Pestalotiopsis chamaeropsis causing leaf spot disease of round leaf mint-bush (*Prostanthera rotundifolia*) in Australia

Azin Moslemi¹ · Paul W. J. Taylor¹

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Abstract *Pestalotiopsis chamaeropsis* was isolated from necrotic leaf spots of round leaf mint-bush -*Prostanthera rotundifolia* (Lamiaceae) in Melbourne, Australia. Pathogenicity was confirmed by obtaining similar symptoms after inoculation of the young leaves and stems with a spore suspension of *P. chamaeropsis*. Molecular analysis of the ITS, *tefl* and TUB genes, individually and combined, identified the pathogen as *P. chamaeropsis*. The pigmentation of the median cells, number of apical and basal appendages were similar to the type isolate of *P. chamaeropsis*. This is the first report of *P. chamaeropsis* being a pathogen of round leaf mint-bush in Australia.

Keywords ITS · *tefl* · TUB · Apical · Type isolate

Prostanthera rotundifolia (Lamiaceae) is a native mint-bush plant growing in cool and temperate regions of Australia such as South Australia, Victoria, New South Wales, north and east of Tasmania (Fulton 2000; Conn 1998). The oil extracted from *P. rotundifolia* contains hexane, ethyl acetate and methanol which are antimicrobial properties and inhibit growth of bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (Dellar et al. 1994).

There are very few reports of plant pathogens causing diseases in *P. rotundifolia*. Formerly, *Phytophthora cinnamomi* was isolated from mint-bush but the plant was recognised as a resistant host and showed no disease symptoms (Barker and Wardlaw 1995). In 2013, young and newly grown leaves and

stems of mint-bush plants growing in the Systems Garden at the University of Melbourne, Australia were found showing brown, water-soaked and sunken round lesions (predominately on the underside), darker in the margins, light brown-red in the centre. Chlorotic tissue occurred on the surface of the leaves but not underside. Typical symptoms of the disease were collected from the mint-bush plant and transferred to the laboratory in plastic bags for culturing and isolate identification.

Leaves were surface sterilised by immersing for 30 s in 80 % ethanol, before transferring to 1 % a.i. sodium hypochlorite for 1 min, followed by washing in sterilised water. Sterilised leaves were then blotted with sterilised paper towel, then 2 mm² of tissue from the margin of the lesion with healthy tissue was excised and plated onto water agar (WA) and incubated for 4 days at 23–25 °C. Mycelia that grew out of the leaves were transferred onto potato dextrose agar (PDA) and incubated for a further 7 days. Single spored colonies were obtained from isolates which were grown on PDA and incubated for 10 days at 22–24 °C. A culture of the isolate was deposited at the Queensland Plant Pathology Herbarium (BRIP), Australia.

Colony growth rate was measured for three PDA cultures at 2-day intervals. The isolate was also grown on synthetic poor nutrient agar (SNA) (Crous et al. 2009) with pieces of sterilized filter papers (double autoclaved at 121 °C for 20 min) on the surface of the agar to induce sporulation. Seven days after culturing, spores were mounted in lactic acid and the conidia shape and size measured.

To confirm pathogenicity, twigs containing rosettes of young healthy leaves and stems were cut from a healthy plant; surface sterilised and then placed in plastic containers. Inoculations were by either spraying leaves with 10⁴ spore/mL, or by pipetting 20 µl of spore suspension onto both sides of the leaves. Containers were sealed then incubated at 23–

✉ Paul W. J. Taylor
paulwjt@unimelb.edu.au

¹ Faculty of Veterinary and Agricultural Science, University of Melbourne, Melbourne, VIC 3010, Australia

25 °C for 7 days. Controls were inoculated with sterilised water. Leaf and stem tissue that developed necrotic lesions were cultured onto PDA until sporulation, then the pathogen's morphological characteristics were recorded.

DNA was extracted from the fungal mycelia scraped directly from 7-day-old single spored cultures on PDA using the DNeasy Plant Mini Kit (Qiagen) following the manufacture's instruction.

The internal transcribed spacer (ITS) regions was amplified using primers ITS1 and ITS4 (White et al. 1990), the partial β -tubulin (TUB) gene was amplified using primers BT2A and BT2B (Maharachchikumbura et al. 2012) and the *tef1* gene was amplified using primers EF1-728 F and EF2 (Maharachchikumbura et al. 2014; O'Donnell et al. 1998; O'Donnell et al. 2010). PCR was performed using the procedure described in Maharachchikumbura et al. (2012), with a slight difference in the amount of the template DNA used, except that 10 ng of template DNA was used for ITS and *tef1*; and 30 ng for TUB genes. DNA amplification was carried out using an Eppendorf thermal cycler. PCR products were then purified using QIAquick PCR purification kit (Qiagen) and an electrophoresis gel was run after purification to test the integrity of the bands. All PCR and purification products were stained in ethidium bromide and visualised under UV light.

Purified amplicons were sent to Australian Genome Research Facility Ltd. for sequencing. Sequence intensity was assessed using ChromasLite MFS computer package and then were aligned using the multiple alignment program ClustalW.

The consensus sequences were obtained by alignment of forward and reverse sequences using MEGA6 (Tamura et al. 2013) and deposited in GenBank (Table 1). BLAST searches in GenBank using the obtained ITS, TUB and *tef1* sequences revealed highest similarity with multiple species of *Pestalotiopsis*. Therefore, to study the taxonomic placement of the isolate within *Pestalotiopsis* species complex, sequences of *Pestalotiopsis* type strains available from GenBank were retrieved for inclusion in the phylogenetic analyses (Maharachchikumbura et al. 2014); The accession numbers of all sequences are listed in Table 1.

Phylogenetic trees were constructed for each gene individually and combined in MEGA6. Trees were analysed using Maximum Parsimony (MP) statistical method. *Seiridium* sp. was used as the outgroup (Maharachchikumbura et al. 2012; Zhang Y et al. 2012). To assess the relative stability of branches, bootstrap analysis with 1000 replicates was performed. Gaps were treated as missing data.

On PDA and mint-bush stems, black, pycnidia-like conidiomata containing septated conidiospores with apical appendages developed which was typical of *Pestalotiopsis* species. The culture characteristics were white fluffy aerial mycelia on PDA that reached a diameter of 35–45 mm after

a week. Black conidiomata scattered on the surface of the mycelia, were produced after 7–10 days of culturing with the reverse side white-pink colour.

The black conidiomata were mostly aggregated, globose, >500 μm diameter. The conidiospores were 4-septate (5 celled) and were fusoid to ellipsoid; second cell from the base was $4.0\text{--}5.7 \times 4.0\text{--}5.3$ μm ; third cell was $3.0\text{--}5.8 \times 5.0\text{--}6.9$ μm ; fourth cell was $4.0\text{--}6.0 \times 4.0\text{--}5.5$ μm with a total length of $19.0\text{--}27.6$ μm . There were three median cells doliiform, $13.0\text{--}17.0$ μm long, concolorous with the two upper median cells slightly darker than the median basal cell. The apical cells were hyaline, $3.5\text{--}5.7 \times 2\text{--}3.9$ μm with 2 to 3 unbranched, unknobed tubular apical appendages (mostly 3) with the average size of 15.19 μm . The basal cell was obconic, hyaline, $3.7\text{--}6.2 \times 2.0\text{--}3.5$ μm , single unbranched with a cylindrical basal appendage, 5.65 μm long (Fig. 1).

Disease symptoms and infection by the mint-bush *Pestalotiopsis* species on both sides of the leaves were significantly higher than on the stems. On mint-bush, fewer symptoms were observed during May–August 2013 and 2014 which may have been due to lower temperatures in autumn and winter.

The underside of young leaves and stems inoculated by either spray or droplets of spores developed globose, brown and water soaked lesions after 1 week. *Pestalotiopsis* spores were obtained from the lesions. Cultures from infected leaves were the same as those of the *Pestalotiopsis* species originally isolated from mint-bush plants.

Nineteen isolate sequences were used to construct phylogenetic trees (Table 1). Combined tree of the three genes was selected out of 5 most parsimonious trees (length=692), consistency index (0.622951), retention index (0.684066), and the composite index (0.570384) for all sites and parsimony-informative sites. There were a total of 1402 positions in the final dataset (Fig. 2). In both individual and combined trees, the mint-bush *Pestalotiopsis* isolate clustered with *P. chamaeropsis*.

Individual and combined phylogenetic trees showed that the *Pestalotiopsis* species causing leaf spot of *P. rotundifolia* clustered with other isolates in the *P. chamaeropsis* species complex. In the ITS tree (unpublished), the mint-bush isolate also clustered with *P. linearis* and *P. intermedia* along with *P. chamaeropsis*. Maharachchikumbura et al. (2014) also reported that *P. chamaeropsis* formed a sister clade with *P. intermedia* and *P. linearis* in the combined phylogeny of the three genes. Maharachchikumbura et al. (2012) reported that these three closely related species could not be separated on spore size and shape because of the large variation in these characters.

Morphological characters of the mint-bush *P. chamaeropsis* showed similar characteristics to the ex-type isolate such as pigmentation of the median cells (concolorous), number of apical appendages (2–3, mostly 3), basal appendages and

Table 1 List of isolates with their accession numbers used in this study; ex-type isolates are in bold type

<i>Pestalotiopsis</i> isolates	Culture collection No.	Host	Location	ITS	TUB	<i>tefl</i>
<i>P. chamaeropsis</i>	BRIP 62468	<i>Prostanthera rotundifolia</i>	Australia Melbourne	KR259104	KR259103	KR259102
<i>P. chamaeropsis</i>	CBS 113604	—	—	KM199323 ^a	KM199389 ^a	KM199471 ^a
<i>P. chamaeropsis</i>	CBS 237.38	—	Italy	KM199324 ^a	KM199392 ^a	KM199474 ^a
<i>P. chamaeropsis</i>	CBS 113607	—	—	KM199325 ^a	KM199390 ^a	KM199472 ^a
<i>P. chamaeropsis</i>	CBS 186.71	<i>Chamaerops humilis</i>	Italy	KM199326 ^a	KM199391 ^a	KM199473 ^a
<i>P. australis</i>	CBS 114193	<i>Grevilliae</i> sp.	Australia; New South Wales	KM199332 ^a	KM199383 ^a	KM199475 ^a
<i>P. camelliae</i>	CBS 443.62	<i>Camelliae sinensis</i>	Turkey	KM199336 ^a	KM199424 ^a	KM199512 ^a
<i>P. colombiensis</i>	CBS 118553	<i>Eucalyptus eurograndis</i>	Colombia	KM199307 ^a	KM199421 ^a	KM199488 ^a
<i>P. grevilleae</i>	CBS 114127	<i>Grevilliae</i> sp.	Australia	KM199300 ^a	KM199407 ^a	KM199504 ^a
<i>P. australasiae</i>	CBS 114141	<i>Protea</i> sp.	Australia; New South Wales	KM199298 ^a	KM199410 ^a	KM199501 ^a
<i>P. hollandica</i>	CBS 265.33	<i>Sciadopitys verticillata</i>	Netherlands	KM199328 ^a	KM199388 ^a	KM199481 ^a
<i>P. novae-hollandiae</i>	CBS 130973	<i>Banksia grandis</i>	Australia	KM199337 ^a	KM199425 ^a	KM199511 ^a
<i>P. intermedia</i>	MFLUCC 12-0259	Unidentified tree	China	JX398993 ^a	JX399028 ^a	JX399059 ^a
<i>P. linearis</i>	NN0471900	<i>Trachelospermum</i> sp.	China	JX398992 ^a	JX399027 ^a	JX399058 ^a
<i>P. rhododendri</i>	RFRDCC 2399	<i>Rhododendron sinogrande</i>	China	KC537804 ^a	KC537818 ^a	KC537811 ^a
<i>P. telopeae</i>	CBS 113606	<i>Telopea</i> sp.	Australia	KM199295 ^a	KM199402 ^a	KM199498 ^a
<i>P. unicolor</i>	MFLUCC 12-0275	Unidentified tree	China	JX398998 ^a	JX399029 ^a	JX399063 ^a
<i>P. scoparia</i>	CBS 176.25	<i>Chamaecyparis</i> sp.	—	KM199330 ^a	KM199393 ^a	KM199478 ^a
<i>Seiridium</i> sp.	—	—	—	JQ683725 ^{ab}	JQ683709 ^{ab}	JQ683741 ^{ab}

^a (Maharachchikumbura et al. 2014), ^b (Maharachchikumbura et al. 2012)

Fig. 1 **a** - brown water soaked lesions on the back of the leaves, **b**- brown pycnidia-like lesions on the young stem, **c**- formation of conidiomata on a mint-bush stem, **d**- Black conidiomata on PDA after 7 days, **e**- conidiogenous cells, **f**- conidiospores. Scale bars: E-F- 20 μ m

Fig. 2 Maximum Parsimony (MP) analysis of the combined ITS, TUB and *tef1* genes. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm. The analysis involved 19 nucleotide sequences. Consistency index (CI)= 0.62; retention Index (RI)= 0.68. * refers to ex-type species. Culture accession numbers were derived from Maharachchikumbura et al. (2014)

septa (4-septate) (Maharachchikumbura et al. 2014). However, the length and width of median, basal and apical cells were shorter. Jeewon et al. (2003) reported that the length of spores, median cells and appendages were not phylogenetically important in identifying the species, however, these factors might be informative in identifying species groups. In contrast, pigmentation of the median cells and appendage tip morphology were informative factors in phylogenetic identification of the species.

Most *Pestalotiopsis* species are pathogenic, while some are endophytes or saprobes (Wei et al. 2007). Wei et al. (2007) reported the colonisation of the leaves of *Podocarpaceae*, *Theaceae* and *Taxaceae* families by endophytic *Pestalotiopsis* species in southern China and suggested that colonisation increased with leaf age and season. High temperatures (tropical regions) favoured endophytic *Pestalotiopsis* species (Liu et al. 2007).

Previously it was assumed that *Pestalotiopsis* species were host specific (Wei et al. 2007). However, Jeewon et al. (2004) and Hyde et al. (2014) demonstrated that most species isolated from the host plant were not necessarily host-specific. Moreover, host specification and geographical location became less informative with the introduction of molecular identification (Hyde et al. 2014). *P. chamaeropsis* was initially isolated from the leaves of dwarf palm (Herrera 1989) (*Chamaerops humilis*) in Italy in 1971 (Maharachchikumbura et al. 2014). Interestingly, the pathogen has not been identified on any other native plants in Australia to date and seems that it is only a pathogen of *Prostanthera rotundifolia*; the Australian mint-bush. However, other *Pestalotiopsis* species such as *P. australasiae*,

P. grevilleae, *P. telopeae* and *P. novae-hollandica* have been recorded as pathogens of native Australian plants (mostly isolated from the plants of Proteaceae) (Maharachchikumbura et al. 2014). *Pestalotiopsis* species are cosmopolite and can be isolated from different substrates. However, bioassays carried out with *P. chamaeropsis* isolated from necrotic spots on native mint-bush, proved the pathogenicity of the isolate for the first time in Australia.

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