Australasian Plant Dis. Notes (2011) 6:87–90
DOI 10.1007/s13314-011-0030-x

First report of *Phoma clematidina* the cause of leaf spot-wilt disease of *Clematis pubescens* in Australia

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Received: 12 April 2011 / Accepted: 5 October 2011 / Published online: 3 November 2011

Abstract Leaf lesions and wilt of *Clematis pubescens* were observed in the Jarrah (*Eucalyptus marginata*) forest of South-Western Australia in 2009. A *Phoma* sp. was consistently isolated from the infected tissues and its morphological characteristics were determined. The identity of the fungus was confirmed by sequencing of the internal transcribed spacer (ITS) region of the rDNA operon and *Phoma clematidina* was identified. A pathogenicity test was conducted and Koch's postulates were fulfilled by re-isolation of the identified fungus. This is the first report of *P. clematidina* causing leaf lesions and wilt of *Clematis pubescens* in Australia.

Keywords Phoma leaf spot-wilt · Clematis · Rehabilitation · Jarrah forest

The cosmopolitan genus *Clematis* is a member of the *Ranunculaceae* family and contains 300 species, mostly climbers or groundcovers. Many of these are grown commercially. *Clematis pubescens* Endl. is found throughout the Jarrah (*Eucalyptus marginata*) forest of South-Western Australia. There are three *Clematis* species present in Western Australia (*C. delicata*, *C. linearifolia*, *C. pubescens*) but only *C. pubescens* occurs in the northern Jarrah forest (Wheeler et al. 2002).

Alcoa of Australia Limited operates two bauxite mines in the northern Jarrah forest and about 600 ha are mined and rehabilitated annually. The rehabilitation aim is to restore a self-sustaining Jarrah forest ecosystem (Elliot et al. 1996; Gardner 2001). Due to low germination and survival rates, it is difficult to establish *C. pubescens* using broadcast seed in rehabilitation areas. The current practice involves planting established seedlings and has been especially developed for rehabilitation of bauxite mines by Alcoa's Marrinup Nursery.

As *C. pubescens* is new to horticultural production, little is known about its diseases although sporadic leaf lesions were observed in 2008. *Ascochyta clematidina* and *Coniothyrium clematidis-rectae* have been reported as the causal organisms of leaf spot and wilt of *Clematis* spp. However, *A. clematidina* was transferred to the genus *Phoma* as *Phoma clematidina* (Thüm.) Boerema, Versl. Meded. Plziektenk. (Woudenberg et al. 2009). *P. clematidina* causes destructive disease on *Clematis* spp. and is widespread in Europe, America and New Zealand (Smith and Cole 1991; van de Graaf et al. 2001).

Symptoms of disease caused by *P. clematidina* were observed on *C. pubescens* in the south-west of Western Australia in 2009 (Fig. 1). Typical symptoms appeared on the leaves of propagated plants at the nursery as purple lesions that eventually coalesced to form large necrotic areas (Fig. 2). Favourable growing conditions during autumn 2009 resulted in the propagated plants having excessively long above-ground stems; too long for carrying into the mine rehabilitation sites during the forthcoming

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Fig. 1 *Clematis pubescens* showing leaf lesions and wilt after transplanting in the mine re-vegetation site of the south–west of Western Australia

rainy season (June to August). To facilitate the transport and transplanting of the propagated plants, the above-ground stems were heavily pruned near the end of the dry season, which was a new cultural practice and has been rarely adopted previously. The outbreak of leaf spots and wilt were pronounced on *C. pubescens* after transplanting. Pruning appeared to have increased the risk of infection via wounded tissues. *P. clematidina* has been reported to be a wound pathogen causing leaf spots on both wilt susceptible and wilt resistant cultivars (Smith and Cole 1991).

Twenty five sections of plant tissue surrounding the leaf lesions were surface-sterilised by immersion in a 1.25% aqueous solution of sodium hypochlorite for 2 min, rinsed in sterile water and dried in a laminar flow cabinet. The sterilised leaf pieces were then either placed on potato dextrose agar (PDA) and incubated at 22±3°C, with developing fungal colonies sub-cultured onto PDA and then single-spored to obtain pure cultures; or placed in trays on moist filter paper and incubated at 25°C with a 12-h dark and light cycle. A *Phoma* sp. was consistently isolated and the colony size, colony morphology and morphological characteristics of the isolated fungus were determined for 10 isolates. The colony was white to pale grey turning olivaceous grey with age. The average colony size was 30–40 mm after 7 days on PDA. Chlamydospores were uni- or multicellular thick-walled, brown, guttulate, in chains and 6–10 μm (unicellular) or 25–40×10–25 μm (multicellular) in diam. Pycnidia were immersed in culture and were formed subepidermally in leaf lesions. They were dark brown, subglobose, ostiolate and 100–125 μm in diam. Conidia were unicellular, variable in shape, subellipsoidal to cylindrical, usually 5–9×2–3.5 μm, occasionally larger with 1 septa and 10–15×3–4.5 μm on malt-extract agar (MEA) (Fig. 3a). However, conidia were distinctly large (12–25×4.5–6.5 μm) with up to two septa in older pycnidia that formed on the leaf lesions (Fig. 3b). Both cultural and morphological characteristics of the fungal isolates were similar to those described for *P. clematidina* (Boerema et al. 2004; Woudenberg et al. 2009).

Two representative isolates of morphologically identified *P. clematidina* were grown on PDA for 2 weeks at 25°C. Total genomic DNA was extracted from fungal mycelium with a DNeasy Plant Mini Kit (Qiagen, Melbourne, Vic., Australia) according to the manufacturer's instructions. Amplification of the internal transcribed spacer (ITS)1

Fig. 2 Leaf lesions on *Clematis pubescens* (*left*) caused by *Phoma clematidina* on the propagated plants in the nursery (*right*)

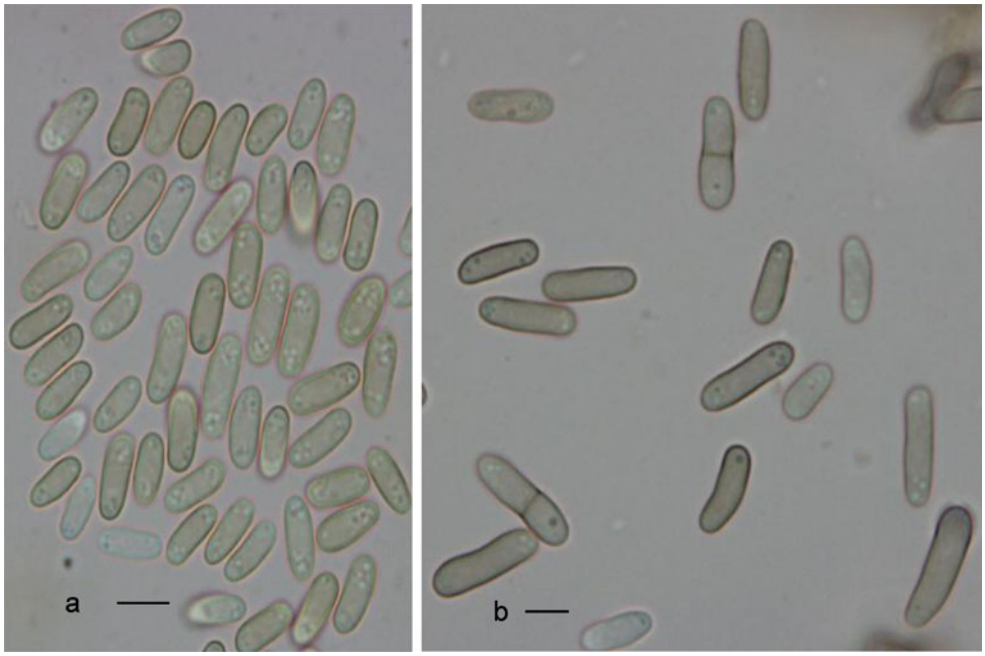

Fig. 3 Conidia of *Phoma clematidina* in vitro (**a**) and in vivo (**b**). *Bars*=5 μm

and ITS2 regions flanking the 5.8S rRNA gene were carried out with universal primers ITS1 and ITS4 according to the published protocol (White et al. 1990). The polymerase chain reaction (PCR) product was purified with Qiagen PCR purification kit (Qiagen, Melbourne, Vic Australia) and sequenced on an ABI 3730 DNA Sequencer (Applied Biosystems, Melbourne, Vic., Australia) at Murdoch University, Perth, WA, Australia. The two sequences of the ITS1 and ITS2 regions were identical to the sequences from the same region of *P. clematidina* (GenBank Accession No. FJ426991) in the NCBI database (www.ncbi.nlm.nih.gov/) as published by Aveskamp et al. (2009).

Pathogenicity tests were performed on 2-month old *C. pubescens* plants using two representative isolates of *P. clematidina*. Leaves and stems of three plants were inoculated by either wounding or non-wounding per isolate. Wounding was used as *P. clematidina* has been reported to be a wound pathogen (Smith and Cole 1991). A conidial suspension (10^6 spores/mL) was prepared in sterile water using a culture of each isolate grown on MEA. For the wounding inoculation test, the conidial suspension was dropped on the leaf and stem sites (5 μL per site/10 sites per plant) after pin-prick wounding using a syringe needle. For the non-wounding inoculation test the conidial suspension was sprayed on the plant surface to runoff. Control plants (one wounded and one non-wounded) were inoculated using sterilised water. All plants were placed under mist for 48 h and then moved to a growth room chamber at 22±3°C. Leaf lesion and wilt symptoms were observed 2–3 weeks post-inoculation. The symptom development was faster and more pronounced on wound-inoculated plants. Control plants remained asymptomatic.

Koch's postulates were fulfilled by re-isolation of *P. clematidina*. A culture of *P. clematidina*, the morphological identity of which was confirmed via ITS sequencing, was deposited in the Western Australia Plant Pathogen Collection (WAC13312). To our knowledge, this is the first report of *P. clematidina* causing leaf spot-wilt disease on *C. pubescens* in Australia.

Acknowledgements The authors would like to thank Ms Paula Mather for technical assistance.

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