Tree host–pathogen interactions as influenced by drought timing: linking physiological performance, biochemical defence and disease severity

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There is increasing concern about tree mortality around the world due to climatic extremes and associated shifts in pest and pathogen dynamics. Yet, empirical studies addressing the interactive effect of biotic and abiotic stress on plants are very rare. Therefore, in this study, we examined the interaction between drought stress and a canker pathogen, *Quambalaria coyrecup*, on the eucalypt – *Corymbia calophylla* (marri), which is experiencing increasing drought stress. We hypothesized that drought stress would increase marri’s susceptibility to canker disease, and cankers would have the largest negative effect on plants that are already drought stressed before pathogen inoculation. To test the hypotheses, in a glasshouse, marri saplings were exposed to drought either before or after pathogen inoculation, or were well-watered or droughted throughout the experiment either with or without inoculation. Canker development was greater in well-watered saplings than in droughted saplings, with the fastest development occurring in well-watered saplings that had experienced drought stress before inoculation. Irrespective of water treatments, marri saplings employed phenol-based localized biochemical defence against the pathogen. Drought reduced photosynthesis and growth, however, a negative effect of canker disease on saplings’ physiological performance was only observed in well-watered saplings. In well-watered saplings, canker-induced loss of sapwood function contributed to reduced whole-plant hydraulic conductance, photosynthesis and growth. The results provide evidence that timing of drought stress influences host physiology, and host condition influences canker disease susceptibility through differences in induced biochemical defence mechanisms. The observations highlight the importance of explicitly incorporating abiotic and biotic stress, as well as their interactions, in future studies of tree mortality in drought-prone regions worldwide.

**Keywords**: biochemical defence, canker disease, drought stress, eucalypt, hydraulic conductivity, tree mortality.

Introduction

Climate change induced tree mortality has been increasingly reported over recent decades in many parts of the world, including Australia (Allen et al. 2010, Carnicer et al. 2011, Matusick et al. 2013). Although most studies have implied heat and/or drought as primary causal factors of tree mortality, tree mortality is a complex process that has been attributed to multiple interacting abiotic and biotic factors, ranging from particular sequences of climate stress, and stressor-driven changes in the dynamics of plants, insects and pathogens, to stand life histories of disease infection (Franklin et al. 1987, Miao et al. 2009, Mitchell et al. 2014). Sturrock et al. (2011) proposed that trees affected by predisposing factors (i.e., long-term changes in soil moisture, precipitation or stand characteristics) or inciting factors (i.e., short-term factors – drought/heat events or defoliating insects, etc.) generally can recover, depending on the duration and intensity of the stress, unless they are also affected by contributing factors such as pests and pathogens. This would imply
that even drought-tolerant plants may be unable to resist drought-induced stress when pest and pathogen attacks occur simultaneously or subsequent to drought events.

Despite the considerable effects of pathogens on tree survival, studies on the relationship between pathogens and tree physiology have been relatively rare (but see McElrone et al. 2003, Rohrs-Richey et al. 2011, Oliva et al. 2014, da Silva et al. 2018). Pathogen infection can indirectly impact the host physiology by down-regulating the genes involved in photosynthetic activities (Bilgin et al. 2010). The extent of a potential direct effect of the pathogen on plant functions may depend on the functional type of pathogen. Biotrophic pathogens, deriving energy from living cells, have very little direct impact on the water and C cycle of the host (Oliva et al. 2014). However, both vascular wilts and necrotrophic canker pathogens, deriving energy from dead cells, can directly affect plant physiological processes (Oliva et al. 2014). Firstly, these pathogens can colonize plant vascular tissues, thereby reducing stem hydraulic conductance and increasing the risk of cavitation (Crist and Schoeneweiss 1975, Madar et al. 1989, Yadeta and Thomma 2013), with the risk of embolism increasing with disease severity (Fukuda et al. 2007). Secondly, host plants often compartmentalize infected areas by forming lignified or suberized callus tissue enriched with phenolics, terpenes and other secondary metabolites. The defence responses can include healthy tissues around the infected area such as the cambial zone and phloem and vascular tissues (Oliva et al. 2014), further increasing cavitation risk and disrupting water and photosynthetic transfer through the stem. Thus, pathogen infection may accelerate drought-induced cavitation risk and tree mortality.

The drought predisposition hypothesis may apply to the decline of marri (Corymbia calophylla) (Schoeneweiss 1983), a keystone eucalypt species in Western Australia, which has increasingly been suffering from stem canker infestations by a pathogenic Quambalaria fungus (Q. coyrecup) (Paap et al. 2008, 2017). As the pathogen is thought to be native to Western Australia, it is likely to have co-evolved with marri. Therefore, the current disease epidemic is likely to be associated with other predisposing/inciting biotic and abiotic factors (Paap et al. 2008, 2017). Marri canker disease was first reported at the end of the 1940s, and more widespread infection and mortality were observed in the 1970s (MacNish 1963, Paap et al. 2008). Interestingly, a pronounced shift towards a drier and warmer climate has also been reported for this region since the 1970s (Bates et al. 2008). Thus, climate change related drought stress may be a principal predisposing factor in the development of the current disease epidemic, especially as drought stress is a well-known predisposing or inciting factor for a range of diseases including cankers in other species (Old et al. 1990, Maxwell et al. 1997).

The degree of water stress experienced by host plants has been suggested to be a crucial factor determining the success of infection and disease progression by most pathogens, although the required drought intensity level appears pathogen-specific (Schoeneweiss 1975, 1981). Under drought stress, cell metabolism is constrained, which may reduce plant defence capabilities against pests and pathogens by restricting the production and translocation of carbohydrates, resins, and other secondary metabolites (Bucci et al. 2016), which may increase disease susceptibility. For instance, increased disease susceptibility as a result of drought stress was reported in pine and poplar trees (Bagga and Smalley 1973, Blodgett and Stanosz 1997) and several Eucalyptus species (Old et al. 1990). The exact timing of stress has also been suggested to influence the outcome of plant–pathogen interactions. Post-infection stress is presumed to be a major factor in the progression of dieback disease (Phytophthora cinnamomi; an introduced water mould) of Eucalyptus marginata in SW Australia (Marks and Smith 1991). Pre-inoculation drought stress was reported to be more critical than post-inoculation stress in canker disease caused by Lasiodiplodia theobromae on Cornus florida (dogwood) in subtropical regions of the USA (Müllen et al. 1991). However, Jacquet et al. (2012) in a general review on the effects of drought stress on pest and pathogen infections, reported no consistent increase in disease incidence under drought. Drought stress can also reduce disease susceptibility in certain woody plant diseases by disrupting the life cycle of plant pathogens as most fungi, for example, require high humidity for spore discharge, germination, and infection (Bendz-Hellgren and Stenlid 1998, Swinfield et al. 2012). Thus, host and pathogen-specific responses to drought, and the particular timing and severity of drought in relation to pathogen infection, prevent any generalization to be drawn.

Although there has been considerable research on the effect of drought on plant disease (Rohrs-Richey et al. 2011, Fernández-Martinez et al. 2013, Barradas et al. 2018), very little is known about the influence of drought timing in relation to infection on plant biochemical defence traits and their impact on plant–pathogen interactions. Empirical studies support the idea that disease severity depends on host biochemical defence capacity (Li et al. 1969, Cahill and McComb 1992, Eyles and Mohammed 2003, Hayes et al. 2014), and drought stress is likely to alter plant biochemistry (McKernan et al. 2014, 2015), thus drought may increase disease susceptibility. To our knowledge, there are no studies that have investigated the effect of drought timing on plant defence responses and disease severity, and their impact on plant physiological performance in a single pathosystem. In this study, we have investigated the physiological and biochemical defence responses of the Q. coyrecup – marri interaction as dependent on the timing of drought. In the present work, we tested the following hypotheses: Hypothesis 1: Drought stress, pre- or post-inoculation, will weaken a plant’s biochemical defence capacity, and thus will increase its vulnerability to canker disease. Hypothesis 2: Canker disease will
significantly impair sapwood hydraulic conductance, resulting in reduced stomatal conductance, photosynthesis and growth, especially in plants already experiencing drought stress.

Materials and methods

Plant material and glasshouse conditions

_Corymbia calophylla_ seedlings were germinated and established in small pots (150 ml) from seeds collected from a native marri stand in Carey, Western Australia (34°35’191’’S 115°84’202’’E) in March 2014. After 8 months in a mesh shadehouse (60% of ambient light intensity), seedlings were transplanted to 1-litre free-draining pots filled with standard potting mix and sand (1:1.5). Several months before the start of experimental treatments, 72 healthy similar-sized saplings (average base diameter: 5.95 ± 0.07 mm, average height: 128 ± 15 cm) were transferred to PVC cylinders (450 mm [ht] * 150 mm [dia]) filled with 10.4 kg of soil mix (potting mix 1:1 sand). Slow-release Osmocote fertilizers (Osmocote® Plus Trace Elements: Native Gardens, Scotts Brand) were also added to the soil mix at the manufacturer’s recommended rate (15 g kg⁻¹ soil). The cylinders had small holes at the bottom to ensure adequate drainage, with a 1 mm nylon mesh covering the cylinder bottoms to prevent soil loss. The top of the cylinders was covered by a layer of gravel (0.7 kg) to reduce soil evaporation. Seedlings were moved to a greenhouse equipped with UV-transmissible glass (reducing visible light by ~10%). Until the experimental treatments started, saplings were watered to soil field capacity with excess water draining out through the holes at the bottom of the cylinders. On 15 September 2016, experimental watering treatments were started, with saplings watered to a set soil water content at least every second day by pot weighing. Glasshouse temperatures were moderated by automated temperature-dependent evaporative cooling and a shade screen on top of the glasshouse. During the experimental period, mean glasshouse air temperature was 23 °C, with a minimum 13 °C and maximum 35 °C, and seedlings were rotated within each bench every second day to minimize spatial effects.

Experimental design

The experiment was conducted between September 2015 and June 2016. At the start of the experimental treatments, average plant diameter at 5 cm above soil line was 7.87 ± 0.18 mm, and total stem length (main stem plus branches) was 151 ± 18 cm. Plants were randomly allocated to six different treatments (details below), and placed on four different benches, each serving as blocks as every treatment combination was equally represented on each bench. Treatments included a two-level factorial design with two levels of watering (droughted and well-watered) and two levels of inoculation (inoculated and non-inoculated). To evaluate the effects of drought timing more specifically, two additional inoculation treatments were included in which plants were droughted either before or after the inoculation but were otherwise well-watered (Figure 1). Plants were inoculated 40 days after onset of the watering treatments, when the droughted plants had displayed reduced transpiration, photosynthesis and growth for several weeks. For the pre- and post-inoculation drought treatments, plants’ watering regimes were switched 20 days after inoculation, when canker disease symptoms were visible. In total, the experiment lasted 180 days, with different physiological variables (i.e., leaf water potential, gas exchange, canker size, plant growth) being measured in six campaigns (Figure 1), followed by a final harvest at 140 days post-inoculation (dpi). Each measurement campaign consisted of 4 days, and all plants within a block were measured on the same day. For all treatment combinations, 9–12 replicate plants were used.

Fungal isolates and inoculation

_Quambalaria coyrecup_ isolate was used to produce inoculum which was collected from a cankered _C. calophylla_ in a native marri population from Woottatting (31°87’730’’S 116°42’580’’E). The inoculum was produced from the original isolate and maintained on potato dextrose agar (½ PDA; Becton, Dickinson, and Company, Sparks, MD, USA) at room temperature (20 °C). As our priority was to obtain maximum infection rates, we only used inoculum plugs bearing mycelium to apply to artificial wounds, as this method was shown to be more successful in infecting marri seedlings than applying a spore suspension to the wound (Yulia et al. 2014). Stems were inoculated at 10 cm height from the ground by applying 12 days old inoculum plugs to the wound. The inoculation area was wiped with 80% ethanol, and a longitudinal 12 mm double slit wound (1 mm apart from each other) was made to cambium depth with a razor blade. Agar plugs (12 mm x 3 mm) of actively growing inoculum were cut and placed on the double slit wound area, with mycelia facing the wound, and secured with sterile moist cotton wrapped with Parafilm (Pechiney Plastic Packaging, Menasha, WI, USA) to prevent cross-contamination. The Parafilm was

![Figure 1. Schematic illustration of the experimental design.](https://academic.oup.com/treephys/article/39/1/6/5123809)
removed after 2 weeks. Procedural control plants (i.e., WC and DC in Figure 1) were wounded similarly but covered with sterile agar plugs.

**Watering treatments**

Well-watered seedlings were maintained at 12% gravimetric soil water content (SWC) throughout the experiment by daily rewatering PVC cylinders with the amount of water lost during the previous measurement interval as determined by pot weighing. The drought treatment was commenced by cessation of watering until the pot SWC reached 4% (confirmed to cause moderate drought stress, based on observations of plant water use and water status; data not shown), after which seedlings were maintained at that level by watering every second day throughout the experiment, or every third day in case of consecutive overcast days and low evaporative demand. Every pot was weighed between 8:00 and 10:00 h to determine water loss. From 80 dpi onwards, we observed visual symptoms of wilting in drought stressed saplings on the second/third day after watering. To prevent plant mortality, from 90 dpi onwards, the minimum SWC of droughted plants was increased from 4% to 5%.

**Canker size**

External disease progression was monitored by regularly measuring the length and width (at 3 mm intervals longitudinally) of the developing canker on the stem. An index of canker size was calculated by multiplying the canker length and the average of width. After harvest, canker-affected stems were cross-sectioned at inoculation point and examined to determine the inner radial colonization by the pathogen. Before sectioning, a toluidine blue solution (0.02%) was forced through the xylem vessels of the stem piece at a low pressure (100 kPa) for 5 min. The stained solution (0.02%) was forced through the xylem vessels of the stem piece at a low pressure (100 kPa) for 5 min. The stained stem was then sliced at the inoculation point and photographs were taken under a compound microscope equipped with a digital camera (Nikon SMZ800, Digital Sight DS-Fi2, Nikon Corporation, Japan). The photographs were analysed using ImageJ v 1.48 (National Institutes of Health, Bethesda, MD, USA) to quantify the total sapwood area and percentage of infected sapwood by the pathogen. All abnormal dark coloured and surrounding unstained tissues (Supplementary Figure S1) were considered as unhealthy-infected sapwood and stained conductive tissues as healthy sapwood in the calculation.

**Plant growth**

Stem diameter (at 5 cm height), plant height and total stem length (height plus all branch lengths) were measured just before inoculation and monthly thereafter until the final harvest. At the end of the experiment, aboveground biomass was determined by measuring dry weights of stems and leaves (after four days in a 70 °C oven). Leaf area of leaves from two branches of each plant was determined with a leaf area meter (Li-cor model LI-3000, LI-COR Inc., Lincoln, NE, USA), after which they were oven dried. These data were used to calculated leaf mass per area (LMA), and estimate whole plant leaf area from whole plant leaf dry weight, and leaf mass ratio (total leaf mass/total aboveground plant mass). At the time of inoculation, terminal leaves of each branch were marked and leaves that developed after inoculation were collected and measured separately to determine leaf area increase after inoculation.

**Plant water use and water status**

Whole plant transpiration was calculated from regular pot weighing, after subtracting the water loss from eight control pots without plants. Midday leaf water potential was measured with a pressure chamber (Model 600, PMS, Albany, OR, USA) at each measurement campaign (Figure 1). Prior to the final harvest, both predawn and midday leaf water potential were measured before sunrise between 4:00 and 5:00 and at midday between 12:30 and 13:30, respectively.

**Gas exchange**

Leaf gas exchange was measured between 8:00 and 10:30 in the days before inoculation, 20 dpi, 50 dpi, 80 dpi, 110 dpi and 140 dpi using a LI-COR 6400 (LI-COR Inc., Lincoln, NE, USA) to assess the effects of drought and inoculation. As droughted plants were watered on alternate days, gas exchange measurements were conducted on the second day after watering, to avoid possible effects of recent watering. A sun-exposed matured leaf was chosen from each replicate plant. For all measurements, we used a constant reference CO2 concentration (400 μmol CO2 mol⁻¹), and constant light intensity (1500 μmol m⁻² s⁻¹) in the cuvette. Cuvette leaf temperature was set based on ambient greenhouse temperature at each measurement campaign and a relative humidity of 50 ± 10% during the measurements. Flow rates were set at 500 μmol s⁻¹ for well-watered plants and 300 μmol s⁻¹ for droughted plants.

CO2 response curves (A–C curves) were measured on 12 randomly chosen well-watered plants (six well-watered control and six well-watered inoculated plants) to determine whether differences in photosynthetic capacity between control and inoculation treatments were associated with biochemical or stomatal limitation. These measurements were limited to well-watered treatments as no significant reduction of photosynthesis due to pathogen inoculation was observed in droughted plants. A–C curves were initiated after equilibrating the leaves for ~10 min at 400 μmol mol⁻¹ CO2, 1500 μmol m⁻² s⁻¹ PAR, and with leaf temperatures maintained close to ambient temperatures. The measurement was continued with a stepwise reduction in CO2 concentrations to sub-ambient (~40 μmol mol⁻¹) and then increased up to 1800 μmol mol⁻¹, in a total of 12 steps. Leaves were maintained at each CO2 level for 120–180 s before being measured. The analysis of the A–C curves followed the mechanistic model of CO2 assimilation using a spreadsheet developed by Sharkey (2015).
Whole plant conductance

Whole plant conductance was estimated for all plants in the glasshouse on a sunny day at ~130 dpi when the canker disease was fully developed. Well-watered plants were watered to their set SWC level on the afternoon before the measurement, whereas droughted plants were watered on the afternoon a day before measurement to ensure an appropriate level of drought stress. To prevent soil evaporation, pots were sealed with plastic at the base of the stem. Whole plant transpiration was determined by weighing (0.01 g accuracy) each pot half an hour before and after the midday water potential measurement. Transpiration was calculated from the water lost over this period divided by total leaf area measured at final harvest as described in the plant growth section. Midday water potential was determined on one light-exposed fully expanded leaf per plant using a pressure chamber (Model 600, PMS, Albany, OR, USA). Immediately after the reweighing, all pots were moved to a dark, humid room (~20 °C) and left for a minimum of 20 h to ensure equilibrium between plant and soil water potential. During the following morning, predawn water potential was measured on one leaf per plant. Whole plant conductance (mmol m⁻² s⁻¹ MPa⁻¹) was calculated by dividing whole plant transpiration by the difference between midday and predawn water potential.

Stem hydraulic conductivity

Before the final harvest, five plants for each treatment combination were randomly selected for stem hydraulic conductivity measurements and biochemical analyses. Before harvesting the main stem, all side branches were removed, after which the main stem was left for ~10 min to alleviate the existing stem tension and reduce the chance of further embolism when cutting the main stem just below the root collar. Then the main stem was uprooted, and transported to the laboratory in a sealed plastic bag with moist paper towels. A 25 cm stem section (~10 cm above and below the inoculation/wound point) was cut under water. Hydraulic conductivity was measured on this stem section using a flow meter (Liqui-Flow™, Bronkhorst High-tech B.V., Ruurlo, the Netherlands) as outlined by Sperry and Tyree (1988). Stem samples were re-cut under water to a length of 24 cm, and ~4 cm of bark and phloem were removed on both ends of the stem. The bark and phloem tissues were wrapped with Parafilm M (Bemis NA, Neenah, WI, USA) and the bottom end of the stem was attached to the flow meter. Native stem hydraulic conductivity ($K_s$) was measured using overhead pressure with the flow meter pressure head containing degassed 20 mM potassium chloride (KCl) solution. The height of the flow meter pressure head was adjusted for droughted (at 45 ± 2 cm height) and well-watered plants (30 ± 5 cm) to ensure maximum flow rates within the scale of the flow meter. The length and cross-sectional area of conductive sapwood (as determined using dye infusion, i.e., excluding diseased/damaged area) were also measured to calculate $K_s$. After the $K_s$ measurement, stem samples were flushed for 15 min at 100 kPa to remove embolisms; flushing time was tested (10, 15, 20 and 25 min) but only negligible changes were observed in conductance after 15 min. After flushing, maximum stem conductivity ($K_{s,max}$) was measured. The percentage loss of conductivity was calculated as: \(\text{PLC} = 100\times(1-K_s/K_{s,max})\).

Biochemical analyses

Five randomly chosen plants of each treatment were used for biochemical analysis. Several young fully expanded leaves from the top of the plant, as well as the stem section containing the canker were sampled and preserved (~80 °C) for subsequent biochemical analysis. Stem samples for analysis consisted of a hemi-cylindrical section, taken from the inoculation point upwards (2 cm length) on the side of the canker. Stem and leaf samples were ground with a Geno-grinder (SPEX SamplePrep 2010, AXT Pty Ltd, Australia).

Terpene analysis

Subsamples of fresh frozen leaf (100 mg) or stem (200 mg) were extracted in a mixture of 1 ml of pentane with 0.05 μl dodecane, and stored for at least 12 h at ~20 °C. The extracts were centrifuged for 3 min at 15,339g, after which 100 μl of the supernatant was transferred to a glass vial for subsequent analysis using gas chromatography (GC) coupled with mass spectrometry (MS) as described in Hayes et al. (2014) and Achotegui-Castells et al. (2015). For the analysis, 1 μl of extracted sample was injected into a GC (Agilent 7890 A GC) coupled to a mass spectrometer (Agilent 5975 C MSD) set up with a silica capillary column (Agilent, model VF-5 + 10 m EZ guard, 30 cm × 0.25 mm × 0.25 um film thickness). Samples were injected at an initial temperature of 40 °C which was maintained for 2 min, after which temperature was increased by 10 °C per min up to a maximum of 260 °C which was maintained for 6 min and total run time was 40 min. The carrier gas used was helium (1 ml min⁻¹) and the MS scan rate was 4.45 scans per second which held in the ion source at 280 °C. The solvent delay was 4.5 min, mass range 35–400 amu and electron impact of 70 eV.

Individual terpene compounds were identified by comparing individual peaks with respect to the National Institute of Standards and Technology (NIST) mass spectral library using AMDIS software (version 2.72) and individual peak area was quantified using the MassHunter Workstation Software for quantitative analysis (version B.07.01/Build 7.1524.0). The total quantity of terpenes was calculated from the summed area of all terpene peaks detected by gas chromatography–mass spectrometry relative to the internal standard (dodecane). Concentrations of individual terpene compounds and total terpene quantity were expressed as μg g⁻¹ DW of leaf or stem materials using the internal standard.
Total phenolic analysis  For total phenolic analysis, a subsample of the fresh frozen leaf (50 mg) or stem (50 mg) samples was extracted twice with 1.5 ml 50% acetone by vortexing (30 s) and centrifuging (3 min; 15,339 g). Extracts were pooled and stored for 24 h at 4 °C in the dark. Total phenols were estimated by the Folin-Ciocalteu method, adapted from Cork and Krockenberger (1991). An extract of 100 μl was diluted with 1900 μl de-ionized water, after which 125 μl of diluted Folin-Ciocalteu reagent (Sigma Chemical Co.) was added, and the sample was left for 3 min, after which 375 μl of 1 M aqueous Na₂CO₃ was added. The solution was shaken and left to react for 45 min. The absorbance of samples was measured at 725 nm with a spectrophotometer (Thermo electron corporation, USA). Concentrations of total phenols were calculated with reference to a gallic acid standard curve (10–200 μg ml⁻¹ dissolved in acetone) by applying regression analysis, and results were expressed as gallic acid equivalent (μg) per mg extracted dry weight (DW) of wood or leaf.

Data analysis
One-way analysis of variance (ANOVA) was used to assess the effects of water and inoculation treatment combinations on growth, total sapwood area, the percentage of infected sapwood, water potential, stem and whole plant conductance, total terpene and phenolic compounds. When significant effects were detected, a Tukey–Kramer test (P < 0.05) was used to identify the significant differences across the treatment combinations. Linear regression models were used to explore the relationships between the watering treatments and canker development, and between infected sapwood percentage and leaf gas exchange parameters (function ‘lm’). Prior to the analysis, data were log-transformed if necessary to correct for non-normality and heteroscedasticity. The software R (v3.4.1; R Foundation for Statistical Computing, Vienna, Austria) was used for statistical evaluation of the data.

Results
For ease of referral, saplings that were well-watered for the majority of the experiment, including the pre-inoculation droughted plants, are referred to as well-watered, whereas the continuously droughted saplings and the post-inoculation droughted saplings are referred to as droughted, unless otherwise indicated.

Plant growth
Prior to the onset of water treatments, saplings allocated to the well-watered and drought treatments were similar in size (total stem length of 467 ± 27 and 445 ± 20 cm, respectively; P = 0.64). However, at the time of inoculation, i.e., ~40 days after the onset of water treatments, total stem length of droughted saplings was 18% smaller than that of well-watered saplings (P = 0.04). After the inoculations (real or mock), droughted saplings showed greatly reduced growth compared to saplings in the well-watered treatments, largely independent of the inoculation treatment (see results for shoot biomass at the final harvest and leaf area or stem length increase in Figure 2a–c). Canker presence reduced growth significantly in the well-watered treatments but had no effect on sapling growth in the drought treatments. The growth of saplings in the pre- or post-inoculation drought treatments tended to be intermediate between growth of the continuously well-watered and droughted inoculated saplings. Similar trends between treatment combinations were observed for other size variables such as height and diameter (results not shown). There were no significant (P > 0.05) differences in LMA and leaf mass ratio amongst the treatment combinations (results not shown).

Figure 2. (a) Total aboveground dry biomass at the final harvest, (b) leaf area increase and (c) total stem length increase of C. calophylla saplings from inoculation time to the final harvest (140 days). Values are means ± SE. Different letters indicate significant (P < 0.05, Tukey–Kramer test) differences between the treatments: WC = well-watered control, WI = well-watered inoculated, DI-W = pre-inoculation drought, WI-D = post-inoculation drought, DI = drought inoculated, and DC = drought control.
Canker development and water treatment effects

Cankers developed in all inoculated plants irrespective of the watering regime. At an early stage (20 dpi), external canker size was higher in well-watered inoculated (WI) plants (50.6 ± 4.6 mm²) than in droughted inoculated (DI) plants (37.5 ± 2.9 mm²; P = 0.03), and this difference increased during the experimental period (Figure 3). At 140 dpi, average canker size was almost 3.5 times larger in the well-watered plants (WI) than in the droughted plants (DI; P = 0.003). The response of pathogen activity to water regime changes in the WI-D and DI-W treatments (20 dpi) was relatively slow. Initially, the canker growth rates remained close to the original watering regimes these treatments received. However, from 50 dpi onward, the new watering regimes started clearly affecting canker growth rates, with newly droughted saplings showing a marked decline in canker growth rate compared to continuously well-watered plants, and newly well-watered saplings showing a strong increase in canker growth rates, compared to continuously droughted saplings (Figure 3). The latter resulted in the pre-inoculation droughted saplings attaining the largest average canker sizes. The differences in canker growth rate resulted in a strong time by treatment interaction, with all treatments responding differently to each other (Table 1).

At the final harvest, well-watered saplings had a larger cross-sectional total sapwood area (128 ± 3.9 mm²) at the inoculation points than droughted saplings (78.8 ± 2.9 mm², P < 0.001, Figure 4a). As expected, affected areas in inoculated saplings were much larger than wounded areas in mock-inoculated control saplings (P < 0.001). Also, well-watered inoculated saplings on average had a higher infected sapwood area and a larger percentage of affected sapwood area (36.0 ± 1.7 mm²; 27 ± 1.5%) than droughted plants (11.9 ± 0.9 mm²; 14 ± 1%) (P < 0.001). Post-inoculation droughted saplings (WI-D) and pre-inoculation droughted saplings (DI-W) had affected sapwood areas that were similar in size to that of continuously droughted (DI) or well-watered (WI) saplings, respectively (P > 0.05; Figure 4b).

Plant water relations

Throughout the experiment, droughted saplings maintained a lower range of water potentials (predawn: −0.6 MPa to −1.0 MPa; midday: −1.7 MPa to −3.1 MPa) than the well-watered saplings (predawn: −0.3 MPa to −0.5 MPa; midday: −0.8 MPa to −1.8 MPa) (results not shown). At 140 dpi, cankers reduced the midday water potential (mean decrease of 15%) in the well-watered treatment (P = 0.05) but had no effect on the saplings in the drought treatment (results not shown). Post- and pre-inoculation droughted saplings did not differ significantly from continuously droughted inoculated saplings or continuously well-watered inoculated saplings, respectively, in any of the measured water relations variables. Drought significantly increased the leaf water potential gradient and reduced transpiration in all drought treatments (P < 0.001, Figure 5a). Droughted saplings also showed greatly reduced whole plant conductance (Kₚ; Figure 5b), and slightly reduced stem hydraulic conductivity (Kₛ; Figure 6a) compared to well-watered saplings. Cankers significantly (P < 0.001) reduced whole plant conductance only in well-watered saplings, although a similar but smaller trend was observed in the droughted

![Figure 3. Changes in external lesion size in saplings of C. calophylla under different watering treatments. Symbols indicate mean canker area ± SE. Dotted lines refer to treatments receiving either continuously reduced watering (DI) or reduced watering post-inoculation (WI-D) only, whereas solid lines represent saplings that were either continuously well-watered, or were well-watered post-inoculation only. The arrow indicates the time of water regime change for the WI-D and DI-W treatments. The inset photo shows an example of a stem canker lesion, where white areas are sporulation and dark areas are kino.](https://academic.oup.com/treephys/article/39/1/6/5123809)

Table 1. Analysis of deviance table showing the contribution of terms to the fitted model for the external canker size development in C. calophylla saplings under different watering treatments over time. Residual values are shown in parenthesis. P values generated by F test and asterisks indicate significance of model terms (* at P < 0.05, ** at P < 0.01 and *** at P < 0.001, ns = non-significant). Treatments: WI = well-watered inoculated, DI-W = pre-inoculation drought, WI-D = post-inoculation drought, DI = drought inoculated.

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<td>All</td>
<td>1 (163)</td>
<td>12.64 (7.67)</td>
<td>3 (160)</td>
</tr>
<tr>
<td>Between treatments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WI-D</td>
<td>1 (83)</td>
<td>5.36 (4.15)</td>
<td>1 (82)</td>
</tr>
<tr>
<td>WI-D-W</td>
<td>1 (88)</td>
<td>11.67 (3.15)</td>
<td>1 (87)</td>
</tr>
<tr>
<td>WI-W-D</td>
<td>1 (78)</td>
<td>4.84 (2.85)</td>
<td>1 (77)</td>
</tr>
</tbody>
</table>

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saplings (Figure 5b). Cankers tended to reduce stem hydraulic conductivity only in well-watered saplings, whereas no such effects were observed in droughted saplings (Figure 6).

Gas exchange
Drought-stressed saplings (DC, DI, WI-D) had lower rates of photosynthesis (2.58–6.47 μmol CO₂ m⁻² s⁻¹) than well-watered (WC, WI, DI-W) saplings (10.04–12.73 μmol CO₂ m⁻² s⁻¹) throughout the experiment, which was strongly related to their lower stomatal conductance (Figure 7), indicating that stomatal closure was responsible for decreased photosynthesis. Cankers reduced photosynthesis and stomatal conductance in well-watered saplings only after 110 dpi, whereas there was no such effect in droughted saplings at any stage of the experiment (P < 0.05) (Supplementary Figure S2). Within the well-watered saplings, cankers had no significant effect on the maximum rate of carboxylation (WC: 99.7 ± 3.7 μmol m⁻² s⁻¹; WI: 93.7 ± 7.7 μmol m⁻² s⁻¹; P = 0.68) and maximum rate of electron transport (WC: 148.3 ± 1.8 μmol m⁻² s⁻¹; WI: 150.6 ± 7.4 μmol m⁻² s⁻¹; P = 0.89). The reduction of stomatal conductance was strongly associated with an increased percentage of infected/wounded sapwood in well-watered saplings (Figure 8). Post-inoculation droughted (WI-D) and pre-inoculation droughted saplings (DI-W) had a similar range of stomatal conductance and photosynthesis to that of continuously droughted (DI) or well-watered (WI) saplings, respectively (Figure 7).

Plant biochemical defence
Inoculation increased the total concentration of phenolics in the stem at least fourfold (P < 0.001), while inoculated saplings which had experienced drought stress tended to have lower stem total phenolics than inoculated well-watered saplings, but had no effect on leaf phenol concentration (Figure 9a and b). In contrast, stem and leaf total terpene concentrations showed no clear patterns, although stems of the well-watered control saplings tended to have the lowest concentrations and leaves of the well-watered inoculated saplings the highest (Figure 9c and d). The total terpene concentration, the concentration of individual terpene compounds and the number of different terpenes detected (49), were substantially higher in leaves than in the stems (9) (P < 0.001). Among the 49 identified terpene compounds in leaves, six compounds, i.e., α-phellandrene, pinocarvone, farnesol, α-terpinol, 1,8 cineole, α-pinene, constituted more than 60% of total leaf terpene in marri saplings, regardless of water and inoculation treatment (Supplementary Table S1). The major identified stem terpenes were γ-terpinene, α-pinene, p-cymene, aromadendrene and squalene, which constituted ~50% of total stem terpene (Supplementary Table S2). In well-watered treatments, some of the terpene compounds clearly showed an increasing or decreasing trend response to inoculation in both leaves and stems. Inoculation increased the concentration of farbesol, α-phellandrene, lomine in the leaf (P < 0.05), whereas limonene, 1,8 cineole and squalene concentrations tended to be higher in infected stem. In contrast, inoculated saplings showed a decreasing trend of the concentration of α-terpinol, epi-globulol, selinene and α-caryophyllene in the leaves only.

Discussion
Our results showed that, contrary to our expectations, canker developed fast only under well-watered conditions and that only under well-watered conditions the development of cankers reduced plant growth, which was associated with a loss in stem hydraulic conductivity and stomatal conductance. Thus, overall, there was no evidence of an additive or multiplicative effect between the abiotic and biotic stress factors. Timing of drought stress did however influence the development of canker: well-
watered saplings that had experienced drought before inoculation displayed the fastest increase in canker size. It appears that conditions for growth of *Q. coyrecup* are more favourable in newly grown well-hydrated sapwood (WI and DI-W), as was observed for *Heterobasidium annosum* in *Picea abies* (Bendz-Hellgren and Stenlid 1998, Madmony et al. 2018). Higher amounts of soluble sugars in newly grown stem tissue may facilitate C access to the fungal pathogen and accelerate disease development, as reported for other pathogens, e.g., *Armillaria mellea* and *Phytophthora quercina* (Wargo 1972, Angay et al. 2014). In contrast, stem growth in the sustained drought-treated saplings was very limited (<40% of the well-watered saplings) and thus most of the sapwood was ‘pre-infection tissue’, which showed very little canker growth. It is possible that this sapwood also had increased sugar concentrations, as is often reported due to osmoregulation and reduced sink strength in drought-stressed plants, but if this was the case, it did not stimulate canker growth (Anderegg et al. 2012).

We observed a higher relative canker growth rate in pre-inoculation droughted plants (DI-W), which had experienced drought before inoculation, compared to sustained well-watered plants (WI), suggesting that drought before infection made the plants more vulnerable to disease. Similarly, a canker disease caused by *Lasiodiplodia theobromae* (fungus) in *Cornus florida* (dogwood) also showed greater canker development in pre-inoculation droughted plants than non-droughted plants (Müllen et al. 1991). Müllen et al. (1991) also reported that non-droughted plants formed bigger reaction zones (callus) which may have slowed down the disease development more than in the drought predisposed plants. Upon pathogen infection, plants tend to form a reaction zone enriched with different secondary metabolites, mainly polyphenols, around the affected area to prevent further disease development (Eyles and Mohammed 2003, Yulia et al. 2014). Secondary metabolites like terpene and...
phenol are known plant defence metabolites against pests and pathogens and are well known in eucalypts, including *Corymbia* (Cahill and McComb 1992, Eyles and Mohammed 2003, Eyles et al. 2010, Hayes et al. 2014). For instance, inoculation with *Phytophthora cinnamomi* caused a rapid and substantial increase of total phenolics in the roots of field resistant *Corymbia calophylla*, but not in the susceptible *Eucalyptus marginata* (Cahill and McComb 1992). In-vitro studies have confirmed that phenol-based biochemical defence compounds can inhibit fungal pathogen growth (Li et al. 1969, Elansary et al. 2017). We found a consistent increase of total phenol in stems around the inoculation point, suggesting that marri employs phenol-based localized biochemical defence. We also found that the infected saplings which had experienced drought stress during inoculation (DI-W and DI) had lower stem total phenolic concentrations than the well-watered inoculated saplings (WI). This indicates that drought may reduce host resistance by reducing plant biochemical defence (Sturrock et al. 2011, McKiernan et al. 2014, 2015). Our observation that cankers grew more rapidly in DI-W plants than in WI plants, whereas infected sapwood was not different in these treatments at the end of the experiment, suggests that the biochemical defence in marri was more effective in halting the inward growth of the pathogen. Overall, while the fast canker growth in pre-drought inoculated well-watered (DI-W) saplings may have been due to poorer defence, the slow canker
growth in permanently drought-stressed saplings was most likely due to drought itself.

Despite the link between pathogen infection and an increase in stem total phenol concentration, we did not observe any substantial changes in total phenol and terpene concentration in leaves. The production of defence compounds in the tissues distal to the inoculation area (systemic defence) is common for eucalypt species (Eyles et al. 2010, Naidoo et al. 2014, Visser et al. 2015). For instance, a systemic increase of terpene compounds in leaves was observed in response to stem inoculation with the canker pathogen Chrysosporium australiacanum in Eucalyptus grandis seedlings (Visser et al. 2015). This would suggest that marri saplings have no systemic defence response to the stem canker pathogen. We cannot exclude the possibility of a systemic response in stem tissue distal to the infection, as we did not analyse such tissue. Since we observed no substantial changes in total terpene concentrations either in leaves or stems in response to pathogen inoculation, terpenes may not be involved in defence response against Q. coycrescup in C. calophylla.

Drought affected all growth parameters in the experiment, but we found no evidence of greater drought stress in diseased saplings compared to non-diseased saplings, as might have been expected for a pathogen that affects sapwood functionality. In fact, a greater percentage of sapwood was affected by the canker in well-watered saplings than in droughted saplings, and only in these well-watered saplings (as opposed to droughted plants) canker-induced loss of sapwood function reduced whole-plant conductance and stomatal conductance. The limited impact of the canker on the water relations of drought-stressed saplings appears to be due to smaller canker growth (Fukuda et al. 2007), presumably due to lack of new tissues, and to physiological adjustment to the lost hydraulic capacity. Leaf water potentials were not significantly lower in diseased saplings than non-diseased saplings, and did not reach extreme values, indicating an efficient stomatal control to adjust to the lost hydraulic capacity. Infected drought-stressed saplings (WI-D and DI) lost ~15% of their conductive sapwood, but the impact on hydraulic conductivity was not statistically significant. Even in well-watered saplings, which lost almost twice as much sapwood, the percentage loss in $K_s$ was less than the percentage sapwood lost. This suggests that there were compensation mechanisms, perhaps involving lateral connectivity of xylem vessels (Schulte and Costa 2010, Halis et al. 2013), or growth of new xylem tissue with higher specific conductivity.

There is no evidence that Q. coycrescup has direct negative effects on leaf photosynthetic capacity, however loss of hydraulic conductance in diseased well-watered saplings caused a reduction in stomatal conductance late in the experiment. This supports the general view that stem canker pathogens colonize plant vascular tissues, thereby reducing stem hydraulic conductance and leaf stomatal conductance (Clemenz et al. 2008). Stomatal conductance was indeed lowest in the saplings with the largest cankers. The temporal measurement throughout the disease development did not show any significant decrease in stomatal conductance until 110 dpi, which indicates plants had redundancy in hydraulic capacity to be able to maintain gas exchange to that point (Supplementary Figure S2) (Tyree and Sperry 1989). The observed reductions in net photosynthesis at the end can be fully attributed to the lower stomatal conductance, as shown by a relationship between photosynthesis and stomatal conductance that was independent of treatment. Although we did not find evidence of an additive effect of the abiotic and biotic stress factors in this study, we expect that more advanced cankers in combination with more severe drought will have serious negative effects on trees. Our data show the predicted trends of lower stomatal conductance and more negative water potentials under such conditions. As with many tree decline phenomena, it is difficult to predict if trees deteriorate primarily as a result of hydraulic failure, or due to unfavourable carbon balance (Mitchell et al. 2013, Johnson et al. 2018). While depleted carbon reserves rarely seem to be the direct cause of mortality, lack of growth may cause loss of tree vigour in the longer term. In this study, we also found evidence that biochemical defence protected existing sapwood, but the growth of new sapwood and leaves was compromised by the pathogen. In such situations, essential physiological functions are increasingly dependent on ageing tissues, which may ultimately be the cause of branch and whole tree death. Further research on mature trees over longer time spans is required to test results obtained using saplings.

In conclusion, our results demonstrate complex interactions between biotic and abiotic stress. While canker growth was fastest in well-watered plants, and negative effects on plant performance were also greatest in well-watered plants, there was also evidence of predisposition due to abiotic stress, as plants that had been drought stressed before and during inoculations showed the fastest subsequent canker growth under well-watered conditions. Further, phenol-based defence tended to be reduced in drought-stressed plants, which may have reduced the ability of the plant to constrain subsequent canker growth when conditions became favourable for the pathogen. This study expands the current knowledge of the impact of drought in plant–pathogen interaction by including both plant physiology and biochemical defence responses for the first time in a single pathosystem study.

**Supplementary Data**

Supplementary Data for this article are available at Tree Physiology Online.

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Quambalaria isolate and preparation of the inoculum that was used in this experiment. The terpene analysis was performed at Metabolomics Australia at the University of Western Australia, Western Australia. We also thank the reviewers for their helpful suggestions.

Conflict of interest

None declared.

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References


