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Carpogenic germination of sclerotia of *Sclerotinia minor* and ascosporic infection of pyrethrum flowers

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Abstract: Carpogenic germination of sclerotia and infection of flowers by ascospores of *Sclerotinia minor* is rare and seldom documented in most hosts. During 2007–2009, *S. minor* isolates were obtained from surface-sterilized pyrethrum flowers collected from fields in Australia. The isolation frequency of *S. minor* from flowers in 2007, 2008 and 2009 was 15.8%, 5% and 1.4%, respectively. During these years, the prevalence of *S. minor* in flowers amongst pyrethrum fields varied between 10.3% and 60%. Sclerotia with apothecia, consistent in size with *S. minor*, were collected in one field. Colonies from individual ascospores from this isolate were identified as *S. minor*. A subsample of 10 *S. minor* isolates was selected for further studies. Phylogenetic analysis based on the internal transcribed spacer region grouped these isolates with *S. minor*, and distinct from published sequences of other *Sclerotinia* spp. Species-specific primers developed previously to differentiate the four major *Sclerotinia* spp. (*S. sclerotiorum, S. minor, S. homoeocarpa* and *S. trifoliorum*) were used to confirm identity. Of the 10 *S. minor* isolates, eight were able to carpogenically germinate *in vitro*. Pathogenicity of *S. minor* to flowers was confirmed in the greenhouse using ascospores. This study is one of the few instances documenting the ability of *S. minor* to infect floral tissues and the first documentation of *S. minor* causing flower disease of pyrethrum. These findings serve as a scaffold for further investigations into the mechanisms of flower infection by *S. minor* and have implications for the management of the Sclerotinia disease complex affecting pyrethrum in Australia.

Keywords: carpogenic germination, pyrethrum, Sclerotinia flower blight, *Sclerotinia minor*, *Sclerotinia sclerotiorum*

Résumé: La germination carpogénique des sclérotes, et l'infection des fleurs par les ascospores de *Sclerotinia minor* qui s'ensuit, ne se produit pas souvent et est rarement documentée chez la plupart des hôtes. Durant la période de 2007 à 2009, des isolats de *S. minor* ont été obtenus de la surface stérilisée de fleurs de pyrèthre collectées dans des champs en Australie. La fréquence d'isolement de *S. minor* pour les fleurs en 2007, 2008 et 2009 était de 15.8%, 5 % et 1.4%, respectivement. Durant ces années, la prévalence de *S. minor* sur les fleurs dans les champs de pyrèthre variait de 10.3% à 60%. Des sclérotes produisant des apothécies conformes en taille à celles de *S. minor* ont été collectées dans un champ. Des colonies issues d'ascospores individuelles provenant de cet isolat ont été reconnues comme *S. minor*. Un sous-échantillon de 10 isolats de *S. minor* a été sélectionné pour procéder à d'autres études. L'analyse phylogénétique basée sur la région de l'espaceur transcrit interne a associé ces isolats à *S. minor* et a permis de les distinguer des séquences publiées des autres espèces de *Sclerotinia*. Des amorces spécifiques d'espèces préalablement conçues pour différencier les quatre principales espèces de *Sclerotinia* (*S. sclerotiorum, S. minor, S. homoeocarpa* et *S. trifoliorum*) ont été utilisées pour en confirmer l'identité. Chez 8 des 10 isolats de *S. minor*, il y a eu germination carpogénique *in vitro*. La pathogénicité de *S. minor* sur les fleurs a été confirmée en serre en utilisant des ascospores. Il s'agit d'une des rares études qui fait état de la capacité de *S. minor* à infecter des tissus floraux et la première confirmation de *S. minor*

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infectant les fleurs du pyrèthre. Ces constatations servent de tremplin à d'autres recherches sur les mécanismes d'infection des fleurs par *S. minor* et ont des répercussions sur la gestion de l'ensemble des maladies causées par *Sclerotinia* sur le pyrèthre en Australie.

Mots clés: brûlure des fleurs, germination carpogénique, pyrèthre, *Sclerotinia minor*, *Sclerotinia sclerotiorum*

Introduction

Pyrethrum (*Tanacetum cinerariifolium* (Trevir.) Sch. Bip.; Family *Asteraceae*) is grown commercially for insecticidal pyrethrins which are produced mainly within the secretory ducts in the flowers (Zito et al. 1983). The pyrethrins are extracted and used within formulations of a range of domestic pest control products (Grdiša et al. 2009). Australia produces over half of the global pyrethrin supply from fields in northern Tasmania and the Ballarat region of Victoria.

In Australia, pyrethrum flowers begin to open in late October (late spring) and mature over a 6-week period (Pethybridge et al. 2008). Pyrethrin yield is a function of the number of harvestable flowers, flower size (number of achenes produced within each flower) and pyrethrin content in individual flowers. Losses from flower diseases are typically incurred from a reduction in the number of harvestable flowers due to abscission prior to maturity or shattering and subsequent loss of achenes during harvest (Pethybridge et al. 2010). There are three main diseases affecting flowers of pyrethrum in Australia: Botrytis flower blight, caused by *Botrytis cinerea*; ray blight, caused by *Stagonosporopsis tanaceti* (Vaghefi et al. 2012); and Sclerotinia flower blight (Pethybridge et al. 2008, 2010).

Sclerotinia flower blight of pyrethrum, and flower infections in many other crops (e.g. beans), have traditionally been attributed to infection by *S. sclerotiorum* ascospores that result from carpogenic germination of sclerotia (Pethybridge et al. 2008, 2010). In contrast, diseases caused by *S. minor* usually result from myceliogenic germination (Abawi & Grogan 1979). Carpogenic germination in *S. minor* has been shown to be important in disease of lettuce (Hawthorne 1976; Clarkson et al. 2004), but is generally considered rare and of minor importance (Abawi & Grogan 1979). Under *in vitro* conditions, *S. minor* ascospores have been found to infect sunflower inflorescences (Ekins et al. 2002), and ascospores have been found on canola petals under field conditions (Hind et al. 2001). Modelling based on long-term average environmental conditions also predicted carpogenic germination in *S. minor* could occur in southern Australia, including the pyrethrum production regions of Tasmania and Victoria (Ekins et al. 2002).

Sclerotinia sclerotiorum and *S. minor* are also associated with Sclerotinia crown rot of pyrethrum which can reduce stand densities and vigour (Pethybridge et al. 2008; Scott et al. 2014). Crown rot results from myceliogenic germination of *S. sclerotiorum* and *S. minor* sclerotia, leading to infection of stems and crowns near the soil surface, wilting, and potentially plant death. As pyrethrum flowers are produced terminally on stems approximately 70–100 cm in height, and carpogenic germination of *S. minor* was considered unlikely in nature (Abawi & Grogan 1979), it has been assumed that *S. sclerotiorum* was the only species causing Sclerotinia flower blight (Pethybridge et al. 2008, 2010). Spatial analyses also have demonstrated a significant association between the presence of *S. sclerotiorum* apothecia and the incidence of flowers with Sclerotinia flower blight (Pethybridge et al. 2010).

The objective of this study was to quantify the role and importance of *S. minor* in Sclerotinia flower blight of pyrethrum. This information is essential for understanding the epidemiology of flower diseases and the Sclerotinia disease complex affecting pyrethrum, and for the design of appropriate management strategies.

Materials and methods

Prevalence and incidence of Sclerotinia *spp. in pyrethrum flowers in commercial fields*

Twenty-five flowers were collected systematically within a diagonal transect across a 240 m^2 arbitrary location within each pyrethrum field. Multiple fields were sampled in each of three successive seasons (number of fields, n = 10 in 2007; n = 15 in 2008; and n = 29 in 2009). In 2007 and 2008, flowers were collected between 2–4 December (budding) and 14–22 December (flowers open). In 2009, only mature flowers were sampled within the latter sampling dates. Flowers were placed in mesh bags and surface-sterilized in 2% sodium hypochlorite in distilled water for 5 min and then immersed in three successive changes of sterile distilled water before drying. Five flowers were placed on fibreglass mesh suspended within sealed plastic trays containing damp tissue to maintain high humidity. Trays were incubated at room temperature and observed for fungal growth after 14 days. Sclerotia produced *in vitro* were surface-sterilized in 4% sodium hypochlorite for 2 min before being

rinsed with three consecutive washes of sterile distilled water. After drying on filter paper, sclerotia were bisected and placed on Petri plates containing potato dextrose agar (PDA) and incubated in the dark at 20°C. Hyphal tips were removed from colonies after 48 h and placed on PDA. Isolates on PDA were identified according to the description of Willetts & Wong (1980), including sclerotial size and distribution on artificial media, and ascospore dimensions. Isolates were stored at −80°C as air-dried PDA-colonized plugs, or as sclerotia at 5°C.

The prevalence and incidence of *Sclerotinia* spp. associated with pyrethrum flowers collected from commercial fields was summarized. Prevalence was defined as the number of fields where *S. minor* and *S. sclerotiorum* was isolated compared with the total number of fields sampled × 100. Incidence was defined as the number of diseased flowers from which either *S. minor* or *S. sclerotiorum* was isolated compared with the total number of flowers sampled × 100.

Pathogenicity of Sclerotinia minor *to pyrethrum flowers*

The pathogenicity of *S. minor* to pyrethrum flowers was tested on 8-month-old plants in replicated experiments. An apothecium from each of four isolates from pyrethrum flowers was excised and placed in 4 mL of sterile distilled water to produce inoculum, with concentrations adjusted to 4.7×10^6 and 2.7×10^9 ascospores mL^{-1} for the first and second experiments, respectively. Germination efficiency (%) was quantified by streaking 100 µL of inoculum across the surface of 10 PDA plates, which were incubated in the dark for 24 h at room temperature. One hundred ascospores were then assessed for viability on each plate from three microscope fields arbitrarily located on the plate surface but avoiding the edges. An ascospore was considered germinated when the germ tube was at least the length of the ascospore.

For each experiment, five flowers on each of 10 pyrethrum plants were inoculated with the ascospore suspension (~ 0.6 mL) containing 0.1% Tween-20 by dipping the individual flowers into the suspension for 20 s. The same number of flowers and plants were also dipped into sterile distilled water as non-inoculated controls. All flowers were of the same maturity when inoculated, with ray florets horizontal and between 0 and 30% of disc florets open. Plants were then maintained under high humidity for 48 h by covering with plastic bags supported with stakes to prevent contact with flowers. Plants were maintained in a glasshouse for an additional 14 days, at which time flowers were examined for disease symptoms. At this time, flowers were also detached and placed in high humidity, and observed for fungal growth after 14 days. Isolations of resultant fungi and sclerotia were then made according to the methods described for the isolations from field flower samples. The effect of inoculation on disease incidence in each experiment was assessed by analysis of variance (Genstat 14; Version 1 (VSN International, Hemel Hempstead, UK). Means were separated using Fisher's protected least significant difference test ($P = 0.05$).

Characterization of Sclerotinia *spp. isolates from pyrethrum flowers*

Ten isolates obtained from pyrethrum flowers putatively identified as *S. minor* on the basis of morphological characters (Willetts & Wong 1980) were selected for further characterization. Nine of these isolates were obtained from flowers collected over three years. The remaining isolate was found within a pyrethrum field at Table Cape, Tasmania, Australia, as a carpogenically germinated sclerotium. For this isolate, the apothecium was placed in 2 mL sterile distilled water and an aliquot (100 µL) of the ascospore suspension was pipetted onto PDA and spread with a sterile glass rod. Plates were incubated in the dark (20°C for 24 h) and observed after 36 h. Germinated ascospores were transferred to PDA and incubated in the dark to obtain monosporic isolates for storage.

Morphological characterization. Mycelial plugs of isolates were removed from storage and incubated at 20°C on PDA. Four replicates of each isolate were then subcultured onto PDA and incubated in the dark at 20°C for 14 days. Sclerotial dimensions for each isolate were measured. Twenty-five sclerotia from each of four PDA plates were measured per isolate ($n = 100$). Sclerotia from each isolate were conditioned according to the method described by Ekins et al. (2005) for measurements of apothecial and ascospore dimensions. Briefly, sclerotia from hyphal-tipped cultures were placed on V8 juice agar, incubated in darkness at room temperature and sub-cultured at 7-day intervals. After three transfers on V8 juice agar, isolates were transferred to wholemeal agar plates and incubated at 20°C in darkness for a further 4 weeks. Sclerotia were scraped onto sterile filter paper and dried for 2 days. Sclerotia ($n = 200$/isolate) were placed in 10 mL of sterile distilled water and incubated at 15°C for 8 h, followed by 10°C for 16 h for 12 weeks. Isolates were then placed at 10°C for a further 14 days in darkness, before exposure to 15W fluorescent daylight tubes illuminated with an 8 h photoperiod at 10°C for

12 weeks. The length and width of 10 mature asci and 100 ascospores from each isolate were measured.

Molecular characterization. For each isolate, mycelia were recovered from a 10–14-day-old potato dextrose broth. Tissue was placed in a Tissue Lyser (QIAGEN Inc., Valencia, CA, USA) for 1 min at 30 hz s^{-1} in the presence of the equivalent of 0.5 ml of 0.5 mm glass beads. DNA was diluted to 2 ng μL^{-1} and stored at −20°C before use. The 5.8S nuclear ribosomal RNA gene and the flanking internal transcribed spacers (ITS) were amplified using the primers ITS1 and ITS4 (White et al. 1990). PCR reactions were performed in a BioRad C1000 Thermal Cycler (BioRad Laboratories Pty Ltd, Gladesville, New South Wales, Australia) in a total volume of 25 µL using a Phusion High-Fidelity PCR Master Mix (Finnzymes, Thermo-Fisher Scientific Australia Pty Ltd, Scoresby, Victoria, Australia) incorporating 12.5 µL of 2 × Master Mix with HF Buffer, 400 µM of each primer and 10 ng genomic DNA. PCR conditions were: (i) initial denaturation at 98°C for 30 s; (ii) 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s; and (iii) 5 min of final extension at 72°C. Amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN) before direct bidirectional sequencing using an AB 3730xl DNA Analyzer and BigDye Terminator v.3.1 chemistry according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

DNA consensus sequences of the ITS region were obtained by alignment of the forward and reverse sequences. Sequences were compared with other *Sclerotinia* spp. available within GenBank (Table S1) and aligned. Phylogenetic relatedness between sequences was tested within maximum parsimony and Bayesian inference frameworks. Maximum parsimony was conducted using PAUP* v4.0.10b (Swofford 2003) using a heuristic search with stepwise branch addition. Indels were treated as missing data. Branch support was assessed by analysing 1000 bootstrap pseudoreplicates (Felsenstein 1985), with values greater than 70% considered significant (Hillis & Bull 1993). Bayesian inference was conducted using MrBayes v3.2.1 (Ronquist & Huelsenbeck 2003), using the SYM model with gamma correction. The evolutionary model was selected based on the Akaike Information Criterion (AIC) following analysis with jModelTest v2.1.4 (Nylander 2004). Within MrBayes, two concurrent analyses of four chains (one cold, three heated) were run for 1 200 000 generations to ensure the average standard deviation of split frequencies fell below 0.01. Trees were sampled every 200 generations. The first 200 000 generations were discarded to ensure the analyses reached stationarity prior to parameter estimation. From the remaining data, a majority rule consensus tree was constructed and posterior probabilities estimated. Branches with posterior probabilities of 0.95 or greater were considered significant. *Monilinia fructicola* (GenBank FJ411109) was used as an outgroup for both analyses.

To further confirm the identity of the *S. minor* isolates, the species-specific PCR assay of Abd-Elmagid et al. (2013) was used. The concentration of DNA within the extractions was quantified using a Qubit® 2.0 Flurometer (Life Technologies, Carlsbad, CA) and a Qubit® dsDNA HS Assay Kit. Aliquots of DNA were then diluted to a concentration of 1 ng μL^{-1} with sterile distilled water. The primer pair, SMlcc2 F and SMlcc2 R, specific for *S. minor* (Abd-Elmagid et al. 2013), was used for PCR amplification. Reactions consisted of 1× TopTaq PCR Buffer including 1.5 mM $MgCl_2$ (QIAGEN), 800 µM of each primer, 200 µM of each dNTP, 1× CoralLoad™ (QIAGEN) and 0.5 U of TopTaq™ DNA polymerase (QIAGEN) in a total volume of 10 µL. Amplification was carried out in a Bio-Rad C1000 thermal cycler with an initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 61°C for 20 s and 72°C for 40 s, with a final extension at 72°C for 7 min. Amplicons were separated by gel electrophoresis in 1.5% agarose gels pre-stained with GelRed™ nucleic acid stain (Biotium Inc., Hayward, CA, USA) and visualized by UV illumination. DNA extracts from *S. minor* (VPRI 21356; National Collection of Fungi, Bundoora Herbarium, Melbourne, Victoria, Australia), *S. sclerotiorum* (BRIP 43020; Queensland Plant Pathology Herbarium, Dutton Park, Queensland, Australia), and *S. trifoliorum* (VPRI 22449; National Collection of Fungi) sourced from culture collections were included as positive controls.

Results

Prevalence and incidence of Sclerotinia *spp. in pyrethrum flowers in commercial fields*

Sclerotinia minor was isolated from diseased and asymptomatic buds and flowers from commercial pyrethrum fields in all years of this study (Table 1). In 2007, *Sclerotinia minor* and *S. sclerotiorum* were found in buds collected from all fields. *Sclerotinia sclerotiorum* was the predominant species in all other samplings and was isolated from buds and flowers in all fields in 2008, and was 5.7 times more abundant than *S. minor* in flowers in 2009. The highest mean incidence of *S. minor* in buds was in 2007. The highest mean incidence of *S. minor* in

Table 1. Prevalence and incidence of *Sclerotinia minor* and *S. sclerotiorum* isolated from buds and flowers in pyrethrum fields in Tasmania, Australia, sampled from 2007 to 2009.

		Sclerotinia minor		*Sclerotinia sclerotiorum*	
Year[a]	Tissue[b]	Prevalence (%)[c]	Mean incidence (%)[d]	Prevalence (%)[c]	Mean incidence (%)[d]
2007 (10)	Buds	100	3.8 (1–7)	100	15.8 (8–27)
	Flowers	60	3.4 (1–14)	100	24.9 (2–59)
2008 (15)	Buds	20	2.5 (1–34)	100	23.1 (6–69)
	Flowers	33	5 (1–60)	100	26.5 (6–60)
2009 (29)	Flowers	10.3	2.5 (1.3–35)	58.6	5.5 (4–36)

[a]Number of fields sampled in each year represented in parentheses.
[b]Twenty-five flowers were collected systematically within a diagonal transect across a 240 m^2 arbitrarily located area within each field.
[c]Prevalence = number of fields where *Sclerotinia minor* and *S. sclerotiorum* was isolated/number of fields sampled × 100; Incidence = number of diseased flowers with either *S. minor* or *S. sclerotiorum*/number of flowers sampled × 100.
[d]Mean incidence across all fields. Minimum and maximum incidence on a per field basis provided in parentheses.

flowers was 5% in 2008; in one field, the highest incidence was 60%. The lowest mean incidence of *S. sclerotiorum* was 5.5% from flowers in the final year of this study. The mean incidence of *S. sclerotiorum* in buds in the first 2 years of this study was 19.5%, and from flowers was 18.9% across the entire study period (Table 1). The *Sclerotinia* sp. isolate germinating carpogenically on the soil in a pyrethrum field was within a 1 m distance of diseased plants (Fig. 1). Diseased plants were wilted and had bleached lesions typical of Sclerotinia crown rot. Flowers on adjacent plants were also diseased.

Pathogenicity of Sclerotinia minor *to pyrethrum flowers*

The viability of ascospores used in the first and second experiments was 97% and 95%, respectively. Inoculated flowers had ray florets that were shrivelled and necrotic (Fig. 2). Disc florets on inoculated flowers became light brown in colour, with sunken areas. Ray florets remained intact and disc florets remained yellow, with approximately two-thirds of disc florets open at the conclusion of the experiment on non-inoculated controls, typical of healthy flowers. All inoculated flowers developed necrotic symptoms and *S. minor* was isolated from 92% and 98% of inoculated flowers in the first and second experiments, respectively. No fungi were isolated from the non-inoculated controls. The effect of inoculation on the disease incidence and reisolation frequency of *S. minor* was significant in both experiments ($P < 0.001$).

Sclerotinia minor *isolate characterization*

Across all *S. minor* isolates from pyrethrum, mean length and width of sclerotia was 1.78 (± standard deviation = 0.19) mm and 1.22 (± 0.11) mm, respectively. Sclerotia were irregularly produced across the surface of PDA cultures of all isolates. Between isolates, mean asci length varied among isolates from 126.5 to 138.8 µm, and width from 8.3 to 11.0 µm.

A 455 bp segment of the ITS region was amplified from all 10 *S. minor* isolates. All isolates had identical sequences with the exception of BRIP 53345 and BRIP 53459, which differed from the remainder by one and two bases, respectively. BLAST comparisons indicated that the greatest sequence similarity of all isolates was to *S. minor*, with 98.4–100% sequence identity with *S. minor* isolates from GenBank (Fig. 3). Both maximum parsimony and Bayesian inference analyses showed isolates in this study formed a monophyletic clade within *S. minor* (Fig. 3). Sequences were deposited in GenBank with accessions ranging from JF279875 to JF279884. These numbers also represent the deposition codes for the Queensland Plant Pathology Herbarium, Indooroopilly, Queensland, Australia.

Further confirmation of the species identity was made from amplification of a 264 bp product from all putative *S. minor* pyrethrum isolates, and the *S. minor* isolate (VPRI21356). DNA from the cultures of *S. trifoliorum* (VPRI 22449) and *S. sclerotiorum* (BRIP 43020) failed to amplify (Fig. 4).

Discussion

Putative *S. minor* isolates were collected from diseased buds and flowers in Australian pyrethrum fields, with morphological and cultural characteristics consistent with previously published descriptions (Purdy 1955; Willetts & Wong 1980; Ekins et al. 2002, 2005). Moreover, all isolates from pyrethrum produced sclerotia

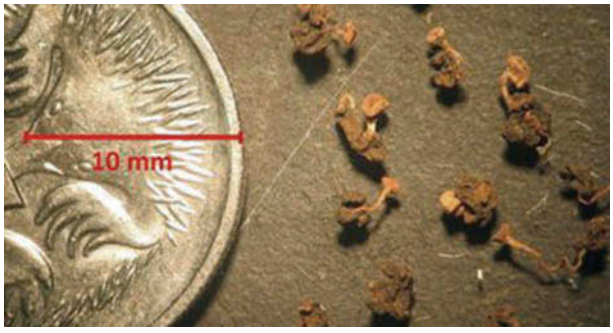

Fig. 1 (Colour online) *Sclerotinia minor* sclerotia with apothecia found on soil associated with plants affected by Sclerotinia crown rot and flower blight in a pyrethrum field in Tasmania, Australia.

across the agar surface when growing *in vitro*, consistent with reported patterns of *S. minor* (Willetts & Wong 1980) and distinct from *S. sclerotiorum*, in which larger sclerotia form at the periphery of colonies (Ekins et al. 2005). Mean ascospore length and width were also within the ranges described for *S. minor* (Purdy 1955; Ekins et al. 2005). Phylogenetic analysis provided additional evidence for identification of the isolates as *S. minor*. Although the ITS regions of *S. sclerotiorum, S. minor, S. trifoliorum* and *Botryis cinerea* have a high degree of sequence similarity (e.g. Holst-Jensen et al. 1998; Mandal & Dubey 2012; Osmundson et al. 2013), phylogenetic analyses indicated that *S. trifoliorum* and *S. minor* sequences formed two separate groups. Sequences of *S. sclerotiorum* were more variable, but also grouped separately from S. *trifoliorum* and *S. minor*. Sequences from isolates obtained from pyrethrum identified as *S. minor* based on morphological characters grouped with other published *S. minor* sequences and were distinct from *S. trifoliorum, S. sclerotiorum* and *B. cinerea*. Phylogenetic analyses also demonstrated low intraspecific variability within the ITS region of the *S. minor* isolates from pyrethrum. Further molecular confirmation of *S. minor* from pyrethrum flowers was obtained using *Sclerotinia* species-specific primers (Abd-Elmagid et al. 2013).

The pathogenicity of *S. minor* to pyrethrum flowers was confirmed by artificial inoculation and subsequent re-isolation, thereby completing Koch's postulates. Disease lesions were indistinguishable to those observed following infection by *S. sclerotiorum*; however, the smaller sclerotial size was indicative of disease caused by *S. minor* (Pethybridge et al. 2008). Sclerotia of *S. sclerotiorum* and *S. minor* were not observed on the same lesion. To the best of our knowledge, this the first report of the contribution of *S. minor* to flower blight in pyrethrum. This finding is consistent with the results of CLIMEX modelling that have previously

Fig. 2 (Colour online) Non-inoculated pyrethrum flower (upper) compared with flower inoculated with ascosporic inoculum of *Sclerotinia minor* after 14 days (lower).

predicted the potential for *S. minor* to germinate carpogenically in southern Australia, including regions where pyrethrum is grown (Ekins et al. 2002). A high proportion of *S. minor* isolates collected in this study were capable of carpogenic germination and produced viable ascospores following conditioning in the laboratory. However, the prevalence and incidence of *S. minor* associated with diseased flowers in the field were generally low compared with *S. sclerotiorum*. The difference in prevalence appears to be related to differences in availability of ascosporic inoculum by *S. minor* when

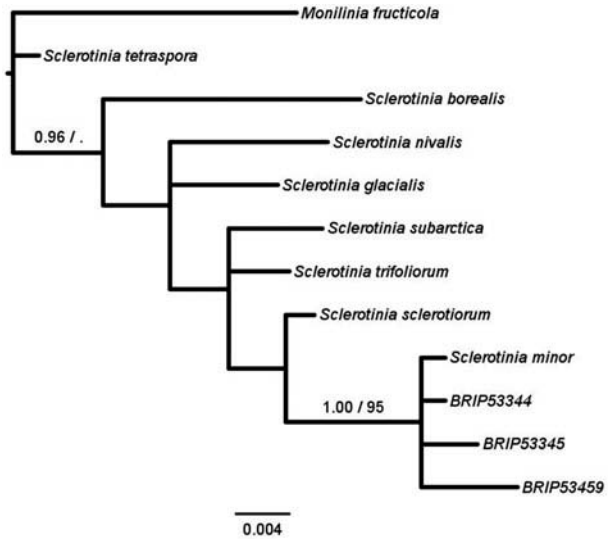

Fig. 3 Bayesian inference dendrogram based on the alignment of the ITS1, 5.8s and ITS2 rDNA region of *Sclerotinia* spp. Individual *Sclerotinia* species represented by a strict consensus sequence generated from alignments of all relevant GenBank sequences for that species (Supplemental Table 1). All putative isolates of *Sclerotinia minor* from Tasmania, Australia, except BRIP 53345 and BRIP 53459, shared 100% genetic homology and are represented by BRIP 53344. Branch values indicate posterior probabilities (PP) from Bayesian inference and bootstrap support (BS) from maximum parsimony analysis (PP/BS). Only branch values greater than 0.95 (PP) and 70% (BS) indicated. Dendrogram rooted using a single isolate of *Monilinia fructicola* (P169). Scale bar indicates the proportional genetic similarity.

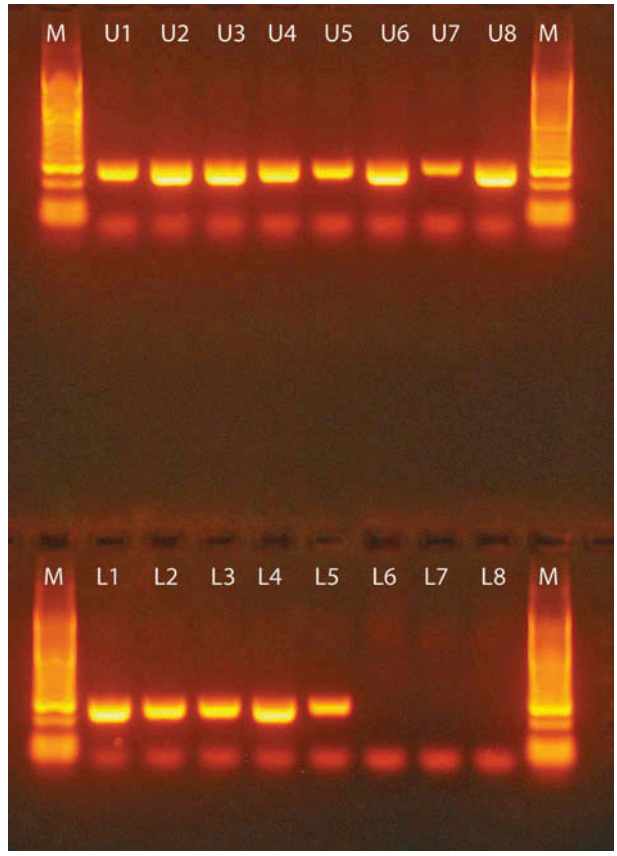

Fig. 4 (Colour online) Gel showing 264 bp polymerase chain reaction product amplified using the *Sclerotinia* species-specific primers described by Abd-Elmagid et al. (2013) for 10 *Sclerotinia minor* isolates (pyrethrum: upper row [U] 1 to 8, and lower row [L] 1 and 2); L3 and L4: Two replicates of *S. minor* culture VPRI 21356; L5: *S. minor* culture BRIP (Queensland Plant Pathology Herbarium, Australia) 42890; L6: *S. trifoliorum* VPRI (National Collection of Fungi, Australia) 22449; L7: *S. sclerotiorum* BRIP 43020; and L8: distilled water. M indicates a molecular size marker. PCR products were separated on a 1.5% agarose gel pre-stained with GelRed™ nucleic acid stain.

flowers are present, as Sclerotinia crown rot, caused by *S. minor* and *S. sclerotiorum*, is common in pyrethrum fields in Tasmania (Scott et al. 2014). The lower isolation frequency of *S. minor* from flowers compared with *S. sclerotiorum* could be partially due to a more stringent temperature range required for the formation of apothecia by the former species. The temperature range for apothecia development for *S. minor* has been reported to range from 11 to 17°C, and temperatures of 20°C or higher are sufficient to inhibit stipe initiation (Hao et al. 2003). In contrast, *S. sclerotiorum* produces apothecia over temperatures ranging from 4°C to 30°C (Schwartz & Steadman 1978; Phillips 1987). In our study, two of the 10 *S. minor* isolates failed to produce apothecia *in vitro* under what should be ideal conditions. This suggests that either some *S. minor* isolates are not subject to conditioning for carpogenic germination or there are substantial differences in optimal temperatures for conditioning and apothecial production within *S. minor* isolates. The mean maximum daily temperature over flowering for the period 1981–2013 was 17.8 and 19.6°C during November and December, respectively, at Devonport, a location representative of the pyrethrum production area in Tasmania, Australia. Similarly, the mean maximum daily temperature in November and December for the period 1981–2013 at Ballarat, representative of the production area in Victoria, Australia, was 20.1 and 22.7°C, respectively. This suggests that mean maximum temperatures over pyrethrum flowering in northern Tasmania in December, and in Ballarat in November and December, may be above the optimum reported by Hawthorne (1976) for stipe formation in *S. minor*. Maximum temperatures over these months in Victoria may be inhibitory to apothecial development of *S. minor*.

Future studies will aim to quantify the losses caused by pathogens infecting pyrethrum flowers and the association between crown rot and flower blight. This information will be used as the basis for a cost-benefit analysis of the current prophylactically applied flowering fungicide programme that underpins disease management. Fungicides will likely remain an important component of durable integrated disease management strategy, but the timing and efficacy of applications may need to consider the composition of *Sclerotinia* spp. likely to be present within and among fields and potential polyetic nature of epidemics. Given the wide host range of *Sclerotinia* spp. (Boland & Hall 1994; Melzer et al. 1997), cultural tactics that reduce the number of infection periods within a season, such as tillage (Ferraz et al. 1999; Gracia-Garza et al. 2002) and canopy manipulation (Williams & Stelfox 1980), may also contribute to disease management. These findings have immediate implications for disease management in pyrethrum. For example, reducing plant densities and row orientation may reduce the relative humidity under the canopy and therefore conditions conducive to carpogenic germination and disease development. These findings also suggest a re-evaluation of the potential for carpogenic germination of *S. minor* may be warranted in other pathosystems. The pathogen may have previously unrecognized roles in other diseases where carpogenic germination has been potentially overlooked.

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Supplemental data

Supplemental data for this article, Supplemental Table 1, can be accessed here: http://dx.doi.org/10.1080/07060661.2015.1036122

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