The Etiology and Epidemiology of European Blackberry (*Rubus anglocandicans*) Decline in the South-West of Western Australia

By

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(B.Sc., M.Sc.)

This thesis is presented for the fulfilment of the requirements for the degree of Doctor of Philosophy, School of Veterinary and Life Sciences, Murdoch University

Perth, Western Australia, December 2013
DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Sonia Aghighi
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Prof. Giles E. St. J. Hardy and Associate Prof. Treena I. Burgess; I am very proud of working in your laboratories. Thank you for your enthusiasm, patience, thoughtful advice and support during my PhD journey.

Dr. John K. Scott; Thanks for your thoughtful advice and accompany during my fieldworks.

If I decide to do another PhD, I would like to work again with Giles, Treena and John!

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ABSTRACT

European Blackberry (*Rubus anglocandicans* A. Newton) is one of the top 20 Weeds of National Significance in Australia. It is a major weed of conservation areas, particularly in wetter regions, and is also a major weed of forestry and agriculture. *Rubus anglocandicans* is the most abundant and widespread species of European blackberry in the south-west of Western Australia (WA). Herbicides and cultural control methods are generally ineffective, or require multiple applications; however, this weed is often located within inaccessible areas, which limits control options. Therefore, biological control has been identified as the main option for the control of blackberry in Australia.

Biocontrol started in the 1980s, initially with the appearance a strain of the host-specific rust *Phragmidium violaceum* in Victoria. This, so called “illegal strain” and later the official strain of the rust were eventually spread to WA, but they failed to provide control. The blackberry species specifically targeted was *R. anglocandicans* – the main species in the Manjimup-Pemberton area and along the Warren and Donnelly Rivers in the south-west of Western Australia. New strains of the rust were released in 2004 and 2005. In some areas the levels of rust developed on blackberry was high, at least initially.

While monitoring for rust control, the presence of blackberry decline was noticed. The extent of the disease, with noticeable changes to vegetation structure, from an impenetrable tangle of vegetation to a parklike setting of trees and grass, following the disappearance of dense blackberry infestations, has lead to it being called “blackberry decline”. More detailed examination of the decline sites suggested that the decline was not due to the inoculated rust, but possibly due to a root pathogen.

Surveys between 2010 and 2012 led to the recovery of ten different *Phytophthora*, nine *Pythium* species and *Cylindrocarpon* species. The surveys also identified other abiotic
and biotic factors such as landforms and grazing that appear to be associated with the decline of blackberry. The *Phytophthora* species isolated included a new species from *Phytophthora* clade 6 which was described as *P. bilorbang* as a part of this study. The other *Phytophthora* species included *P. cinnamomi* from decline-free sites, and *P. amnicola, P. cryptogea, P. inundata, P. litoralis, P. multivora, P. taxon personii, P. thermophila*, and a *P. thermophila-amnicola* hybrid from decline sites. Primocane under-bark inoculations and pot infestation trials in the glasshouse provided evidence of the pathogenicity of *P. bilorbang* and *P. cryptogea* to *R. anglocandicans*. In a dual combination trial to examine synergistic effects between *Phytophthora* species, disease severity increased by combining at least two to three species including *P. amnicola, P. bilorbang, and P. cryptogea* under a regime of regular waterlogging. In an *in planta* under-bark inoculation trial in the field to confirm the pathogenicity of *Phytophthora* species in the blackberry decline with and without application of phosphite, phosphite reduced the size of lesions caused by all *Phytophthora* species. Extensive ‘on-ground’ surveys showed the “decline” to extend along at least 64 km of riverbank, and at present is only known from the Warren and Donnelly River Catchments.

In this project, the etiology and epidemiology of the decline distribution have been investigated and a conceptual model, a “blackberry decline spiral” is proposed to describe the key factors that are hypothesised to be involved in the decline phenomenon of *R. anglocandicans*. This model includes predisposing, inciting and contributing factors. It is assumed that predisposing or stress factors such as periodic flooding set the stage for inciting factors (e.g. lack of genetic potential in *R. anglocandicans* and grazing by animals). Whilst contributing factors (e.g. *Phytophthora* species as root pathogens and leaf rust) included in the blackberry decline spiral all have a role in this syndrome, the involvement of the hypothesized predisposing and inciting factors are also essential for the expansion of the decline.
This thesis has shown blackberry decline to be a complex syndrome made up of a number of factors, most significant of which are periodic flooding and damage to the roots by at least two *Phytophthora* species, *P. bilorbang* and *P. cryptogea*.

Publications resulting from the thesis

Journal papers


Conferences presentations (2010-2013)


• Aghighi S, Burgess TI, Scott JK, and Hardy GESrJ, 2012. 3rd Iranian PhD Students Conference in Australia and New Zealand. 27-29 April, Brisbane. Poster presentation.


• Aghighi S, Fontanini L, Yeoh PB, Scott JK, Burgess TI and Hardy GESrJ, 2012. Studies toward understanding the cause of blackberry decline in the South-West of Western Australia. 7th Australasian Soilborne Diseases Symposium. 17-20 September 2012, Notre Dame University, Perth, Australia. Poster presentation.

• Aghighi S, Hardy GESJ, Scott JK and Burgess TI, 2012. *Phytophthora bilorbang*, a new species associated with declining *Rubus anglocandicans* (blackberry) in the natural ecosystem of South-West of Western Australia. **Australasian Plant Pathology Society annual student symposium** October 26th, Department of Agriculture and Food, Perth. Oral presentation, **won the peer award**.


• Aghighi S, Fontanini L, Yeoh PB, Burgess TI, Scott JK and Hardy GESJ, 2013. The Association of *Phytophthora* Species in the Blackberry (*Rubus anglocandicans*) Decline in the Natural Ecosystems of South-Western Australia, **Die-back Information Group, with all the bells and whistles conference** (June 28th), State library of WA, Perth, Australia. Oral presentation.

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<td>Chapter 7</td>
<td>138</td>
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<td>150</td>
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</tbody>
</table>
CHAPTER 1

General Introduction

&

Literature Review

A part of this Chapter published as:


Minor authors’ contributions:
Giles Hardy, Treena Burgess and John Scott were supervisors. Lee Fontanini and Paul Yeoh provided field assistance.
Introduction

*Rubus anglocandicans* is the most common species of European blackberry in Western Australia (WA) and one of the few weeds of national significance in the south-west of WA. It is a major weed of conservation areas, forestry and agriculture. Exotic strains of the blackberry rust *Phragmidium violaceum* have been introduced to WA as biological control agents, but in most areas it seems that they are not effective, possibly due to climate.

In 2007 while monitoring establishment of the released rust strains, unexplained dead and diseased blackberry plants were discovered at two locations, along the Warren River near Pemberton and the Donnelly River near Manjimup in the south-west of WA. The extent of the disease, with noticeable landscape changes due to the disappearance of dense blackberry infestations, has lead to it being called “blackberry decline”. The organism or organisms responsible for killing the blackberry plants are so effective that within a couple of years, previously impenetrable stands of well established blackberry have been completely killed for at least several km from the initial sightings of disease symptoms. This chapter outlines the history of this invasive species with emphasis on its impact in Western Australia and its “decline” phenomenon in the south-west of WA.

*Rubus* is a cosmopolitan genus that includes raspberry, blackberry and numerous berry fruit cultivars for horticultural production (Evans *et al.*, 2007). *Rubus anglocandicans* A. Newton is considered as a biotype taxon of *R. fruticosus* L. aggregate (Table 1.1) which is known as European blackberry (USA) or bramble (UK) (Evans & Weber, 2003). *Rubus* originates from the Latin *ruber* meaning red and refers to the red immature berries and Anglocandicans refers to its origin in England [European Blackberry aggregate (*Rubus fruticosus* L. aggregate),
European blackberries and other exotic *Rubus* species are important weeds of agriculture, forestry and natural ecosystems in Australia, New Zealand, North America and South Africa (Parsons & Cuthbertson, 1992; Amor *et al.*, 1998). Blackberries include semi-deciduous, scrambling shrubs with tangled, prickly stems that form impenetrable thickets several metres high (Fig. 1.1).


<table>
<thead>
<tr>
<th>Taxonomic tree</th>
<th>Taxonomic position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domain</td>
<td>Eukaryota</td>
</tr>
<tr>
<td>Kingdom</td>
<td>Tracheobionta-vascular plants</td>
</tr>
<tr>
<td>Super-division</td>
<td>Spermatophyta-seed plants</td>
</tr>
<tr>
<td>Division</td>
<td>Magnoliophyta-flowering plants</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida-Dicotyledons</td>
</tr>
<tr>
<td>Sub-class</td>
<td>Rosidae</td>
</tr>
<tr>
<td>Order</td>
<td>Rosales</td>
</tr>
<tr>
<td>Family</td>
<td>Rosaceae-Rose family</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Rubus</em></td>
</tr>
<tr>
<td>Sub-genus</td>
<td><em>Rubus</em> (formerly <em>Eubatus</em>)</td>
</tr>
<tr>
<td>Species</td>
<td><em>Rubus fruticosus</em> L. aggregate</td>
</tr>
</tbody>
</table>
Figure 1.1 Blackberries are semi-deciduous, scrambling shrubs that form dense thickets (a, b) (photos kindly provided by Lee Fontanini, images along Warren River in south-west Western Australia).
There are 26 known introduced *Rubus* species in Australia a number of which belong to the *R. fruticosus* L. aggregate (Table 1.2). The other 10 are classed as other introduced weedy *Rubus* species and originate from either North America or Asia (Table 1.3). There are also 10 native *Rubus* species present in Australia and none of which are present in WA. Fig. 1.2 and Fig. 1.3 represent distribution map of blackberry (*R. fruticosus* L. aggregate) globally and in Australia, respectively.

**Table 1.2** Identified distinct species of European blackberry (*R. fruticosus* L. aggregate) in Australia until 2007 (NSW Department of Primary Industries, 2009).

<table>
<thead>
<tr>
<th>Species/State</th>
<th>WA</th>
<th>NT</th>
<th>SA</th>
<th>QLD</th>
<th>NSW</th>
<th>VIC</th>
<th>TAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubus anglocandicans</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. leucostachys</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. polyanthemus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. laciniatus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. ulmifolius var. ulmifolius</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. ulmifolius var. anoplothyrsus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. vestitus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. leightonii</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. erythrops</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. cissburiensis</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. echinatus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. rubritinctus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. phaeocarpus</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. riddelsdellii</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. sp. Tasmania</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>R. sp. Scott Creek</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 1.3 Other introduced *Rubus* species in Australia as recorded in 2008 (Evans *et al.*, 2007; NSW Department of Primary Industries Weed Management Unit, 2009).

<table>
<thead>
<tr>
<th>Species/State</th>
<th>WA</th>
<th>NT</th>
<th>SA</th>
<th>QLD</th>
<th>NSW</th>
<th>VIC</th>
<th>TAS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. laudatus</em> (Bundy/Plains blackberry)</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. philadelphicus</em> (lawtonberry)</td>
<td></td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. loganobaccus</em> (loganberry)</td>
<td>√</td>
<td></td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. ellipticus</em> (yellow Himalayan raspberry)</td>
<td></td>
<td></td>
<td></td>
<td>√</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. idaeus</em>** (raspberry) in the cooler region of the southern States</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. rugosus</em>** (keriberry)</td>
<td>√</td>
<td></td>
<td></td>
<td>√</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. roribaccus</em>** (dewberry, youngberry and boysenberry)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. alceifolius</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. odoratus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>√</td>
</tr>
<tr>
<td><em>R. niveus</em></td>
<td>√</td>
<td></td>
<td>√</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.2 Global distribution map of *R. fruticosus* L. aggregate in 2011 (CAB International, 2013, http://www.cabi.org/)
Figure 1.3 Blackberry (*Rubus fruticosus* L. aggregate) in Australia. (a) Abundance and distribution (Australian Government National Land and Water Resources Audit, 2008). (b) Potential distribution based on the climatic suitability (Victorian Department of Primary Industries, 2008 derived using CLIMATE® modelling system).
Morphology

Leaves
Compound leaves are arranged singly, consisting of 5 shortly-stalked oval leaflets. Single leaves often occur around the flowering area. They usually have a dark green above, with a lighter green under surface. Some taxa have their under surface covered in pale hairs. Short “prickles” are found covering the leaf veins and stalks.

Flowers, fruits and seeds
Clusters of white (rarely with a tinge of pink) flowers, which are 2-3cm in diameter (from late November to late February) (Fig. 1.4a). The fruit is a berry, changing color from green to red to black as it ripens, with a diameter of 1-3cm (Fig. 1.4b). Each berry consists of an aggregate of fleshy segments, each containing one seed. Fruiting occurs from late December to April. The number of seeds in a berry depends on the taxon, but there can be as many as eighty. The deeply and irregularly pitted seeds are oval, light to dark brown, 2-3mm long.

Roots
The main root grows vertically to a maximum depth of 1.5m depending on soil type, from a woody crown up to 20cm in diameter. Secondary roots grow horizontally from the crown for 30-60cm, and then grow down vertically. Many thin roots grow in all directions from the secondary roots (Evans & Weber, 2003; NSW Department of Primary Industries Weed Management Unit, 2009).
Figure 1.4 Blackberry morphology. (a) Flowers, and (b) fruiting (photos kindly provided by Lee Fontanini).
Table 1.4 Description of selected characters for *Rubus anglocandicans* (Australian biotype) (Evans & Weber, 2003).

<table>
<thead>
<tr>
<th>Characters</th>
<th><em>R. anglocandicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stem</strong></td>
<td></td>
</tr>
<tr>
<td>diameter (mm)</td>
<td>6-10</td>
</tr>
<tr>
<td>hairs per 5 cm</td>
<td>0 (-2)</td>
</tr>
<tr>
<td>surface in summer *</td>
<td>dark wine-red</td>
</tr>
<tr>
<td>Angles</td>
<td>dark wine-red</td>
</tr>
<tr>
<td>base of prickles</td>
<td>dark wine-red</td>
</tr>
<tr>
<td><strong>Terminal leaflet</strong></td>
<td></td>
</tr>
<tr>
<td>Base</td>
<td>rounded to truncate</td>
</tr>
<tr>
<td>in living state</td>
<td>often ± undulate</td>
</tr>
<tr>
<td><strong>Inflorescence</strong></td>
<td></td>
</tr>
<tr>
<td>Shape</td>
<td>Narrow</td>
</tr>
<tr>
<td>prickles on rachis</td>
<td>significantly curved</td>
</tr>
<tr>
<td><strong>Petals</strong></td>
<td></td>
</tr>
<tr>
<td>Colour</td>
<td>white (rarely with pink tinge)</td>
</tr>
<tr>
<td>length (mm)</td>
<td>commonly 13-19</td>
</tr>
<tr>
<td><strong>Anthers</strong></td>
<td>glabrous</td>
</tr>
<tr>
<td><strong>Aggregate fruit size</strong></td>
<td>small to medium</td>
</tr>
</tbody>
</table>

* Assuming the stem is exposed to full sunlight. In mid-late summer or autumn the surface, angles and bases of prickles become more or less uniformly red.

**Biology, propagation and ecology**

Blackberry has a two-year growth pattern (Fig. 1.5), consisting of a scrambling, semi-prostrate to almost erect plant, of biennial canes (stems), and a perennial root system (Fig. 1.6).
Figure 1.5 Flow diagram of blackberry life-cycle (modified from Agriculture and Resource Management Council of Australia and New Zealand, 2001).

Figure 1.6 Growth pattern and reproductive morphology of *R. fruticosus* L. aggregate species (Bruzzese & Lane, 1996).
Blackberries in Australia and New Zealand

Blackberry occurs on wasteland, hedgerows, fence lines, roadsides, steep banks, hillsides, scrubby hillsides, forest, plantations, scrub margins, clearings, fen land, swamps, damp places, creeks, stream sides, river banks, river flats, river terraces, damp and neglected pasture (Webb et al., 1988).

In Australia, blackberry was evidently planted in New South Wales by the late 1830s. In 1842 blackberry was first recorded as being deliberately introduced from Europe into Adelaide, South Australia for its fruit. It was included in the sale catalogue of a Tasmanian nursery by 1845. Blackberry was recognised to have become a significant weed by the 1880s, and first proclaimed a noxious weed in Gippsland, Victoria in 1894 (Agriculture and Resource Management Council of Australia and New Zealand, 2001).

The initial introduction to New Zealand was probably as a food plant by early settlers and other introductions can be traced back to distributions of plants from the Melbourne Botanic Gardens in the mid 1800s (Webb et al., 1988). In New Zealand, the initial spread of blackberry was intentional by planting for use as a food source and to form hedges, with unintentional distribution via humans, sheep and particularly by introduced birds, and by horticultural escape (Healy, 1952). In New Zealand, it is on a list of 110 species of National Surveillance Plant Pests, prohibited from propagation, sale, distribution, and commercial display throughout the country (Pennycook, 1998).

European blackberry (R. anglocandicans) is ranked as one of the top 20 Weeds of National Significance in Australia with wide distribution (Fig. 1.7) and high invasiveness in conservation areas and particularly in forestry and agriculture.
Figure 1.7 Distribution of *R. anglocandicans* in Australia (adopted from Atlas of Living Australia: http://bie.ala.org.au/species/Rubus+anglocandicans; Evans & Weber, 2003).

**Blackberry as a weed in Western Australia**

European settlement of Western Australia (WA) started in 1829 and blackberries were probably introduced soon afterward. At least three species of weedy *Rubus* (blackberry) are established in the south-west of WA; *R. anglocandicans* A. Newton, *R. laudatus* A. Berger and *R. ulmifolius* Schott (Evans *et al.* 2007). In addition, the cultivated species *R. loganobaccus* L.H. Bailey (loganberry) is locally established (Evans *et al.* 2007) and a population of a different *Rubus* species has been recently discovered and is in the process of being identified (Fontanini, unpublished observations).
*Rubus anglocandicans* is the most widespread *Rubus* species over all of Australia (Evans & Weber 2003) and the most common in WA. It originates from England so it was probably the first species to be introduced into WA. American blackberries (includes *R. laudatus*) were present by 1875 (Anonymous, 1875), but their distribution is more localized, being found mainly north of Bunbury (Yeoh *et al.* 2006b). *Rubus ulmifolius* is widespread, but only in small populations.

The first record of blackberry establishment in the Warren Catchment (part of the study area of this paper) was in 1893 by a journalist (L.L.C.) of the newspaper, The West Australian, who provided the following quote by the pioneer farmer Thomas Muir:

"In the kitchen garden almost every kind of culinary vegetable is to be found and also a number of small fruits such as gooseberries, currants, strawberries, and alas! the blackberry. It was an evil day when this last named berry got a foothold in the district."

The location of the farm Deeside where blackberry was present in 1983 is shown in Fig. 1.8. By the 1950s blackberry was well established and the subject of public demands for its control. Today blackberry is widespread in the south-west of WA in areas with more than 800 mm rainfall.

In general, the greatest impact of blackberries in WA is along watercourses (Hancock *et al.*, 1996). Yeoh *et al.* (2006a) also report a reduction of about 50% in plant species diversity associated with sites invaded by *Rubus anglocandicans*. Where there are rivers, the most important recreational fishing is for marron (a species of freshwater crayfish, *Cherax cainii*) and the dense thickets of blackberry prevent people from being able to fish for marron. Away from the river bank, *R. anglocandicans* is also a weed, albeit minor, of agriculture and plantation forestry. However, Crackel & Roberts (1987) in a cost benefit analysis concluded that it was not economical to control blackberry in WA using Government resources. Commercial production of weedy varieties of
blackberries is prohibited under state laws (Yeoh et al. 2006b) and the only positive impact, wild fruit collection, is a minor activity.

**Current methods for management of invasive blackberries in Australia**

Management options for blackberry include a combination of biological, chemical and mechanical control methods. Herbicides and cultural control methods are generally ineffective, or require multiple applications; however, the weed is often located within inaccessible areas, which limits control options. Therefore, biological control has been identified as the main strategy for control of blackberry. Opportunities exist to investigate additional means of control. This could include use of more biological control agents (eg. develop a suite of agents for several taxa) and combinations of other chemical and/or mechanical methods. Potential grazing management afforded by goats and deer may be an option on land already used for farming them, although the need to prevent feral populations being created is to be a priority. The challenge is to expand the acceptance and implementation of best management practices to control blackberry (Agriculture and Resource Management Council of Australia and New Zealand, 2001).

**Known diseases of blackberries**

Blackberry is generally more resistant to disease compared to other *Rubus* species. A range of fungi, oomycetes and viral diseases have been associated with blackberry across the world (Table 1.5).
Figure 1.8 Location of the Donnelly and Warren River catchments in Western Australia, including their main rivers, towns and the two sites (black open squares) where blackberry decline was first recorded in 2007. Blackberry is found along all rivers and tributaries apart from the upper reaches of the Donnelly, Perup and Tone. Deeside (near the junction of the Perup and Warren Rivers) is the location of the first known blackberry in these catchments.
Table 1.5 Known diseases of blackberry (Ellis et al., 1991).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Causal agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthracnose</td>
<td><em>Elsinoe veneta</em></td>
</tr>
<tr>
<td>Armillaria root rot</td>
<td><em>Armillaria mellea</em></td>
</tr>
<tr>
<td>Blackberry rust</td>
<td><em>Phragmidium violaceum</em></td>
</tr>
<tr>
<td>Botrytis fruit and cane rot</td>
<td><em>Botrytis cinerea</em></td>
</tr>
<tr>
<td>Cane and leaf rust</td>
<td><em>Kuehneola uredinis</em></td>
</tr>
<tr>
<td>Cane and leaf spot</td>
<td><em>Septoria rubi</em></td>
</tr>
<tr>
<td>Crown and cane gall</td>
<td><em>Agrobacterium tumefaciens</em> and <em>A. rubi</em></td>
</tr>
<tr>
<td>Downy mildew</td>
<td><em>Peronospora sparsa</em></td>
</tr>
<tr>
<td>Dry berry</td>
<td>Unknown</td>
</tr>
<tr>
<td>Orange rust (rare)</td>
<td><em>Arthuriomyces peckianus</em> and <em>Gymnoconia nitens</em></td>
</tr>
<tr>
<td>Phytophthora root rot</td>
<td><em>Phytophthora</em> spp.*</td>
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<tr>
<td>Powdery mildew</td>
<td><em>Sphaeroteca macularis</em></td>
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<tr>
<td>Purple blotch</td>
<td><em>Septocyta ruborum</em></td>
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<tr>
<td>Raspberry Bushy Dwarf Virus</td>
<td><em>RBDV</em></td>
</tr>
<tr>
<td>Stamen blight</td>
<td><em>Hapalosphaeria deformans</em></td>
</tr>
</tbody>
</table>

Biological control of blackberry in WA

Recent biological control of blackberry in WA has focused on releases of additional strains of the rust fungus *Phragmidium violaceum* (Morin et al., 2011). Initially, single releases of the nine rust strains were made in autumn 2004 at a site on Lefroy
Brook, a tributary of the Warren River and in spring 2004 at a tributary of the Donnelly River.

**Blackberry decline in the south-west of WA**

In November 2006, at the Warren River “decline site”, canes throughout the site looked normal but not particularly healthy and at the time it was assumed that plants were just slow to regrow after winter senescence. Cane densities were 73% of that observed at the same time of the season in the previous year. Cane densities at the other three healthy sites on same river were approximately the same as in the previous season.

Being a semi-deciduous plant, foliage cover varies throughout the year, peaking each summer and senescing each winter. New season foliage started to be produced in October. The “decline site” differed slightly from the healthy sites in November 2006 because not as many new leaves had reappeared on the previous year’s canes at the start of the new season. In November 2006, plants in the decline site only had 10% of the foliage cover seen the previous summer in contrast to the other healthy sites that at the same time, all had over 60% of their previous summer foliage levels (Fig. 1.9a).

In October 2007, the blackberry population at the “decline site” had crashed (Fig. 1.9b) and the initial reaction was to assume that the plots had been sprayed with herbicide rather than reacting to the rust. Dead canes were everywhere. The defoliation was more severe and rapid than anything observed previously, even when plants were inoculated with high densities of rust spores under optimal laboratory conditions. The few new primocanes produced at the decline site the following season were thin and weak and surviving floricanes failed to produce fruit. Dead
canes fell to the ground, rotted quickly and disappeared within a year, in contrast to those at healthy sites, or at sites sprayed with herbicide, where dead stems often stayed hard and upright for several years. Even the hard woody crowns of the decline plants rotted quickly and disappeared (Yeoh & Fontanini, personal communication). The possibility of herbicide use was excluded after a search found lack of vehicle evidence on site, investigation with local authorities, the remoteness of locations and absence of non-target damage to native plant species.
Figure 1.9 Site on the Warren River, WA. (a) October 2005 before blackberry decline symptoms were observed. (b) October 2007 following the peak of the decline, which killed most blackberry canes (Yeoh & Fontanini, personal communication).
After detecting the decline at the Warren River site, all previous release sites were revisited in November 2007. Decline at this time was also evident at the site of the 2004 spring release on the Donnelly River. At this site, there was no previous plant density or cover records, but like the decline site on the Warren River site, areas that were impenetrable previously could be comfortably traversed. In 2007, P.B. Yeoh and L. Fontanini noticed that blackberry plants in the Donnelly and Warren River catchments (Fig. 1.9b) were dying in a spectacular manner in areas that they were monitoring following the release in previous years of additional strains of *Phragmidium violaceum* for blackberry biological control. This disease became known as "blackberry decline". Surveys of blackberry were made throughout the south-west of WA during 2004 prior to the releases of additional strains of the rust fungus. This involved obtaining 432 samples for 93 locations by 30 collectors (mostly land managers), without any reports of unusual mortality of blackberry. In the following years “rust release kits” containing the nine rust strains were sent to 116 people throughout the south-west of WA between October 2006 and November 2008. It would be expected that unusual deaths of blackberry would have been reported at that stage because people were monitoring their rust release sites, but none were reported. To date blackberry decline has only been reported in relatively inaccessible sites and appears to have been active along the Donnelly River before or at a similar time to the release of the additional rust strains in 2004. For these reasons it is believed that blackberry decline is a relatively recent phenomenon. As of 2012 the decline of blackberry has spread extensively along the Donnelly and Warren rivers.
Related species

There are no native *Rubus* species in the south-west Australia (Wheeler *et al.*, 2002; Evans *et al.*, 2007), indeed the only native species in the family Rosaceae in the south-west is *Acaena echinata*, which possibly originates from eastern Australia (Wheeler *et al.*, 2002). Thus it seems unlikely that the pathogen causing blackberry decline is a native species associated with the Rosaceae.

Aside from *Rubus*, the introduced Rosaceae established in south-west Australia are in the genera (number of species) *Aphanes* (1), *Acaena* (1 or 2), *Cotoneaster* (2), *Prunus* (1), *Rosa* (5) and *Sanguisorba* (1) (Wheeler *et al.*, 2002). The area also has many species of Rosaceae in horticulture and grown as ornamental species (e.g. peaches, roses). There have been no reports so far of these introduced species exhibiting decline symptoms similar to that of blackberry, including among commercial *Rubus* crops. Thus, there is no evidence of a source host from amongst the introduced species.

Aims of the project

The appearance of "blackberry decline" offers possible opportunities for the biological control of blackberry in WA. At present it is only known from the Warren and Donnelly Catchments. This research project aimed to:

- Survey, sample, isolate, identify and determine the pathogenicity of putative pathogenic species to *R. anglocandicans* (Chapter 2).
- Describe new *Phytophthora* species associated with blackberry decline using classical and molecular identification methods (Chapter 3).
• Examine pathogenicity of recovered *Phytophthora* species to *R. anglocandidans* through under-bark inoculations and pot infestation trials under controlled glasshouse conditions (Chapter 4).

• Assess disease severity and synergism through a dual pot infestation trial with putative pathogenic species in the glasshouse (Chapter 5).

• Conduct mapping of disease fronts and epidemiology of blackberry decline extension along the Warren and Donnelly Rivers where major decline occurs and develop a conceptual model to describe the abiotic and biotic factors involved in blackberry decline (Chapter 6).

• Conclusions and recommendations for future research (Chapter 7).
CHAPTER 2

Disease Surveys, Sampling, Isolation and Preliminary Pathogenicity Screening of Putative European Blackberry Pathogens
Introduction

As outlined in Chapter 1, blackberry decline has been observed along Warren and Donnelly River Catchments in the south-west of Western Australia. The purpose of this Chapter was to survey the extent of blackberry decline along two Rivers; to sample from non-decline, declining and decline sites over 2010, 2011 and 2012 seasons; to isolate possible involved pathogen(s); to identify recovered pathogen(s) through classical and molecular methods; to do a preliminary pathogenicity screening of putative pathogens through under-bark inoculation of excised *Rubus anglocandicans* primocanes. The hypotheses that could explain the presence of the disease and possible pathogens are discussed here.

Materials and Methods

Survey, sampling and isolation techniques

Sites around the Warren and Donnelly rivers where blackberry decline has been reported on *R. anglocandicans* (Aghighi et al., 2012a, Chapter 1), the most common species of blackberry in WA, were visited over different seasons between 2010 and 2012 including autumn (April), winter (June), spring (September and November) 2010, winter (August) and spring (September) 2011 and autumn (May) 2012. Rhizosphere soil and diseased roots from both dying and healthy plants were collected. Briefly, plants were dug out and as no obvious disease symptoms were detected on foliage and canes, these were excluded and roots with crowns were placed into plastic bags and kept in an insulated box to protect samples from high temperatures and direct sunlight and carried to the laboratory for further examination and isolation. In August and September 2012, samples were collected for isolation
from five decline sites and five non-decline sites (Fig. 2.1). Rhizosphere soil and roots from both declining and healthy plants were collected. In addition, in spring 2011, six streams and rivers close to the decline sites (Fig. 2.1) were baited (Fig. 2.2) using the ‘Fishing for Phytophthora technique’ (Hüberli et al., 2013).

Isolation of oomycetes

Both rhizosphere soil and diseased root samples were baited with youngest fully expanded leaves of *Alnus* sp., *Grevillea* sp., *Pittosporum* sp., *Prunus persica, Quercus suber, Q. ilex, R. anglocandicans*, and petals of *Hibbertia* sp. and *Rosa officinalis*. After 2-7 days, leaves with brownish lesions were blotted dry, and the lesions cut into ~1-2 mm sections and plated onto *Phytophthora* selective media including a modified recipe of NARPH (Hüberli et al., 2000) from which pentachloro-nitrobenzene was excluded [990 mL/L distilled water, 17 g CMA (Becton, Dickinson and Company, Sparks, USA),

1 mL nistatin (nilstat 22.7 mg/mL, oral drop), 0.1 g ampicillin, 0.5 mL rifampicin (rifadin, 100 mg/5 mL), all from Symbion® Pharmacy Services, Symbion Pty Ltd., Perth, Western Australia, 0.05 g hymexazol (Tachigaren, Agricultural Chemicals Research Laboratories, Sankyo Co., Ltd., Japan), all antibiotics plus hymexazol were mixed with 10 mL sterile distilled water and amended to the medium immediately after autoclaving and before pouring plates], and a modified recipe of PARPHN (Jung et al., 2000) from which pentachloro-nitrobenzene was excluded and medium was made by using Corn Meal Agar (CMA) or V8 juice Agar (V8A) [990 mL/L distilled water, 17 g CMA, 0.4 mL pimaricin (2.5% aqueous suspension, Sigma-Aldrich, Australia), 2.019 mL nistatin (nilstat 22.7 mg/mL, oral drop), 0.2 g ampicillin, 0.4 mL rifampicin (rifadin, 100 mg/5 mL), 0.025 g hymexazol),
antibiotics and hymexazol were amended to the medium as described above. To make this medium V8A was used instead of CMA: 100 mL/L filtered vegetable juice (Campbells V8 vegetable juice; Campbell Grocery Products Ltd., Norfolk, UK), 890 mL/L distilled water, 0.1 g/L CaCO₃, Bacteriological Agar or Grade A Agar 16g (Becton, Dickinson and Company, Sparks, USA)]. Also, sections (~2-5 mm) of both asymptomatic and necrotic roots were rinsed with tap water, re-washed with distilled water, blotted dry and plated directly onto PARPHN or surface sterilised with 70% ethanol for 20-45 s (according to the thickness of root materials) and rinsed three times in distilled water, blotted dry and plated as above. Plates were incubated in the dark at 20 ± 1°C and checked regularly for *Phytophthora* hyphae. Aseptate hyphal colonies growing from the plated lesion sections were transferred to vegetable juice agar (V8A) [100 mL/L filtered vegetable juice (Campbells V8 vegetable juice; Campbell Grocery Products Ltd., Norfolk, UK), 900 mL/L distilled water, 0.1 g/L CaCO₃, pH adjusted to 7 and 17 g Grade A Agar (Becton, Dickinson and Company, Sparks, MD, USA)] for confirmation of hyphae typical of oomycetes. Pure cultures grown on half-strength PDA (Becton, Dickinson and Company, Sparks, USA) were maintained under long-term storage in McCartney bottles filled with 2/3rd sterile distilled water and deposited at the Centre for *Phytophthora* Science and Management (CPSM), Murdoch University Culture Collection.
Figure 2.1 Location of ‘Fishing for Phytophthora’ and sampling sites during blackberry decline surveys.
**Fishing for Phytophthora species at six tributaries**

In order to bait for *Phytophthora* from streams, fishing was conducted in spring (September) 2011. Bait bags were prepared using polyvinyl chloride (PVC) coated fibreglass insect screen mesh (Cyclone Industries a Division of ITW Australia Pty Ltd.) shaped into an A4 envelope (Fig. 2.2). Fishing for *Phytophthora* was conducted as described by Hüerli *et al.* (2013). Briefly, this involved placing leaves of different baits such as *Metrosideros excelsus* (New Zealand Christmas tree), *Prunus armeniaca* (plum), *Pittosporum undulatum*, and *Quercus* spp. inside the bags. Each bag was attached to a rope tied to the tributary bank (Fig. 2.2). Bags were placed in six tributaries located along the Warren and Donnelly Rivers (2 bags per tributary) (Fig. 2.1). To ensure the bait bags floated rivers just below the surface of waterways, buoyant polyurethane material was sown along one side of the bait bags. Baits were collected after six days and lesions were plated onto the two *Phytophthora* selective media described above. Potential isolates were maintained under long term storage as mentioned previously.
Figure 2.2 Fishing for Phytophthora species from tributaries with a bag containing youngest fully expanded leaves of different plants as bait.

**Isolation of fungi**

In order to process root samples for isolation of fungi, roots collected from decline and non-decline sites were rinsed with running tap water to remove soil particles. To wash out phenolic compounds, roots were soaked in distilled water (DW) for up to 48 hours and water changed regularly. Roots were then examined to observe any type of sporulation or fruiting bodies of possible pathogens using stereo (SZH10, Olympus) and compound (EL SMX234, Olympus) microscopes. To isolate possible pathogenic fungi, well washed symptomatic and non-symptomatic roots and crowns were surface sterilized with 70% ethanol for 20-45 s, rinsed three times in distilled water, blotted dried using paper towel and cut into small pieces (~2-5 mm) and
plated onto half-strength PDA plus Streptomycin [19.5 g potato dextrose agar; 7.5 g Grade A agar/L distilled water (all from Becton, Dickinson and Company, Sparks, USA) and 10 mL/L Streptomycin sulphate (Sigma-Aldrich, Australia) from stock solution (1 g/75 mL DW) was amended to the medium using 0.20 µm Millipore filter (Millipore, Merck Pty. Ltd., Australia) immediately after autoclaving the medium and before pouring plates]. Plates were incubated at 25 ± 1°C, in the dark and the emergent colonies were transferred to PDA (39 g/L DW). Single spore cultures were produced, and pure cultures were maintained on PDA at 25 ± 1°C.

In another method, Granny Smith apples were used as baits for necrotic roots. Briefly, two holes were made with sterile cork borer (10 mm width and 30 mm length) in each apple, the column taken out and surface sterilised necrotic roots were placed into each hole and blocked with the removed apple column and sealed with parafilm (Parafilm®, Pechiney Plastic Packaging; Menasha, WI, USA). Each apple was kept for 5-7 days in a separate zip-lock bag (Sandvik, Australia) at 25 ± 1°C and brown discoloured lesions around holes were plated onto half-strength PDA plus Streptomycin and PARPHN as described previously. Cultures were maintained as described above.

Recovered putative oomycetes and fungal isolates were identified through both classical morphology and molecular identification.

**DNA isolation, amplification and sequencing of recovered species**

Recovered isolates were grown on half-strength PDA or PDA at 20°C for 1-2 weeks and the mycelium was harvested by scraping from the agar surface with a sterile blade and placed in a 1.5 mL sterile Eppendorf® tube. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder and genomic DNA was extracted
according to Andjic et al. (2007). The region spanning the internal transcribed spacer (ITS1-5.8S-ITS2) region of the ribosomal DNA was amplified using the primers ITS-6 (5’ GAA GGT GAA GTC GTA ACA AGG 3’) (Cooke et al. 2000) and ITS-4 (5’TCC TCC GCT TAT TGA TAT GC 3’) (White et al. 1990). The PCR reaction mixture and PCR conditions were as described by (Andjic et al. 2007). The clean-up of products and sequencing were as described by Sakalidis et al. (2011).

**Excised primocane under-bark inoculation**

*Rubus anglocandicans* primocanes (~15 mm in diameter) were selected from a non-blackberry decline site in Manjimup-Pemberton in the south-west of WA. Primocanes were cut into 30 cm lengths, and all foliage and side shoots were removed before both ends were immediately sealed with melted paraffin before inoculation. At about the mid point of each cane, a sterile scalpel was used to cut a flap approximately 8 mm long and 5 mm wide through the outer bark without damaging the cambial tissue underneath. A 5 mm diameter inoculum disc cut from the actively growing margin of a 10-day-old half-strength PDA culture of each of the selected oomycete isolates and a 7-day-old culture of fungal isolates (Table 2.1) was inserted under the bark flap (mycelial surface face down) and the wound sealed with parafilm to prevent desiccation. For controls, non-inoculated agar plugs were used. There were ten replicate stems per isolate and stems were kept in the zip-lock bags (with one replicate per isolate per zip-lock bag) with a wet cotton ball inside and incubated at 25°C in the dark. After two weeks, a thin layer of bark from the point of inoculation along the primocanes was removed and the lesion lengths were measured.
Table 2.1 Tested oomycetes and fungal isolates in blackberry excised primocane under-bark inoculation.

<table>
<thead>
<tr>
<th>Putative oomycets and fungi/isolate code</th>
<th>Source</th>
<th>Method of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cylindrocarpon</em> aff. <em>pauciseptatum</em> (SA245)</td>
<td>Root</td>
<td>Granny Smith apple</td>
</tr>
<tr>
<td><em>Cylindrocarpon</em> sp. (SA076)</td>
<td>Root</td>
<td>Direct plating</td>
</tr>
<tr>
<td><em>Cylindrocarpon</em> sp. (SA195)</td>
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<td><em>Fusarium</em> sp. (SA246)</td>
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<td><em>Phlebia acerina</em> (SA032)</td>
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<td><em>Pythium vexans</em> (SA094)</td>
<td>Soil</td>
<td>Baiting (<em>Quercus ilex</em>)</td>
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<tr>
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<td><em>Eucalyptus marginata</em></td>
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<tr>
<th>Species</th>
<th>Habitat</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. litoralis</em> (SA072)</td>
<td>Root</td>
<td>Direct plating</td>
</tr>
<tr>
<td><em>P. multivora</em> (SA134)</td>
<td>Soil</td>
<td>Baiting (R. sp., American blackberry)</td>
</tr>
<tr>
<td><em>P. multivora</em> (SA136)</td>
<td>Soil</td>
<td>Baiting (R. sp., American blackberry)</td>
</tr>
<tr>
<td><em>P. multivora</em> (SA150)</td>
<td>Soil</td>
<td>Baiting (<em>R. anglocandicans</em>)</td>
</tr>
<tr>
<td><em>P. multivora</em> (SA151)</td>
<td>Soil</td>
<td>Baiting (<em>R. anglocandicans</em>)</td>
</tr>
<tr>
<td><em>P. multivora</em> (SA153)</td>
<td>Soil</td>
<td>Baiting (<em>R. anglocandicans</em>)</td>
</tr>
<tr>
<td><em>P. taxon personii</em> (SA278)</td>
<td>Root</td>
<td>Baiting (<em>Grevillea</em> sp.)</td>
</tr>
<tr>
<td><em>P. thermophila</em> (SA399)</td>
<td>Water</td>
<td>Fishing</td>
</tr>
</tbody>
</table>

Data analysis

Statistical analysis of data was carried out in STATISTICA software package Version 5 (Statsoft 1984–1999). The mean lesion length for each treatment was recorded. Differences in lesion lengths between different isolates were assessed using one-way analysis of variance (ANOVA). Tukey’s post hoc honest significant difference test for equal sizes was used to determine which groups of isolates were different from others. Data were analysed using Univariate Test of Significance for Planned Comparison. Planned comparisons were used to compare lesion lengths specifically between groups of isolates belonging to one species with groups of isolates from another species.

Results

Isolation and identification

More than 453 oomycetes and fungi were isolated and identified. These included ten *Phytophthora* species (*Phytophthora amnicola*, *P. taxon oaksoil* (described in Chapter 3 as *P. bilorbang*), *P. cryptogea*, *P. inundata*, *P. litoralis*, *P. multivora*, *P. taxon personii*, *P. thermophila*, *P. thermophila-amnicola* hybrid from decline sites...
and P. cinnamomi from non-decline sites (Fig. 2.3). Incidence of Phytophthora species recovery and seasonal activity showed that P. amnicola, P. bilorbang and P. cryptogea were isolated more consistently from blackberry decline and declining sites (Table 2.2 and Table 2.3). Baiting for rhizosphere soil with oak (Q. ilex and Q. suber) youngest fully expanded leaves was the most successful method for isolation of the majority of Phytophthora species. Nine Pythium species were recovered from both decline and non-decline sites including Pythium sp. 1, Py. sp. 2, Py. anandrum, Py. citrinum, Py. dissotocum, Py. helicoides, Py. irregulare, Py. macrosorum, Py. vexans.

True fungi were also recovered from both decline and healthy sites including Neonectria radicicola (Cylindrocarpon destructans) and Cylindrocarpon aff. pauciseptatum, Fusarium spp., Mortierella spp., Phlebia acerina, Trichoderma spp. and Gliocladium spp. After sequencing, pure cultures of all isolates were maintained at constant temperature room (20 ± 1°C) under long-term storage in McCartney bottles filled with ~ 2/3rd sterile distilled water as a part of CPSM culture collection, Murdoch University.
Figure 2.3 Incidence of *Phytophthora* species (n=162 isolates) recovered from blackberry decline survey. *P. cinnamomi* from non-decline sites and other species were recovered from decline or declining sites.
Table 2.2 Presence or absence (+/-) and/or number of recovered isolates of *Phytophthora*, *Pythium* and *Cylindrocarpon* species isolated from the blackberry survey sites during disease surveys in 2010 to 2012.

<table>
<thead>
<tr>
<th>Species/Site</th>
<th>C1</th>
<th>D1</th>
<th>C2</th>
<th>D2</th>
<th>C3</th>
<th>D3</th>
<th>H4</th>
<th>H5</th>
<th>H6</th>
<th>H7</th>
<th>H8</th>
<th>H9</th>
<th>H10</th>
<th>D11</th>
<th>F1,2,3</th>
<th>F4,5,6</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phytophthora amnicola</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td><em>P. bilorbang</em></td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. cinnamomi</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. cryptogea</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>32</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. inundata</em></td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. litoralis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. multivora</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>13</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. taxon personii</em></td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. thermophila</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>P. thermophila-amnicola hybrid</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>Pythium</em> spp.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>Cylindrocarpon</em> spp.</td>
<td>5</td>
<td>35</td>
<td>+</td>
<td>8</td>
<td>17</td>
<td>43</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*C*: Sites adjacent to the decline sites; *D*: Decline sites; *H*: Healthy sites far away from decline sites, and *F*: Fishing for *Phytophthora* in the streams. *NA*: Data not available. Surveyed sites include: *C1* and *D1*: Up stream from Banister Bridge, Warren River; *C2*: Tributary to Lefroy Brook, Cascade area, Warren Catchment; *D2*: Down stream of Gloucester Road Bridge, south bank, Warren River; *C3*: East Brook crossing on Spring Gully Rd, Warren Catchment; *D3*: Collins Road crossing and 2 Km north of Collins Road on Warren River; *H4*: Scabby Gully Dam, Warren Catchment; *H5*: Smith Brook Road, Warren Catchment; *H6*: Palings Road Bridge, Donnelly River; *H7*: Gordon Road Bridge corner Gregory Road, Donnelly River; *H8*: Pine plantation next to Foresters Wood, Donnelly Catchment; *H9*: Yanmah Brook, Donnelly Catchment; *H10*: Northcliffe-Windy Harbour Road, corner Double Bridges Road, Gardner River; *D11*: Rory Dean, Donnelly Catchment; *F1,2,3*: Fishing in Warren tributaries; *F4,5,6*: Fishing in Donnelly tributaries.
Table 2.3 Seasonal activity, isolation method and source of *Phytophthora* species recovered from blackberry decline sites in the Warren and Donnelly river catchments between April 2010 to May 2012 and in February 2013.

<table>
<thead>
<tr>
<th>Phytophthora species</th>
<th>% of each species recovered across seasons</th>
<th>Isolation Method (%)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring</td>
<td>Summer</td>
<td>Autumn</td>
</tr>
<tr>
<td><em>P. amnicola</em></td>
<td>22</td>
<td>78</td>
<td>2</td>
</tr>
<tr>
<td><em>P. bilorbang</em></td>
<td>4</td>
<td>23</td>
<td>73</td>
</tr>
<tr>
<td><em>P. cryptogea</em></td>
<td>31</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td><em>P. inundata</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. litoralis</em></td>
<td>25</td>
<td>75</td>
<td>50</td>
</tr>
<tr>
<td><em>P. multivora</em></td>
<td>4</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td><em>P. taxon personii</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. thermophila</em></td>
<td>80</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td><em>P. thermophila-amnicola</em> hybrid</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

*Representing seasons in the Southern hemisphere (spring: September, October, November; summer: December, January, February); autumn: March, April, May); winter: June, July, August).
Sporulation of *Cylindrocarpon* species on necrotic roots

During microscopic examination of roots, conidiogenesis of *Cylindrocarpon* species were observed frequently from samples collected from different sites (Fig. 2.4). The fungal propagules were found in both healthy and symptomatic roots but the number of propagules including hyphal mass, macro and microconidia and chlamydospores were considerably higher in diseased than healthy plants. This anamorphic stage was isolated successfully after surface sterilisation of roots and also from Granny Smith apples.

**Figure 2.4** *In situ* observation of *Cylindrocarpon* species sporulation from *Rubus anglocandicans* necrotic roots. (a-c) Conidiogenesis. (d) Two-celled microconidia (white arrow) and four-celled macroconidia (black arrow) at 400x magnification.
*In vitro* pathogenicity screening of recovered fungal and oomycete isolates

The preliminary pathogenicity trial using excised stems showed that all *Phytophthora* species were able to cause moderate to extended lesions in primocanes compared to the control inoculations, *Pythium* species (except for *Py. dissotocum* (SA370) and fungal isolates (Fig. 2.5). There was no significant (*p* = 0.05, df = 1) difference in lesion lengths between the different *P. bilorbang* and/or *P. cryptogea* isolates after paired analysis of the two species. A significant difference was observed after paired comparison between *P. bilorbang* with *P. inundata* (*p* = 0.005, df = 1) or *P. cryptogea* with *P. inundata* (*p* = 0.00008, df = 1). Paired comparisons for other species were not made. There was a variation between pathogenicity of different tested fungal and oomycetes isolates. For instance, *P. bilorbang* (SA092) and *P. cryptogea* (SA167) caused the largest lesions. Accordingly, *P. multivora* (SA153) caused smaller lesions in primocanes compared to other *P. multivora* tested isolates.

**Discussion**

More than 453 oomycetes and fungi were recovered over the disease surveys between 2010 and 2012. Of these 210 belonged to *Phytophthora* species and 157 out of 210 were identified (as representative of total recovered isolates) through classical and molecular identifications. *P. amnicola, P. cryptogea, P. multivora* and *P. bilorbang* were recovered more frequently than other *Phytophthora* species, respectively.
Figure 2.5 Mean (± SE) of lesion lengths (mm) in *Rubus anglocandicans* (blackberry) in excised primocanes under-bark inoculation with a range of Pythiaceous and fungal isolates obtained from declining blackberry, except for *P. cinnamomi* (MU9448) which was used as positive control. The control was an agar plug which was placed under-bark. Isolates were examined over a number of trials.
With regards to the isolation of *P. amnicola*, the fishing method led to the recovery of this species from water; however baiting from soil samples also resulted in high recovery of this species. *P. cinnamomi* was the only *Phytophthora* species isolated from two blackberry non-decline sites. *Cylindrocarpon* species were also frequently isolated after direct plating of surface-sterilised roots. Hence, the main focus of this thesis was targeted to *Phytophthora* species (Chapters 3 and 4) and *Cylindrocarpon* (Chapter 5).

There was a high recovery of different *Pythium* species. In excised primocane under-bark inoculation except for *Pythium dissotocum* (SA370), other *Pythium* species did not produce lesions in the canes; however, they may not develop lesions in canes but under inundation can cause damage to the roots. Due to time limitations, *Pythium* species were excluded from this project. Further research is recommended to investigate pathogenicity of recovered *Pythium* species to *R. anglocandicans*.

Compared to the excised primocane under-bark inoculation method, it is assumed that the fungal and oomycetes isolates could cause more extended lesions *in planta* as the result of consistent sap flow. Oomycetes in particular are very dependent on water to grow and cause disease symptoms; therefore, low moisture in excised primocanes could be reason why smaller lesions developed in excised primocanes compared to intact primocanes. More work is required to examine the tested pathogens *in planta* to confirm the result of excised primocanes under-bark inoculation (Chapters 4 and 5).
CHAPTER 3:

*Phytophthora bilorbang* sp. nov., a New Species

Associated with the Decline of *Rubus anglocandicans* (European Blackberry) in

Western Australia

This Chapter was published as:


Minor authors’ contributions:

Giles Hardy, Treena Burgess and John Scott were supervisors.
**Introduction**

*Rubus anglocandicans* is the most widespread and invasive species in the *Rubus fruticosus* aggregate (European blackberry) found in Australia (Evans & Weber, 2003) and one of the few weeds of national significance widespread in the south-west of Western Australia (WA). It is a major weed of conservation areas (particularly in wetter regions), forestry and agriculture because of its high degree of invasiveness, potential for spread, and economic and environmental impacts. Herbicides and cultural control methods are generally ineffective against blackberry, or require multiple applications and have proved to be expensive and difficult to apply in natural habitats and inaccessible areas invaded by blackberry (Amor et al., 1998).

European blackberry in Australia has been targeted by biological control since the 1980s. Most of this effort has focused on introducing exotic strains of the host-specific leaf rust, *Phragmidium violaceum*. New strains of the rust selected for *R. anglocandicans* and other European blackberries (Morin et al., 2011) were released in 2004 and 2005 in WA. In some areas the levels of rust developed on blackberry was high, at least initially, but it seems that it does not have enough potential to control the weed, and is possibly limited by climatic factors (Morin & Evans, 2012).

During surveys established to assess the releases of the rust fungus in 2005, dead and diseased blackberry plants were found at two locations along the Warren and Donnelly Rivers in WA (Yeoh, personal communication). However, the disease could not be attributed to the release of the biological control agent, the rust fungus. Over the next few years, the extent of the disease increased within the Warren and Donnelly river catchments, with noticeable landscape changes due to disappearance of dense blackberry thickets. This has lead to the disease being called “blackberry decline”. The
disease appears to be due to root pathogen/s and during preliminary sampling several
Phytophthora species were isolated (Hardy, unpublished data).

In order to investigate the cause(s) of blackberry decline and the potential role of
Phytophthora species in the disease, field surveys were carried out over 2010 and 2011
in the decline and non-decline sites along the Warren and Donnelly Rivers. During
these surveys, isolates with identical ITS sequence to $P$. taxon oaksoil were recovered
and are described here as Phytophthora bilorbang sp. nov., a new taxon within the ITS
Clade 6 of Phytophthora.

Materials and Methods

Isolation procedure

Rhizosphere soil and roots were collected from five sites with dying Rubus
anglocandicans and three non-decline sites with apparently healthy blackberry in the
south-west of Western Australia. Both soil and root samples were baited with $R$. 
anglocandicans and Alnus sp., Quercus suber, Q. ilex juvenile leaves, Rosa and
Hibbertia petals and Eucalyptus sieberi cotyledons following the modified method of
Rea et al. (2010). After 3-7 days, baits with brownish lesions were blotted dry, and the
lesions cut into 1–2 mm sections and plated onto Phytophthora selective media
including NARPH (Hüberli et al., 2000) and a modified recipe of PARPHN (Jung et al.,
2000) from which pentachloronitrobenzene was excluded. Plates were incubated in the
dark at 20°C and checked regularly for Phytophthora hyphae. Colonies growing from
the plated lesion sections were transferred to V8 agar [0.1 L filtered V8 juice, 0.1 g
CaCO$_3$ and 0.9 L distilled water and pH adjusted to 7, 17 g Grade A agar (Becton,
Dickinson and Company, Sparks, MD, USA)] for confirmation of hyphae typical of
Phytophthora species. Cultures were maintained under long-term storage in water at the Murdoch University Culture Collection.

**DNA isolation, amplification and sequencing**

The *Phytophthora* isolates were grown on half-strength potato dextrose agar PDA (Becton, Dickinson and Company, Sparks, USA, 19.5 g PDA, 7.5 g of agar and 1 L of distilled water) at 20°C for 2 weeks and the mycelium was harvested by scraping from the agar surface with a sterile blade and placed in a 1.5 mL sterile Eppendorf® tube. Harvested mycelium was frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted according to the method of Andjic *et al.* (2007). The region spanning the internal transcribed spacer (ITS1-5.8S-ITS2) region of the ribosomal DNA was amplified using the primers DC6 (Cooke *et al.*, 2000) and ITS-4 (White *et al.*, 1990). The PCR reaction mixture and PCR conditions were as described previously (Andjic *et al.*, 2007). The mitochondrial gene *cox1* was amplified with primers FM84 and FM83 (Martin & Tooley, 2003). The PCR reaction mixture was the same as for the ITS region, but the PCR conditions were as described previously (Martin & Tooley, 2003). Heat shock protein 90 (HSP90) was amplified with HSP90-F1 and HSP90-R2 primers (Blair *et al.*, 2008). The PCR reaction mixture was the same as for the ITS region, but the PCR conditions were as described previously (Blair *et al.*, 2008). β-tubulin (BT) was amplified with primers TUBU-F2 and TUBU-R1 and NADH dehydrogenase subunit 1 was amplified with NADH-F1 and NADH-R1 primers according to Kroon *et al.*, (2004).

For all gene regions except HSP, templates were sequenced in both directions with primers used in amplification. Additionally, for *cox1* templates were also sequenced with primers FM 85 and FM 50 (Martin & Tooley, 2003). For HSP, templates were sequenced in both directions with primers HSP90-F1 and HSP90-R1.
The clean-up of products and sequencing were performed as described previously (Sakalidis et al., 2011). All sequences derived in this study were deposited in GenBank and accession numbers are given in Table 3.1.

**Phylogenetic analysis**

The data set comprised of sequences of *P. bilorbang* sp. nov. and those of closely related species in ITS Clade 6, sub-clade II, that were either sequenced for this study or obtained from GenBank (http://www.ncbi.nlm.nih.gov/). ITS sequence data were assembled and manually edited as described previously (Jung & Burgess, 2009). There were no gaps in the *cox1*, NADH, BT, and HSP90 alignments. Trees were rooted to species from ITS sub-clade I (*P. inundata* and *P. humicola*) and sub-clade III (*P. taxon asparagi*).

Parsimony analysis was performed in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2003) and Bayesian analysis with MrBayes v. 3.1 (Ronquist & Heuelsenbeck, 2003) as described previously (Jung & Burgess, 2009). Alignment files and trees can be viewed on TreeBASE (http://www.treebase.org/).
Table 3.1 Identity, host, location, isolation information and GenBank accession numbers for *Phytophthora* isolates considered in the phylogenetic study.

<table>
<thead>
<tr>
<th>Reference collection no.</th>
<th>Other collection no.</th>
<th>Identity</th>
<th>Substrate</th>
<th>Host</th>
<th>Location</th>
<th>Isolated by</th>
<th>Date</th>
<th>GenBank Accession No.</th>
<th>ITS</th>
<th>Cox1</th>
<th>HSP90</th>
<th>BT</th>
<th>NADH</th>
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</thead>
<tbody>
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<td><em>P. asparagi</em></td>
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<td><em>Lomandra sonderi</em></td>
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<td>2007</td>
<td>EU301168 HQ012845 HQ012891</td>
<td>JN547592</td>
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<td><em>P. bilorbang</em></td>
<td>Soil</td>
<td><em>Rubus anglocandicans</em></td>
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<td>S Aghighi</td>
<td>2010</td>
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<td>S Aghighi</td>
<td>2010</td>
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<td>Soil</td>
<td><em>R. anglocandicans</em></td>
<td>Australia, WA, Warren River</td>
<td>S Aghighi</td>
<td>2010</td>
<td>JN547623 JN547645 JN547656 JN547584</td>
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1 Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; IMI = CABI Bioscience (Imperial Mycological Institute), UK; VHS = Vegetation Health Service Collection, Department of Environment and Conservation, Perth, Australia; DDS = earlier prefix of VHS Collection; TCH = TC Hill, in VHS Collection; MJS = MJC Stukely, in VHS Collection; HSA = Hart, Simpson and Associates, in VHS Collection; DCE = EM Davison, in VHS Collection; MUCC = Murdoch University Culture Collection.

2 isolates in bold, italics were sequenced during this study.
Colony morphology

Colony growth patterns were described from 7-day-old cultures grown at 20°C in the dark on V8A, 2% malt-extract agar MEA, half-strength PDA (19.5 g PDA, 7.5 g agar and 1 L distilled water), carrot agar (CA) (0.1 L filtered carrot juice, 17 g agar and 1 L distilled water) and corn-meal agar (CMA) (17 g CMA and 1 L distilled water) (all media and agar were sourced from Becton Dickinson Co. Sparks MD 21152 USA). Colony morphologies were described according to the patterns described previously (Erwin & Ribeiro, 1996; Jung et al., 2011).

Growth rates and cardinal temperature

All isolates were sub-cultured onto V8A plates and incubated at 20°C to initiate growth for 24 hours. Three replicate plates for each isolate were then transferred to incubators set at 4, 10, 15, 20, 25, 30, 32.5, 35 and 37.5°C (± 0.5°C), and radial colony growth was measured after 7 days. Max/min thermometers were placed in all the incubators. Plates showing no growth above 30°C were returned to 20°C to determine isolate viability.

Morphology of sporangia and gametangia

Sporangia, hyphal swellings and gametangia of five isolates of *P. bilorbang* sp. nov. were produced on V8A measured using the methods described in Jung et al. (1999). Sporangia were produced by flooding 15 x 15 mm agar squares taken from growing margins of 7-d-old colonies, so that their surfaces were covered with distilled water in 90 mm Petri dishes which were incubated at room temperature around 22°C in natural daylight. The water was decanted and replaced again after 2 and 8 h. Two mL of diluted non-sterile soil extract was added at 8 h. The soil extract was made from 100 g of pine bark potting mixture suspended in 1 L distilled water, incubated for 24 h at 20°C, filtered through cheesecloth and refiltered through Whatman no. 1 paper. After 15-24 h,
dimensions and characteristic features of 50 mature sporangia, 25 exit pores and zoospore cysts per isolate were chosen at random and measured. Likewise, after 3-7 days, 25 hyphal swellings were also measured. All measurements were made at x400 magnification (BX51, Olympus). Isolates grown in the dark on V8A and CA plates at 20°C for 14-28 d were examined and 50 mature oogonia, and oospores together with 35 antheridia per isolate chosen at random were measured at x400 magnification (BX51, Olympus).

Glasshouse pot trial

In order to investigate pathogenicity of *P. bilorbang* sp. nov., three isolates [SA092, SA142 and SA262 (CBS161653)] were tested in a soil-infestation pot trial. *Rubus anglocandicans* daughter plants (less than 1 month old) were collected from sites that have remained disease-free in the Manjimup region and placed into 130 mm free-draining polyurethane pots containing a commercial bark based substrate (Soils Aint Soils, Perth, Western Australia). The plants were grown in an evaporatively-cooled glasshouse (11-25°C) for five months prior to use.

Two different types of inocula were made. Firstly, plugs were prepared as described previously (Butcher *et al.*, 1984; Rea *et al.*, 2010) except that tree lucerne (*Cytisus palmensis*) was used for the plugs instead of *Pinus radiata*. Secondly, vermiculite inoculum (vermiculite 1 L, millet seeds 10 g and 600 mL V8 broth) was produced as described previously (Jung *et al.*, 1996)

The vermiculite inoculum was mixed with pasteurized and washed river sand in a ratio of 40 g/L sand and healthy blackberry plants and attached soil were transferred from the 130 mm pots into 170 mm free-draining pots filled with 1 L of the infested sand
(control pots received non-inoculated vermiculite). Each pot also received four colonised or non-colonised (control) tree lucerne plugs. There were 11 replicate pots for each isolate and the pots were placed in a randomized design in a glasshouse (12°C min - 25°C max). The plants were watered to container capacity daily, and fertiliser (water soluble, Thrive®, Yates Company, Australia) was applied at half the manufacturer’s recommended rate in the second and third weeks after inoculation. The pots were flooded in 9 L buckets twice at 2 and 7 weeks after inoculation for 15-17 h. After four months, infested soil was baited to recover Phytophthora species, and the plants were rated as asymptomatic, declining or dead. Necrotic roots were plated directly on PARPHN. Symptomatic roots were baited with Q. suber and Q. ilex juvenile leaves, and baits with lesions were plated onto PARPHN as described previously.

Results

Recovery of P. bilorbang sp. nov.

Fourteen isolates of P. bilorbang sp. nov. were isolated from four decline sites and it was not recovered from non-decline sites. Juvenile leaves of R. anglocandicans, Alnus sp., Quercus suber and Q. ilex, but not petals, were effective at recovering this species.

Phylogenetic analysis

The aligned datasets for ITS, HSP90, BT, cox1 and NADH consisted of 846, 895, 914, 1192 and 813 characters, respectively. Based on partition homogeneity tests in PAUP, the datasets were congruent (P = 0.12) and were concatenated, resulting in a combined dataset of 4646 characters. Additional relevant sequences were available on GenBank only of the ITS region and are presented in addition to the concatenated dataset.
There were 103 informative characters in the ITS dataset and significant phylogenetic signal (P < 0.01, g1 = -0.75). Heuristic searches resulted in 39 most parsimonious trees of 191 steps (CI = 0.67, RI = 0.86). The trees from the Bayesian analysis had similar topology to those from the parsimony analysis, but generally provided greater support for deeper branches (Fig. 3.1, TreeBASE 12248). Isolates of *P. bilorbang* sp. nov. reside in a strongly supported terminal clade together with several isolates designated as *P. taxon oaksoil* in Europe and northern America and an isolate designated as *P. taxon riversoil*. There is weak support for the clustering of the *P. bilorbang* sp. nov. isolates to the *P. gregata - P. gibbosa* species group. The other cluster contains the remaining species within sub-clade II with the exclusion of *P. taxon salixsoil* and *Phytophthora* sp 2 (HM004225) which are the basal species.

There were 580 informative characters in the concatenated ITS, HSP90, BT, *cox1* and NADH dataset and significant phylogenetic signal (P < 0.01, g1 = -0.98). Heuristic searches resulted in a single most parsimonious tree of 1115 steps (CI = 0.66, RI = 0.83). The tree from the Bayesian analysis had similar topology to the ITS dataset (Fig. 3.1), but generally provided greater support for deeper branches (Fig. 3.2, TreeBASE 12248). The isolates of *P. bilorbang* sp. nov. were identical and reside in a strongly supported terminal clade, basal to the majority of described species and designated taxa in ITS Clade 6, sub-clade II with the exclusion of *P. taxon salixsoil* which is the basal species to the whole sub-clade.
Figure 3.1 Bayesian inference tree based on rDNA ITS sequences showing phylogenetic relationships between *Phytophthora bilorbang* and other species in ITS Clade 6. Numbers above the branches represent posterior probability based on Bayesian analysis; numbers below the branches represent the bootstrap support based on parsimony analysis. *P. taxon asparagi* was used as outgroup taxa.
Figure 3.2. The most parsimonious tree based on the concatenated ITS, HSP90, BT, coxl and NADH dataset showing phylogenetic relationships between *Phytophthora bilorbang* and other species in ITS Clade 6. Numbers above the branches represent posterior probability based on Bayesian analysis; numbers below the branches represent the bootstrap support based on parsimony analysis. *P. inundata, P. humicola* and *P. taxon asparagi* were used as outgroup taxa (not shown).
Taxonomy

*Phytophthora bilorbang* sp. nov.

MycoBank no. MB563863; Fig. 3.3 and 3.4.

*Etymology:* The chosen name of the new species refers to a Noongar (south-west Australian Aboriginal) word for a person living on the banks of a river.

*P. bilorbang* is homothallic. Oogonia globose and averaged $33.5 \pm 4.4 \, \mu m$. Oospores highly plerotic to slightly aplerotic, averaged $31.3 \pm 4.1 \, \mu m$. Oospore wall, thick with a mean of $3.0 \pm 0.8 \, \mu m$. Antheridia paragynous and globose to cylindrical ($12.4 \pm 2.9 \times 14.1 \pm 2.9 \, \mu m$). Sporangia abundant in liquid cultures and also in V8A, persistent, terminal, nonpapillate, limoniform, limoniform with a tapering base, ellipsoid, ovoid and less obpyriform, peanut shaped and club shaped ($51.6 \pm 6.4 \times 29.0 \pm 4.6 \, \mu m$) with a length/breadth ratio $1.8 \pm 0.3 \, \mu m$. Conspicuous basal plug common. Sporangiophores simple, sometimes with basal swelling or swelling along the sporangiophore. Sporangial proliferation external and internal observed both in liquid culture and V8A. Hyphal swellings globose to ellipsoid, catenulate or angular with radiating hyphae. Chlamydospore not formed. Colony in V8A stellate to petaloid (carnation shape). Optimum temperature on V8A 25°C, average radial growth rate $4.2 \pm 0.1$, maximum temperature 32.5°C.

*Typus:* Western Australia, Pemberton, banks of Warren River, from rhizosphere soil of dying *Rubus anglocandicans*, 2010, collected by S. Aghighi, **holotypus** MURU 470; cultures ex-type CBS 161653; ITS, *coxl*, HSP90, BT and NADH sequence JQ256377, JQ256375, JQ256376, JQ256374 and JQ256378, respectively.
**Sporangia, proliferations and hyphal swellings**

Sporangia of *P. bilorbang* were observed on solid agar media including V8A, CA and CMA and were produced abundantly in V8A when flooded with non-sterile soil extract. Sporangia were borne terminally on unbranched sporangiophores, often in chains of internally extended or non-extended proliferating sporangia. They were non-caducous and non-papillate (Fig. 3.3), although in all isolates a fragile twist was infrequently observed in the pedicle (Fig. 3.3e, g). Sporangial shape was variable ranging from limoniform (41.6%; Fig. 3.3a-c) to ellipsoid (27.6%; Fig. 3.3d), ovoid (23.2%; Fig. 3.3e-i), and less frequently was obpyriform (3.6%; Fig. 3.3j), peanut shaped (3.2%; Fig. 3.3k-l) and club shaped (0.8% Fig. 3.3m). Features such as a tapering base (Fig. 3.3b, k) or a conspicuous basal plug were common (Fig. 3.3c). Sporangia usually proliferated internally in both a nested and extended way (Fig. 3.3o-s). External proliferation was observed as a short extension just behind the primary sporangium (Fig. 3.3n). In all isolates, two extended internal proliferating sporangia were sometimes observed (Fig. 3.3q-r). Occasionally an externally proliferating sporangium was produced from an internal extended proliferation while was still in the primary sporangium (Fig. 3.3s). Direct germination of sporangia and germination of zoospores inside sporangia were observed on agar media.

Sporangial dimensions of the five *P. bilorbang* isolates averaged 50.3 ± 10.6 x 27.3 ± 5.5 µm with a range of 43.8 – 59.6 x 25.0 – 30.0 µm. The length/breadth ratio of the sporangia averaged 1.87 ± 0.34. Zoospores were discharged through exit pores 10.4 - 13.4 µm wide (av. 11.6 ± 2.5 µm) (Fig. 3n-s). They were limoniform, ovoid to reniform whilst motile, becoming spherical (av. diam = 10.9 ± 1.7 µm) on encystment (Fig. 3w). Cysts often germinated with up to three hyphae (Fig. 3x-z). In V8A flooded with non-sterile soil extract, globose, ellipsoid or angular hyphal swellings, frequently catenulate
and with radiating hyphae or forming branching points, were regularly formed (Fig. 3t-u). Hyphal swellings had a mean diameter of $18.6 \pm 4.8 \, \mu m$. Chlamydospores were not seen. Coiled hyphae were observed on agar media.
**Figure 3.3** Morphology of asexual structures of *Phytophthora bilorbang* on V8 agar flooded with diluted soil extract. (a-c) Limoniform sporangia with (b) tapering base and (c) conspicuous basal plug, d. an ellipsoid sporangium; e-i. ovoid sporangia with (h) hyphal swelling on sporangiophore or (i) a swollen base; j. an obpyriform sporangium; k-l. peanut shaped sporangia with (k) tapering base; m. elongated club shaped sporangium; n-s. an empty sporangium with (n) a short external proliferation, (o) extended internal proliferation (p) nested internal proliferations (q-r) two extended internal proliferating sporangiophores in just one empty sporangium (s) an external proliferation arising from the internally proliferating sporangiophore; t-u. hyphal swellings (t) elongated and radiate and (u) catenulate and globose; v. sporangium releasing zoospores; w. encysted zoospores; x-z. encysted zoospores germinating through up to three germ tubes. Scale bar = 50 µm.

**Oogonia, oospores, antheridia and aggregations**

*Phytotophthora bilorbang* is homothallic and all the five isolates readily produced oogonia in single culture on CA and V8A and oospores matured within 3 - 5 weeks. Oogonial shape was globose with smooth walls (Fig. 3.4a-k). Oogonial stalks were sometimes long (Fig. 3.4i-j). Oogonial diameters averaged 33.9 ± 6.0 µm with a range of 26.5 – 37.4 µm. Most oogonia contained oospores with a large ooplast (Fig. 3.4b-g) (aborted oospores = 3.4%) (Fig. 3.4n-o) or with two ooplasts (Fig. 3.4h-i). Oospores were highly plerotic (Fig. 3.4b, d and g-h), slightly aplerotic (Fig. 3.4e-f) to aplerotic (Fig. 3.4j-k, t) and averaged 32.2 ± 6.1 µm in diameter with relatively thick oospore walls (av. 2.7 ± 0.7 µm; range 2.0 – 3.0 µm) and a high mean oospore wall index of 0.42 ± 0.07. Abnormal shaped oospores were formed, rarely (Fig. 3.4m).

Antheridia were one-celled, hyaline and globose to cylindrical, with a range of isolate means of 11.00 - 12.43 x 12.19 - 14.09 (av. 11.4 ± 2.28 x 13.0 ± 2.51). They were paragynous (Fig. 3.4g) and up to 6 finger-like projections were observed (Fig. 3.4c-d, h, j, q-t). Generally, there was more than one antheridium attached per oogonium (Fig.
Small hyphal aggregations were produced on CMA. Large aggregations were formed on malt extract agar and on selective media (Fig. 3.4u).

**Figure 3.4** Morphology of *Phytophthora bilorbang* sexual structures (a-t) on carrot agar and hyphal aggregation (u) of malt extract agar. a. young oogonium; b. three oospores with large ooplast bodies and thin walls; c. an apleorotic oospore and paragynous antheridium with finger like projections; d. a pleorotic oospore and paragynous and spherical antheridium with a short projection; e-g. oospores with two nuclei; h-i. oospores with two ooplast bodies (h-i) and three nuclei (h); j. oospore and antheridium with several projections; k. oospore with a long oogonium stalk; l-m. abnormal shaped oospores; n-o. aborted oospores after fertilisation; p. oospore with twisted oogonium stalk and paragynous antheridium; q-t. paragynous antheridia with short finger-like projections; u. large size aggregation. Scale bar = 50 μm,
Colony morphology, growth rates and cardinal temperatures

All five *P. bilorbang* isolates formed stellate to petaloid (carnation shape) colonies with sparse to limited aerial mycelium on V8A, carrot agar and malt extract agar, and petaloid, dense-felty and dome shape at the centre on half-strength PDA (Fig. 3.5). Colonies on corn meal agar were sparse and almost invisible.

Among the temperatures tested, *P. bilorbang* had an optimum and maximum temperature for growth of 25°C and 32.5°C, respectively (Fig. 3.6). All isolates failed to grow at 32.5°C, and did not resume growth when plates incubated for 7 d at 32.5°C were transferred to 20°C. Isolates grew slowly at 4°C. The average radial growth rate on V8A at 25°C was 4.17 ± 0.21 mm d⁻¹.
Figure 3.5 Colony morphology of (top to bottom) *Phytophthora bilorbang* CBS161653, *P. gregata*, *P. gibbosa* and *P. megasperma* after 7 days growth at 20°C on different agar (left to right); V8A, CA, MEA and half-strength PDA. *P. megasperma* was not grown on CA.
**Figure 3.6** Mean radial growth rates of *Phytophthora bilorbang* (five isolates) compared to *P. gregata*, *P. gibbosa* and *P. megasperma* on V8 agar at different temperatures. Standard error bars for *P. bilorbang* were smaller than the symbols.

**Additional specimens examined**

WESTERN AUSTRALIA, Manjimup, from rhizosphere soil of dying *Rubus anglocandicans*, 2010, collected by S. Aghighi SA92, SA142, SA143, SA146.

**Notes:** *P. bilorbang* most closely resembles other homothallic species in Clade 6; *P. gregata*, *P. gibbosa* and *P. megasperma*, but can be easily distinguished based on a combination of molecular and morphological differences (Table 3.2). In a multigene phylogeny of the ITS, HSP90, BT, NADH and *coxI* gene regions, *P. bilorbang* differs from *P. gregata* by 183 steps (3.23%), *P. gibbosa* by 178 steps (3.14%), *P. megasperma* by 199 steps (3.51%). All four species have been isolated from soil in Western
Australia; *P. gregata* and *P. gibbosa* are only known from WA, while *P. megasperma* and *P. bilorbang* have been isolated elsewhere. In comparison to these species, *P. bilorbang* has the ability to produce chains of nested and extended proliferating sporangia, external proliferation, production of secondary lateral sporangia and forming branched sporangiophores in primary sporangia. *P. bilorbang* produces smooth-walled oogonia, whilst *P. gibbosa* forms ornamented oogonia. Moreover, *P. bilorbang* has the ability to form aggregations on agar media but *P. gibbosa* and *P. megasperma* do not. Additionally, zoospores of *P. bilorbang* can germinate with up to three germ tubes. In comparison with *P. gregata*, *P. gibbosa* and *P. megasperma*, *P. bilorbang* grows very slowly on PDA and moderately slowly on other media (Fig. 3.5), and unlike these species it produces stellate colonies on carrot and V8A.
Table 3.2. Morphological characters, dimensions and temperature-growth relations of Phytophthora bilorbang, P. gibbosa, P. gregata and P. megasperma. Characters decisive for species discrimination are highlighted in bold.

<table>
<thead>
<tr>
<th>Character</th>
<th>P. bilorbang</th>
<th>P. gibbosa&lt;sup&gt;1&lt;/sup&gt;</th>
<th>P. gregata&lt;sup&gt;1&lt;/sup&gt;</th>
<th>P. megasperma&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of isolates/source</td>
<td>5</td>
<td>4</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Sporangia</td>
<td>Limoniform, ellipsoid, ovoid shaped, club shaped, nonpapillate</td>
<td>Ovoid, ellipsoid, limoniform, nonpapillate, <em>some semipapillate</em></td>
<td>Ovoid, limoniform, obpyriform, nonpapillate, <em>some semipapillate</em></td>
<td>Ovoid, obpyriform, nonpapillate</td>
</tr>
<tr>
<td>Lxb mean (µm)</td>
<td>50.3±10.57x27.3±5.48</td>
<td>48.8±9.6x30.8±5.4</td>
<td>51.0±13.8x30.5±2.9</td>
<td>59.3±8.8x42.8±4.5</td>
</tr>
<tr>
<td>Total range (µm)</td>
<td>22.8–80.7x11.7–40.4</td>
<td>24.8–71.1x17.4–48.0</td>
<td>25.7–102.3x14.8–50.7</td>
<td>37–84x35-56</td>
</tr>
<tr>
<td>Isolate means (µm)</td>
<td>43.8–59.6x25.0–30.0</td>
<td>44.8–52.2x27.9–33.0</td>
<td>37.3–72.7x25.6–35.0</td>
<td>59.3±8.8x42.8±4.5</td>
</tr>
<tr>
<td>L/b ratio</td>
<td>1.87±0.34</td>
<td>1.58±0.15</td>
<td>1.67±0.32</td>
<td>1.39±0.2</td>
</tr>
<tr>
<td>Exit pores Width (µm)</td>
<td>11.6±2.49</td>
<td>12.7±3.5</td>
<td>10.7±2.7</td>
<td>12.4±1.2</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Internal extended and nested, external, some sporangiophores branched in sporangium</td>
<td>Internal extended &amp; external, never nested</td>
<td>Internal extended &amp; external, never external, sporangiophore partly branching in sporangium</td>
<td>Internal extended &amp; external, never nested, sporangiophore partly branching in sporangium</td>
</tr>
<tr>
<td>Hyphal swellings</td>
<td>Globose, elongated, partly angular, some radiated (or branched), catenulate</td>
<td>Subglobose, elongated&lt;sup&gt;1&lt;/sup&gt;, never catenulate</td>
<td>Globose, elongated, angular, partly catenulate</td>
<td>Globose or angular, catenulate or clustered</td>
</tr>
<tr>
<td>Mean diam (µm)</td>
<td>18.6 ± 4.8</td>
<td>18.7 ± 5.0</td>
<td>14.8 ± 3.8</td>
<td>25.4 ± 1.8</td>
</tr>
<tr>
<td>Hyphal aggregations</td>
<td>present</td>
<td>absent</td>
<td>Abundant, up to 170µm</td>
<td>absent</td>
</tr>
<tr>
<td>Clamydospores</td>
<td>absent</td>
<td>absent</td>
<td>Abundant, up to 170µm</td>
<td>absent</td>
</tr>
<tr>
<td>Sexual system</td>
<td>Homothallic</td>
<td>Homothallic</td>
<td>Homothallic or inconsistently homothallic</td>
<td>Homothallic</td>
</tr>
<tr>
<td>Oogonia</td>
<td>Smooth</td>
<td>ca. 50% Ornamented</td>
<td>Smooth</td>
<td>Smooth</td>
</tr>
<tr>
<td>Mean diam (µm)</td>
<td>33.87±6.0</td>
<td>38.1±5.4</td>
<td>36.8±4.1</td>
<td>41.8±2.4</td>
</tr>
<tr>
<td>Total range (µm)</td>
<td>19.6–47.3</td>
<td>27.0–49.9</td>
<td>23.9–50.9</td>
<td>27–52</td>
</tr>
<tr>
<td>Oospores</td>
<td>Mean diam (µm)</td>
<td>32.2±6.1</td>
<td>31.4±4.6</td>
<td>31.6±4.0</td>
</tr>
<tr>
<td>Total range (µm)</td>
<td>18.1–44.7</td>
<td>18.9–39.4</td>
<td>21.4–45.3</td>
<td>23–42</td>
</tr>
<tr>
<td>Isolate means (µm)</td>
<td>24.2–36.3</td>
<td>30.0–33.0</td>
<td>27.8–35.5</td>
<td>23–42</td>
</tr>
<tr>
<td>Wall thickness (µm)</td>
<td>2.65±0.69</td>
<td>3.17±0.69</td>
<td>2.65±0.81</td>
<td>3.31±0.4</td>
</tr>
<tr>
<td>Oospore wall index</td>
<td>0.42±0.07</td>
<td>0.49±0.06</td>
<td>0.42±0.09</td>
<td>0.46±0.06</td>
</tr>
<tr>
<td>Abortion rate of isolates</td>
<td>&lt;5%</td>
<td>16–37%</td>
<td>52.5–99.8%</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Antheridia</td>
<td>Paragynous</td>
<td>Amphigynous</td>
<td>Predominantly paragynous</td>
<td>Paragynous and amphigynous</td>
</tr>
<tr>
<td>Ixb mean (µm)</td>
<td>11.4±2.3x13.0±2.5</td>
<td>13.6±2.4x14.0±2.0</td>
<td>17.1±3.0x11.0±1.8</td>
<td>13±1.5x10±1.3</td>
</tr>
<tr>
<td>Total range (µm)</td>
<td>6.7–20.1x6.3–24.9</td>
<td>10.6–24.9x7.6–17.8</td>
<td>10.6–24.9x7.6–17.8</td>
<td>10.7–15.8x8.1–13</td>
</tr>
<tr>
<td>Maximum temp. (ºC)</td>
<td>30+±32.5</td>
<td>33–&lt;35</td>
<td>33–35</td>
<td>33</td>
</tr>
<tr>
<td>Optimum temp. (ºC)</td>
<td>25</td>
<td>30</td>
<td>25 (1 isolate 30)</td>
<td>22.5–25</td>
</tr>
<tr>
<td>Growth rate on V8A at optimum (mm/d)</td>
<td>4.2±0.2</td>
<td>6.3±0.3</td>
<td>6.5±0.7</td>
<td>6.7±0.1</td>
</tr>
<tr>
<td>Growth rate on V8A at 20ºC (mm/d)</td>
<td>3.8±0.1</td>
<td>5.2±0.1</td>
<td>5.2±0.6</td>
<td>6.6±0.1</td>
</tr>
</tbody>
</table>

<sup>1</sup> from Jung *et al.* (2011)
**Pathogenicity**

The three *P. bilorbang* isolates differed in their aggressiveness to *R. anglocandicans*. Those plants inoculated with isolates SA092 and CBS161653 showed severe wilting and extensive root lesions; whereas plants inoculated with SA142 revealed only moderate symptoms of wilting. Also, 54.5%, 18.2% and 45.4% of plants inoculated with isolates SA092, SA142 and CBS161653, respectively were dead or with moderate to severe symptoms of decline by the end of the trial while non-inoculated control plants showed no symptoms. *P. bilorbang* was recovered only from plated necrotic lesions on fine roots and also from baited roots of inoculated blackberry plants satisfying Koch’s postulates.

**Discussion**

A new homothallic species from *Phytophthora* Clade 6 with identical ITS sequence to the undescribed *P*. taxon oaksoil was described here as *P. bilorbang*. This new species has been isolated from baited rhizosphere soil and roots of dead and dying European blackberry (*R. anglocandicans*) and not from healthy plants at four out of five decline sites, in the south-west of Western Australia. Phylogenetic analysis shows *P. bilorbang* to be a unique species residing in sub-clade II of ITS Clade 6. *P. bilorbang* is easily distinguished from related species by a range of morphological criteria. In a soil infestation glasshouse trial, *P. bilorbang* was reisolated from necrotic diseased roots on selective medium indicating that it is a pathogen of *R. anglocandicans*.

A single unique *Phytophthora* isolate was obtained in 1998 from soil under oak trees in Illwald Forest of Alsace, France (Hansen & Delatour, 1999). In 2003, P1055 was informally designated as *P*. taxon oaksoil by Brasier *et al.* (2003a) and introduced as sexually sterile. Isolates with identical sequence to P1055 were more recently identified from stream water in Oregon (Reeser *et al.*, 2011a and b). Additionally, there are ITS
sequence data on GenBank for several isolates designated as *P*. taxon oaksoil obtained in Poland. These are not linked to any formal publication and no more information is available for these isolates. In addition to isolates designated as *P*. taxon oaksoil, the study of Brasier *et al.* (2003a) also reported a single isolate, P1044 of *P*. taxon riversoil isolated from a riverbank in Worcestershire, UK which also has identical ITS sequence to *P*. taxon oaksoil. However, these two species were reported as sexually sterile (Brasier *et al.*, 2003a), whereas all the isolates recovered in WA were homothallic. On carrot agar *P*. taxon oaksoil isolates formed flat featureless colonies (Brasier *et al.*, 2003a), while *P*. bilorbang produced stellate colonies.

The known distribution of *P*. bilorbang in Western Australia is limited to the Warren and Donnelly river catchments. *P*. bilorbang was recovered from damp regions along the banks of these rivers. According to the climate statistics for Australian locations, the long term mean monthly maximum temperature in this region is 20.3°C in summer and 9.7°C in winter (Bureau of Meteorology of Australian Government, http://www.bom.gov.au/). These temperatures are within the range favourable for growth of *P*. bilorbang and if it was considered as a biological control agent it could be active throughout the year.

During a re-evaluation of the *Phytophthora* collection maintained by the Vegetation Health Service of the Department of Environment and Conservation in WA, many new undescribed taxa and unique isolates were identified (Burgess *et al.*, 2009) within what is known as ITS Clade 6 (Cooke *et al.*, 2000). Many of Clade 6 taxa are associated with riparian ecosystems or with forest soils prone to waterlogging. Those species known to cause disease, such as *P*. inundata, only do so when sites are flooded (Brasier *et al.*, 2003a and b; Jung *et al.*, 2011). During the winter rainfall months in the south-west of Western Australia both the Warren and Donnelly rivers are subjected to periods of
flooding. These flooding events are likely to provide the conductive conditions that favour *P. bilorbang* and potentially cause the massive decline events being observed for European blackberry in this region. These field observations were supported by the glasshouse trial where flooding led to the mortality of plants.

*Phytophthora* root rot can be an extremely destructive disease on susceptible cultivars of *Rubus* spp. such as red, purple and black raspberries, where conditions favour its development. *Phytophthora* species reported on raspberry include *P. cactorum*, *P. citricola*, *P. gonapodyides*, *P. taxon raspberry*, *P. megasperma*, *P. cryptogea*, *P. rubi*, *P. idaei* and *P. bisheria* (Duncan & Kennedy, 1987; Washington, 1988; Ellis *et al*., 1991; Wilcox *et al*., 1993; Kennedy & Duncan, 1995; Wilcox & Latorre, 2002; Brasier *et al*., 2003a; Abad *et al*., 2008; Jung *et al*., 2011). In contrast, many blackberry cultivars appeared to be highly tolerant to *Phytophthora*. However, a *Phytophthora* root rot in blackberries in Kentucky has been recorded by Ellis *et al.* (2004, http://ohioline.osu.edu/b861/pdf/ch03_70-73.pdf) but further information is unavailable. The association of *P. bilorbang* with the decline of European blackberry in the south-west of WA appears to be the first formally documented *Phytophthora* disease of blackberries.

In conclusion, a novel taxon, *P. bilorbang* is described. Based on GenBank accession data for ITS gene region, this species has been isolated from other parts of the world, and may have been introduced to the south-west of WA. Further investigations are required to better understand the epidemiology of the disease in the field and other possible factors which are involved in the decline. A host-range study of *P. bilorbang* is recommended to evaluate the biological control potential of this new species as a part of integrated management strategy of the noxious *R. anglocandicans*. 
CHAPTER 4

Pathogenicity of *Phytophthora* species to *Rubus anglocandicans*
**Introduction**

European blackberry is a complex species belonging to the *Rubus fruticosus* L. aggregate (Morin & Evans, 2012) and is one of the 20 Weeds of National Significance in Australia (Thorp & Lynch, 2000), because of its high degree of invasiveness, potential for spread, and economic and environmental impacts (Sagliocco & Bruzzese, 2004). Blackberry thickets restrict recreational access to waterways and adversely affect indigenous plants and animals. *Rubus anglocandicans* A. Newton (hereafter “blackberry”) is the most widespread species within the *R. fruticosus* aggregate in Australia (Evans & Weber 2003), and the most widespread and abundant *Rubus* species in Western Australia (WA). It originates from England so it was probably the first species introduced into WA. Herbicides and cultural control methods are ineffective, or require multiple applications; however, the weed is often located within inaccessible areas limiting control options.

A disease recorded as ‘blackberry decline’ has been observed in some blackberry sites in WA since 2006 (Aghighi *et al*., 2012a, b, Chapters 1 and 3). In order to isolate and identify root-associated pathogen(s), a disease survey (Chapter 2) was conducted in the Manjimup-Pemberton region along the Warren and Donnelly river catchments in WA in 2010. The survey led to the isolation of nine *Phytophthora* species including *P. amnicola*, *P. bilorbang* (Aghighi *et al*., 2012b, Chapter 3), *P. cryptogea*, *P. inundata*, *P. litoralis*, *P. multivora*, *P. taxon personii*, *P. thermophila* and a *P. thermophila-amnicola* hybrid after direct plating of roots, baiting roots and rhizosphere soil from declining *R. anglocandicans*, and by ‘fishing’ for *Phytophthora* in the streams close to the decline sites (Chapter 2).
During disease surveys between 2010 and 2012, 210 *Phytophthora* isolates were recovered from decline and adjacent decline-free sites identified as belonging to ten species (Chapter 2).

Phosphite has been widely used to control a range of oomycete pathogens in both horticultural crops and in natural ecosystems (Hardy *et al.* 2001). In addition, Scott (2011) used phosphite to improve the health of tuart (*Eucalyptus gomphocephala*) in tuart woodlands, which in turn resulted in *Phytophthora multivora* being linked to tuart decline. Therefore, phosphite has the potential to be used as a tool to determine if a *Phytophthora* species is likely to be involved in the blackberry decline syndrome.

This chapter aimed to investigate the pathogenicity of isolates belonging to the nine recovered *Phytophthora* species to *R. anglocandicans* in four separate trials. It also aimed to use phosphite to provide further evidence of a *Phytophthora* species being involved in blackberry decline.

**Materials and Methods**

*In planta* primocane under-bark inoculation in the glasshouse

Preliminary trials with excised stems (Chapter 2) found that *P. cryptogea* and *P. bilorbang* produced the largest lesions. *Rubus anglocandicans* daughter plants (one month old) were collected from disease-free sites and placed into 150 mm free-draining polyurethane pots containing a steam pasteurised commercial pine (*Pinus radiata*) bark based container substrate (SOILS AINT SOILS, Western Australia). Plants were grown in an evaporatively cooled glasshouse (14-25°C) for 7 months. *Phytophthora bilorbang* (SA092 and CBS161653) and *P. cryptogea* (SA014 and SA261) were grown on V8A for a week prior to inoculation. In September 2012 primocane under-bark inoculation was conducted as described in Chapter 2. The primocanes were 1 m in height with stem
diameters ranging from 10 to 15 mm and were inoculated approximately halfway along
the main cane. There were three replicate plants per isolate. Plants were watered daily
with deionised water to container capacity. After 10 days, inoculation points were
inspected for the presence of lesions, which were measured and then photographed with
a Canon camera (Canon INC., Digital IXUS 990 IS, 12.1 Mega Pixels, Japan).

**In planta primocane under-bark inoculation in the field with and without the
application of phosphite**

In the middle of spring (October) 2012, a field based pathogenicity trial was conducted
using nine isolates from six *Phytophthora* species on naturally growing blackberries. In
addition, phosphite was included to determine if (1) phosphite can be used to control the
different *Phytophthora* species, and (2) to further confirm if *Phytophthora* species are
involved in the blackberry decline syndrome. Isolates tested included *P. bilorbang*
(isolates SA092 and CBS161653), *P. cryptogea* (isolates SA014 and SA261), *P.
amnicola* (isolates SA326 and SA388), *P. taxon personii* (isolate SA278), *P. inundata*
(isolate SA285) and *P. multivora* (isolate SA134). Primocanes were inoculated using
the *in planta* under-bark inoculation method described except that silver duct tape
(Packmate®, China) was wrapped around the inoculation point to protect it from direct
sunlight and prevent desiccation. For each of the phosphite treated or phosphite not-
treated treatments, there were nine replicate primocanes for each of the nine isolates and
the non-inoculated control treatment. The control consisted of a V8A alone. Inoculation
treatments were distributed with a randomized replicate block design. There were nine
blocks each for the phosphite treatments and non-phosphite treatments, with one
replicate of each inoculation treatment per block. The primocanes were between 1 and
1.5 m in height with stem diameters of 10-20 mm and were inoculated approximately
halfway along the main stem at least 1 m above ground level.
For the phosphite treatments, three weeks post-inoculation the blackberry plants were sprayed to run-off with 0.5% 600g/L potassium phosphite (Agri-Fos® Agrichem Manufacturing Industries Pty Ltd., Loganholme, Queensland, Australia) plus 0.25% Pulse® (Nufarm Ltd., Australia) as a wetting agent.

**Harvest and determination of pathogenicity**

Three weeks after phosphite application, the inoculated and control primocanes treated or not treated with phosphite were harvested and returned to the laboratory for the measurement of lesions and plating onto *Phytophthora* selective agar. Colonization of *Phytophthora* species and Koch’s postulates was examined through plating 1-cm stem sections from the visible lesion and for 5 cm beyond the lesion onto PARPHN agar [a modified recipe of PARPHN (Jung et al., 2000) from which pentachloro-nitrobenzene was excluded and Corn Meal Agar CMA (Becton, Dickinson and Company, Sparks, USA; 17 g CMA and 1 L of distilled water) was used instead of V8 juice Agar (V8A)]. The lesion lengths for the different isolates of *Phytophthora* in the phosphite treated and not-treated plants were assessed using two-way ANOVA. The factors were isolates plus control (10 levels) and treatment (sprayed or not sprayed with phosphite). Data conformed to all assumptions of ANOVA; therefore, no transformations were required. Post hoc Tukey’s HSD tests for unequal sample sizes were used to identify significantly different groupings within significant effects.

**Glasshouse pot trials to determine the pathogenicity of selected *Phytophthora* species**

Two glasshouse pot infestation trials were conducted to test the pathogenicity of selected isolates from blackberry decline surveys.
**Glasshouse trial 1**

In order to investigate the pathogenicity of *P. bilorbang* and other selected *Phytophthora* species, three isolates of *P. bilorbang* (isolates SA092, SA142 and CBS161653), three isolates of *P. cryptogea* (isolates SA014, SA167 and SA261), one isolate of *P. litoralis* (isolate SA072), and as a positive control one isolate of *P. cinnamomi* (isolate MU 9448) were tested in a soil-infestation pot trial.

*Rubus anglocandicans* seedlings were collected in November 2010 from disease-free sites and placed into 130 mm free-draining polyurethane pots containing a steam pasteurised commercial bark based container substrate (SOILS AINT SOILS, Western Australia). The plants were grown in an evaporatively cooled glasshouse (11-25°C) for five months prior to use.

Two different types of inocula were used. The first used tree lucerne (*Chamaecytisus palmensis* (H. Christ) F. A. Bisby & K. W. Nicholls) plugs as the inoculum source. These were prepared as described by Rea *et al.* (2010). Briefly, stem sections (plugs) ~1.0 cm in diameter and 1.5-2 cm in length were cut from young debarked branches of tree lucerne. The tree lucerne plugs were then washed and 50 plugs were placed into each 1-L flask and autoclaved three times over three consecutive days at 121°C for 20 min. Flasks were then stored at room temperature in the dark until required. Thirty 9 mm diameter agar plugs cut with a sterile cork borer from 10-day old *Phytophthora* cultures grown on vegetable juice agar (V8A) plates at 25°C in the dark were added to the flasks containing the sterilised lucerne plugs. Each isolate was inoculated into a separate flask. Flasks were incubated at 20°C for eight weeks before use. The flasks were shaken weekly to facilitate even colonisation of the lucerne plugs. Control flasks received non-colonised agar plugs.
The second inoculum source contained vermiculite (1L), millet (*Panicum miliaceum*) seeds (10 g) and 600 mL V8 broth which were sterilised in 500 mL flasks (1L of inoculum divided by 4 flasks and each flask was filled with 250 mL) by autoclaving two times over two consecutive days at 121°C for 20 min and then placed in a laminar flow with the light source left on overnight and inoculated the following day. Fifteen 9 mm diameter agar plugs from 7 day-old cultures of the selected *Phytophthora* isolates grown on V8A plates were added to the separate flasks. Control flasks received non-colonised agar plugs. The flasks were incubated for eight weeks at 20°C in the dark, and shaken weekly to facilitate even colonisation of the substrate.

The vermiculite inoculum was mixed with pasteurized and washed river sand in a ratio of 40g/L sand and healthy blackberries were transferred from the 130 mm free-draining polyurethane pots into 170 mm free-draining polyurethane pots filled with 1L of the infested sand. Each pot also received four colonised or non-colonised (control) tree lucerne plugs. There were six replicate pots for each *Phytophthora* isolate tested and the pots were placed in a complete randomized design in a controlled temperature glasshouse (12°C min-25°C max). The plants were watered to contained capacity daily, and fertiliser (water soluble, Thrive®, Yates Company, Australia) was applied at half the manufacturer’s recommended rate in the second and third weeks after inoculation. The pots were flooded in 9L buckets twice at 2 and 4 weeks after inoculation for 15-17 h to stimulate the production of sporangia and zoospores and to mimic temporary inundation as would occur in riparian zones where blackberry naturally occur.

After three and a half months, pots were sub-sampled to analyse the roots for disease symptoms using WinRHIZO (WinRHIZO software, version Pro, 2007d (Regent instruments Inc., Quebec, Canada). Each pot was sub-sampled randomly three times from around each plant with an Aluminium soil borer (30 mm diameter x 200 mm length). The soil cores were washed carefully to remove the sand from roots. The
washed roots were placed in 1L plastic take away containers (11.5 mm width x 16.5 mm length x 7.5 mm depth, GENFAC® plastics Pty Ltd., Melbourne, Australia) and covered with de-ionised water and sealed with a lid and scanned as they were harvested. Root samples were plated on PARPHN and baited with *Q. ilex* youngest fully expanded leaves to fulfil Koch’s postulates. In order to scan, each root sample was dispersed in de-ionised water in a transparent tray - a component of the WinRHIZO positioning system (30 cm x 20 cm), covered with a blue background to avoid shadows and to improve contrast, and scanned (EPSON Expression XL 10000) (Sturite *et al*., 2005; Scott *et al*., 2012) with a resolution of 600 dpi using reflected (flatbed) light. Saved images were analysed with WinRHIZO software by the method of object separation from background and classification of pixel colours. Images were analysed choosing three groups, these were for background colour, healthy and discolored roots, and for each group three colour classes were selected. The colour classes were blue for background; white, cream and grey for healthy roots; brown, and dark brown and black for damaged or diseased roots. Orphan pixels (pixels with colours that did not fall into any of the defined colour classes) were allocated to the closest defined class (Sturite *et al*. 2005).

All variables were analysed in STATISTICA (STAT Soft Inc., version 7.1, 1984-2006, Tulsa, Oklahoma, USA) using a non-parametric multivariate analysis of variance (PERMANOVA). Also, a Univariate Test of Significance for Planned Comparison was applied to compare groups of different isolates of the same species against other species.

**Glasshouse trial 2**

The second trial was included as more *Phytophthora* species were recovered after further disease surveys. Those *Phytophthora* isolates that caused significant lesions in
the field and had more impact on roots in glasshouse trial 1 were selected for the glasshouse trial 2.

*Rubus anglocandicans* daughter plants were collected in May 2012 from disease-free sites and placed into 150 mm free-draining polyurethane pots containing washed and pasteurized white river sand. At the time of potting the plants, two inoculum delivery tubes (Poly pipe, 12 cm length x 2 cm width) were inserted into the pots. The plants were grown in an evaporatively cooled glasshouse (12°C min-25°C max) for five months prior to infestation. Vermiculite inoculum was prepared as described previously.

Three isolates of *P. bilorbang* (isolates SA092, SA142 and CBS161653), two isolates of *P. cryptogea* (isolates SA014 and SA261), two isolates of *P. amnicola* (isolate SA326 and SA388), and one isolate each of *P. taxon personii* (isolate SA278), *P. inundata* (isolate SA285), *P. thermophila* (isolate SA399), and *P. multivora* (isolate SA134) were tested in a sand-infestation pot trial. These isolates were selected from the previous pot trial and primocane under-bark inoculation in the field.

At the time of inoculation, the inoculation tubes were removed and 40g of inoculum (20g/L sand) was placed in the holes (20g/hole) and covered with pasteurised sand. Controls received inoculum without *Phytophthora*. There were ten replicate pots for each treatment and pots were placed in a randomized design in an air-conditioned glasshouse (14-32°C). Electrical Conductivity (EC) and pH of substrate were measured post-inoculation. Briefly, EC was measured using a Conductivity Meter (HANNA instruments®; HI8733, Conductivity Meter, Singapore), 8 weeks post inoculation (Smith & Doran, 1996; Zhang & Wienhold, 2002). Measurement of pH of sand collected from the root area (without disturbing roots) was achieved by using a soil pH test kit (MANUTEC® Pty. Ltd. South Australia), 4 weeks after inoculation and also by a pH meter (HANNA instruments®, HI8424, Microcomputer pH Meter, Singapore), 8 weeks
post inoculation (Smith & Doran, 1996; Zhang & Wienhold, 2002). Plants were watered daily with deionised water to container capacity, and fertiliser (water soluble, Thrive®, Yates Company, Australia) was applied fortnightly at half the manufacturer’s recommended rate. Pots were flooded in 9L buckets five times at weeks 2, 4, 15, 19, and 21 post-inoculation for 48-72 h to stimulate the production of sporangia and zoospores. During inundation, water was baited with juvenile leaves of Scholtzia involucrata and monitored regularly for lesions which were then excised and plated onto PARPHN, 3-7 days after baiting to confirm the presence of zoospores.

Number of primocanes per pot were counted and diameter of primocanes were measured using an electronic digital caliper (Measuring Vernier Craftright® 150 mm S/s Caliper Dgtl, PA90113, China) four months post inoculation and also before harvesting the trial at six and a half months post inoculation. At this time, the shoots were excised at the soil line and discarded, whilst the roots were harvested in a completely blind design. Briefly, pots were coded randomly from 1-12 (according to 12 treatments) within each of the 10 replicates. Replicates were harvested sequentially, thus while the replicate was known, the assigned treatment was ‘blind’. Roots were washed using tap water to remove sand and inoculum, placed in 1L plastic take away containers and covered with de-ionised water and sealed with a lid. Fresh weight and root volume were recorded. The water displacement method was used to measure root volume according to Pang et al., 2011. Disease rating was visually assessed to compare the impact of the different Phytophthora species and isolates on root health (3 = Severe root damage with lots of necrotic roots and loss of feeder roots; 2 = moderate damage (less necrotic roots and more normal feeder roots); 1 = slight damage (minimum damage and few necrosis along the roots with lots of normal feeder roots); 0 = no damage). The same rating system was conducted on the controls to assess damage caused by inundation in the
absence of the *Phytophthora* species. Damage is a rank variable, so the data were analysed with a Kruskall-Wallis test.

Roots with lesions were plated on PARPHN and incubated for up to 10 days in the dark at 20°C and examined daily for the presence of hyphae typical of *Phytophthora* species. Recovered *Phytophthora* hyphae were sub-cultured to V8A in order to fulfil Koch’s postulates. Roots from flooded control plants were also plated to confirm they were *Phytophthora* free. Harvested roots were placed in paper bags and dried at 40 °C for two weeks and dry weight was measured and recorded.

Data were analysed using STATISTICA (STAT Soft Inc., version 7.1, 1984-2006, Tulsa, Oklahoma, USA) and PAST (version 2.15, Hammer *et al*., 2012).

**Results**

*In planta* primocane under-bark inoculation in the glasshouse

All *P. bilorbang* and *P. cryptogea* isolates produced lesions in blackberry primocanes under glasshouse conditions. Both species produced much larger lesions than those produced on excised primocanes (Fig. 4.2). *P. cryptogea* caused more extensive lesions along the inoculation points (Fig. 4.2b) than *P. bilorbang* whilst lesions caused by *P. bilorbang* isolates were darker in colour (4.2a) and developed beyond phloem into xylem.
Figure 4.2 *Rubus anglocandicans* primocane under-bark inoculation in the glasshouse with two *Phytophthora* species, 10 days post inoculation. Extended lesions in (a) *P. bilorbang* (SA092), and (b) *P. cryptogea* (SA014). Arrows show length of lesions.

**Primocane under-bark inoculation in the field with and without the application of phosphite**

All *Phytophthora* species were recovered from plated visible lesions fulfilling Koch’s postulates and not from non-symptomatic sections beyond the lesions. No lesions were formed in controls (Fig. 4.4a) and *Phytophthora* was not isolated from plated sections. Plants sprayed with phosphite had significantly ($F_{1,137} = 23.3$, $p < 0.01$) shorter lesions than those that were not sprayed. Isolates also varied significantly ($F_{8,137} = 21.6$, $p < 0.01$) in the length of lesions produced. The longest lesion lengths in unsprayed plants were produced by *P. cryptogea* (SA014) and *P. bilorbang* (CBS161653 and SA092) (Fig. 4.3 and Fig. 4.4d,e), while the longest lesions in plants sprayed with phosphite were produced by *P. cryptogea* (SA261 and SA014). *P. cryptogea* (SA261) was unusual.
as the lesions it produced were longer in sprayed plants than those in unsprayed plants, but this was not significant ($F_{8,137} = 1.8, p = 0.07$) (Fig. 4.3). Based on the result of this experiment, *P. cryptogea* and *P. bilorbang* isolates were more pathogenic than other tested species.

**Figure 4.3** Mean (± SE) of lesion lengths (mm) in *Rubus anglocandicans* (blackberry) primocanes in the field after under-bark inoculation with six *Phytophthora* species ($n = 9$ isolates); without (grey bars) and with (black bars) foliar application of phosphite. The control was a plain agar plug, which was placed under-bark.
Figure 4.4 *Rubus anglocandicans* under-bark inoculated with *Phytophthora* species in the field (a) Control, (b) *P. taxon personii* (SA278), (c) *P. amnicola* (SA326), (d) *P. cryptogea* (SA014), (e) *P. bilorbang* (CBS161653) and (f) *P. inundata* (SA285).

Glasshouse trial 1

After each flooding event, all *Phytophthora* species were isolated from the baited water/soil of *Phytophthora* treated pots. No *Phytophthora* species were recovered from the control pots. None of the *Phytophthora* treated blackberries caused mortality at the time of harvest; however, *Phytophthora* treated plants were less vigorous compared with the flooded controls. All *Phytophthora* isolates were re-isolated by direct plating and baiting washed roots from *Phytophthora* infested pots after harvest, fulfilling...
Koch’s Postulates (Fig. 4.5). *Phytophthora* species were not recovered from harvested and washed root samples of control pots. *P. bilorbang* and *P. cryptogea* caused extensive necrotic lesions along all parts of the roots (Fig. 4.6a, c) compared to flooded control (Fig. 4.6b) and other tested *Phytophthora* species e.g *P. litoralis* (Fig. 4.6d). In *P. cryptogea* inoculated roots, lesions were chocolate brown to brown in colour while for *P. bilorbang*, lesions appeared darker in colour. *P. bilorbang* and *P. cryptogea* hyphae were observed to appear on the selective agar plates faster than the other species. *P. bilorbang* was isolated from necrotic fine roots up to 1mm in diameter, but not from thick roots. Statistical analysis of WinRHIZO data including all variables together (Fig. 4.7 and Fig 4.8) in a non-parametric multivariate analysis of variance (PERMANOVA) showed that there was no significant ($F_{(7,56)} = 0.99, p = 0.45$) difference between isolates; However, a Univariate Test of Significance for Planned Comparison revealed a significant ($p = 0.03, df = 1$) difference in total root length as a dependent variable when *P. bilorbang* isolates were grouped together and when compared against the other *Phytophthora* species (Fig. 4.8b). The same result was obtained when *P. bilorbang* together with *P. cryptogea* isolates were compared against other *Phytophthora* species ($p = 0.03, df = 1$).

**Figure 4.5** Baiting for blackberry (*Rubus anglocandicans*) roots with *Quercus ilex* leaves showing lesions (arrows) caused by *Phytophthora bilorbang* (CBS161653) after harvesting pot infestation trial 1 in the glasshouse.
Figure 4.6 Comparison of harvested *Rubus anglocandicans* root samples treated with *Phytophthora* species (a,c and d) and non-treated (b) in pot infestation trial 1 and preparation of samples to be analysed with WinRHIZO software. (a) *P. bilorbang* (SA092) with lots of necrotic roots, (b) *P. cryptogea* (SA014), (c) flooded control with lots of healthy roots, and (d) *P. litoralis* (SA072).
Figure 4.7 Root lesion length representing percentage of control root classes. (a) 0-0.5 mm, (b) 0.5-1 mm, (c) 1-1.5 mm, and (d) 1.5-2 mm according to the root diameter after harvesting Phytophthora pot trial 1 and analysing scanned root images with WinRHIZO software. Error bars represent standard errors of the means. Green bars: P. cryptogea (SA014, SA167 and SA261), red bars: P. bilorbang (SA092, SA142 and CBS161653), blue bar: P. litoralis (SA072), orange bar: P. cinnamomi (MU9448).
Figure 4.8 Number of root tips (a), and total root length with length of discoloured or damaged (in dark shade or top bar) and healthy roots (in light shade or bottom bar) (b) after harvesting Phytophthora pot trial 1 and analysing scanned root images with WinRHIZO software (Error bars represent standard errors of the means). White/black bars: Flooded control. Green bars: *P. cryptogea* (SA014, SA167 and SA261), red bars: *P. bilorbang* (SA092, SA142 and CBS161653), blue bar: *P. litoralis* (SA072), orange bar: *P. cinnamomi* (MU9448).
Glasshouse trial 2

During inundation, all *Phytophthora* species were isolated from the baited water/sand of *Phytophthora* infested pots. No *Phytophthora* species were recovered from the control pots. No significant difference was observed related to cumulative primocane measurements in the middle of February and before harvest in April (Fig. 4.9). There was a significant (*p = 0.005*) difference in root dry weights when *P. bilorbang* and *P. cryptogea* were compared against the flooded control treatments using Multivariate Test of Significance for Planned Comparisons (Fig. 4.10b). Root volume data led to the same result as dry weight (Fig. 4.10a). Moreover, a significant (*p = 0.01*) difference was obtained in a Planned Comparison of all *Phytophthora* species against the flooded control. However, Multivariate Test of Significance resulted in no significant difference between dry weight of each treatment and to that of flooded control.

EC of collected sand samples was 6.1 µs/m (0.061 ds/m). pH of sand collected from root area was 6.5 when recorded by pH Kit and 6.2 using the pH meter.
Figure 4.9 Mean (± SE) cumulative primocanes diameter (mm) (a) and number of primocanes (b) in a sand-infestation pot trial 2 over 2012-2013. (grey and black bars represent measurements in the middle of February and end of April 2013, respectively).
Figure 4.10 Mean (± SE) of root volume (a) and dry weight (b) of *Rubus anglocandicans* (blackberry) in sand-infestation pot trial 2 over 2012-2013.
Damage rating of harvested roots

Based on the visual assessment, *P. cryptogea* (SA014), *P. inundata* (SA285) and all *P. bilorbang* isolates caused the most damage to the *R. anglocandicans* roots; however, *P. bilorbang* (SA142 and SA092) and *P. cryptogea* (SA014) had highest impact on the roots volume and dry weight (Fig. 4.10). In contrast, *P. taxon personii* caused similar levels of root damage to the flooded control (Fig. 4.11). No significant difference was observed between different treatments (Table 4.1), indicating that all *Phytophthora* species are able to cause damage to the roots after regular inundations. Moreover, regression relationship between dry weight and disease rate of roots resulted in a significant difference (*p* = 0.03). In general, the higher the root damage rating, the lower root weight (Fig. 4.12).

![Diagram](image)

**Figure 4.11** Percentage root damage rating of *Rubus anglocandicans* six and a half months after inoculation with seven *Phytophthora* species in the glasshouse pot trial 2. Error bars represent standard errors of the means.
Table 4.1 Analysis of root damage after harvesting *Rubus anglocandicans* roots in pot infestation trial 2 by Kruskall-Wallis test.

<table>
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<th>Isolate *</th>
<th>SA092</th>
<th>SA142</th>
<th>CBS161653</th>
<th>SA014</th>
<th>SA261</th>
<th>SA326</th>
<th>SA388</th>
<th>SA134</th>
<th>SA399</th>
<th>SA278</th>
<th>SA285</th>
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<td>7</td>
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<td>3</td>
<td>6</td>
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<td>-0.1</td>
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<td>1.8</td>
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<td>0.8</td>
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<tr>
<td>&gt; Median: observed</td>
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<td>5</td>
<td>5</td>
<td>8</td>
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<td>3</td>
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<td>7</td>
<td>4</td>
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</tr>
<tr>
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<td>120</td>
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<tr>
<td>Dependent: damage</td>
<td>Median Test, Overall Median = 2</td>
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<tr>
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<td>Chi-Square = 10.2, df = 11, p = 0.5</td>
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</table>

Discussion

*Phytophthora bilorbang* and *P. cryptogea* were more pathogenic than the other tested *Phytophthora* species across the under-bark inoculation and glasshouse soil infestation trials. There was a positive correlation between all under-bark inoculations and the soil infestation pot trials for *P. bilorbang* and *P. cryptogea*. However, in both trials the pathogenicity between different isolates of same species was variable. Koch’s postulate was satisfied for both species.

Generally, all *Phytophthora* species were able to cause damage to the *R. anglocandicans* roots under flooding conditions, although unlike *P. bilorbang* and *P. cryptogea* their pathogenicity was inconsistent in under-bark inoculations compared to
pot trials. For instance, *P. inundata* (SA285) caused small lesions in under-bark inoculations but high root damage was observed. This could indicate that using underbark stem inoculation for pathogenicity screening is not appropriate for all *Phytophthora* species.

In primocane under-bark inoculation of excised stems (Chapter 2, Fig.2.5) and intact field plants, the largest lesions were recorded for *P. cryptogea* (SA014) and *P. bilorbang* (CBS161653). Lesions caused by *P. cryptogea* and *P. bilorbang* isolates were larger than those caused by the other species. Inoculation in the field led to more extended lesions compared to the excised primocanes experiment possibly due to sap flow being more extensive in the intact tissues due to photosynthesis. Application of phosphite in the field after primocane under-bark inoculation reduced the size of lesions caused by *Phytophthora* species. This provides strong evidence that one or more *Phytophthora* species are involved in the blackberry decline syndrome. However, a phosphite field trial now needs to be undertaken on a blackberry site exhibiting early symptoms of decline. If phosphite treated areas remain healthy compared to non-phosphite treated areas, this will provide further and more definitive evidence of the role *Phytophthora* species in the decline.

In the pot trials, no mortality of blackberries was observed for any of the *Phytophthora* species, although their vigour was compromised particularly for *P. bilorbang* and *P. cryptogea*. However, it is likely that if the duration of the flooding event was extended mortality would have occurred as observed by Scott *et al.* (2012). In their study, they have shown that in a soil infestation trial to assess pathogenicity of *P. multivora* to *Eucalyptus gomphocephala* and *Eucalyptus marginata*, above-ground symptoms were not observed at the end of the trial although the plants had poorly developed root systems. Such plants would not survive in the field, but do so in glasshouse because of regular watering that reduces stress to the plant from a lack of roots.
It seems that duration of inundation is very important in the blackberry decline scenario as blackberries on the river banks in the natural ecosystems in the south-west of WA are frequently flooded for few weeks in duration (Fontanini, personal observation). Based on the results of glasshouse experiments, all *Phytophthora* species were able to cause damage to the *R. anglocandicans* roots under regular flooding events; however, their impact on the root biomass (root dry weight and volume) was different. This finding is in accordance with studies conducted by Duncan & Kennedy (1989). They have investigated different waterlogging regimes on several *Phytophthora* species involved in red raspberry root rot and found duration of waterlogging had a noticeable effect on the pathogenicity of even non-pathogenic *Phytophthora* species to red raspberry.

It is noteworthy that European blackberries are genetically identical (Evans *et al.*, 2011), and therefore have a low evolutionary potential and a lower chance to express resistance to pathogens. This feature is an advantage that facilitates reliability of research conducted in nature (*in planta*) and in glasshouse trials. Further work is required to determine how the pathogenicity of *Phytophthora* species (in particular *P. bilorbang* and *P. cryptogea*) to *R. anglocandicans* is affected by the interaction of other biotic and abiotic factors in natural ecosystems in the south-west of WA.

According to the findings of the present study, *Phytophthora* species, and in particular, *P. bilorbang* and *P. cryptogea* are pathogenic to *R. anglocandicans* when the plants are subjected to prolonged waterlogging. More detailed trials have yet to be conducted to examine the possibility of synergism between *P. bilorbang* with *P. cryptogea*. Duration of experiments should be extended for longer as the decline phenomenon in blackberry appears to have a complex etiology and all components interact with each other to cause the decline syndrome. Role of predisposition and stress factors should not be ignored specifically for plant diseases with complex etiology nature (Schoeneweiss, 1975; Manion, 1981). Consequently, a maximum of six months trial under glasshouse...
conditions would not have reflected the real situation occurring in blackberries populations in the remote forests in the south-west of WA. Further research is required to assess pathogenicity of recovered *Phytophthora* spp. to native species in the south-west of Western Australia before establishment of any rehabilitation project in blackberry decline sites.
CHAPTER 5

Possible Synergism between *Phytophthora* and *Cylindrocarpon* Species to European Blackberry
Introduction

During disease surveys between 2010 and 2012 (Chapter 2), several *Phytophthora* species were recovered and pathogenicity of these species and their aggressiveness to *Rubus anglocandicans* was investigated (Chapter 4). Of the *Phytophthora* spp., *P. bilorbang* and *P. cryptogea* were the most pathogenic. In addition to the *Phytophthora* spp., several isolates of *Cylindrocarpon* spp. including *C. destructans* and *C. aff. pauciseptatum* were regularly isolated from diseased and healthy blackberries roots (Chapter 2, Table 2.1). *Cylindrocarpon* spp. were recovered from both decline and non-decline sites, although the number of isolates from decline sites and necrotic roots was much higher than from non-decline sites (Chapter 2, Table 2.1).

The anamorphic genus *Cylindrocarpon* Wollenw. is in the Nectriaceae along with *Fusarium*. *Cylindrocarpon* spp. are soil inhabitants, and occur as endophytes of woody plant species (Sieber, 2002), include both pathogenic and opportunistic root invaders, have a wide host range, and are the main cause and/or have association in several plant diseases in herbaceous and woody plant species (Brayford, 1992). *Cylindrocarpon* root diseases include black foot disease of grapevine (Gubler et al., 2004; Halleen, 2005; Alaniz et al., 2007; Mohammadi et al., 2009; Probst et al., 2012), root rot of avocado (Dann et al., 2012; Vitale et al., 2012), root rot of subterranean clover in the lower south-west of Western Australia (Barbetti, 2005). *Cylindrocarpon* species have been involved in root death of *Pinus sylvestris* (Unestam et al., 1989) and *Eucalyptus regnans* seedlings (Iles et al., 2010), disappearing root rot on ginseng (Rahman & Punja, 2005), and apple replant disease (Tewoldemedhin et al., 2011).

The most widespread species is *Cylindrocarpon destructans* (Crous et al., 2009). *Cylindrocarpon destructans* is a natural root coloniser of thimbleberry (*Rubus parviflorus*), and associated with root rots when *R. parviflorus* were treated with
glyphosate (Wall & Shamoun 1990a). Cedeño et al. (2004) reported the association of *C. destructans* var. *destructans* with black foot rot of blackberry (*Rubus glaucus* Benth.) in Mérida, Venezuela.

There are studies which consider *Cylindrocarpon* spp. to predispose plants to further infections by other pathogens. For example, Dann *et al.* (2012) noted *Cylindrocarpon* and *Fusarium* spp. stress avocado trees which then become more susceptible to *Phytophthora cinnamomi* and *Rosellinia necatrix*. In another study, when grapevines are infected with both *Phytophthora* spp. and *Cylindrocarpon destructans*, death was more rapid and the symptoms look different (Gubler *et al.* 2004) than when infected with *Phytophthora* alone. *Phytophthora* infected plants developed extensive root rot but when *C. destructans* further invaded, the tissues turned to the black and caused rapid dieback and death of the vines.

This chapter examines the pathogenicity of *Cylindrocarpon* species in a sand-infestation pot trial in the glasshouse and in a dual combination trial with several *Phytophthora* species shown to be pathogenic to blackberry (Chapter 4) to investigate possible synergism between the two taxa. This trial will determine if *Phytophthora* species together with *Cylindrocarpon* species result in increased disease severity in blackberry.

**Materials and Methods**

**Pot trial 1: Sand-infestation pot trial with selected *Cylindrocarpon* species**

*Rubus anglocandicans* daughter plants were collected in November 2010 from disease-free sites along the Warren River and placed into 130 mm free-draining polyurethane pots containing a steam pasteurised commercial bark based container substrate (SOILS AINT SOILS, Western Australia). Blackberries were grown in an evaporatively cooled glasshouse (11-25°C) for five months prior to use.
**Inoculum preparation**

In order to investigate pathogenicity of *Cylindrocarpon* species, five monoconidial cultures of isolates SA195 (*Neonectria* sp.), SA245 (*C. aff. pauciseptatum*), SA263 (*Neonectria radicicola*), SA264 and SA265 (*Cylindrocarpon* sp.) were tested in a sand-infestation pot trial.

Barley grains were used as the inoculum substrate (Cëdeno et al., 2004) for the selected isolates. Briefly, 150 g barley grains were placed in a 500-mL flask, 50 mL DW added and flasks were sealed with cotton protected with gauze and sterilised twice, with an interval of 24 h, for 20 min at 121°C. Each flask was inoculated with 15 discs (5 mm diameter) cut from an actively growing margins of a 7-day old monoconidial culture grown on PDA plates. Control flasks received non-colonised agar plugs. Flasks were incubated at 25 ± 2°C for 3 weeks before soil infestation and were shaken weekly to facilitate even colonisation of the barley grains.

**Sand infestation**

The barley grains inoculum were mixed with pasteurized and washed river sand in a ratio of 20g/L sand and healthy blackberries were transferred from the 130 mm pots into 175 mm free-draining pots filled with 1L of the infested sand. There were seven replicate pots for the five *Cylindrocarpon* isolates tested plus seven replicates for the two controls (one group flooded and another group not-flooded). Pots were placed in a completely randomized design in a controlled temperature glasshouse (12°C min-25°C max). Fertiliser (water soluble, Thrive®, Yates Company, Australia) was applied twice in the second and third weeks after inoculation. The plants were watered to container capacity daily. Except for the not-flooded control, all the pots were flooded as a predisposing factor (Unestam et al. 1989) three times. Pots were placed in 9L buckets at three, six and twelve weeks after inoculation for 15-17 h for the first and second
flooding and a week for the last flooding event. Plants were checked regularly for any symptoms and mortality for duration of seven months.

**Pot trial 2: Dual sand-infestation pot trial with Phytophthora species and Cylindrocarpon aff. pauciseptatum**

*Rubus anglocandicans* daughter plants were collected in September 2012 from disease-free sites and placed into 150 mm free-draining polyurethane pots containing washed and pasteurized white river sand. At the time of potting the plants, four inoculum delivery tubes (Poly pipe, 12 cm length x 2 cm width) were inserted into the pots. The plants were grown in an evaporatively cooled glasshouse (12-25°C) for seven months prior to infestation.

**Trial design**

The *Phytophthora* isolates selected were those that produced the largest lesions in the primocane under-bark inoculation in the field trial (Chapter 4). These isolates were *P. cryptogea* (SA014), *P. bilorbang* (CBS161653), and *P. amnicola* (SA326). The trial consisted of the following treatments:

- Control plants without flooding
- Control plants with flooding
- *C. aff pauciseptatum* (SA245)
- *P. bilorbang* paired with *P. cryptogea*
- *P. bilorbang* together with *P. cryptogea* and *P. amnicola*
- All three *Phytophthora* species combined with *C. aff. pauciseptatum*
**Inoculum preparation**

Wheat grains were used as substrate for the selected isolate of *C. aff. pauciseptatum* (Barbetti, 2005). Briefly, 100 g wheat grains were soaked in a 250 mL flask in 150 mL DW overnight and water drained day after; flasks were sealed with cotton protected with gauze and sterilised twice, with an interval of 24 h, for 20 min at 121°C. Each flask was inoculated with 10 discs (5 mm diameter) cut from an actively growing margin of a 7-day old monoconidial culture grown on PDA plates. Flasks were incubated at 25 ± 2°C for 3 weeks before sand-infestation and were shaken weekly to facilitate even colonisation of the wheat grains. For *Phytophthora* spp., Vermiculite-millet seed-V8 broth inoculum was prepared as described in Chapter 4.

**Sand infestation**

At the time of inoculation, the inoculation tubes were removed and 25g of *Phytophthora* inoculum for each species (1.25% inoculum:sand w/v) was placed in the holes in each pot according to the trial design for each treatment and covered with pasteurised sand. Pots to be treated with *Cylindrocarpon*, received 12g (0.6% inoculum:sand w/v). Controls received no inoculum and after removing tubes holes were filled with pasteurised sand. There were eight replicates for each treatment and pots were placed in a randomized design in an air-conditioned glasshouse (14-32°C). Plants were watered daily with deionised water to contained capacity, and water soluble fertiliser (*Thrive*®, Yates Company, Australia) was applied fortnightly at half the manufacturer’s recommended rate. Pots were inundated pre and post-inoculation. As a predisposing factor, pots were flooded with deionised water twice for duration of 24 h two weeks before infestation (once per week). After inoculation, pots were flooded in 9L buckets six times at weeks 2 and 4 for 72 h, and weeks 6, 10, 12 and 17 for 48 h to stimulate the production of sporangia and zoospores. Controls without inundation were excluded.
during each flooding event and watered daily to container capacity. At each flooding event, the water in the buckets was baited with juvenile leaves of *Scholtzia involucrata* and monitored regularly for lesions which were then excised and plated onto PARPHN, 3-7 days after baiting. Plants were checked for disease symptoms and mortality daily.

**Harvest**

After five months, the shoots were excised at the soil line and discarded and roots were harvested. Roots were washed using tap water to remove sand and inoculum, and the fresh weight was recorded. Root necrosis was visually assessed to compare the impact of the different treatments on root health on a rating scale of 0-3 (3 = severe root damage with lots of necrotic roots and loss of feeder roots; 2 = moderate damage (less necrotic roots and more normal feeder roots); 1 = slight damage (minimum damage and few necrosis along the roots with lots of normal feeder roots); 0 = no damage). Damage is a rank variable, so the data were analysed with a Kruskall-Wallis test.

Roots with lesions were plated onto PARPHN (Chapter 4) for *Phytophthora* species and on half-strength PDA plus Streptomycin (Chapter 2) for *C. aff. pauciseptatum* and incubated for up to 7 days in the dark at 25°C and examined daily for the presence of hyphae typical of *Phytophthora* and *Cylindrocarpon* species to fulfil Koch’s postulates. Roots from flooded and non-flooded control plants were also plated. Harvested roots were placed in paper bags and dried at 40°C for two weeks and dry weight was recorded. Univariate Tests of Significance was carried out to compare dry weight of different treatments. STATISTICA (STAT Soft. version 7.1, 1984-2006, Oklahoma, Tulsa, USA) software was used for data analysis.
Results

Pot trial 1: Sand infestation trial with *Cylindrocarpon* species

None of the five isolates were pathogenic to blackberries and there was no difference between flooded controls and *Cylindrocarpon* treated plants in terms of foliage growth. All of the plants were able to grow normally even seven months post inoculation. When roots were inspected for possible symptoms, sporadic and slightly discoloured (brown) lesions were infrequently observed (Fig. 5.1).

Figure 5.1 Comparison of blackberry (*Rubus anglocandicans*) root necrosis caused by *Cylindrocarpon* and *Phytophthora* species in 2 separate pot infestation trials in the glasshouse. (a) Sporadic necrosis with cream colour observed in roots treated with *Cylindrocarpon* aff. *pauciseptatum* (SA245) in pot trial 1 (this Chapter), (b) lots of brown to dark brown nectotic roots caused by *Phytophthora cryptogea* (SA261) and (c) lots of dark brown to black necrotic roots in roots treated with *Phytophthora bilorbang* (CBS161653) in pot trial 1 in Chapter 4.

Pot trial 2: Dual sand infestation pot trial

*Phytophthora* species were recovered from the buckets used to flood the plants during all six flooding events, for all the treatments which included *Phytophthora* species. No *Phytophthora* species were isolated from the flooded control, non-flooded control or the *C. aff. pauciseptatum* treatment. All *Phytophthora* species and *C. aff. pauciseptatum* were reisolated and identified from plated roots and not from controls. *Phytophthora*
species were recovered only from necrotic lesions on fine feeder roots up to 1mm
diameter and not from thicker roots. In contrast, *Cylindrocarpon* was isolated from all
root sizes. The highest impact on the roots were caused by the combined treatments
(*Phytophthora* together with *Cylindrocarpon*) and the controls with flooding compared
to roots treated only with *Cylindrocarpon* or the controls without flooding (Fig. 5.2, Fig.
5.4).

Data were heteroscedastic and there was a strong correlation between means and
variances across the cells of the design. These problems were corrected with logarithmic
transformation. Univariate Test of Significance for logarithmic dry weight of roots
revealed significant difference between different treatments (df = 5, p = 0.03). The
lowest blackberry root fresh (Fig. 5.3a) and dry weight (Fig. 5.3b) caused by *P. bilorbang*
and *P. cryptogea* treatment, and *P. amnicola*, *P. bilorbang* and *P. cryptogea*
treatment, respectively. Flooding alone, had a great impact on the roots health (Fig. 5.3).
Figure 5.2 Impact of different treatments on blackberry (*Rubus anglocandicans*) roots after harvesting pot infestation trial 2 in the glasshouse. (a) Control without flooding, (b) control with flooding, (c-d) roots treated with *Phytophthora bilorbang* and *Phytophthora cryptogea*, (e) roots treated with *Phytophthora amnicola*, *P. bilorbang* and *P. cryptogea*, and (f) roots treated with *P. amnicola*, *P. bilorbang*, *P. cryptogea*, and *Cylindrocarpon aff. Pauciseptatum*. Arrows show necrotic lesions.
Figure 5.3 Mean (± SE) of fresh weight (g) (a) and dry weight (b) of *Rubus anglocandicans* roots after harvesting a pot trial with different *Phytophthora* and *Cylindrocarpon* treatments in a glasshouse. Cp: *Cylindrocarpon* aff. *pauciseptatum* (SA245); Pb+Pc: *Phytophthora bilorbang* (CBS161653) and *P. cryptogea* (SA014); Pa+Pb+Pc: *P. amnicola* (SA326), *P. bilorbang* (CBS161653) and *P. cryptogea* (SA014); Pa+Pb+Pc+Cp: *P. amnicola* (SA326), *P. bilorbang* (CBS161653), *P. cryptogea* (SA014) and *Cylindrocarpon* aff. *pauciseptatum* (SA245).
Root damage ratings

A significant difference was detected between root damage caused by the different treatments including the two control groups (p = 0.03, df = 5) (Table 5.1). The greatest damage to the blackberry roots caused by the combined *Phytophthora* treatment (*P. amnicola*, *P. bilorbang* and *P. cryptogea*). Control with flooding had same damage rate as caused by *C. aff. pauciseptatum* alone (Fig. 5.4), indicating that flooding alone impacts blackberry root health as was shown previously (Fig. 5.3).

![Figure 5.4](image-url) Comparison of percentage root damage rating of *Rubus anglocandicans* with different treatments of *Phytophthora* species and *Cylindrocarpon* aff. *pauciseptatum* in sand-infestation pot trial 2 in the glasshouse. Error bars represent standard errors of the means. Cp: *Cylindrocarpon* aff. *pauciseptatum* (SA245); Pb+Pc: *Phytophthora bilorbang* (CBS161653) and *P. cryptogea* (SA014); Pa+Pb+Pc: *P. amnicola* (SA326), *P. bilorbang* (CBS161653) and *P. cryptogea* (SA014); Pa+Pb+Pc+Cp: *P. amnicola* (SA326), *P. bilorbang* (CBS161653), *P. cryptogea* (SA014) and *Cylindrocarpon* aff. *pauciseptatum* (SA245).
Table 5.1 Analysis of root damage in harvested *Rubus anglocandicans* by Kruskall-Wallis test.

<table>
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<th>Treatment</th>
<th>Non-flooded control</th>
<th>Flooded control</th>
<th>Cp*</th>
<th>Pb+Pc</th>
<th>Pa+Pb+Pc</th>
<th>Pa+Pb+Pc+Cp</th>
<th>Total</th>
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<td>4.0</td>
<td>6.0</td>
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<td>1.5</td>
<td>-1.5</td>
<td>-2.5</td>
<td>-0.5</td>
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</tr>
<tr>
<td>&gt; Median: observed</td>
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<td>2.0</td>
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<tr>
<td>obs.-exp.</td>
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<td>0.5</td>
<td>-1.5</td>
<td>1.5</td>
<td>2.5</td>
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<tr>
<td>Total: observed</td>
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<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>48.0</td>
</tr>
</tbody>
</table>

* Cp: Cylindrocarpon aff. pauciseptatum (SA245); Pb+Pc: Phytophthora bilorbang (CBS161653) and *P. cryptogea* (SA014); Pa+Pb+Pc: *P. amnicola* (SA326), *P. bilorbang* (CBS161653) and *P. cryptogea* (SA014); Pa+Pb+Pc+Cp: *P. amnicola* (SA326), *P. bilorbang* (CBS161653), *P. cryptogea* (SA014) and Cylindrocarpon aff. pauciseptatum (SA245).

Median Test, Overall Median = 2.5
Dependent: damage, Independent (grouping) variable: treatment
Chi-Square = 11.93, df = 5, p = 0.03
Discussion

Neither of the *Cylindrocarpon* species caused mortality in the first trial and root lesions were infrequent and sporadic. Dumroese *et al.* (2005) have shown the ability of *Cylindrocarpon* to cause serious root damage without foliage symptoms. Furthermore, Unestam *et al.* (1989) have discussed predisposing factors are necessary for pathogenicity of *Cylindrocarpon* species and therefore testing a particular species in a single trial might fail in the absence of predisposing factors. On the other hand, screening a wide number of isolates could assist to select more pathogenic isolates and consideration should be taken into wide screening for pathogenicity of different isolates in future research. To support this statement, researchers have postulated variability in the morphology and pathology between different isolates of the same *Cylindrocarpon* species (Halleen *et al.*, 2004; Halleen, 2005).

Surprisingly, in the combined trial *Cylindrocarpon* treated plants showed high root mass equivalent to the both the flooded and non-flooded control treatments. It seems that the type of inoculum and rate of application can affect plant growth and influence the pathogen’s impact on disease expression and severity. With regards to inoculum, a study by Strauss & Labuschagne (1995) has shown that inoculum type affects disease expression caused by *Fusarium solani*. The most severe root rot occurred when millet seed inoculum was used, while inoculation with conidial suspension or vermiculite containing mycelium did not lead to significant root rot. Consequently, they have indicated a food base containing inoculum is preferred. In the current study, barley and wheat grains were used as the inoculum substrate and this was found to be adequate; however, using wheat grains resulted in higher root mass compared to controls in the pot infestation trial 2.
Waterlogging alone had great impact on the blackberries and damaged the roots. Duncan and Kennedy (1989) investigated duration of waterlogging and have shown inundation for 4-8 days causes purplish colour in red raspberry roots as the result of stress and low oxygen in the absence of *Phytophthora* species and red raspberry plants exhibited wilting but survived. In another trial with the presence of moderately pathogenic to non-pathogenic *Phytophthora* species, death of red raspberries occurred after 4 days of inundation.

In the dual combination trial, treatments with 2 or 3 *Phytophthora* species combined, resulted in greater disease ratings than the other treatments and in particular the non-flooded controls. Also, the combination of *Phytophthora* species together with *C. aff. pauciseptatum* resulted in a high disease rating together with less root dry weight.

It is concluded that more than one *Phytophthora* species (in particular *P. amnicola*, *P. bilorbang* and *P. cryptogea*) have an association in the blackberry decline syndrome, with the presence of *Cylindrocarpon* species acting as possible secondary pathogens but the most damage they cause is under waterlogging conditions and in the presence of other stress factors.
CHAPTER 6

Epidemiology, Disease Description and a Conceptual Model to Describe the Decline of *Rubus anglocandicans* (European Blackberry), a Weed of National Significance in Australia

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**Minor authors’ contributions:**

Giles Hardy, Treena Burgess and John Scott were supervisors. Lee Fontanini and Paul Yeoh assisted in the field.
Introduction

Human activities have had an adverse impact on ecosystems on a global scale and have caused an unprecedented redispersal of organisms, with both plants and pathogens moving from their regions of origin to other parts of the world (Brasier, 2008; Alexander, 2010; Giraud et al., 2010; Morin et al., 2013). Invasive plants are a potential threat to ecosystems globally and their management costs tens of billions of dollars per annum (Culliney, 2005).

*Rubus anglocandicans* A. Newton (European blackberry, previously included in the aggregate taxon *Rubus fruticosus* L. aggregate) is a serious invasive species in Australia (Amor et al., 1998). It is one of the original 20 ‘Weeds of National Significance’ and a major weed of conservation, forestry and agriculture, particularly in wetter regions (NSW Department of Primary Industries Weed Management Unit, 2009). Herbicide and cultural control methods are generally inefficient or require multiple applications (Amor et al., 1998; Bruzzese et al., 2000). Therefore, a biological control program using stem and leaf rust strains is the main option in Australia (Scott et al., 2002; Evans et al., 2004; Barreto et al., 2012). However, biological control using rusts has been patchy, as host factors, climate and weather can alter the impact of the rust at different locations (Pigott et al., 2003; Evans et al., 2005; Evans et al., 2011; Morin & Evans, 2012). In 2007, Yeoh and Fontanini noticed that blackberry plants (*R. anglocandicans*) on the banks of the Donnelly and Warren Rivers in the south-west of Western Australia were dying in areas that were being regularly monitored for the impact of rust as biological control agent. The symptoms on blackberry became known as the disease "blackberry decline".
The epithets “dieback” or “decline” have been given to many perennial plant diseases to describe either the most significant criterion or the common nature of the specific disease syndrome and, in most cases, the causes are unknown or incompletely understood (Houston, 1992, [but see Ostry et al. (2011) for a contrary view]). Therefore, continuous and intensive investigations are required to discover the different biotic and abiotic components associated with specific declines in plant populations. There are numerous examples of decline phenomena in natural ecosystems (McDougall et al., 2002; Elmer et al., 2013; Matusick et al., 2013) including the determination of possible causes through classical and molecular pathology and ecological studies. This has led to the development of specific models for each decline phenomenon. For instance, Flory and Clay (2013) hypothesise the ecological consequences of pathogen accumulation for introduced hosts and encapsulate this in the Pathogen Accumulation and Invasive Decline (PAID) model. Elmer et al. (2013) have shown the involvement of biotic stressors (Fusarium species, nematodes and herbivores) and abiotic factors such as cyclic drought and rise of the sea level in the sudden vegetation dieback in Atlantic and Gulf Coast salt marshes.

Blackberries are generally more resistant to plant diseases than other Rubus species (Ellis et al., 1991). A dieback disease of blackberries affecting primarily the Boysen and Young trailing blackberries was prevalent in nearly all berry growing regions of California during the winters of 1947-8 and 1948-9 but the cause(s) remained unclear (Wilhelm et al., 1952). Wall & Shamoun (1990a) investigated diseases of Rubus parviflorus (thimbleberry) in British Columbia and cited the association of Cylindrocarpon destructans, Naematoloma fasciculare, Resinicium bicolour and a Verticillium sp. with root rots. Also, a blackberry root rot by Rhizoctonia solani has been reported by Cedeño et al. (1993). The association of Cylindrocarpon destructans
var. destructans and Neonectria discophora var. rubi with black foot rot on blackberry (Rubus glaucus) has been reported from Merida, Venezuela by Cedeño et al. (2004). Hence, based on the literature there are only a few records of root pathogens involved in blackberry diseases across the world. This Chapter describes blackberry decline and disease symptoms, epidemiology and its distribution in south-west of WA. We also present a conceptual model to explain the presence of the disease on blackberry.

Blackberry as a Weed in Western Australia

Blackberries were probably introduced to south-west of WA soon after European settlement in 1829. At least three species of weedy Rubus (blackberry) have established; R. anglocandicans (the most widespread species), R. laudatus and R. ulmifolius (Evans et al., 2007). In addition, the cultivated species R. loganobaccus (loganberry) and an unidentified blackberry are locally established (Yeoh et al., 2006a; Evans et al., 2007).

Blackberry is now widespread in the south-west of WA in areas with more than 800 mm rainfall and the impact being along watercourses (Hancock et al., 1996). Rubus anglocandicans infestation can reduce plant species diversity by 50% (Yeoh et al., 2006a). The most important recreational river fishing is for marron (a species of freshwater crayfish, Cherax cainii) and the dense thickets of blackberry prevent access to the river banks. In agricultural and forestry situations, R. anglocandicans is occasionally a major weed. Commercial production of weedy varieties of blackberries is prohibited under state laws (Yeoh et al., 2006a) and the only positive impact, wild fruit collection, is a minor activity.
Control by herbicides is effective but limited to accessible areas and regular reapplication. Therefore, biological control has been identified as the main strategy for control of blackberry.

**Blackberry Biological Control**

The only agent so far introduced to Australia for the biological control of blackberry is the rust *Phragmidium violaceum* (Schultz) G. Winter (Morin & Evans, 2012). An unknown strain of this rust was found to be widespread in Victoria in 1984 (Marks *et al.*, 1984). This is referred to as the illegal strain because there was neither prior host-specificity testing of this strain nor any official sanctioning of its release.

Another strain of this rust, (F15) was selected for its virulence and reproductive fitness under laboratory conditions and was officially approved for release and intentionally released in 1991 in Australia (Bruzzese & Lane, 1996). However, Evans *et al.* (2000) using specific molecular markers showed that the F15 strain did not become well established, as the illegal strain predominated in nearly all populations examined.

In an attempt to find more virulent and better-adapted rust strains capable of affecting Australian blackberry species growing under field conditions, trap gardens with blackberry plants transplanted from Australia were established at Montpellier, France (Morin *et al.*, 2011). Eight potential effective new strains were identified. These were approved for release in Australia in 2004 with releases of these strains together with F15 beginning in the same year.

In south-west of WA, the nine strains of rust were released in autumn and spring 2004 in the Manjimup region (Morin *et al.*, 2006). As part of a one year study to gather
baseline data on the blackberry infestations within these areas, and to investigate the possibility of integrating chemical and biological control methods, releases of the nine rust strains were made at six sites in the Warren River Catchment as well as two sites in the Blackwood River Catchment (situated immediately north of the Donnelly River Catchment) during the spring of 2005 (Yeoh et al., 2006a and b).

Eight sites used for experiments on treatments with fungicide and/or rust in 2004 and 2005 consisted of dense stands of healthy *R. anglocandicans*; in 2005, sites had on average 9 canes/m² or 5.4 plants/m² (Yeoh et al., 2006a). Healthy control plants not inoculated with the rust or sprayed with herbicide were approximately 12 m long, with 95 fruits and 1800 seeds by the end of the autumn (April). In spring 2006, a sub-set of the eight experimental sites from 2005 was used to setup new experiments designed to measure the impact of the blackberry rust. As part of this study, the fungicide 4Farmers Tebuconazole 430 [active ingredient 439g/L Tebuconazole was applied to half the plots (including eight plots, 3 x 2 m)] in 2006 onwards to control rust (a single application in October 2006 at the future Warren River decline site, followed by monthly applications October 2007 onwards).

**Description of the Study Area: the Donnelly and Warren Catchments**

The Donnelly and Warren Catchments are 1,688 km² and 4,315 km², respectively and located in the south-west of WA. This region has a strongly seasonal humid Mediterranean climate (Bureau of Meteorology of Australian Government, http://www.bom.gov.au/), with most of the rain falling in winter (June to August) and summer (December to February) being dry and hot. Most of the two catchments are covered with *Eucalyptus* forests of karri (*Eucalyptus diversicolor*) or mixed jarrah (*E. marginata*) - marri (*Corymbia calophylla*) with the remainder being farmland. The main
towns in the two catchments are Manjimup and Pemberton and the main industries are farming (started in the 1850s), horticulture, vineyards, forestry and, tourism.

**Initial Detection of Decline**

In November 2006, at the Warren River “decline site”, canes throughout the site looked normal but not particularly healthy and cane densities had fallen to 73% of overall in previous year. Cane densities at the three control healthy sites on same river were approximately the same as in the previous season (Fig. 6.1).

![Figure 6.1](chart.png)

**Figure 6.1** Seasonal changes in coverage of blackberry [Mean % cover (± SE) (n=4 plots/site)] in a non-decline site and decline site (both on the Warren River, WA). Half the plots at the decline sites were treated with the fungicide Tebuconazole from October 2006 to April 2009.
Being a semi-deciduous plant, foliage cover varies throughout the year, with a higher level of cover in summer (December to February) than in winter (June to August). In our study area, new season foliage was produced in October (spring) each year. In November 2006, plants in the decline sites only had 10% of the foliage cover seen the previous year, in contrast to the healthy sites that at the same time had over 60% of their previous year foliage levels (Fig. 6.2A).

In October 2007 the blackberry population at the decline sites had crashed as evidenced by a profusion of dead canes. The damage was more severe and rapid than anything previously observed by even after high inoculations of *P. violaceum* under laboratory conditions. The few new primocanes produced at the decline sites in the following season were thin and weak and the surviving floricanes failed to produce fruit. Dead stems fell to the ground (Fig. 6.2B), rotted quickly and disappeared within one year. Even the hard woody crowns of the decline plants also rotted quickly. In contrast, at healthy sites, or at sites sprayed with herbicide, the dead stems often stayed hard and upright for two to three years.

The application of the fungicide did not protect the plants in the decline site from further decline to near zero values by 2008. Since the fungicide is specific to true fungi (ascomycetes and basidiomycetes) we considered the decline might be caused by one or more oomycetes, such as *Phytophthora*. 
Figure 6.2 Blackberry at the Collins Road site, Warren River, WA: A, Extensive healthy blackberry in November 2005; B, typical blackberry decline at the same site, with dead canes on the ground in August 2008 after winter floods.
Surveys to Determine Extent and Distribution of the Decline Syndrome

All surveys were made between 15 March 2011 and 22 March 2012 by at least two observers and always included Lee Fontanini. A hand-held global positioning system (GPS) unit (GARMIN GPS, eTrex, high sensitivity) was used for geo-referencing and mapping. Date, location, waypoint number, coordinates were recorded at the start and end of an assessment area and when plant condition changed. The following were also recorded separately for each river bank: Health of blackberries [1 = healthy/vigorous, 1-2 = signs of poor vigour, 2 = plants stressed by foliar pathogens or grazing, 2-3 = evidence of recent herbicide use 3 = dead or dying (due to decline)]; density [(percentage of vegetation cover) 1 = absent, 2 = <1%, 3 = 1 – 10%, 4 = 11 – 50%, 5 = >50% on the riverbank] and any photo numbers and comments. The riparian zone, across both sides, was up to 100 m wide (but usually about 30 m wide) and is clearly defined both by a distinct change in ground height (up to 2 m) and in the vegetation changing from riparian species to either karri or other forest types along with an associated change in soil type. Both sides of the river were assessed at the same time (these rivers are often not very wide and reduced to about 1m in summer). Walking surveys were carried out in the area where the major decline was observed at the few road crossings, covering a distance of about 40 km of river (80 km of river bank).

Spot surveys along the rest of the river were made at vehicle access points. A waypoint was taken at the vehicle crossing/access point and surveys, both sides, 100 m upstream and 100 m downstream from the crossing/access point were made on foot. The same information was recorded as described for the walking surveys.
Collected data and points from GPS were imported to ArcGIS, ArcMap v. 10.0 (ESRI, Redlands, California, USA) and maps (Fig. 6.3 to Fig. 6.5) created. Blackberries are found extensively along both the Donnelly and Warren Rivers, and tributaries such as the Lefroy Brook (Fig. 6.3 and Fig. 6.5). The distribution of blackberries is limited by the 750 to 800 isohyet as shown by the distribution on the Tone, Perup and Donnelly Rivers (Fig. 6.3). Blackberry is found downstream, almost to the coast, and into areas where the rainfall has averaged 1600 mm (p.a.). Throughout this area the blackberry has a percentage cover of over 50%, typically forming dense thickets up to several metres high.

The initial two locations where decline was detected are 29 km apart on the Donnelly and on the Warren Rivers (Fig. 6.3). The decline areas (Fig. 6.4) were found throughout the distribution of blackberry in the Donnelly and Warren Catchments. Covering over 50 km of riverbank on the Warren river and 14 km on the Donnelly River (almost certainly an under estimate because large sections of the Donnelly River have not been surveyed). So far, the disease has mostly been found along the major watercourses due to the difficulty of river access in the densely forested areas.

For the walked sections of the Donnelly and Warren Rivers with only native forest, decline presence was extensive, but not equivalent on both sides of the river. Distinct disease fronts were present and these extended over time (Fig. 6.5).

A preliminary search in the neighbouring catchment, the Shannon, returned only very healthy blackberries. The catchment to the north (the Blackwood) was subjected to widespread community releases of the rust fungus and stakeholders were asked to respond with information on the effectiveness of the rust releases. No reports of decline from rust release or other causes were received. Further a field in WA, dying
blackberries have been reported, but have since proved to be due to the rust being particularly effective in certain seasons and locations.

**Description of the “Blackberry Decline” Syndrome**

At each decline and non-decline site, plants were visually assessed for disease symptoms and other stress factors before digging roots and collecting soil samples. Decline symptoms included a dramatic change in the dense stands of blackberry and browning of the canes and foliage compared with healthy sites. Dead canes fall on the ground or remained 2-3 m high in forks of native trees. Dying and healthy plants were collected from adjacent declining and non-decline sites, respectively. Diseased and healthy roots and crowns were examined using a sharp scalpel to make horizontal and vertical slices to observe length and depth of lesions in the plant tissues, presence or absence of discoloration and necrosis, pathogen fruiting bodies and insect activities. Since, no evidence of disease was observed in canes, these were excluded. Crowns and roots were kept in the plastic bags in insulated boxes and moved to the laboratory for further processing. Crowns and roots were rinsed with tap water to remove soil particles and root symptoms were assessed, photographed and recorded. Crowns, roots and soil materials from healthy and diseased plants were baited and plated on different selective and non-selective media in order to isolate possible pathogens (Aghighi *et al*., 2012b, Chapter 3). Several *Phytophthora* spp. (only from decline and adjacent sites), *Pythium* and *Cylindrocarpon* species (from both decline and healthy sites) were recovered (Aghighi *et al*., 2012a and b, Chapters 1 and 3).
No lesions were observed in healthy blackberry roots and crowns (Fig. 6.6A and 6.6B) which were white internally compared with reddish-purple discoloration of coarse roots and crowns together with grey to black streaks in the vascular tissues of declining plants (Fig. 6.6C and 6.6E). The roots and crowns of dead plants affected by root rot showed dark chocolate brown to black necrotic lesions in all parts of the roots including fine roots, root hairs and root tips (Fig. 6.6D and 6.6F). Necrosis of lateral roots was seen where they are attached to the main roots. The cortex of diseased roots could be sloughed off with ease in all diseased plants (Fig. 6.6F). Root biomass and production of root hairs was greatly reduced and also in most of the symptomatic roots, shortened root clusters were formed.

Canes that had been defoliated through rust infection, thrip damage and activity of secondary fungi were observed. The impact of the decline on seeds was not measured. Likewise, it is not known if the decline affects seed in the soil. No mortality of seedlings was observed in decline areas; however, at most of the sites there was very little if any seedling development observed which suggests pre-emergence damping-off was occurring.
Figure 6.3 Distribution of blackberry and its density (% cover) along the Donnelly and Warren Rivers and major tributaries during walked (Walked sites) and river crossing surveys (Access sites) in 2011-2013. The red zigzag lines and numbers show bands of average annual rainfall in mm. The Walked sites (squares) are shown in more detail in Fig. 6.5.
Figure 6.4 Distribution of decline symptoms (red dots and squares) and other types of damage to blackberries observed along the Donnelly and Warren Rivers and tributaries during walked and river crossing surveys in 2011-2013. The black squares show the location of the first two discoveries of blackberry decline. The red zigzag lines and numbers show bands of average annual rainfall in mm. The Walked sites (squares) are shown in more detail in Fig. 6.5.
Figure 6.5 Distribution of blackberry decline symptoms along a section of the Warren River detected during walked surveys in 2010-2011. Nearby Access sites surveys (Fig 6.4) are shown as dots. Blackberry absent: 30.72 km; blackberry healthy: 15.50 km; blackberry sprayed: 2.98 km; possible blackberry decline: 26.03 km; blackberry decline: 4.18 km; total Riverbank 79.41 km (ie 39.71 km of Warren River surveyed by walking).
Figure 6.6 Healthy blackberry crowns and roots (A,B) and blackberry decline symptoms (C,D,E,F): A, longitudinal section of a healthy crown with cream to white tissues; B, healthy root ball; C, reddish-purple discoloration in a crown; D, a diseased root with dark chocolate brown to black necrotic lesions in all parts of the roots; E, grey to black streaks in the vascular tissues; F, severe root damage with cortex sloughing off symptoms (arrows).
Comparison with Herbicide Affected Plants

Populations of blackberries dying due to herbicide can initially look very similar to those dying due to the decline syndrome. In both cases, the crowns of dying plants can look similar with the red-purple layer being present. However, herbicide treated canes are hard and ‘woody’ and persist for up to three years, whilst those killed by the decline syndrome are soft and disappear from the site within one year. In addition, herbicides are not specific to blackberry so off-target damage occurs and is usually evident on adjacent native plant species. This ‘collateral damage’ was not observed in decline sites.

Blackberry Decline ‘Conceptual Model’

We have identified numerous factors that may contribute towards the decline. These are summarised in the conceptual ‘blackberry decline’ model (Fig. 6.7) showing a decline spiral (Manion, 1981; Houston, 1992). Other components included in our spiral were based on our observations and from the literature. The factors are grouped into predisposing, inciting and contributing factors.
Figure 6.7 Conceptual model of possible factors involved in blackberry decline [based on the tree decline model of Manion (1981)].
**Predisposing factors**

Climate or site factors are always a major predisposing component to a decline syndrome (Manion, 1981). In south-west of WA the climate is expected to become warmer and dryer, but with more extreme weather events (Matusick *et al*., 2013). Wild fires in natural ecosystems in Australia kill blackberries and allowing natives and other competitor species to grow (NSW Department of Primary Industries Weed Management Unit, 2009). Furthermore, the topography of the *R. anglocandicans* decline sites consist of broad river valleys facilitating cyclic inundation following large rainfall events (including rainy days over summer), which raise the water levels in the Donnelly and Warren Rivers, often causing temporary flooding. These flooding events can last for 10 days or more and will lead to temporary waterlogging of the riparian zone (flood plain) normally above the water level. In contrast, in the non-decline sites where temporary inundation does not occur, *R. anglocandicans* remains healthy. Occasional dense stands of *R. anglocandicans* were observed on steep well-drained banks of rivers in the decline sites, which further supports the ‘waterlogged soil’ predisposition hypothesis underlying the decline syndrome. Blackberries do not like waterlogging (Ellis *et al*., 1991) and this stress predisposes them to infection by root pathogens. Flooding also distributes soil-borne pathogens and their propagules.

Shading and low light reduces photosynthesis, in turn stresses plants and eventually causes mortality due to carbohydrate starvation (Marshall & Waring, 1985; Waring, 1987; McDowell, 2011). A combination of shading with mechanical abrasions, and other factors can alter photosynthate allocation (Waring, 1987) and susceptibility to root pathogens. For instance, Matson & Waring (1984) have demonstrated significant relationship between shading and susceptibility of mountain hemlock
(Tsuga mertensiana) forests in the Oregon Cascades to dieback and laminated root rot caused by Phellinus weirii.

Predisposing stress components are long-term factors in the conceptual tree decline model (Manion, 1981; Houston, 1992), and have the potential to enhance susceptibility of healthy plants to pathogens or injury-inducing factors. These stresses set the stage for inciting factors (Castello et al., 1995).

**Inciting factors**

Lack of genetic potential has been recognized to have a role in blackberry decline. In a few taxa within the Rosaceae such as Rubus alceifolius (Amsellem et al., 2001) and R. anglocandicans (Evans & Weber, 2003), the reproduction system is largely apomictic and consequently these taxa lose their genetic variability. This switch occurs between their native centre of origin and their area of introduction. Evans & Weber (2003) have described R. anglocandicans as the most widespread taxon of European blackberry in Australia and mentioned that this species is a facultatively apomictic taxon and exhibits little genetic diversity. Vegetatively propagated species with self-pollination are generally more severely attacked by diseases than cross-pollinating ones (Harper, 1977; Dinoor & Eshed, 1984); therefore, in Australia a lack of genetic potential in R. anglocandicans populations is likely to have a strong involvement in the decline syndrome.

Grazing may also be an inciting factor, and ringtail possums (Pseudocheirus occidentalis), quokkas (Setonix brachyurus) and grey western kangaroos (Macropus fuliginosus) were photographed grazing in blackberry sites (Fontanini, personal observations). Exotic mammals (goats) were also captured on camera feeding on
blackberry. The role of herbivores and overgrazing in similar scenarios was illustrated in a recent study by Elmer et al. (2013) on sudden vegetation dieback in Atlantic and Gulf Coast salt marshes.

Competition of native species and other invasive weeds for water, nutrients and light should not be ignored. Competition with other introduced species was listed as a cause for population crashes of four invasive species showed by Simberloff and Gibbons (2004). Native bracken (*Pteridium esculentum*) and sedges (*Lepidosperma effusum*) can be considered as competitors in blackberry infested sites in the south-west of WA (Fontanini, personal observations).

Slashing or mowing (removal of live top-growth) is one of the Integrated Weed Management (IWD) strategies to control blackberries population in Australia (Bruzzese et al., 2000; NSW Department of Primary Industries Weed Management Unit, 2009). It forces the plant to regrow and may deplete crown and root reserves. Consequently, plants become more susceptible to death from other causes. Furthermore, during summer, the impact of blackberry leaf rust can be enhanced as juvenile leaves and newly emerged foliage (present as the consequence of slashing) are more susceptible to the rust isolates (Bruzzese et al., 2000; NSW Department of Primary Industries Weed Management Unit, 2009). Controlled burns before herbicide application is also used to reduce blackberry thickets to a more manageable size and burning after herbicide spray clears away dead canes (NSW Department of Primary Industries Weed Management Unit, 2009).

The application of herbicides can induce root disease problems (Lévesque & Rahe, 1992) and other diseases of plants (Johal & Huber, 2009). For example, fungal colonization of tomato roots occurs rapidly after glyphosate is applied (Bramhall &
Higgins, 1988). Furthermore, a combination of a low dose of glyphosate together with *Cylindrocarpon destructans*, a natural colonizer of roots of thimbleberry (*R. parviflorus*), caused mortality and wilting of the plants (Wall & Shamoun, 1990b).

**Contributing factors**

Leaf rust (*Phragmidium violaceum*) has been identified as a successful biological control tool in some locations in Australia with direct and indirect impacts on blackberry growth. Continuous attack on the leaves by such fungal foliar pathogens weakens plants by depleting root reserves of photosynthates. Leaf rust infection during sequential growing seasons can lead to more defoliation and allow other plants to compete particularly over autumn and winter, when blackberries are in a dormant stage, which in turn can limit blackberry growth through shading (Bruzzone & Lane, 1996). Stem rust caused by *Kuehneola uredinis* (Shivas, 1989) has less impact on blackberries. Thrip damage (common greenhouse thrip: *Heliothrips haemorrhoidalis*) was regularly observed and can lead to blackberry defoliation. Red berry mite (*Acalitus essigi*) (Scott et al., 2008) and leaf spot (*Septoria rubi*) (Ellis et al., 1991) were recorded regularly and can weaken blackberries or reduce seed dispersal.

In the decline sites, ‘grey mould’ caused by a *Botrytis* sp. and other secondary fungi grew on leaves and canes of *R. anglocandicans* containing stem and leaf rusts. Synergistic effects have been observed between necrotrophic fungi and rusts resulting in more rapid cell death (De Nooij & Paul, 1992; Halett & Ayres, 1992). Secondary pathogens invade rust (*Puccinia poarum*) pycnia andaecia on coltsfoot (*Tussilago farfara*) which has led to death of host leaves (De Nooij & Paul, 1992).
During our disease surveys, nine *Phytophthora* species were isolated from decline sites including *P. amnicola*, *P. bilorbang*, *P. cryptogea*, *P. inundata*, *P. litoralis*, *P. multivora*, *P. taxon personii*, *P. thermophila* and *P. thermophila-amnicola* hybrid. *Phytophthora bilorbang* and *P. cryptogea* were recovered consistently from baited roots and associated rhizosphere soil collected from decline and adjacent declining sites over different seasons. Furthermore, different *Pythium* species, *Cylindrocarpon destructans* and *C. pauciseptatum* were also recovered more commonly from blackberry decline sites than non-decline sites. The involvement of different *Phytophthora* species has been assessed through laboratory, glasshouse and field trials and the strong association of *P. bilorbang* (Aghighi et al., 2012b, Chapter 3) and *P. cryptogea* in the blackberry decline has been postulated (Chapter 4). However, the possible synergism between different *Phytophthora* species and other weak pathogens should not be ignored. Synergism between *Phytophthora* spp. and *Cylindrocarpon destructans* has been demonstrated for black foot in grapevine (Gubler et al., 2004). Moreover, in other disease complex scenarios such as apple replant disease, Tewoldemedhin et al. (2011) have highlighted the importance of synergistic interactions between recovered *Phytophthora*, *Pythium* and *Cylindrocarpon* species using co-inoculation approaches. Hence, we are investigating interactions between putatively pathogenic *Phytophthora* species and *C. pauciseptatum* in an ongoing soil-infestation pot trial in the glasshouse (Chapters 4 and 5).

*Phytophthora* species are best known as invasive pathogens destroying trees and crops worldwide. Plant diseases caused by *Phytophthora* species are devastating to agriculture and natural ecosystems (Hansen et al., 2012; Kroon et al., 2012). As of 2012 there are 121 described *Phytophthora* species with 4384 distinct host-pathogen
associations distributed in 138 countries (Scott et al., 2013). Phytophthora species are one of Australia’s most serious plant pathogens and the majority of Phytophthora species have been introduced to Australia and widely distributed (Irwin et al., 1995). We believe that oomycetes and in particular P. bilorbang and P. cryptogea have strong involvement in the blackberry decline and are able to cause severe damage to the roots after temporary inundation creating conducive conditions in the declining sites across Donnelly and Warren Rivers. Waterlogging in conjunction with invasion by Phytophthora species may be especially effective and thereafter Cylindrocarpon species may invade the weakened roots and produce toxins as has been postulated by Unestam, et al. (1989).

Plants mediate interactions between different above- and below-ground biota and respond to stressors in several ways. Eventually these responses may result in negative effects on plant itself (Heil, 2011). We believe that contributing factors included in the blackberry decline spiral all have a role in this syndrome, but the involvement of the hypothesized predisposing and inciting factors are also essential for the expansion of the decline.

**Summary**

Blackberry decline is now a major feature in the ecology and control of blackberry with an impressive impact on previously infested banks along the Donnelly and Warren Rivers. To date, there are no reports of blackberry decline in neighbouring catchments (e.g. Shannon, Blackwood). Likewise, we are not aware of any similar “decline” syndromes from elsewhere in the world, despite blackberry being a very widespread species. Therefore, to our knowledge, this is the first record of such a
decline of European blackberry worldwide. At this stage, we have not observed any
deaths of native plants in areas where blackberry decline is present. Worldwide
decline syndromes are being observed in natural ecosystems (e.g. McDougall et al.,
2002; Elmer et al., 2013; Matusick et al., 2013) and the conceptual approach that we
have adopted could have wider applicability. More work is required to understand
the decline syndrome to determine if severely infested riparian zones can be
manipulated to initiate the decline syndrome as a management tool for blackberry
control. This would require detailed assessment of the host range of the pathogens
involved including susceptibility of plant species to be used in restoration of decline
sites. The biotic (contributing) factors discovered in this research have yet to be
investigated in more complex trials to examine their interactions in conjunction with
other stress enhancing factors. Finally, another challenge is to manage these decline
sites, to ensure the return of native riparian species and to prevent other exotic weedy
species replacing blackberry.
CHAPTER 7

Conclusions & Future Research
To my knowledge this is the first record of such a dramatic and sustained decline of European blackberry worldwide. In Australia, European blackberry (*Rubus anglocandicans*) has been targeted as one of the top 20 weeds of national significance; therefore, the outcomes of this research have relevance nationally. This research has led to the description of *Phytophthora bilorbang* and based on the pathogenicity experiments in the laboratory, glasshouse and field it has, together with *P. cryptogea* and possibly *P. amnicola* a strong association with the blackberry decline following periods of temporary inundation of blackberry along riparian zones. As a part of this thesis, a synergism trial was conducted to assess disease severity in the presence of more than one *Phytophthora* species. Based on the result of this experiment, it is assumed that more than one *Phytophthora* species are involved in the blackberry decline as disease severity was increased by combining *P. bilorbang*, *P. amnicola* and *P. cryptogea* together with periodic waterlogging. Pathogenicity of different isolates of *P. bilorbang* and *P. cryptogea* varied across the different trials indicating that different isolates appeared to vary in their aggressiveness. It is evident that decline is associated with only *Rubus anglocandicans*, as there was no evidence of disease symptoms in native flora growing in association with blackberry. Furthermore, and importantly the decline syndrome is persistent as no recruitment of *R. anglocandicans* seedlings was observed at the decline sites.

During this research, nine *Phytophthora* species were isolated and identified; all were associated with blackberry decline sites. Many of these species have been associated with diseased plants in Australia or worldwide (Table 7.1). What is of interest is that the majority (seven of the nine) of the species belong to ITS Clade 6.
Importantly, the majority of these species have been shown to cause disease in single/multiple host(s) based on other studies in the literature (Table 7.1). Members of ITS Clade 6 have mostly been isolated in riparian ecosystems. Most species in clade 6 are infectious on roots or present in the rhizosphere (Kroon et al., 2012). For instance, *Phytophthora gibbosa*, *P. gregata*, *P. litoralis* and *P. thermophila* appear to be opportunistic pathogens under favourable episodic conditions such as flooding (Jung et al., 2011). *P. inundata* is a parasite of woody hosts in riparian ecosystems, and causes severe disease outbreaks on susceptible hosts such as ornamental *Aesculus* and *Salix*, or commercially cultivated *Olea* or *Prunus*, after extremely wet periods (Brazier et al., 2003b). Furthermore, a few species in this clade are hybrids and involve clade 6 parents, as for example the *P. thermophila-amnicola* hybrid observed in the current study (Chapters 2 and 4). In addition, other hybrid isolates in Western Australia have been obtained from the rhizosphere soil of dying plants; consequently they should be regarded as potential threats to plant health (Nagel et al., 2013).

*P. bilorbang* as a member of clade 6 is homothallic and readily produces thick-walled oospores on agar media (Chapter 3). In a preliminary experiment, when soil samples containing *P. bilorbang* recovered from a blackberry decline site (Banister Bridge, Fig. 7.1), were dried at room temperature for more than 4 months and then rebaited, *P. bilorbang* was reisolated. It is assumed that *P. bilorbang* survives periodic drought by producing thick-walled oospores. Moreover, the abortion rate of oospores of *P. bilorbang* isolates *in vitro* was less than 5% suggesting that probably *P. bilorbang* is able to produce a large number of oospores as survival structures; however, this feature needs to be tested further *in situ*. In addition, the recovery rate
Table 7.1 *Phytophthora* species recovered from blackberry decline sites, their known hosts and source of isolation.

<table>
<thead>
<tr>
<th>Phytophthora species</th>
<th>Clade</th>
<th>Known pathogen</th>
<th>Host</th>
<th>Source</th>
<th>Origin Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. amnicola</em></td>
<td>6</td>
<td>+</td>
<td><em>Patersonia</em> spp.</td>
<td>Soil/still water</td>
<td>Burgess &amp; Jung, 2012</td>
</tr>
<tr>
<td><em>P. bilorbang</em></td>
<td>6</td>
<td>+</td>
<td><em>R. anglocandicans</em></td>
<td>Rhizosphere soil</td>
<td>Aghighi <em>et al</em>., 2012b</td>
</tr>
<tr>
<td><em>P. inundata</em></td>
<td>6</td>
<td>+</td>
<td>Multiple</td>
<td>Roots</td>
<td>Brasier <em>et al</em>., 2003b</td>
</tr>
<tr>
<td><em>P. litoralis</em></td>
<td>6</td>
<td>?**</td>
<td>Unknown</td>
<td>Soil</td>
<td>Jung <em>et al</em>., 2011</td>
</tr>
<tr>
<td><em>P. taxon personii</em></td>
<td>6</td>
<td>?</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>P. thermophila</em></td>
<td>6</td>
<td>?</td>
<td>Unknown</td>
<td>Soil/roots</td>
<td>Jung <em>et al</em>., 2011</td>
</tr>
<tr>
<td><em>P. thermophila-amnicola</em> hybrid</td>
<td>6</td>
<td>?</td>
<td>NA</td>
<td>water</td>
<td>Nagel <em>et al</em>., 2013</td>
</tr>
<tr>
<td><em>P. cryptogea</em></td>
<td>8</td>
<td>+</td>
<td>Multiple/<em>Rubus idaeus</em></td>
<td>Roots/foliage</td>
<td>Washington, 1988</td>
</tr>
<tr>
<td><em>P. multivora</em></td>
<td>2</td>
<td>+</td>
<td>Multiple</td>
<td>Rhizosphere/foliage</td>
<td>Scott <em>et al</em>., 2009</td>
</tr>
</tbody>
</table>

* More information was provided in Chapter 2 (Table 2.2). ** Unknown. *** Data not available.
of *P. bilorbang* from rhizosphere soil was less (15%) when compared to *P. amnicola*, *P. cryptogea* and *P. multivora*. There is a possibility that *P. bilorbang* is a weak competitor therefore more sampling and baiting will increase the recovery rate of this species.

Based on the literature, the other species isolated in the present study, including *P. cryptogea* and *P. multivora* have multiple hosts (Table 7.1). Both these species were recovered frequently from blackberry decline sites. Although, there was no evidence of dead or dying native species in the blackberry decline sites, other than blackberry, all of the *Phytophthora* species isolated should be regarded as potential threats to native flora of the south-west of WA. In particular, since its description in 2009 (Scott *et al.*, 2009), the host range of *P. multivora* has increased and continues to increase worldwide (Scott, 2011), and is likely to continue to do so in the future. For example, Barber *et al.* (2013) showed *P. multivora* to be the most frequently isolated *Phytophthora* species from symptomatic hosts in the Perth metropolitan region. Therefore, it is recommended that all the *Phytophthora* species isolated in this study should be screened against native plant species being considered for out planting along the decline sites in future restoration programs. Those that are shown to be susceptible then can be withdrawn from such restoration programs, which will save costs of seedling production, out planting and subsequent maintenance.

More work is required to understand the decline syndrome to determine if healthy sites can be manipulated to initiate the decline syndrome as a management tool for blackberry control in severely infested riparian zones. As the result of this PhD project, other projects have been initiated in relation to the rehabilitation of blackberry decline sites in collaboration between Murdoch University (Centre for *Phytophthora* Science and
Major findings and outcomes of this project

- Recovery of *P. amnicola*, *P. bilorbang*, *P. cryptogea*, *P. inundata*, *P. litoralis*, *P. multivora*, *P. taxon personii*, *P. thermophila*, and *P. thermophila-amnicola* hybrid, all associated with blackberry decline.
- Description of *P. bilorbang*.
- Proven pathogenicity of *P. amnicola*, *P. bilorbang* and *P. cryptogea* to *Rubus anglocandicans* especially in the presence of temporary waterlogging and postulation of the involvement of more than one *Phytophthora* species in a dual pot infestation trial under controlled glasshouse conditions.
- Showed the possible involvement of *Cylindrocarpon* spp. and in particular *C. aff. pauciseptatum* working synergistically with *Phytophthora* species contributing to blackberry decline.
- Comprehensive mapping of blackberry decline along the Warren and Donnelly rivers to provide a map of the distribution of the decline syndrome.
- Development a “decline spiral” as a conceptual model, to describe blackberry decline as a disease complex.

Future research

- **Host range study of isolated *Phytophthora* species to native flora**
  A major management challenge is to now to restore the decline sites with native plant species as rapidly and effectively as possible, before other exotic weedy
species replace blackberry. However, there is a lack of knowledge on the susceptibility of the majority of native plant species to the nine *Phytophthora* species isolated during the current study. Consequently, it is necessary to determine the susceptibility or resistance of those native plant species which will be considered in rehabilitation projects to these nine *Phytophthora* species. Therefore, it is highly recommended that pathogenicity trials using soil infestation (not under-bark inoculation methods) under controlled glasshouse conditions are conducted on all native plant species that are likely to be considered in the restoration of the decline sites. This will ensure species susceptible to one or more of the *Phytophthora* species are not planted into the infested areas.

- **Determination of how long all recovered *Phytophthora* species remain present in a site post disappearance of blackberry**

There is a need to find out how long all recovered *Phytophthora* species and in particular *P. bilorbang* and *P. cryptogea* survive once all blackberries have disappeared from along the river banks. This will require on-going soil and water baiting for *Phytophthora* from these sites. In addition, it will be useful to determine if the different *Phytophthora* species can survive saprophytically or in symptomless host species. This knowledge can be used to further inform restoration programs of decline sites.
Pathogenicity assessment of recovered *Phytophthora* species with more isolates

It is recommended that more isolates of all nine *Phytophthora* species need to be screened in pathogenicity trials to compare the “inter and intra” species variation in blackberry. This study can be conducted in under-bark inoculation preferably in the field and pot infestation trials in the glasshouse. It is necessary to conduct both soil infestation and underbark trials, until a better understanding of how well underbark inoculation trials correlate with soil infestation trials for each of the species in question.

Evaluation of blackberry soil seed-banks in the presence and absence of *Phytophthora* species

*In situ* field trials at both healthy and declining sites are recommended to examine pre- and post-emergent damping off. This will provide knowledge on the longevity of the soil seed bank for blackberry and on whether the different *Phytophthora* species are potential damping off pathogens. In addition, continued soil baiting of these sites are required to help determine how long each of the different *Phytophthora* species can survive once adult blackberry plants have disappeared. Also, controlled glasshouse trials should be conducted to determine if any of the nine *Phytophthora* species are damping off pathogens, in order to support any observations made in the field.

Baiting in more streams around different blackberry sites

It is important to conduct ‘fishing for *Phytophthora*’ in all streams and tributaries of the river systems where blackberry is an environmental issue in the south-west of
Western Australia. This should be conducted monthly to capture annual and seasonal activity of the *Phytophthora* species found in the stems close to decline and healthy sites. This will provide further insights into the epidemiology of these *Phytophthora* species and their role in blackberry decline.

➢ **Application of 454-pyrosequencing and meta-analyses**

In order to identify all microorganisms from blackberry roots, sampling from decline, declining and control sites is recommended using the 454-pyrosequencing technique (Buée et al., 2009). This will help identify all the microorganisms associated with roots and rhizosphere of blackberries and help to get a better understanding of population frequencies of these species on healthy and declining blackberries. An advantage of using this technology is that there is no limitation for seasonal recovery of each species as pathogen dormancy is not an issue using this technique. Moreover, application of this method will be very helpful for recovery of less competitive pathogens, or putative pathogens that are obligate biotrophs (cannot be isolated on agar media).

➢ **Determination of centre of origin of *P. bilorbang***

As more isolates come available from the studies recommended above, from elsewhere in Australia and overseas it will be worth determining where the origin of *P. bilorbang* is actually from. A key question remains, “has it been introduced to WA”? However, it is likely to have been introduced, as other isolates of *P. bilorbang* are now listed on GenBank from other parts of the world. Although, it is possible that it has been ‘moved’ from Australia overseas.
- **Installation of remote cameras in different blackberry sites**
  
  There are other blackberry sites in inaccessible and accessible areas. To obtain a more complete understanding of how the decline syndrome develops at a site and how quickly it kills plants, it is suggested to install remote cameras in accessible blackberry sites (even healthy looking) and monitor them over time to capture the progression of the decline syndrome, along with timing and duration of temporary inundation events. The inclusion of weather stations, soil and water temperature probes and depth gauges (to measure river levels) alongside the cameras would also be advantageous to improve our knowledge on the epidemiology of this decline syndrome.

- **Mapping of other blackberry sites in order to capture further decline sites**
  
  Ground based mapping is recommended for other readily accessible blackberry sites to monitor if any decline is occurring at sites that have not yet been surveyed.

- **Interaction studies between *Phytophthora* species and blackberry leaf and stem rust diseases under controlled conditions**
  
  Blackberry leaf and stem rust pathogens are almost everywhere in the studied sites. Rust impact and its role in the decline should not be ignored (as was discussed in Chapter 6). It is recommended to set up a trial under controlled glasshouse conditions to investigate the interaction of leaf rust with *Phytophthora* species. Trials can be established by using positive and negative controls and a combination of leaf rust on foliage and *Phytophthora* as root pathogens. Alternatively, sites with early onset of symptoms typical of decline, with both *Phytophthora* species and rust present could be treated or not-treated with
fungicides specific to rust. This would provide information on how important the rust disease is in contributing towards the decline syndrome.

- **Pathogenicity trials with different *Pythium* species**

  *Pythium* species were found infrequently in healthy sites but with higher species diversity and more frequent recoveries in decline sites. However, the majority of the recovered *Pythium* species did not produce lesions in primocane under-bark inoculation trials (except for *Pythium dissotocum* SA370). Since, we have a poor understanding of how well under-bark inoculation of stems and corresponding lesion lengths correlates with root necrosis for *Pythium* species, it is also recommended that pathogenicity trials are conducted using soil infestation tests together with temporary periods of waterlogging under controlled conditions. Such studies will indicate whether *Pythium* species are also contributing to the decline syndrome or not.

- **Temporary damming of rivers to stimulate the decline syndrome**

  Since, temporary inundation appears to play such a dominant role in the blackberry decline syndrome and that blackberry is so hard to control by other methods consideration should be given to the temporary damming of rivers during winter months, to mimic major rainfall events. Briefly, where *P. bilorbang* and/or *P. cryptogea* and possibly one or more of the other *Phytophthora* species are present along a river, dams could be built that allowed rapid but controlled inundation of a river or section of river to be inundated for 2-3 weeks at a time, and for two or three periods a winter season. Since none of the river systems in the south-west of WA are large (deep or wide), such a procedure could likely be cost
effective. It is also unlikely to have severe impacts on other biota, as these river systems have evolved to undergo periodic inundation events. However, close consideration and monitoring of such a method on other biodiversity values should not be neglected. Possibly a cost-benefit analysis could be undertaken before such a strategy was implemented.

**Concluding remarks**

This research has investigated the etiology and epidemiology of European blackberry (*R. anglocandicans*) decline in two river systems (Warren and Donnelly River Catchments) in the south-west of Western Australia. It was concluded that blackberry decline is a complex syndrome and *Phytophthora* species and in particular *P. bilorbang* and *P. cryptogea* together with temporary inundation are considered as major biotic and abiotic factors, respectively contributing to blackberry decline.
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