-l** asci containing eight ascospores. Scale bars **b-g** and **h** 20 μ m; **i** 100 μ m; **j-l** 50 μ m

A. infectoria (BRIP 65180) claded with the holotype strain *A. infectoria* (CBS 210.86) within the *A. infectoria* species group with bootstrap value of 100% (Fig. 4). However, in individual trees of *EF1* and *GAPDH*, the isolate claded with *A. conjuncta*.

Stemphylium herbarum (BRIP 65181) claded with the type strain of *S. herbarum* (CBS 191.86) in the ITS-*EF1-RPB2-GAPDH* phylogenetic tree (Fig. 4) with 100% bootstrap value and in ITS-*EF1-GAPDH* phylogenetic tree (Fig. 5) with 88% bootstrap support confirming that this was a different species to *S. botryosum* that had been previously reported on pyrethrum. Individual trees showed similar results to the combined phylogenetic trees (individual trees were not shown). Isolate BRIP 65181 clustered closely with related species to *Stemphylium* spp. group C (*S. herbarum* group; Inderbitzin et al. 2009) and next to the type strain of *S. herbarum* (CBS 191.86).

All the five isolates cultured from leaf lesions were identified as *A. alternata* and clustered in the *Alternaria* species complex in the individual *EF1* tree with 98% bootstrap support (tree was not shown). ML bootstrap supports obtained from MEGA6 multigene analysis were similar to those obtained in PhyML analysis.

In the combined maximum likelihood tree of ITS-*EF1-RPB2-GAPDH* constructed for the species of *Alternaria*, a total of 2035 (ITS: 510, *EF1*: 248, *RPB2*: 829, *GAPDH*: 448) positions in the final data set were obtained with 1201 constant sites (ITS: 386, *EF1*: 133, *RPB2*: 372, *GAPDH*: 310) and 613 (ITS: 97, *EF1*: 83, *RPB2*: 353, *GAPDH*: 80) parsimony informative sites and 221 uninformative variable characters.

In the combined maximum likelihood tree of ITS-*EF1-GAPDH* constructed for the species of *Stemphylium*, a total of 1342 (ITS: 394, *EF1*: 437, *GAPDH*: 511) positions in the final data set were obtained with 851 constant sites (ITS: 342,

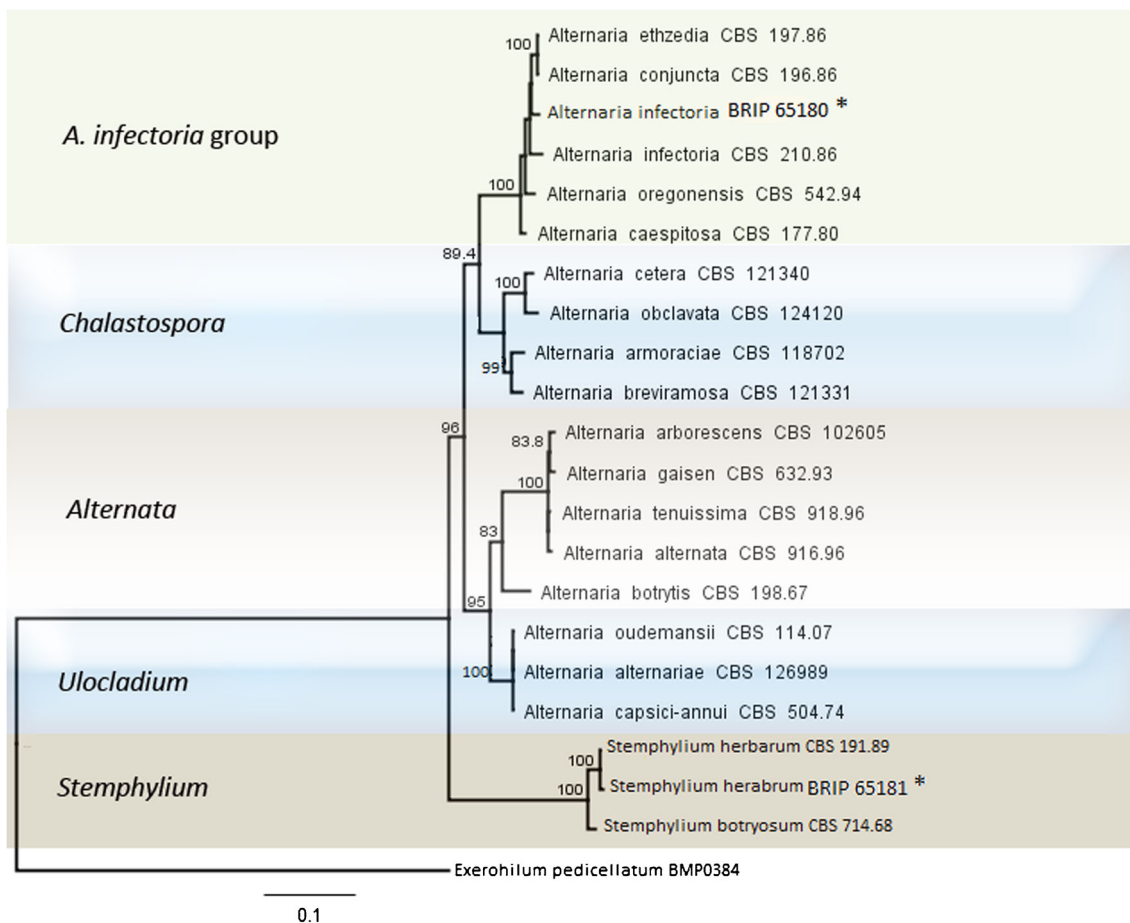


Fig. 4 Maximum likelihood PhyML combined phylogenetic tree of ITS-*EF1-RPB2-GAPDH* for *Alternaria* and *Stemphylium* spp. Highest log likelihood -8961.6128. The analysis involved 22 nucleotide sequences. The tree was rooted to *Exerohilum pedicellatum* BMP0384. Bootstrap

values less than 80% were deleted. * refers to strains recovered from the base of the pyrethrum flower stems. Scale bar indicates expected changes per site

EF1: 150, *GAPDH*: 359); 376 (*ITS*: 133, *EF1*: 116, *GAPDH*: 127) parsimony informative sites and 115 uninformative variable characters.

Pathogenicity tests

Alternaria infectoria and *S. herbarum* were isolated from necrotic leaf lesions produced in both in vivo and in vitro experiments. *Alternaria infectoria* was able to produce small black spots on the surface of the leaves 4 days after inoculation with 10^6 spore/mL spore suspension in detached leaf bioassay and 2 weeks after inoculation in in vivo glasshouse experiments. Lesions were mostly small with regular margins and did not expand further beyond 14 days after inoculation in both experiments (Fig. 6A).

Stemphylium herbarum also caused reddish-brown necrotic leaf lesions on both sides of the leaves after 3 days in in vitro and 5 days after inoculation in in vivo bioassays. Lesions expanded to encompass the entire leaf surface (Fig. 6B). No controls were found infected in in vivo and in vitro experiments for both species.

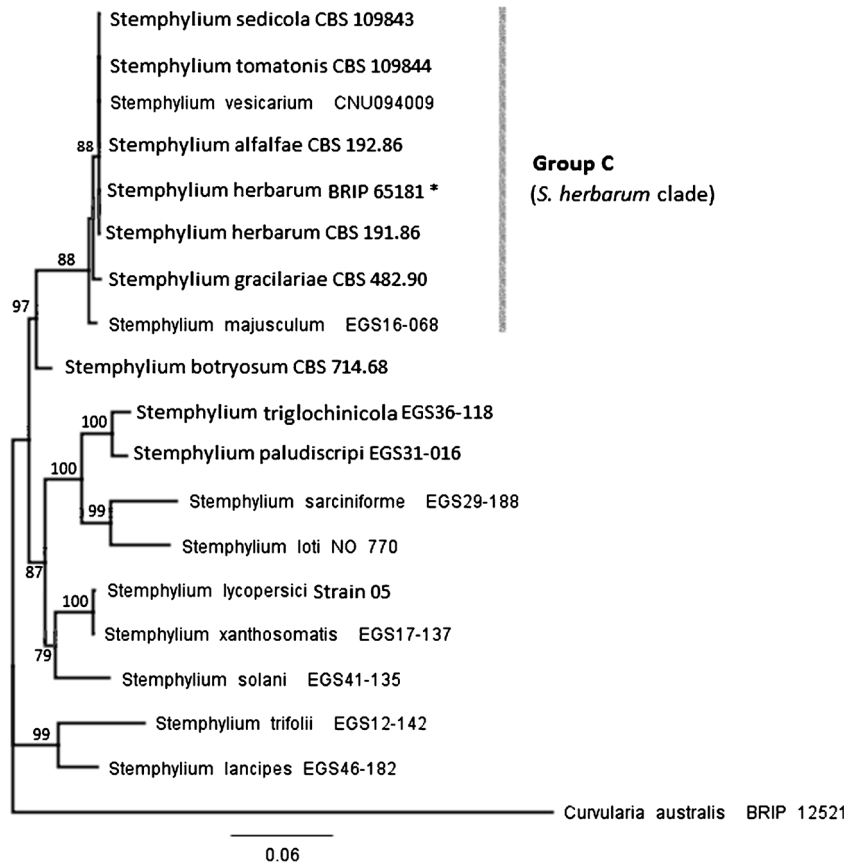
Discussion

Alternaria infectoria and *Stemphylium herbarum* have been identified as new pathogens of pyrethrum in Australia. Both pathogens were isolated from the perithecia collected from dead pyrethrum flower stems in plants affected by yield-decline syndrome in northern Tasmania.

Alternaria infectoria caused black necrotic lesions on both sides of the leaves in the glasshouse and detached leaf pathogenicity bioassays. However, leaf symptoms on plants in the glasshouse were not severe and developed slowly after 2 weeks. Low isolation frequency of this pathogen from leaf lesions of field plants and morphological similarities of conidia shape and size to other *Alternaria* species isolated from pyrethrum might have been the main reasons that *A. infectoria* was not identified in previous field surveys.

Pyrethrum winter blight disease has been reported to be caused by *A. tenuissima* (Pethybridge et al. 2003; Pethybridge et al. 2008a). Pethybridge et al. (2004) reported that *A. tenuissima* was a minor pathogen but had the potential

Fig. 5 Maximum likelihood PhyML combined phylogenetic tree of ITS-*EF1*-GAPDH for *Stemphylium* spp. Highest log likelihood -5364.0913. The analysis involved 19 nucleotide sequences. The tree was rooted to *Curvularia australis* BRIP 12521. Bootstrap values less than 75% were deleted. * refers to the *S. herbarum* strain recovered from the base of the pyrethrum flower stems. Group C created according to Inderbitzin et al. (2009). Scale bar indicates expected changes per site

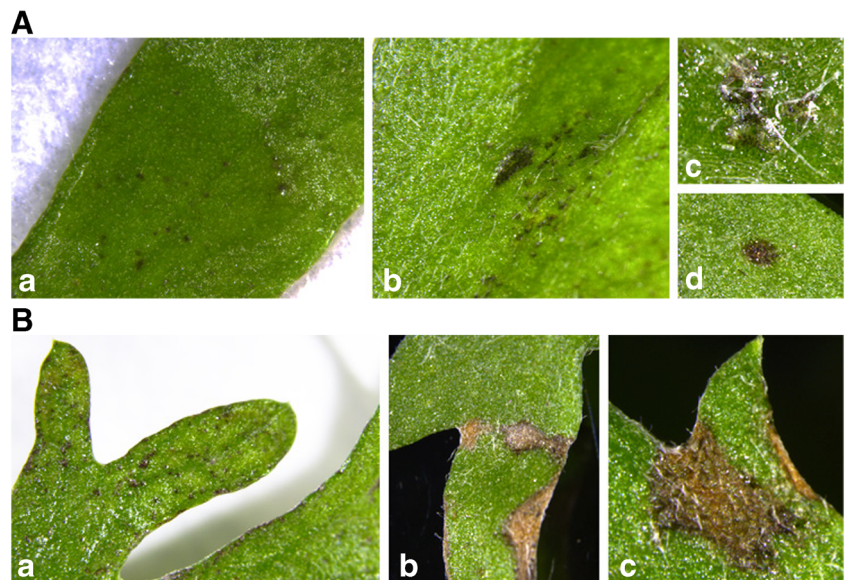


to reduce the leaf area and hence affect photosynthesis and flower production.

Perithecia of plant pathogens on dead plant material are important sources of inoculum with ascospores dispersed by wind or water splash (Thomma 2003). However, the sexual morphs for most species of *Alternaria* have not been identified (Thomma 2003). Isolates of *A. alternata* from leaf lesions

were in the *Alternata* species complex in which no sexual stage has been recorded. More extensive surveys of dead pyrethrum flower stems and trash need to be undertaken to assess the incidence of *A. infectoria*. In the study by Hay et al. (2015), different species of *Alternaria* were reported to be associated with pyrethrum leaf spots but the species were not identified. More investigations need to be carried out on

Fig. 6 **A** Symptoms caused by *Alternaria infectoria* on pyrethrum in in vivo and in vitro trials. *a-c* necrotic and black spots produced 4 days after inoculation in vitro on both sides of the leaves. *d* necrotic lesions produced two weeks after inoculation in vivo. **B** Symptoms caused by *Stemphylium herbarum* on pyrethrum in in vivo and in vitro trials. *a* necrotic spots produced 3 days after inoculation in vitro bioassay *b-c* necrotic and reddish brown irregular lesions produced 5 days after inoculation in in vivo experiment on both sides of the leaves



Alternaria spp. as foliar pathogens of pyrethrum in Australia and the role they have in yield-decline of pyrethrum. Isolation of *A. alternata* was also reported by Pethybridge et al. (2004), however, this was shown to be a saprophyte colonizing the dead pyrethrum plant material and was unable to infect pyrethrum leaves in glasshouse bioassays. Nevertheless, *A. alternata* has been reported to be pathogenic to chrysanthemum (Pethybridge et al. 2008a).

The *A. infectoria* species group comprises many taxa which are morphologically differentiated from taxa in other *Alternaria* sections by the production of shorter and branched conidia (Andersen et al. 2009). *Alternaria infectoria* is taxonomically distinct from other closely related genera and clustered with other members of the sexual *Alternaria* lineage (Andersen et al. 2009; Woudenberg et al. 2013). In individual trees of *EF1* and *GAPDH* the isolate from the base of the old flower stems claded with *A. conjuncta*, however, *A. infectoria* was morphologically different to *A. conjuncta* as it produced longer conidiospores and short conidiophores which grow between conidia (Andersen et al. 2009). Nevertheless, morphological characters of taxa within the *A. infectoria* species group are similar; so molecular phylogenetic studies are required to differentiate many members of the group.

Pathogenicity of *S. herbarum* on pyrethrum was also confirmed with symptoms very similar to those of *S. botryosum* (Pethybridge et al. 2004). *Stemphylium herbarum* is morphologically different to *S. botryosum* as it produces longer, narrower conidia. However, no information exists about the teleomorph of *S. botryosum* (syn. *P. tarda*). Pathogenic *Stemphylium* spp. survive on the debris of the host plants where perithecia are produced. Perithecia discharge ascospores under suitable environmental conditions. Penetration occurs through the epidermis or preferably stomata of the host plant and then hyphae enter the cells resulting in production of necrotic, brown lesions on the surface of the leaves (Kohl et al. 2009; Ahmed 2014). BLAST searches of the gene sequences of *S. herbarum* (BRIP 65181) also showed high similarity to *S. vesicarium*, however, the sexual stage of *S. vesicarium* is morphologically different to the sexual stage of *S. herbarum* where the width of asci is smaller and ascospore arrangement within asci is different. Other taxa within group C such as *S. alfalfae* showed genetic similarity to *S. herbarum* (Inderbitzin et al. 2009) but were morphologically different to *S. herbarum* as they produce long conidia and different ascospores with unpredictable longitudinal septa. Species grouped in *S. herbarum* clade (group C) were closely related and multilocus phylogenetic analyses have not been able to distinguish intra-species relationships, although they resolved new lineages. Hence, Camara et al. (2002) divided *Stemphylium* species into different groups according to differences in their nucleotide sequences, bootstrap value obtained by Bayesian analyses and phenotypic differences based on asexual spores.

Diseased pyrethrum plants frequently showed leaf lesions on both sides of the leaves and perithecia at the bases of the dead flower stems at different times of the year. This raises the question as to whether *S. botryosum* that was reported previously as a pathogen of pyrethrum does actually exist in pyrethrum fields of northern Tasmania. Hence, more field surveys and sample collections are needed to identify all *Stemphylium* spp. and *Alternaria* spp. existing in the fields and associated with pyrethrum leaf diseases; and to determine their effect on pyrethrum yield reduction.

The symptoms caused by *Alternaria* spp. and *Stemphylium* spp. have been reported to be very similar (Pethybridge et al. 2008a). Care must be taken in isolation and identification of the causal agent as many foliar pathogens in pyrethrum fields cause similar symptoms and can be easily confused.

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