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## Isolation and pathogenicity of *Phytophthora* species from declining *Rubus anglocandicans*

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### Abstract

*Rubus anglocandicans* is the most widespread and abundant blackberry species within the European blackberry (*Rubus fruticosus*) aggregate in Western Australia (WA). European blackberry is also one of the 32 Weeds of National Significance in Australia. A disease recorded as 'blackberry decline' was first observed in some blackberry (*R. anglocandicans*) sites in WA in 2006. A disease survey was conducted in the Manjimup-Pemberton region along the Warren and Donnelly river catchments in WA between 2010 and 2012.

*Phytophthora amnicola*, *P. bilorbang*, *P. cryptogea*, *P. inundata*, *P. litoralis*, *P. multivora*, *P. taxon personii*, *P. thermophila*, and a *P. thermophila-amnicola* hybrid were recovered from declining and adjacent decline-free sites, as well as from streams and rivers. *P. cinnamomi*

was isolated from dying *Banksia* and *Eucalyptus* species from two non-decline sites. Of these  
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species, *P. bilorbang* and *P. cryptogea* were more pathogenic than the others in under-bark inoculations using excised stems (primocanes), *in planta* primocane inoculations in blackberry growing wild in native forest stands, and in glasshouse pot trials. 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 major biotic and abiotic factors, contributing to blackberry decline.

*Keywords:* European blackberry, decline, pathogenicity

## **Introduction**

*Rubus anglocandicans* A. Newton (hereafter “blackberry”) is a species within the *Rubus fruticosus* L. aggregate (Morin & Evans, 2012) and is one of the 32 Weeds of National Significance in Australia (Thorp & Lynch, 2000; www.weeds.org.au), 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. It 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, and was probably introduced into WA by the early settlers from this country. Herbicides and cultural control methods are ineffective, or require multiple applications; furthermore, the weed is often located within inaccessible areas limiting control options. Since 2006 a disease recorded as ‘blackberry decline’ has been observed in some blackberry sites in WA (Aghighi *et al.*, 2012a) where it has provided spectacular level of control (see photos in Aghighi *et al.* 2014). A rust fungus, *Phragmidium violaceum*, had been released in WA, become widespread, but

was not proving effective as a biological control agent (Aghighi *et al.*, 2014). Preliminary surveys had indicated that foliar pathogens, other than *P. violaceum*, were not present, and initial isolations from diseased roots in blackberry decline areas had indicated the presence of more than one *Phytophthora* species (Aghighi *et al.*, 2014).

Blackberries are one of Australia's most important weeds making any potential control options worth investigation. Between 2010 and 2012 more detailed surveys were conducted in the Manjimup-Pemberton region in the Warren and Donnelly river catchments in WA to determine if root-associated pathogens were involved in the blackberry decline syndrome. Several *Phytophthora* species were isolated. Here we investigate the pathogenicity of these *Phytophthora* species to *R. anglocandicans* in field and glasshouse trials, to determine their role in the decline syndrome. If pathogenic, they have the potential to be applied as biological control agents to combat this notorious weed. We also applied phosphite to provide further evidence of a *Phytophthora* species being involved in blackberry decline. Phosphite is widely used to control oomycete pathogens in horticultural crops and in natural ecosystems Hardy *et al.* (2001). For example, Scott *et al.* (2013) 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.

## **Materials and methods**

### **Field survey and sampling**

Sites around the Warren and Donnelly rivers where blackberry decline has been reported on *R. anglocandicans* (Aghighi *et al.*, 2012a) were visited over different seasons between 2010 and 2012 (Fig. 1). Rhizosphere soil and root samples from dying and healthy plants were collected. Plants were dug out and as no obvious disease symptoms which might indicate a

foliar pathogen were detected on foliage and canes, these were excluded. Roots with crowns were placed into plastic bags, kept in an insulated box to protect samples from high temperatures and direct sunlight, and carried to the laboratory for further examination and isolation.

### **Isolation of *Phytophthora* species**

Both soil and diseased root samples were baited following the modified method of Rea *et al.* (2010). Briefly, samples were placed in 1L plastic take away containers (11.5 mm width x 16.5 mm length x 7.5 mm depth, GENFAC<sup>®</sup> plastics Pty Ltd., Melbourne, Australia), covered with distilled water (1:2 v/v soil/water) and baited by floating youngest fully expanded leaves (including *Alnus* sp., *Grevillea* sp., *Pittosporum undulatum*, *Prunus persica*, *Quercus suber*, *Q. ilex*, *R. anglocandicans*) and petals (*Hibbertia scandens* and *Rosa officinalis*) over the surface of water. After 2-7 days, leaves and petals with brownish lesions were blotted dry, and the lesions cut into ~1-2 mm sections and plated onto *Phytophthora* selective media. These included modified recipes of NARPH (Hüberli *et al.*, 2000) and PARPHN (Jung *et al.*, 2000) from which pentachloro-nitrobenzene was excluded. The modified PARPHN was made by using Corn Meal Agar (CMA) [990 mL/L distilled water, 17 g CMA, bacteriological Agar or Grade A Agar 16 g (Becton, Dickinson and Company, Sparks, USA), 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), and 0.025 g hymexazol. Antibiotics and hymexazol were amended to the medium before pouring plates]. 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 and NARPH or surface sterilised with 70% ethanol for 20-45 sec (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  $20 \pm 1^\circ\text{C}$  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 (Campbell's V8 vegetable juice; Campbell Grocery Products Ltd., Norfolk, UK), 900 mL/L distilled water, 0.1 g/L  $\text{CaCO}_3$ , 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 10 mL McCartney bottles filled with  $2/3^{\text{rd}}$  sterile distilled water and deposited in the Murdoch University Culture Collection.

### **Fishing for *Phytophthora* species**

In order to bait for *Phytophthora* from streams, 'fishing' was conducted in 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. Fishing for *Phytophthora* was conducted as described by Hüberli *et al.* (2013) by 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 riverbank. Bags were placed in six locations along the Warren and Donnelly Rivers or tributaries (2 bags per location). Buoyant polyurethane material was sown along one side of the bait bags to ensure they floated just below the surface of the water. Baits were collected after six days and lesions were plated onto the two *Phytophthora* selective media as described above. Potential isolates were maintained under long term storage as mentioned previously.

## **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 (Cooke *et al.*, 2000) and ITS-4 (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).

## ***In vitro* excised primocane under-bark inoculation**

*Rubus anglocandicans* primocanes (~15 mm in diameter) were selected from a non blackberry-decline site. 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 to prevent desiccation. At about the mid-point of each cane a flap approximately 8 mm long and 5 mm wide through the outer bark was made with a sterile scalpel 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 *Phytophthora* isolates (Table 1) was inserted under the bark flap (mycelial surface face down) and the wound sealed with Parafilm® to prevent desiccation of the mycelium. For controls, non-inoculated agar plugs were used. There were ten replicate primocanes per isolate and primocanes were kept in the zip-lock bags, with one replicate per isolate per zip-lock bag, with a moist 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.

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

In October 2012, a field based pathogenicity trial was conducted at site H6 (Fig. 1) using nine isolates from six *Phytophthora* species on primocanes of naturally growing blackberries. Phosphite was included to determine if (1) it 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 are listed in Table 1 and included *P. amnicola* (SA326 and SA388), *P. bilorbang* (SA092 and CBS161653), *P. cryptogea* (SA014 and SA261), *P. inundata* (SA285), *P. multivora* (SA134) and *P. taxon personii* (SA278).

Primocanes were inoculated using the *in planta* under-bark inoculation method described above, except that silver duct tape (Packmate<sup>®</sup>, China) was wrapped around the inoculation point to protect it from direct sunlight and prevent desiccation. For each of the phosphite or non-phosphite treatments, there were nine replicate primocanes for each of the nine isolates and the non-inoculated control treatment. The control consisted of a non-colonised half-strength PDA plug. 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 had 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% 600 g/L potassium phosphite (Agri-Fos<sup>®</sup> Agrichem Manufacturing Industries Pty Ltd., Loganholme, Queensland, Australia) plus 0.25% Pulse<sup>®</sup> (Nufarm Ltd., Australia) as a wetting agent.



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 lesion lengths. Colonization by *Phytophthora* species and Koch's postulates were examined through plating 1-cm stem sections from the edge of the visible lesion and for 5 cm beyond the lesion onto PARPHN agar. The lesion lengths for the different isolates of *Phytophthora* in the phosphite treated and not-treated plants were assessed using two-way ANOVA. The independent variables were *Phytophthora* isolates plus control (10 levels) and treatment [sprayed (0.5%) or not sprayed with phosphite]. The dependent variable was lesion length and the grouping was produced using Tukey's HSD test for unequal samples sizes (see the statistical approaches at the end of this section for more details).

#### **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. In the first glasshouse trial we investigated the pathogenicity of three isolates of *P. bilorbang* (SA092, SA142 and CBS161653), three isolates of *P. cryptogea* (SA014, SA167 and SA261), one isolate of *P. litoralis* (SA072), and as a positive control one isolate of *P. cinnamomi* (MU 94-48) in a soil-infestation pot trial.

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

Two different types of inocula were used. The first used tree lucerne (*Chamaecytisus palmensis*) plugs as the inoculum source. These were prepared as described by Rea *et al.* (2010). The second inoculum source contained vermiculite (1 L), millet (*Panicum miliaceum*)

seeds (10 g) and 600 mL V8 broth (120 mL V8 juice, 480 mL distilled water and 2 g CaCO<sub>3</sub>, pH adjusted to 7) which were sterilised in 500 mL flasks (1 L of inoculum divided by 4 flasks and each flask was filled with 250 mL) by autoclaving twice 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 substrates.

### **Glasshouse trial 1**

Blackberry 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.

This trial used both inocula types. The vermiculite inoculum was mixed with pasteurized and washed river sand in a ratio of 40 g/L sand and healthy blackberries were transferred from the 130 mm free-draining polyurethane pots into 170 mm free-draining polyurethane pots filled with 1 L of the infested sand. Each pot also received four colonised or non-colonised (for control treatments) 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 container capacity daily, and fertiliser (water soluble, Thrive<sup>®</sup>, Yates Company, Australia) was applied twice in the second and third weeks after inoculation. The pots were flooded in 9

L buckets twice at 2 and 4 weeks after inoculation for 15-17 h to stimulate the production of sporangia and zoospores as would occur in riparian zones.

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). WinRHIZO scans the roots and measures their length and diameter as described below.

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 and sub-samples from each pot were aggregated. The washed roots were placed in 1 L plastic containers (11.5 mm width x 16.5 mm length x 7.5 mm depth, GENFAC<sup>®</sup> plastics Pty Ltd., Melbourne, Australia) and covered with deionised water and sealed with a lid and scanned as they were harvested. In order to scan, each root sample was dispersed in deionised water in a transparent tray - a component of the WinRHIZO positioning system (30 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. Root samples were also plated on PARPHN and baited with youngest fully expanded leaves of *Q. ilex* to fulfil Koch's postulates.

The hypothesis that total root length varied between different *Phytophthora* isolates was tested in one-way ANOVA. Under-bark inoculations in the field found strong responses to *P. bilorbang* isolates and *P. cryptogea* isolates, with and without phosphite applications. Therefore, a priori planned comparisons were then used to compare: (i) all *P. bilorbang*

isolates together against the other *Phytophthora* species, and (ii) all *P. bilorbang* isolates plus all *P. cryptogea* isolates against other *Phytophthora* species.

### **Glasshouse trial 2**

Blackberry daughter plants (runners) 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 air-conditioned cooled glasshouse (12°C min-25°C max) for five months prior to infestation. Vermiculite inoculum was prepared as described previously.

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

At the time of inoculation, the inoculation tubes were removed and 40 g of vermiculite inoculum was placed in the holes (20 g/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). Plants were watered daily with deionised water to container capacity, and fertiliser (water soluble, Thrive<sup>®</sup>, Yates Company, Australia) was applied fortnightly at half the manufacturer's recommended rate. Pots were flooded in 9 L buckets five times at weeks 2, 4, 15, 19 and 21 post-inoculation for 48-72 hrs to stimulate the production of sporangia and zoospores, and to

mimic temporary inundation as occurs in riparian zones. 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.

After six and a half months post inoculation, the shoots were excised at the soil line and discarded, whilst the roots were harvested in a completely blind design. 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 1 L plastic containers and covered with deionised water and sealed with a lid. Root volume was 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 ( $\geq 70\%$ ); 2 = moderate damage with less necrotic roots ( $\leq 50\%$ ) and more normal feeder roots; 1 = slight damage with minimum damage and little evidence of necrosis along the roots with lots of normal feeder roots ( $\leq 20\%$ ); 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.

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 recorded.

Differences in dry weight of roots and volume of roots (dependent variables) between different isolates (independent variable) were assessed using multivariate analysis of variance (MANOVA). Based on results of *in planta* experiments in the field, *P. bilorbang* and *P. cryptogea* were suspected of being highly pathogenic isolates. Therefore, planned comparisons were used to compare root dry weight and root volume specifically between these combinations of isolates: (i) *P. bilorbang* and *P. cryptogea* were combined and compared against the flooded controls, and (ii) all *Phytophthora* species combined were compared against the flooded controls.

### **Statistical approaches**

The different statistical analyses described in the sections below were all carried out using STATISTICA (STAT Soft Inc., version 7.1, 1984-2006, Tulsa, Oklahoma, USA). The common approach to parametric analyses was to test for underlying assumptions such as normality of residuals or homoscedascity. If transformations were needed, they are reported in the results. In ANOVA, if main effects or interactions were significant post hoc Tukey's tests for equal sample sizes were used to identify significantly different levels. In some cases, a priori contrasts were specified to test hypotheses regarding particular groupings of levels within an effect. When contrasts were specified, the Bonferroni-Holm method of sequential correction was used to adjust for the multiple tests (Milliken & Johnson, 2009). Where there were multiple dependent variables to be tested, MANOVA was used with Wilks' lambda as the test statistic. The F approximation of Wilks' lambda is presented in the results.

## Results

### Isolation and identification

The 162 isolates found during the surveys were grouped based on colony morphology and identified using classical and molecular methods described by Aghighi *et al.* (2012b) as *Phytophthora amnicola*, *P. bilorbang*, *P. cryptogea*, *P. inundata*, *P. litoralis*, *P. multivora*, *P. taxon personii*, *P. thermophila*, *P. thermophila-amnicola* hybrid from decline and adjacent decline-free sites and *P. amnicola* and *P. cinnamomi* from non-decline sites (Fig. 1).

Incidence of *Phytophthora* species recovery and seasonal activity showed that *P. bilorbang* and *P. cryptogea* were mostly isolated consistently from blackberry rhizosphere soil and from decline and declining sites (Table 2). Baiting of soil with youngest fully expanded leaves of oak (*Q. ilex* and *Q. suber*) was the most successful method for isolation of the majority of *Phytophthora* species.

### *In vitro* pathogenicity screening of recovered *Phytophthora* isolates

The preliminary pathogenicity trial using excised stems showed that all *Phytophthora* species were able to cause moderate to extensive lesions in primocanes whereas the control inoculations produced no lesions. The length of lesions ranged from 7 to 25 mm with *P. cryptogea* and *P. bilorbang* producing the longest lesions, respectively.

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

All *Phytophthora* species were recovered from plated visible lesions (Fig. 2) fulfilling Koch's postulates and were not recovered from non-symptomatic sections beyond the lesions. No necrotic lesions developed on controls (Fig. 2 and Fig. 3) and no *Phytophthora* was isolated. A few plants died and were excluded from data analysis including two plants or replicates

inoculated with *P. amnicola* (isolate SA326) without phosphite spray and one plant of each inoculated with *P. taxon personii* (isolate SA278), *P. bilorbang* (isolate SA92), and three replicates of *P. cryptogea* (isolate SA014), sprayed with phosphite. Therefore, the grouping was produced using Tukey's HSD test for unequal samples sizes as mentioned above. Plants sprayed with phosphite had significantly ( $F_{1,137} = 21.6, p < 0.01$ ) shorter lesions than those that were not sprayed. Isolates also varied significantly ( $F_{8,137} = 23.3, p < 0.01$ ) 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. 2d,e and Fig.3), while the longest lesions in plants sprayed with phosphite were produced by *P. cryptogea* (SA261 and SA014). *Phytophthora cryptogea* (SA261) was unusual as the lesions it produced were longer in sprayed plants than those in unsprayed plants, but this interaction was not significant ( $F_{8,137} = 1.8, p = 0.07$ ) (Fig. 3). Based on the result of this experiment, *P. cryptogea* and *P. bilorbang* isolates were more pathogenic than other tested species.

### **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 had died at the time of harvest; however, *Phytophthora* treated plants were less vigorous (visibly with less above ground biomass) compared with the flooded controls. All *Phytophthora* isolates were re-isolated by direct plating and baiting washed roots from *Phytophthora* infested pots after harvest. *Phytophthora* species were not recovered from harvested and washed root samples of control pots. *Phytophthora bilorbang* and *P. cryptogea* were re-isolated from plated and baited roots, fulfilling Koch's postulates. Both of these species caused extensive necrotic lesions along all parts of the roots compared to the flooded control plants and other tested *Phytophthora*



species e.g. *P. litoralis*. Lesions in roots from plants grown in the presence of *P. cryptogea* were chocolate brown to brown in colour while for *P. bilorbang*, lesions appeared darker in colour. *Phytophthora bilorbang* and *P. cryptogea* hyphae both appeared and grew faster from roots on selective agar plates than other species. *Phytophthora bilorbang* was isolated from necrotic fine roots up to 1 mm in diameter, but not from thick roots.

Planned comparisons, incorporating the Bonferroni-Holm sequential correction for multiple tests, revealed a significant ( $F_{1,45} = 4.6$ ,  $p = 0.04$ ) difference in total root length as a dependent variable when *P. bilorbang* isolates were grouped and compared against the other *Phytophthora* species (Fig. 4 and 5a). The same result was obtained when *P. bilorbang* together with *P. cryptogea* isolates were compared against other *Phytophthora* species ( $F_{1,45} = 7.1$ ,  $p = 0.01$ ). Number of root tips was decreased more in plants treated by *P. bilorbang* (SA142 and SA092) and *P. cryptogea* (SA261) compared to other treatments (Fig. 5b).

### **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. Considering root dry weight and volume together, the contrast of *P. bilorbang* and *P. cryptogea* against the flooded control treatments using MANOVA was significant ( $F_{(2, 107)} = 5.54$ ,  $p = 0.005$ ). A significant ( $F_{(2, 107)} = 4.07$ ,  $p = 0.01$ ) difference was also obtained when comparing all *Phytophthora* species against the flooded control.

Based on the visual assessment, *P. cryptogea* (SA014), *P. inundata* (SA285) and all *P. bilorbang* isolates caused the most damage to the blackberry roots; however, *P. bilorbang* (SA142 and SA092) and *P. cryptogea* (SA014) had highest impact on root volume (Fig. 6a)

and dry weight (Fig. 6b). After excluding the controls, root dry weight and lesion length were not significantly ( $F_{(1,8)} = 0.89$ ,  $p = 0.37$ ) related in linear regression. Dry weight and damage rating of the roots (including the flooded controls) were significantly ( $F_{(1,10)} = 7.27$ ,  $p = 0.02$ ,  $R^2 = 0.42$ ) related, with damage higher for plants with lower dry weight.

## Discussion

Nine *Phytophthora* species were isolated and identified as species already known from Australia, although *P. bilorbang* has recently been described from blackberry and is associated with blackberry decline (Aghighi *et al.* 2012b). The other species were also associated with blackberry decline sites in this study. Many of these species have been associated with diseased plants in Australia or worldwide (Table 3). Of these, *P. amnicola*, *P. bilorbang*, *P. cryptogea*, and *P. multivora* were recovered more frequently than other *Phytophthora* species. *Phytophthora cinnamomi* was the only *Phytophthora* species isolated from two blackberry non-decline sites (in association with dying *Banksia littoralis*) and never from decline sites.

Interestingly, the majority (seven of the nine) of the species belong to Clade 6 (Cooke *et al.*, 2000; Blair *et al.*, 2008). 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 3). Members of 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. littoralis* and *P. thermophila* appear to be opportunistic pathogens under favourable episodic conditions such as flooding (Jung *et al.*, 2011). *Phytophthora 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 (Brasier *et al.*, 2003). Furthermore, a few species in this Clade are hybrids and involve Clade 6 parents, as for example the *P. thermophila-annicola* hybrid observed in the current study. 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).

*Phytophthora bilorbang* as a member of Clade 6 is homothallic and readily produces thick-walled oospores on agar media (Aghighi *et al.*, 2012b). *Phytophthora bilorbang* was never isolated from roots in naturally infected blackberries suggesting that *P. bilorbang* is a weak competitor, thus difficult to isolate from plant tissues if other *Phytophthora* or fungal species are present and more sampling and baiting will likely increase the recovery rate of this species. The host range of *P. bilorbang* is unknown whereas *P. cryptogea* has a worldwide distribution and wide host range (Erwin & Ribeiro, 1996) and has been reported on raspberry in Australia (Washington, 1988).

Based on the literature, the other species isolated in the present study, including *P. multivora* have multiple hosts (Table 3). However, *P. multivora* was recovered from blackberry decline sites, but only from bulk soil. Thus there is no direct association of this species with blackberry decline. 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, an increasing number of hosts have been reported for *P. multivora* since its original description (Scott *et al.*, 2009). 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 restoration of blackberry decline sites. Those species shown to be susceptible then can be withdrawn from such restoration programs, which will save costs of seedling production, planting out and subsequent maintenance.

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 blackberry, as there was no evidence of disease symptoms in native flora growing in association with blackberry.

*Phytophthora bilorbang* and *P. cryptogea* were more pathogenic than the other tested *Phytophthora* species across the under-bark inoculation and glasshouse pot trials. In primocane under-bark inoculation of excised stems and intact field plants, lesions caused by *P. cryptogea* and *P. bilorbang* isolates were larger than those caused by the other species. Application of phosphite in the field after primocane under-bark inoculation reduced the size of lesions caused by *Phytophthora* species. This provides strong evidence for the involvement of *Phytophthora* species in the blackberry decline syndrome, since phosphite is well known to reduce the severity and impact of *Phytophthora* species in a range of plant hosts (Hardy *et al.*, 2001; Wilkinson *et al.*, 2001; Scott *et al.*, 2013). 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 both glasshouse pot trials, the pathogenicity between different isolates of the same species was variable. In fact, under controlled conditions in the glasshouse, all *Phytophthora* species were able to cause damage to the blackberry roots under flooding conditions, although unlike *P. bilorbang* and *P. cryptogea* their pathogenicity was inconsistent between under-bark inoculations and pot trials. For instance, *P. inundata* caused small lesions in under-bark inoculations but high root damage was observed.

Plant vigour was compromised particularly for *P. bilorbang* and *P. cryptogea*. However, it is likely that if the duration of the flooding events 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 the glasshouse because of regular watering.

It seems that duration of inundation is very important in the blackberry decline scenario as blackberries on the riverbanks in the natural ecosystems in the south-west of WA are frequently flooded for a few weeks in duration (Fontanini, personal observation). Based on the results of glasshouse experiments, all *Phytophthora* species were able to cause damage to the blackberry roots under regular flooding events of 48-72 h duration; 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 investigated different waterlogging regimes on several *Phytophthora* species involved in red raspberry root rot and found that duration of waterlogging increased the pathogenicity to red raspberry of normally non-pathogenic *Phytophthora* species.

It is noteworthy that there is little genetic variation in blackberry due to reproduction by apomixis (Evans *et al.*, 2011), and therefore it has 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.

According to the findings of the present study, *Phytophthora* species, and in particular, *P. bilorbang* and *P. cryptogea* are pathogenic to blackberry when the plants are subjected to intermittent waterlogging. The duration of experiments should be extended as the decline phenomenon in blackberry appears to have a complex etiology and all components interact with each other to cause the decline syndrome. The role of predisposition and stress factors should not be ignored specifically for plant diseases with complex etiology in nature (Schoeneweiss, 1975; Manion, 1981; Aghighi *et al.*, 2014). Consequently, a six-month trial under glasshouse conditions is unlikely to reflect 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 projects in blackberry decline sites, and 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.

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**Figure 1** Location of sampling sites, 'Fishing for *Phytophthora*', and *Phytophthora* species composition for each site during blackberry decline surveys\*. **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. 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, F2, F3:** Fishing in Warren River tributaries; **F4, F5, F6:** Fishing in Donnelly River tributaries. \* April, June, September and November (2010): C and D sites; August and September (2011): C, D, H and F sites; May (2012): C, D, H6 and H8; five samples per site (excluding F sites) was collected each month.

**Figure 2** *Rubus anglocandicans* (blackberry) primocanes 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). Necrotic lesions are visible in all cases except for (a) and (f), control and *P. inundata* treatments, respectively.

**Figure 3** Mean ( $\pm$  SE) of lesion lengths (mm) in *Rubus anglocandicans* (blackberry) primocanes (stems) in the field after under-bark inoculation with *Phytophthora* species ( $n = 9$  isolates); without (bars with pattern) and with (grey bars) foliar application of phosphite. amn (SA326, SA388): *P. amnicola*; bil (SA092, CBS161653): *P. bilorbang*; cry (SA014, SA261): *P. cryptogea*; inu (SA285): *P. inundata*; mul (SA134): *P. multivora*; per (SA278): *P. taxon personii*.

**Figure 4** Root length of *Rubus anglocandicans* (blackberry) within four root classes after inoculation with *Phytophthora* species. Root classes are based on the root diameter after harvesting pot trial 1 and analysing scanned root images with WinRHIZO software. Error bars represent standard errors of the means. bil (SA092, SA142, CBS161653): *P. bilorbang*; cin

(MU9448): *P. cinnamomi*; cry (SA014, SA167, SA261): *P. cryptogea*; lit (SA072): *P. litoralis*.

**Figure 5** Total root length of *Rubus anglocandicans* (blackberry) with length of discoloured or damaged (top bars with grey colour) and healthy roots (in bottom bars with pattern) (a), and number of root tips (b), after harvesting *Phytophthora* pot trial 1 and analysing scanned root images with WinRHIZO software (error bars represent standard errors of the means). bil (SA092, SA142, CBS161653): *P. bilorbang*; cin (MU9448): *P. cinnamomi*; cry (SA014, SA167, SA261): *P. cryptogea*; lit (SA072): *P. litoralis*.

**Figure 6** Mean ( $\pm$  SE) of root volume (a) and dry weight (b) of *Rubus anglocandicans* (blackberry) in sand-infestation pot trial 2. amn (SA326, SA388): *P. amnicola*; bil (SA092, SA142, CBS161653): *P. bilorbang*; cry (SA014, SA261): *P. cryptogea*; inu (SA285): *P. inundata*; mul (SA134): *P. multivora*; per (SA278): *P. taxon personii*; the (SA399): *P. thermophila*.

**Table 1** Tested *Phytophthora* isolates in *Rubus anglocandicans* (blackberry) excised primocane under-bark inoculation.

**Table 2** Seasonal activity, isolation method and source of *Phytophthora* species recovered from *Rubus anglocandicans* (blackberry) decline sites in the Warren and Donnelly river catchments between April 2010 to May 2012 and in February 2013.

**Table 3** *Phytophthora* species recovered from *Rubus anglocandicans* (blackberry) decline sites, their known hosts and source of isolation.

Putative <i>Phytophthora</i> /isolate code	Source	Method of isolation
<i>Phytophthora amnicola</i> (SA290)	Root	Baiting ( <i>Quercussuber</i> )
<i>P. amnicola</i> (SA388)*	Water	Fishing
<i>P. amnicola</i> (SA326)*	Root	Baiting ( <i>Q. suber</i> )
<i>P. bilorbang</i> (SA092)*	Soil	Baiting ( <i>Quercus ilex</i> )
<i>P. bilorbang</i> (SA142)*	Soil	Baiting ( <i>Rubusanglocandicans</i> )
<i>P. bilorbang</i> (SA144)	Soil	Baiting ( <i>R. anglocandicans</i> )
<i>P. bilorbang</i> (SA146)	Soil	Baiting ( <i>R. anglocandicans</i> )
<i>P. bilorbang</i> [SA262 (CBS161653)]*	Soil	Baiting ( <i>Alnus</i> sp.)
<i>P. cinnamomi</i> (MU94-48)*	Collar	<i>Eucalyptus marginata</i>
<i>P. cryptogea</i> (SA014)*	Soil	Baiting ( <i>R. anglocandicans</i> )
<i>P. cryptogea</i> (SA167)*	Root	Direct plating
<i>P. cryptogea</i> (SA261)*	Soil	Baiting ( <i>Alnus</i> sp.)
<i>P. inundata</i> (SA279)	Root	Direct plating
<i>P. inundata</i> (SA285)*	Root	Direct plating
<i>P. litoralis</i> (SA072)*	Root	Direct plating
<i>P. multivora</i> (SA134)*	Soil	Baiting ( <i>Rubuslaudatus</i> , American blackberry)
<i>P. multivora</i> (SA136)	Soil	Baiting ( <i>R. sp.</i> , American blackberry)
<i>P. multivora</i> (SA150)	Soil	Baiting ( <i>R. anglocandicans</i> )
<i>P. multivora</i> (SA151)	Soil	Baiting ( <i>R. anglocandicans</i> )
<i>P. multivora</i> (SA153)	Soil	Baiting ( <i>R. anglocandicans</i> )
<i>P. taxon personii</i> (SA278)*	Root	Baiting ( <i>Grevillea</i> sp.)
<i>P. thermophila</i> (SA399)	Water	Fishing

\* Isolates were examined for pathogenicity tests in the glasshouse pot trial 1 and trial 2.

<i>Phytophthora</i> species	% of each species recovered across seasons				Isolation Method (%)			Source		
	Spring*	Summer	Autumn	Winter	Direct plating	Baiting	Fishing	Root	Soil	Water
<i>P. amnicola</i>	22			78	2	59	39	√	√	√
<i>P. bilorbang</i>	4		23	73	0	100	0		√	
<i>P. cryptogea</i>	31		2	67	7	73	0	√	√	
<i>P. inundata</i>				100	100	0	0	√		
<i>P. litoralis</i>			25	75	50	50	0	√	√	
<i>P. multivora</i>		4	48	48	0	100	0		√	
<i>P. taxonpersonii</i>				100	0	100	0		√	
<i>P. thermophila</i>	80	20			0	0	100			√
<i>P. thermophila-amnicolahybrid</i>	50		50		0	50	50		√	√

\*Representing seasons in the Southern Hemisphere (spring: September, October, November; summer: December, January, February; autumn: March, April, May; winter: June, July, August).

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

\* Data not available.









