Water relations during drought and the recovery from drought in two *Eucalyptus* species with contrasting water-use strategies

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Abstract

In recent decades, climate change has led to drought and heat-induced mortality events in forest ecosystems worldwide. Our current understanding of physiological mechanisms leading to drought-induced tree death is limited, which prevents us from accurately predicting responses of forest to a warmer and drier climate in the future. Since carbon and water relations are inevitably linked through stomatal control, it has been suggested that trees can die from hydraulic failure or carbon starvation. This PhD project aimed at exploring the effects of drought on carbon and water relations in seedlings of two *Eucalyptus* species. The two species, *Eucalyptus marginata* Sm. and *Eucalyptus wandoo* Blakely, were chosen because they are known to exhibit contrasting water use strategies. *Eucalyptus marginata* is a relatively isohydric species and has been shown to reduce its water loss in response to dry conditions to maintain higher leaf water potentials. *Eucalyptus wandoo*, on the other hand, develops distinctly low water potentials before substantially reducing its stomatal conductance and it is thus considered a relatively anisohydric species. Consequently, the two species were expected to show distinct responses to drought, which could affect carbon relations.

In the first experiment, I aimed to test the capacity of *Eucalyptus* seedlings to repair embolisms when they were rewatered after a 20-day drought treatment, and to study the coordination of embolism repair with the recovery of leaf water potential (\(\Psi_L\)), relative water content and stomatal conductance (\(g_s\)). During the drought treatment, both species lost over 70% of their conductivity through embolisms. Upon rewatering, both species showed a fast recovery of \(\Psi_L\) (from c. -3 to -1 MPa) and relative leaf water content, as well as an almost 30% decrease in the percentage loss in conductivity (PLC), but \(g_s\) did not recover to control levels in *E. wandoo* within eight hours after rewatering. A fast recovery of stem hydraulic conductivity and leaf water potentials is thus not always followed by a recovery of transpiration.

In the second experiment, the two species were exposed to a long and progressive drought, to study embolism repair in relation to carbon pools upon rewatering. Contrasting water-use strategies in the two species were evident from differences in the lowest observed \(\Psi_L\) and timing of stomatal closure. Seedlings of both species, however, accumulated large amounts of soluble sugars in leaves, stems, and bark including
cambium but not in roots, while starch pools remained largely unaffected. Within 18 hours after rewatering, PLC in the more isohydric species, *E. marginata*, recovered from over 50% to levels of well-watered controls (~20%) along with a complete recovery in $\Psi_L$ and $g_s$. In *E. wandoo*, $\Psi_L$ mostly recovered while $g_s$ remained as low as in droughted seedlings. Unexpectedly high PLC in rewatered *E. wandoo* seedlings were most likely a result of ~50% higher maximum sapwood-specific conductivity, compared to droughted seedlings, but similar initial sapwood-specific conductivity, which may indicate that hydrogels had a role in the recovery of hydraulic conductivity or that the removal of embolism during pressurisation was inadequate in droughted seedlings. In both species, soluble sugar pools decreased to levels close to well-watered controls upon rewatering. I suggest that an accumulation of soluble sugars could be significant for osmoregulation and embolism repair in *Eucalyptus* seedlings during drought.

The aim of the third experiment was to assess how drought in two different soil types affected water relations, in particular the hydraulic system, in two *Eucalyptus* species with contrasting water-use strategies and soil preferences. Both species accumulated embolisms in response to soil water deficit but *Eucalyptus wandoo* displayed a more than 20% higher xylem vulnerability in the sandy compared with the loamy soil while xylem vulnerability was the same in *E. marginata*. I propose that adaptations to soil types in their native habitat led to higher vulnerability in *E. wandoo* due to an inability to adjust its water use to higher critical soil water potentials in sandy soils. The survival of some tree species in areas with favourable future climates could thus be threatened if their hydraulic system is affected by the soil substrate.

In summary, *Eucalyptus* seedlings showed a fast, but not always complete, recovery of most hydraulic parameters in response to rewatering after drought. I found no evidence for carbon starvation. Rather, increased soluble sugar concentrations, irrespective of water use strategy, may contribute to drought tolerance through a role in osmoregulation and embolism repair.
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Thesis structure

This thesis consists of a general introduction, three experimental chapters, and a general discussion. The central part of this thesis is a compilation of three experimental chapters in research paper format. The chapters are prepared for publication in international peer-reviewed journals and will be submitted in the near future. Each experimental chapter follows the format commonly used by peer-reviewed journals and thus has its own title, abstract, keywords, and references as well as an introduction, methods, results, and discussion section. In the general introduction, I review the current literature, provide additional, but less specific, background knowledge, and present the hypotheses of my research chapters. In addition, I outline the structure of each experimental chapter and show how the chapters are linked to each other. I then summarise the main findings from the experimental chapters in the general discussion, and relate them to each other to draw general conclusions.

Thesis declaration

This thesis was completed while I was enrolled in the School of Plant Biology at The University of Western Australia. It has not been previously accepted for a degree at this or any other institution.

I hereby declare that this thesis is solely my own work and was completed under the supervision of Assoc. Prof. Erik J. Veneklaas and Prof. Hans Lambers.
Chapter 1

General introduction

This introductory chapter provides a broad background for the following experimental chapters, and reviews current knowledge relevant to this thesis. Here, I briefly outline past and current changes in climatic conditions that are claimed to be responsible for forest mortality events. Furthermore, I introduce the commonly proposed mechanisms that lead to drought-related mortality, and discuss how drought can affect carbon and water relations. I then change the focus from the effects of drought to the current knowledge of the recovery from drought with an emphasis on embolism repair. The theoretical background of the conducted research is then followed by a description of the two study species as well as an outline of the aims of each of the experimental chapters.

Climate change and forest decline

The increase in greenhouse gas emissions due to anthropogenic activities, such as the burning of fossil fuels and land-use changes, alters the global climate. Since 1950, the mean land and ocean surface temperature has risen by 0.4-0.7°C, and a higher frequency in extreme weather and climate events has been observed (IPCC, 2014). Globally, episodes of drought and heat have occurred more frequently in the last decades, and this trend is predicted to continue (IPCC, 2007). Changes in climatic conditions have led to a number of forest mortality events in a variety of habitats around the globe (Allen & Breshears, 2007). Allen et al. (2010) found that 88 out of 150 forest mortality events reported in the literature since 1970 were climate-induced. They also show that the number of published reports of climate-related forest tree mortality has increased in the period between 1985 and 2009, and suggest that this is a result of global climate change.
Tree mortality associated with extended or unseasonal periods of dry and hot conditions has occurred on all vegetated continents and in all major biomes (McDowell et al., 2008; Allen et al., 2010). However, Mediterranean-type ecosystems are predicted to be most likely to undergo severe changes in the future (IPCC, 2007). Globally, Mediterranean-type biomes cover less than 5% of the land area, but harbour about 20% of all vascular plants (Cowling et al., 1996). South-western Australia is one of the five areas on Earth with a Mediterranean-type climate, and is also one of 34 global biodiversity hotspots (Mittermeier et al., 2004). The climate in this area became warmer and drier as the mean annual temperature has increased by 0.9°C since 1900, and average annual rainfall has declined by 17% since 1970 (BOM, 2014). In addition, the duration, frequency, and intensity of heat waves have all increased. Based on recent models, temperatures in south-western Australia are predicted to rise between 1 and 5°C, and annual precipitation is expected to decline by up to 30% by 2070 (BOM, 2014). Using current climate change scenarios, Klausmeyer and Shaw (2009) predict that the area with a Mediterranean-type climate in south-western Australia will shrink to about 49-77% of its current size by 2100. A climatic shift in south-western Australia could have severe consequences for the more than 8000 plant species in this biodiversity hotspot (Myers et al., 2000).

Mechanisms leading to drought-induced tree mortality

The occurrence of tree death around the world has sparked great interest in the mechanisms leading to drought-induced tree mortality. Currently, the exact mechanisms that cause climate-related tree death are not clear (McDowell, 2011), and several mechanisms have been proposed. Hydraulic failure has been identified as a primary reason of drought-induced tree mortality (Brodribb & Cochard, 2009) and is a result of progressive embolisms in the xylem. Water in the xylem of plants is in a meta-stable state and exceedingly negative pressures in the water-conducting conduits can lead to the formation of gas bubbles when liquid water changes to vapour (Tyree & Sperry, 1989). The formed gas bubbles then get trapped in the vessels and impair water transport from roots to leaves, leading to tissue desiccation and eventually death, due to the failure of the entire hydraulic system (i.e. hydraulic failure). Plants can avoid very negative xylem pressures through stomatal closure, however, minimising the water loss by reducing stomatal conductance to avoid tissue desiccation has consequences for a plant’s carbon metabolism. When stomata are closed, plants do not only reduce water
loss, but also CO₂ uptake, as reduced gas-exchange limits photosynthesis (Farquhar & Sharkey, 1982). This inevitable link between water loss and CO₂ uptake has led to the hypothesis that plants can suffer from carbon starvation during drought events. Death from carbon starvation occurs when cellular and defensive processes are not maintained due to inadequate carbohydrate supply through photosynthesis or the use of stored carbohydrates (McDowell et al., 2011). When photosynthesis is limited due to stomatal closure, plants rely on the mobilisation of stored carbon, but these carbon reserves can get depleted if the drought is prolonged. It has thus been suggested that carbon starvation is more likely during long-term, low-intensity drought events, while short but severe drought periods would cause hydraulic failure (McDowell & Sevanto, 2010).

Apart from the intensity and duration of the drought stress, the different water-use strategies of tree species might predispose them to either hydraulic failure or carbon starvation (McDowell et al., 2010). According to McDowell et al. (2008), isohydric species are prone to die from ‘hydraulically-mediated carbon starvation’ during prolonged drought, because they avoid hydraulic failure by reducing the stomatal conductance and thus risk a depletion of carbon pools. Anisohydric species, on the other hand, maintain relatively high stomatal conductances and allow substantial decreases in leaf – and consequently xylem – water potentials, which makes hydraulic failure a likely cause of death during intense drought periods. Mitchell et al. (2014) used species with contrasting water-use strategies to test the idea that water-use strategies determine the cause of death. In their study, relatively isohydric Pinus radiata plants reduced their water loss early in the drought, resulting in substantial decreases in total non-structural carbohydrate (NSC) concentrations (i.e. 41% on average) in roots, stems, and leaves at the time of mortality. In contrast, the two more anisohydric Eucalyptus species (i.e. Eucalyptus globulus and Eucalyptus smithii) depleted the soil water, which led to very low water potentials and a complete loss of hydraulic function. The carbon pools on a whole-plant level, however, remained largely unaffected and were not depleted at mortality in the two Eucalyptus species. Their findings thus confirm the ideas put forward by McDowell et al. (2008). In a study by Hartmann et al. (2013a), soluble sugar as well as starch pools decreased by over 70% in roots of Picea abies trees in response to a c. three-months drought, but were similar to levels of well-watered control trees in all other tissues. They concluded that hydraulic failure could not be excluded as a mortality mechanism, despite a substantial depletion of root carbon pools, as a
complete loss of water transport to the canopy was apparent in droughted trees. At the same time, they suggested that root death as a consequence of reduced carbon pools due to impaired phloem functioning and carbon translocation might have occurred prior to hydraulic failure. Their results highlight the difficulty to disentangle carbon and water relations and their relative contributions to tree death.

The inherent link between carbon and water relations makes it difficult to study the contribution of carbon starvation and hydraulic failure during drought independently of each other. Recently, several elegant approaches have been used to disentangle the two processes and obtain a better insight into carbon and water relations during drought. Methods used include altered carbon dioxide (CO₂) concentrations in the growth environment and different light regimes. Hartmann et al. (2013b) manipulated the carbon balance of Picea abies seedlings by withdrawing either water or CO₂. In their study, droughted saplings died earlier than CO₂-starved saplings, most likely due to hydraulic failure. They found a significant depletion, but not complete exhaustion, of carbon pools in CO₂-starved saplings at the time of mortality, which proves that death can occur under well-watered conditions and as a result of carbon limitations, even though carbon pools were not completely depleted. They concluded that the translocation, rather than the availability of carbon was responsible for mortality in the well-watered but CO₂-starved saplings. This is in support of a third process where reduced phloem transport and consequently impaired translocation of carbon could cause mortality, as a direct mechanism or through carbon starvation or hydraulic failure. This process has not received much attention, possibly because it is largely unknown how environmental stress influences phloem physiology (Sala et al., 2010). However, modelling has revealed that phloem failure occurs when growth ceases before photosynthesis is downregulated (Mencuccini et al., 2015).

Sevanto et al. (2014) came to similar conclusions as Hartmann et al. (2013b) using a different approach to understand mortality mechanisms. In their study, a shade and drought treatment was used to cause carbon starvation and hydraulic failure, respectively. In droughted Pinus edulis trees, death was induced through hydraulic failure as evident from total loss of hydraulic conductivity prior to tree death, while non-structural carbohydrate (NSC) pools did not decrease. Shaded trees, on the other hand, showed substantial decreases in NSC pools, while hydraulic conductivity
remained above zero at the time of mortality, suggesting that death occurred due to carbon starvation. They also found that longer survival times coincided with lower NSC concentrations at the time of death and proposed that carbohydrates played a role in the observed rapid declines in water potentials prior to tree death. They concluded that carbohydrates are important for osmoregulation, and that reductions in carbon pools or impaired carbon translocation could cause hydraulic failure through inadequate osmoregulation and the inability to repair embolisms. In a study by O’Brien et al. (2014), an experimental manipulation of carbon pools through a combination of light and shade treatments revealed that the survival of seedlings of tropical tree species was prolonged when carbon pools were large. When exposed to dry conditions, *Pinus edulis* and *Juniperus monosperma* trees with a higher starch content survived longer (Dickman et al., 2015). This confirms the findings by Sevanto et al. (2014) and highlights the importance of maintaining high carbohydrate concentrations for osmoregulation and potentially embolism repair. The experimental manipulation of carbon relations has increased the understanding of the role of carbohydrates during drought, and revealed that hydraulic failure as well as carbon starvation (potentially mediated through impaired phloem transport to sink tissues) can lead to tree death. However, it remains unclear if carbon starvation commonly leads to drought-induced mortality in trees growing in their natural environment. In addition, it is necessary to understand not only the processes that lead to drought-induced mortality, but also the recovery from drought. This is especially important if we aim to predict responses of forests to a future warmer and drier climate (Xu et al., 2013).

**Mechanisms involved in embolism repair**

The ability to repair embolisms might be more important during drought and the survival of drought than just maintaining water potentials above a certain threshold (Klein et al., 2014). In accordance with the development of embolisms as a result of high xylem tension (i.e. negative pressure), the reversal of embolisms is thought to occur when the tension is reduced and gases dissolve. The reversal of embolisms has also been proposed to be facilitated by a positive pressure as a result of root pressure at night (Konrad & Roth-Nebelsick, 2003). However, it is unlikely that positive root pressure is common in tree species (Tyree et al., 1999). In a study by Fisher et al. (1997), positive predawn root pressures were observed in 61 out of 109 studied species but most of them were not trees but lianas and hemi-epiphytes. It has also been argued
that root pressure would not be sufficient to remove embolisms in trees above a certain height, as root pressure dissipates by 10 kPa m\(^{-1}\) due to gravity (Fisher et al., 1997). The highest root pressure values measured by Fisher et al. (1997) would be sufficient to push water up a height of 14 m, but would not be enough to create positive pressure along the entire height of the stem of the plant. According to Zwieniecki & Holbrook (2009), root pressure can only occur when plants are not transpiring and soil water is available. In addition, embolism reversal has been shown to occur while the xylem pressure was lower than what could be compensated for with root pressure as well as in the absence of root pressure (e.g., in *Laurus nobilis*, Tyree et al., 1999; in *Eucalyptus pauciflora*, Martorell et al., 2014). Alternative explanations are thus needed to explain embolism reversal under conditions contradicting the existing paradigm.

Recent evidence suggests that embolism repair occurs while the xylem is under substantial tension (Salleo & Lo Gullo, 1989; Salleo et al., 1996; Stiller et al., 2005; Salleo et al., 2009) and while plants are transpiring (Canny, 1997). In addition, the time required to repair embolisms is much shorter than previously expected, and ranges from less than one hour (Salleo et al., 2004) to several hours (Holbrook et al., 2001; Kaufmann et al., 2009; Martorell et al., 2014). All these findings imply that an active process is involved in the refilling of embolised conduits. In their review, Nardini et al. (2011) discussed several mechanisms to explain the recent findings including (1) the development of an osmotic gradient through the secretion of solutes into conduits, which would then drive refilling and (2) a phloem-driven transport of water into embolised conduits. The creation of an osmotic gradient due to the secretion of solutes by living vessel-associated cells would draw water into embolised vessels and lead to refilling (Nardini et al., 2011). It has been shown that potassium ions could be involved in the refilling of embolised vessels in *Laurus nobilis* (Tyree et al., 1999), but also sugars and even proteins are potentially secreted to facilitate osmotically-driven refilling. Améglio et al. (2002) showed a correlation between increased sucrose concentrations in the xylem sap of walnut trees and increased xylem pressures. They suggested that starch depolymerisation, followed by the secretion of sucrose or other sugars into xylem vessels, was responsible for embolism repair through the creation of positive xylem pressure (Améglio et al. 2004). Starch depolymerisation also occurred in refilling *Laurus nobilis* stems, but was interpreted as a signal for phloem unloading (Salleo et al., 2009). Salleo et al. (2009) suggested that the depolymerisation of starch
in the wood parenchyma cells would make them strong sinks for phloem unloading and that wood parenchyma cells would then actively transport sugars to the xylem to generate the driving force for refilling. In contrast, Zwieniecki & Holbrook (2009) propose a continuous secretion of sugars into vessels by the xylem parenchyma. They suggest that the small amount of secreted sugars would get translocated in water-conducting vessel but accumulate in embolised vessels. The accumulation of sugars would then generate the osmotic pressure needed for refilling. These findings highlight that carbon does not only play an important role during drought events, but also during the recovery from drought.

The current hypotheses of refilling under tension may require a hydraulic isolation of vessels, since water secreted into an embolised vessel could move to adjacent water-filled vessels with more negative water potentials (Wegner, 2014). It has thus been suggested that vessels remain hydraulically independent from each other during the refilling process so that refilling can occur in some conduits, while others remain under negative pressure (Salleo et al., 2009). The structure and chemical composition of bordered pit membranes could provide a barrier, so water cannot leave a refilling vessel prematurely (Zwieniecki & Holbrook, 2009). It has been shown that xylem anatomy, especially the structure of pit membranes, is associated with embolism resistance (Broddribb et al., 2012), and similar features may facilitate embolism repair through the hydraulic isolation of embolised conduits during the refilling process.

Ecology and physiology of the two study species

The two study species, *Eucalyptus marginata* Sm. and *Eucalyptus wandoo* Blakely, are both endemic to south-western Australia and are common forest and woodland species. They have relatively distinct distribution ranges due to contrasting soil preferences (Verboom & Pate, 2015). *Eucalyptus marginata* grows on a variety of soil types ranging from gravely and sandy soils to red loams (McDonald et al., 2006), but is most abundant on deep or loamy sands that often occur over clay (Boland et al., 2006). In contrast, *Eucalyptus wandoo* predominantly occurs on heavier soils such as loamy sands and sandy loams, often over a saline and clayey subsoil. In the jarrah (*Eucalyptus marginata*) forest region, where both species co-occur, *E. marginata* occurs on lateritic soils overlaying kaolinitic clays with sandy topsoils, while *E. wandoo* prefers the finer-textured valley floors (Doley, 1967). In addition to different preferences for soil
substrates, the two *Eucalyptus* species also grow in areas with different annual precipitation ranges. *Eucalyptus marginata* is dominant in areas with comparatively higher rainfall (600-1250 mm annual precipitation; Boland *et al.*, 2006) than *E. wandoo* (400-700 mm annual precipitation; Boland *et al.*, 2006). As a consequence of a Mediterranean-type climate with wet winters and a long dry summers in south-western Australia, both species have developed strategies to sustain long periods with no or minimal rainfall.

*Eucalyptus* species are generally thought to have extensive root systems to maintain water supply to their foliage; they increase the root to shoot ratio in response to decreasing annual rainfall averages (Bell & Williams, 1997). Relatively rapid transpiration rates were observed in *E. marginata* during late spring and summer, despite low soil water availability in the topsoil, and it was concluded that *E. marginata* trees had access to deep moisture through cracks in the laterite (Doley, 1967). Farrington *et al.* (1996) used the deuterium content of water to study seasonal changes in water sources of *E. marginata* trees and found that *E. marginata* preferably took up water from the upper layers of the soil during winter, when the soil moisture in the upper layers was abundant, and from lower layers of the deep sand in summer. However, they found no evidence that the roots of *E. marginata* trees had access to the 14 m-deep groundwater. In a study by Poot & Veneklaas (2013), *E. marginata* reduced its water loss in response to dry conditions, possibly due to limited access to water in deeper soil layers. *Eucalyptus wandoo* may not have access to groundwater, due to its relatively shallow root system, but this species is known to develop very low leaf water potentials before stomatal conductance is reduced (Colquhoun *et al.*, 1984; Poot & Veneklaas, 2013) which may allow *E. wandoo* to extract more water from the soil.

Poot & Veneklaas (2013) compared the water-use strategies of the two *Eucalyptus* species in an area where they co-occurred as well as in areas that represented a more typical habitat for each species. They found greater stomatal conductances and lower predawn and midday leaf water potentials in *E. wandoo* compared with *E. marginata*, when the two species were studied in the same habitat. In addition, the xylem vulnerability was less in *E. wandoo* compared with *E. marginata*, as evidenced by more negative xylem water potentials of -3.3 MPa in *E. wandoo* compared with -1.3 MPa in *E. marginata* at the point of 50% loss of conductivity. Differences in transpiration, but
not water status, that were observed when the two species co-occurred, largely disappeared when each species was studied in its typical habitat. Poot & Veneklaas (2013) attributed this finding to adaptations to characteristics of their preferred habitat, such as soil properties. This indicates that water relations in *Eucalyptus* species are not only affected by climate, but also by the interaction of the plants with the soil substrate.

**Thesis aims and scope**

The overall aim of this thesis was to contribute to our current understanding of carbon and water relations during drought and the recovery from drought. Two *Eucalyptus* species with contrasting water-use strategies were used to determine effects of water-use strategies on carbon metabolism during drought and subsequent rewatering, and also in response to growth in contrasting soil types.

In Chapter 2, I studied embolism repair, and its temporal coordination with water relation parameters, in response to rewatering after drought in seedlings of *E. marginata* and *E. wandoo*. The aim of the experiment was to determine (1) if embolisms are repaired, (2) the timing of embolism repair, and (3) the timing of the recovery of stomatal conductance, leaf water potentials, and leaf relative water content. Both species were expected to reduce their water use through a reduction of stomatal conductance; however, this was expected to occur at a lower soil water availability in *E. wandoo* than in *E. marginata*. In response to reduced soil water availability, both species were expected to exhibit lower leaf water potentials and consequently accumulate embolisms. I hypothesised that *E. wandoo* would exhibit a higher percentage loss in sapwood hydraulic conductivity compared with *E. marginata*, as a result of faster soil water depletion and more negative xylem water potentials, not higher xylem vulnerability. Assuming that sugars were important for embolism repair, I also anticipated an accumulation of sugars in the sapwood of seedlings of both species.

The second experiment (Chapter 3) was designed to study carbon and water relations over a long period of progressive drought. During this time, I conducted several harvests to investigate the relationship between carbon pools and the ability of *E. marginata* and *E. wandoo* seedlings to recover from drought and repair embolisms after rewatering. In accordance with differences in water-use strategies, I hypothesised that carbon pools and water transport would be affected differently in the two *Eucalyptus*
species. I anticipated that the more isohydric water-use strategy in *E. marginata* would lead to a depletion in carbon pools as a consequence of reduced stomatal conductance to maintain high leaf water potentials. If carbohydrates were involved in embolism repair, a reduction in carbon pools was then thought to reduce the ability of *E. marginata* seedlings to repair embolisms upon rewatering. In contrast, *E. wandoo* would be more likely to risk hydraulic failure because of high stomatal conductances and low leaf water potentials, in accordance with its more anisohydric water-use strategy. I hypothesised that *E. wandoo* would show enhanced embolism repair in response to rewatering compared with that in *E. marginata*, as carbon pools would not be depleted and thus readily available for embolism repair.

The experiment in the fourth chapter was designed to test the ability of *E. marginata* and *E. wandoo* to tolerate soil drying in two different soil substrates, a sandy soil and a loamy soil. Based on the observations by Poot & Veneklaas (2013), I aimed to assess whether the two *Eucalyptus* species could adjust their water use or adapt their water-use strategies to contrasting soil substrates. I anticipated that, in response to drought, both species would reduce their stomatal conductance and lower their shoot water potentials. I also hypothesised that both species would accumulate more embolisms and show a greater loss in sapwood hydraulic conductivity in drying sandy soils compared with that in the loamy soils at a given soil water potential.
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Chapter 2

Stem hydraulic conductivity recovers quickly when drought-stressed *Eucalyptus* seedlings are rewatered but is not always followed by a recovery of transpiration

**Summary**

- Seedlings of *Eucalyptus marginata* Sm. and *E. wandoo* Blakely were subjected to a 17 to 20-day drought, and subsequently rewatered to study the temporal coordination of embolism repair and associated water relations parameters such as stomatal conductance, relative leaf water content and water potentials. It is important to understand the link between hydraulic recovery and the recovery of water relations parameters since they directly affect physiological processes such as photosynthesis and growth.

- Throughout the experiment, I monitored soil water content, whole plant transpiration, and stomatal conductance. When plants were harvested, water potentials and stem hydraulic conductivity were measured, and sapwood samples were taken for non-structural carbohydrate analyses.

- Both species showed a fast recovery after rewatering as evidenced by a reduction in the percentage loss in conductivity, and increases in leaf water potential and relative water content. Upon rewatering, stomatal conductance initially increased in both species, but then decreased after four hours in *E. wandoo*. I observed higher glucose and lower starch concentrations in the sapwood of *E. marginata* compared with that of *E. wandoo*, but the fructose and sucrose concentrations were the same. Concentrations of monosaccharides were approximately twice as high in seedlings with predawn water potentials of -4 MPa compared with those at -2 MPa.

- *Eucalyptus* seedlings partly reversed embolisms within 2-6 hours after rewatering while actively transpiring. Transpiration rates remained high in *E. marginata*, but declined in *E. wandoo* despite a simultaneous recovery of stem hydraulic conductivity and leaf water status. Monosaccharides that accumulated in the sapwood of drought-stressed plants may have contributed to embolism repair.
Introduction

Decreased xylem water transport as a result of embolisms has long been recognised as a major threat to plant survival during periods of severe drought stress. The cohesion-tension theory has provided a framework explaining how and why xylem embolisms occur, based on biophysical processes that lead to the formation of gas bubbles when the xylem is under extreme tension (Dixon & Joly, 1894; Tyree, 1997). The theory also implies that embolism reversal occurs when the tension in the water conduits is relaxed and gases can dissolve. For this process to occur, the pressure in the xylem has to reach pressures greater than atmospheric pressure (Tyree et al., 1999). More recently, however, it has been shown that embolisms can be reversed under conditions that contradict the more than 100-year old cohesion-tension theory (Zwieniecki & Holbrook, 2009). There is increasing evidence that embolisms can be repaired while the xylem is under tension (Salleo & Lo Gullo, 1989; Salleo et al., 1996; Stiller et al., 2005; Salleo et al., 2009; Nardini et al., 2011) and even while plants are rapidly transpiring (Canny, 1997). Furthermore, refilling has occurred in the absence of root pressure (e.g., in Laurus nobilis, Tyree et al., 1999; in Eucalyptus pauciflora, Martorell et al., 2014). Embolism repair is thus thought to be an active process, rather than a simple reversal of biophysical conditions (Tyree et al., 1999), and it has been suggested that the depolymerisation of starch to glucose is involved in this process (Bucci et al., 2003; Secchi & Zwieniecki, 2011).

While the mechanisms leading to refilling are still under debate, it seems very unlikely that the dissolution of gas alone can explain the observed rates of refilling. Depending on the severity of the drought treatment, the study species and tissue as well as the methodology, the time required for embolism reversal ranges from less than one hour (Salleo et al., 2004) to several hours (Holbrook et al., 2001; Martorell et al., 2014; Kaufmann et al., 2009). However, Delzon & Cochard (2014) argue that the evidence for rapid refilling is in some cases biased due to methodological artefacts. The main issues are (1) that xylem pressure was measured on leaves that were not hydraulically connected to the measured segments (Sperry, 2013), and (2) that cutting the stem while the xylem is under tension might introduce additional embolisms when xylem tension is high, and thus purport embolism repair upon rewatering (Wheeler et al., 2013). In addition, many studies fail to provide supporting evidence to prove that water relations improve after rewatering. If refilling of embolisms occurs and stem hydraulic
conductivity recovers, the leaf water potential as well as the leaf relative water content should increase at some stage after rewatering. Once the water status has recovered, associated processes should follow and the plant should increase its stomatal conductance and start to transpire. This would then prove that the recovery from drought is not restricted to embolism reversal, but that an improved water status is followed by a recovery of physiological processes known to be affected by drought.

Many of the studies claiming to show embolism repair artificially introduced embolisms, which could lead to a misinterpretation of the mechanisms involved in embolism repair, even when avoiding methodological artefacts. The accumulation of soluble sugars in the stem tissue to create an osmotic force or the energy for refilling, for example, would be a process that requires time for the adjustment of plant metabolism. An artificial induction of embolism would therefore not allow sufficient time for adjustment of the plant’s metabolic processes, and thus not provide a realistic view of the role of soluble sugars during embolism repair. In order to understand hydraulic recovery upon rewatering, it is crucial to study embolisms that were formed in response to water deficit. In addition, the refilling of embolisms should lead to improved water relations, which need to be monitored in order to ensure that the refilling was successful. If successful refilling of embolisms is not followed by an improvement of water relations and associated physiological processes (e.g. photosynthesis), the recovery of the hydraulic system may not be a good predictor for survival.

*Eucalyptus* species in south-western Australia can display a remarkable resistance to drought. While mature trees may have extensive root systems that help them avoid water shortage to some extent, seedlings and saplings need additional mechanisms to recover from periodic drought. I used seedlings of two *Eucalyptus* species to study how water-use strategies can influence the response to drought and the subsequent recovery after rewatering. The two species, *E. marginata* Sm. and *E. wandoo* Blakely, are native to south-western Australia. They have considerably overlapping geographical distributions and co-occur in certain woodland habitats. *Eucalyptus marginata* is a widespread species growing on a variety of soil types, ranging from gravelly and sandy soils to red loams with a deep clay subsoil (McDonald & Boland, 2006). Since *E. marginata* spans a wide precipitation range (500-1250 mm year⁻¹; McDonald & Boland,
genetic differences in morphology and physiology may exist. Seasonal soil drying in *E. marginata* habitats requires conservative water use during its juvenile stage when deep soil moisture is not yet accessible. *Eucalyptus wandoo* occupies areas of comparatively lower annual precipitation (400-700 mm year\(^{-1}\); McDonald & Boland, 2006), and grows in heavy soils (loamy sands or sandy loams), often over saline subsoil. It typically has a shallow root system and does not rely on groundwater as it might not be present or accessible (Greenwood & Beresford, 1979). *Eucalyptus wandoo* operates at much more negative leaf water potentials than *E. marginata* does, but maintains higher summer transpiration when individuals of both species grow side-by-side (Colquhoun *et al*., 1984; Poot & Veneklaas, 2013). The lower water potential allows *E. wandoo* to extract more soil water, but this can lead to embolisms in water-conducting tissues as tension builds up. However, xylem vulnerability curves obtained through pressurisation of stems showed that *E. wandoo* displays a smaller loss of conductivity over most of the tested pressure range in comparison with *E. marginata* (Poot & Veneklaas, 2013). The water potential at which loss of 50% conductivity occurs (\(\Psi_{50}\)), calculated from curves of individual branches, is reached at -1.3 MPa in *E. marginata* and at -3.3 MPa in *E. wandoo*, demonstrating that the same level of xylem tension impairs xylem water transport more in *E. marginata* than in *E. wandoo* (Poot & Veneklaas, 2013).

Here, I present a study of embolism repair in response to rewatering after a drought treatment and its temporal coordination with stomatal conductance, leaf water potentials and leaf relative water content in seedlings of two *Eucalyptus* species. I expected that both species would accumulate embolisms in response to soil drying, but that *E. marginata* would reduce its stomatal conductance at higher leaf water potentials than *E. wandoo*. I expected that both species would accumulate embolisms leading to similar losses in conductivity in response to soil drying, although *E. wandoo* maintains transpiration at lower soil water content and develops lower xylem water potentials than *E. marginata*, since it is also less vulnerable to embolisms. Upon rewatering, seedlings of both species were expected to repair embolisms within several hours, expressed as a reduction in the percentage loss of hydraulic conductivity. In addition, I hypothesised that refilling would coincide with improved water status and improved water flux as expressed by (1) increased water potential and relative water content and (2) increased
stomatal conductance. I also expected an accumulation of monosaccharides, especially glucose, assuming that these are involved in embolism repair.

**Materials and Methods**

*Plant material and experimental setup*

One and a half year old *E. marginata* and *E. wandoo* seedlings were obtained from Muchea Tree Farm (Muchea, Western Australia, Australia). Twenty-eight seedlings per species were transplanted into storm-water pipe pots (300 mm height, 100 mm diameter) filled with ~1 kg of gravel (to ensure proper drainage) and 4.1 kg of soil mix. The soil mix consisted of one part sand and two parts of a loamy sand collected at the University of Western Australia farm (Ridgefield: S 32° 30’ 23” – E 116° 59’ 31’”, West Pingelly, Australia). Both soils were dried and sieved through a 4 mm mesh before they were mixed. The pots with the seedlings were then watered to field capacity and the soil surface was covered with plastic beads to minimise soil evaporation. The seedlings were grown in a glasshouse at the University of Western Australia for three months before they were moved into a controlled-environment room. Seedlings were moved into a controlled-environment room to ensure that measurements were taken under the same light and temperature conditions throughout the course of the experimental drought. Air relative humidity and temperature were continuously monitored using a logger (DT-172, CEM, Shenzhen, China). Over the course of the experiment, the mean temperature in the controlled-environment room was 26°C during the 12-hour days in artificial light and 19°C during the 12-hour nights. Along with the temperature, the vapour pressure deficit changed from 1.1 kPa at daytime to 0.2 kPa at night.

*Treatments and harvests*

Before the start of the experiment, plants were watered every 2-3 days to keep them well-watered. After six weeks, withholding water started the drought treatment. No water was added during the drydown period to avoid refilling of embolised vessels upon watering. To monitor soil water content (SWC) and whole plant transpiration, all pots were weighed every 2-3 days. Abaxial stomatal conductance was measured every 2-3 days using a porometer (SC-1 leaf porometer, Decagon Devices Inc., Pullman, WA, USA). For *E. wandoo*, which, unlike *E. marginata*, also has stomata on the adaxial side
of its leaves (see Chapter 3), the measured abaxial stomatal conductance values were multiplied with the empirically-obtained factor 1.86 to estimate total stomatal conductance. The pots were allowed to dry down for 17 to 20 days until they reached a SWC of below 8% (w/w), and were then rewatered. The rewatering treatment and physiological sampling was done over four days. One leaf per plant was sampled for predawn water potential ($\Psi_{PD}$) measurements and a leaf disc (16 mm diameter) was removed to obtain relative water content (RWC) of the leaf. One leaf per plant was placed in a foil-laminated bag at predawn for later xylem potential ($\Psi_{X}$) sampling. $\Psi_{PD}$, $\Psi_{X}$, and leaf water potential at harvest ($\Psi_{L}$) were measured using a Scholander-type pressure bomb (Model 1000 pressure bomb, PMS instruments, Albany, OR, USA). A total of three plants per species were not rewatered; these were harvested before the artificial lights in the controlled-environment room were turned on (i.e. predawn conditions). The rest of the plants were rewatered with 250 mL of water (restoring SWC to well-watered conditions) 15 minutes before the light went on to mark the start of daytime in the controlled environment room. During the drought recovery, I measured $g_{s}$ and harvested the plants at hourly intervals. At harvest, $\Psi_{L}$ and $\Psi_{X}$ as well as fresh weights for leaf RWC were measured immediately before the hydraulic conductivity measurements were carried out.

**Hydraulic conductivity measurements**

Intact plants were transported to the laboratory in the pots so the time lag between the removal of the shoot and the measurement was minimal. Immediately after the stem was cut at the base to remove the seedling, a 50 mm piece of the stem was cut under water. The second cut was made at least 40 mm above the first one to avoid the ‘tension-cutting artefact’ (Venturas et al., 2015). Five mm of bark and phloem were removed on either end of the stem segment and wrapped with Parafilm M (Bemis NA, Neenah, WI, USA). Hydraulic conductivity ($K_{h}$ in kg s$^{-1}$ m MPa$^{-1}$) is a measure of the bulk flow rate (F in kg s$^{-1}$) of a liquid relative to its driving force (i.e. pressure drop due to pressure head in MPa) and adjusted for the length of the flow path (i.e. length of the measured stem segment in m). Hydraulic conductivity was measured as outlined by Sperry et al. (1988); however, I used a flow meter (Liqui-Flow™, Bronkhorst High-tech B.V., Ruurlo, the Netherlands) instead of a balance. The flow meter was positioned between the pressure head (400 mm, 3.915 kPa) on one side and the stem segment on the other side. The flow rate of a 20 mM potassium chloride (KCl) solution through the
tubing was then measured by the flow meter. The flow meter was connected to a computer to assess when the flow through the stem segment was stable. Native conductivity was measured before embolisms were removed. To remove embolisms, the stem segment was flushed several times using a syringe filled with a KCl solution to remove all gas bubbles. To achieve a high and relatively stable pressure while flushing, the syringe was placed in a caulking gun and connected to a pressure gauge (100 kPa). Maximum hydraulic conductivity was then measured and PLC calculated. After the hydraulic conductivity measurements were completed and bark was removed, sapwood samples for non-structural carbohydrates (NSC) were snap-frozen in liquid nitrogen. The rest of the shoot was separated into leaves and stems and dried in an oven (80°C, 72 h).

**Non-structural carbohydrates**

The sapwood samples were kept in a -20°C freezer for four weeks before they were dried under vacuum (Benchtop K, VirTis, SP Industries Inc., Warminister, PA, USA). Samples were then ground using a ball mill grinder (Retsch, Haan, Germany). Fifty mg of ground sample material was extracted with 500 µL of 80% (v/v) ethanol in a hot water bath (30 min, 80°C). Samples were centrifuged (30 min at 6450 x g; Biofuge 13, Heraeus Instruments, Hanau, Germany) before the supernatant was transferred into Eppendorf tubes. The pellet was then resuspended in 500 µL of 80% (v/v) ethanol and the second extraction was done in the same way as the first one. Supernatants of both extractions were combined and 400 µL were transferred into HPLC tubes. The extracts were analysed for glucose, fructose and sucrose contents using HPLC (Chow & Landhäusser, 2004). The pellet was dried in a fume hood before the starch was solubilised with 350 µL of 0.2 M NaOH in a hot water bath (95°C, 30 min). The solution was neutralised with 140 µL of HCl/acetate buffer (0.5 M HCl and 0.1 M acetate). A subsample of 80 µL was taken from the solution and added to 600 µl of enzyme mix. The enzyme mix consisted of 2000 µL of 10% amylglucosidase solution and 20 µL of 10% α-amylase (Roche Diagnostics, Indianapolis, USA) in 50 mL of acetate buffer (50 mM). Samples were then incubated for 15 hours at 37°C (Hoch et al., 2002) and subsequently centrifuged at 6450 x g for 20 min. The supernatant was transferred into HPLC tubes for analysis. The starch concentration (g g⁻¹) of the samples was calculated by multiplying the glucose concentration with 0.9. A Waters 717 autosampler and 600E dual head pump (Waters Corp., Milford, USA) were used in
combination with an evaporative light scattering detector (Grace, Columbia, USA). Separation was performed on a Grace Prevail™ ES Carbohydrate column (250 mm x 4.6 mm) at 30°C with 5 µm particle size. The mobile phase consisted of 75% acetonitrile in milli-Q water (at a flow rate of 1 mL min⁻¹).

**Statistical analysis**

All statistical analyses were performed using R (R Development Core Team, 2010). Differences between treatments and species were tested using linear models. Whenever the selection criteria for a linear model (such as homogeneity of variance and normality) were violated, generalised additive models were used to model the response curves. Xylem recovery curves were fitted with binomial generalised linear models. For the analysis of percentage loss of conductivity as well as relative leaf water content against time after rewatering, data points were pooled to 2-hour intervals.

**Results**

Whole plant transpiration rates in both species decreased similarly with decreasing SWC ($P < 0.001$; Fig. 1a). Overall, transpiration rates were significantly faster in *E. wandoo* compared with *E. marginata* (almost 25%, $P < 0.001$). Transpiration rates were fastest when the SWC was around 12% (*E. marginata*: 25.4 g H₂O d⁻¹; *E. wandoo*: 28.5 g H₂O d⁻¹) corresponding to 12.4% faster transpiration rates in *E. wandoo* ($P = 0.016$). When the SWC reached around 6%, whole plant transpiration rates were reduced by over 50% in both species (58.5% in *E. marginata*: 8.1 g H₂O d⁻¹; 67.9% in *E. wandoo*: 11.8 ± 0.8 g H₂O d⁻¹), but *E. wandoo* continued to transpire more (45.6%, $P = 0.013$).

The differences in rate of water loss cannot be attributed to differences in leaf dry matter, as the leaf dry mass was 2.9 g in *E. marginata* and 2.4 g in *E. wandoo* ($P = 0.513$). Overall, the relationship between $\Psi_\text{PD}$ and SWC was strong ($r^2 = 0.56$, $P < 0.001$, $n = 45$; Fig. 1b) and the $\Psi_\text{PD}$ of both species responded similarly to the SWC.

However, *E. wandoo* used more water than *E. marginata*, resulting in a significantly lower SWC at harvest (*E. wandoo*: 6.0 ± 0.1% g H₂O g⁻¹ soil; *E. marginata*: 6.9 ± 0.4% g H₂O g⁻¹ soil; $P < 0.01$, $n = 48$). In response to differences in SWC, $\Psi_\text{PD}$ differed between the two species (*E. marginata*: -2.6 ± 0.3 MPa; *E. wandoo*: -3.3 ± 0.2 MPa; $P = 0.022$, $n = 45$). Since plants were harvested once the SWC had reached 8% or less,
some differences in $\Psi_{PD}$ between plants were observed before rewatering. Plants were therefore allocated to different harvest times to represent a similar distribution of $\Psi_{PD}$ within each group. There were no differences in $\Psi_{PD}$ between plants sampled at different times after rewatering.

**Figure 1** (a) Whole plant transpiration against soil water content for *Eucalyptus marginata* (open symbols) and *E. wandoo* (closed symbols). Solid lines represent means and dashed lines represent standard errors for each species fitted with a generalised additive model using a smoother for soil water content (SWC), respectively. (b) Predawn water potentials ($\Psi_{PD}$) against soil water content for *E. marginata* (open symbols) and *E. wandoo* (closed symbols). Lines are fitted with linear regressions (*E. marginata*: $P < 0.001$, $r^2 = 0.76$; *E. wandoo*: $P < 0.001$, $r^2 = 0.63$; $n = 434$).

Both species responded to the drying down of the soil by reducing their $g_s$ ($P < 0.001$; Fig. 2a). Overall, $g_s$ was higher in *E. wandoo* than in *E. marginata* ($P < 0.001$), and there was an interaction between SWC and species ($P < 0.001$). *Eucalyptus wandoo* exhibited 63% higher $g_s$ values than *E. marginata* when the SWC was 13 ± 1 % (*E. marginata*: 398 ± 31 mmol m$^{-2}$ s$^{-1}$, *E. wandoo*: 651 ± 46 mmol m$^{-2}$ s$^{-1}$, $P < 0.001$), but conductance values converged at low SWC. After rewatering, $g_s$ increased in both species with time after rewatering ($P < 0.001$; Fig. 2b). The species responded differently overall and in response to time after rewatering (significant interaction term; $P < 0.001$). During the hour before rewatering, $g_s$ in both species was around 50 mmol m$^{-2}$ s$^{-1}$ (46 ± 5.8 mmol m$^{-2}$ s$^{-1}$ in *E. marginata*; 52 ± 5.7 mmol m$^{-2}$ s$^{-1}$ in *E. wandoo*) and close to the lowest values previously measured throughout the drought period. After rewatering, $g_s$ was highest in *E. wandoo* after three hours (185 ± 29.5 mmol m$^{-2}$ s$^{-1}$) and then steadily declined to pre-rewatering levels. In *E. marginata*, $g_s$ initially increased and then stabilised four hours after rewatering. In this species, the highest $g_s$ was
recorded five hours after rewatering ($g_s$ after five hours: $266 \pm 50 \text{ mmol m}^{-2} \text{ s}^{-1}$), but did not significantly differ from values four, six or seven hours after rewatering.

![Figure 2](image)

**Figure 2** (a) Stomatal conductance against soil water content for *Eucalyptus marginata* (open symbols) and *E. wandoo* (closed symbol). Lines are fitted with linear regressions. (b) Progression of stomatal conductance after rewatering for *E. marginata* (open symbols) and *E. wandoo* (closed symbols). Vertical bars are standard errors of means.

As a consequence of the drought treatment, both species accumulated embolisms and showed large losses in hydraulic conductivity (Fig. 3a). The percentage loss in conductivity was $75 \pm 8\%$ in *E. marginata* seedlings and $93 \pm 3\%$ in *E. wandoo*. Both species showed a reduction in PLC (i.e. partial recovery from embolisms) two hours after rewatering. *Eucalyptus marginata* showed a continuous and significant reduction in PLC with time after rewatering ($P = 0.020$) and exhibited a PLC of $50 \pm 5\%$ six hours after rewatering. In *Eucalyptus wandoo* PLC initially decreased from almost $93 \pm 3\%$ to $56 \pm 22\%$ within the first two hours after rewatering. PLC then stabilised between $56 \pm 22\%$ and $66 \pm 6\%$ PLC after rewatering. $\Psi_L$ increased with time after rewatering indicating a recovery of $\Psi_L$ in response to rewatering ($P < 0.001$; Fig. 3b). $\Psi_L$ increased from $-2.6 \pm 0.7 \text{ MPa}$ before rewatering to around $-0.9 \text{ MPa}$ four hours after rewatering in *E. marginata* and from $-3.4 \pm 0.6 \text{ MPa}$ to about $-1.2 \text{ MPa}$ in *E. wandoo*. The RWC was $84 \pm 1\%$ in *E. marginata* seedlings before rewatering and increased to $96 \pm 1\%$ and $92 \pm 2\%$ four and six hours after rewatering, respectively. Time after rewatering did not significantly influence the RWC in *E. marginata* ($P = 0.068$); however, the RWC was significantly higher four and six hours after rewatering compared with before rewatering or two hours after rewatering ($P = 0.015$). In *E. wandoo*, RWC ranged between $85 \pm 10\%$ and $93 \pm 3\%$, and was not affected by time after rewatering. Xylem-recovery curves using PLC and $\Psi_X$ at harvest (i.e. after rewatering) showed that plants that achieved higher $\Psi_X$ after rewatering displayed lower PLC, particularly in *E.*
marginata (Fig. 4a). In *E. wandoo*, almost all plants achieved high $\Psi_X$, but not all of those displayed low PLC (Fig. 4b). The relationship between PLC and $\Psi_X$ was significant for *E. marginata* ($P < 0.001$), but not significant for *E. wandoo*.

**Figure 3** (a) Mean percentage loss in conductivity (PLC) against time after rewatering for *Eucalyptus marginata* (white bars) and *E. wandoo* (black bars). Vertical lines represent standard errors. (b) Leaf water potentials ($\Psi_L$) at time of harvest against the time after rewatering. Solid lines represent means and dashes lines represent 95% confidence intervals for each species fitted with a generalised additive model, respectively. (c) Relative water content (RWC) against time after rewatering for *E. marginata* (white bars) and *E. wandoo* (black bars). Vertical lines represent standard errors. Seedlings at time 0 hours were not rewatered.

The two *Eucalyptus* species exhibited similar fructose and sucrose concentrations in the wood tissue while showing significantly different concentrations of glucose and starch (Fig. 5a; $P = 0.021$ for glucose; $P = 0.039$ for starch). The glucose concentration was more than 50% greater in *E. wandoo* compared with that in *E. marginata* (4.6 ± 0.9 mg g$^{-1}$ DW in *E. marginata*; 7.1 ± 0.6 mg g$^{-1}$ DW in *E. wandoo*; $P = 0.021$). At the same time, the starch concentration was almost 30% greater in *E. marginata* compared with that in *E. wandoo* (6.8 ± 0.4 mg g$^{-1}$ DW in *E. marginata*; 4.9 ± 0.7 mg g$^{-1}$ DW in *E. wandoo*; $P = 0.039$). Fructose concentrations in wood tissues of *Eucalyptus* seedlings did not correlate with PLC or $\Psi_L$ at harvest. However, wood tissue fructose concentration was significantly greater when the $\Psi_{PD}$ was more negative (Fig. 5b) in *E. marginata* ($P < 0.001$, $r^2 = 0.74$) as well as *E. wandoo* ($P < 0.01$, $r^2 = 0.37$). There was no difference in the way the two species responded to decreasing $\Psi_{PD}$. Similar to fructose, the glucose concentration in wood tissue samples was not correlated with PLC or $\Psi_L$ at harvest. There was, however, a strong correlation between the glucose concentration and $\Psi_{PD}$ in *E. marginata* ($P < 0.001$, $r^2 = 0.77$; Fig. 5c) and *E. wandoo* ($P = 0.020$, $r^2 = 0.26$). Higher sucrose concentrations were observed when $\Psi_{PD}$ were lower, and there was a good correlation with $\Psi_{PD}$ in *E. marginata* ($P < 0.001$, $r^2 = 0.69$), but
not in *E. wandoo* (Fig. 5d). Starch concentrations did not correlate with any of the measured physiological parameters in either of the *Eucalyptus* species.

**Figure 4** Percentage loss in stem hydraulic conductivity (PLC) against xylem water potential at time of harvest (i.e. after rewatering) for *Eucalyptus marginata* ((a); open circles) and *E. wandoo* ((b); closed circles). Solid lines represent fitted values for the binomial generalised linear model; dashed lines represent corresponding 95% confidence intervals.

**Discussion**

The results of this experiment on two eucalypt species confirm fast recovery of stem hydraulic conductivity and leaf water status in both species, but contrasting patterns of recovery of transpiration. As a consequence of low soil water availability and very negative $\Psi_{PD}$, both species accumulated embolisms leading to almost 75 and over 90% loss in conductivity in *E. marginata* and *E. wandoo*, respectively. PLC was higher in *E. wandoo* than in *E. marginata*, opposite to reported vulnerabilities in field-sampled branches (Poot & Veneklaas, 2013), but this was most likely due to significantly lower SWC and $\Psi_{PD}$ before rewatering in *E. wandoo* compared with those in *E. marginata*. It is also possible that the short but intense experimental drought caused a somewhat different stress compared to seasonal stress in the field.

Upon rewatering, seedlings of both species showed a fast recovery of PLC, indicating that refilling of embolised xylem vessels started rapidly when soil water availability increased and while the xylem was under substantial tension. In *E. marginata*, recovery from embolisms as evidenced by a reduction in PLC was more gradual than in *E. wandoo*. PLC recovered from almost 75 to around 50% in only eight hours in
Figure 5 Concentrations of the non-structural carbohydrates (NSC) fructose, glucose, sucrose and starch in sapwood of *Eucalyptus marginata* (white bars) and *E. wandoo* (black bars) seedlings, respectively (a). Fructose (b), glucose (c), and sucrose (d) concentrations in *E. marginata* (open symbols) and *E. wandoo* (closed symbols) against predawn water potential. Lines (where present) represent linear regression fitted models.

*E. marginata*. Interestingly, PLC decreased substantially from over 90 to under 60% in *E. wandoo* within only two hours after rewatering and was ~65% towards the end of the experiment. These rates of recovery are similar to what has been found for *Eucalyptus pauciflora* where PLC decreased from almost 90% to control levels (~30%) within only 6 hours after rewatering (Martorell et al., 2014). Even though *E. wandoo* had significantly higher PLC before rewatering, it recovered to values similar to what was observed in *E. marginata* two and four hours after rewatering. However, *E. marginata* had significantly lower PLC than *E. wandoo* six hours after rewatering, suggesting that a more complete recovery from embolisms had occurred in *E. marginata*. The shape of the ‘xylem-recovery curves’ after rewatering supports this idea (Fig. 4). *Eucalyptus*
*Eucalyptus marginata* showed a curve that resembled xylem-vulnerability curves obtained by drying down branches in a laboratory environment. Nonetheless, hydraulic conductivity did not recover completely as the PLC values were still relatively high at high $\Psi_X$ in *E. marginata*. *Eucalyptus wandoo*, on the other hand, showed high variability in PLC when $\Psi_X$ were relatively high. When $\Psi_X$ was between -2.5 MPa and almost 0 MPa, PLC ranged between ~20 and over 90% in *E. wandoo*. This could be an indication that the refilling was delayed in some seedlings, but very efficient in others. I show that at least in seedlings, rapid refilling can occur and drought survival does not depend on the formation of new xylem as reported for *Callitris* species (Brodribb *et al.*, 2010).

It remains unclear what conditions led to variation in PLC at high water potentials found in *E. wandoo*, but I surmise that monosaccharides might be involved in the successful recovery after drought in both species. I found that both fructose and glucose concentrations increased substantially in response to more negative $\Psi_{PD}$. Lower $\Psi_{PD}$ was significantly associated with higher concentrations of both monosaccharides, fructose and glucose, sampled a few hours later. This supports the ideas put forward by Nardini *et al.* (2011; and references therein) that soluble sugars secreted into embolised xylem could be the driving force for refilling. It has also been suggested that the depolymerisation of starch is triggered by vessel wall vibrations associated with embolisms (Salleo *et al.*, 2008). It seems plausible that the accumulation of soluble sugars is greater with lower $\Psi_{PD}$, as the depolymerisation of starch would have started earlier or been more intense in response to a more intense soil drought. The correlation between $\Psi_{PD}$ and the concentration of monosaccharides was found in both species, but particularly strong in *E. marginata*, where refilling was more gradual and consistent. In *E. marginata*, but not in *E. wandoo*, the sucrose concentration also correlated with $\Psi_{PD}$.

It has been suggested that sucrose could trigger refilling, as it accumulates in stems even after artificial induction of embolisms (Secchi & Zwieniecki, 2011). These authors also showed that the presence of sucrose in the xylem after plant roots had been submerged in a sucrose solution led to starch depolymerisation in the wood tissue and suggested that sucrose could “induce a response similar to that which occurs after embolism induction” (Secchi & Zwieniecki, 2011).

I hypothesised that not only PLC but also other water relations parameters such as leaf water potential and stomatal conductance would increase in response to rewatering. In
both species, leaf water potential increased rapidly and substantially with time after rewatering and tended to stabilise around -1 MPa. 4 hours after rewatering. This shows that refilling of water-conducting vessels relaxed some of the tension in the soil to leaf continuum. Stomatal conductance increased quickly and continuously in *E. marginata* and reached pre-drought levels four to five hours after rewatering. In *E. wandoo*, however, the initial increase in stomatal conductance came to a halt after only three hours. Thereafter, stomatal conductance decreased rapidly and reached levels close to pre-rewatering at the end of the experiment. It has been shown before that stomatal opening can be delayed after rewatering, even when PLC has recovered (Lovisolo *et al.*, 2008). Martorell *et al.* (2014) found that stomatal conductance in *Eucalyptus pauciflora* seedlings had not recovered ten days after rewatering, even though PLC reached control levels after only six hours and attributed stomatal closure to a residual abscisic acid (ABA) signal in leaves. However, I found that *E. wandoo* seedlings initially increased stomatal conductance and it is therefore unlikely that a residual ABA signal in leaves was responsible for the subsequent reduction in stomatal conductance. I surmise that the translocation of accumulated ABA from roots or even the soil (increase of ABA in soils when water was limiting; Hartung *et al.*, 1996) could be responsible for the observed stomatal conductance patterns. It has been proposed that ABA leaks into the soil and could be taken up by roots with the soil solution once the soil water availability increases (Sauter *et al.*, 2001; and references therein). When water transport is hindered due to embolisms in water-conducting vessels, ABA transport from roots to leaves might be impaired. Upon rewatering, ABA could then be taken up from the soil and exported from the roots to leaves leading to a delayed stomatal response.

**Conclusions**

I show that seedlings of two *Eucalyptus* species can repair embolisms within hours after rewatering and while rapidly transpiring. In this study, the water status of *E. marginata* seedlings recovered steadily, but not completely, within eight hours after rewatering. Apart from PLC, the seedlings’ leaf water potential and leaf RWC recovered to levels recorded under well-watered conditions while stomatal conductance reached pre-treatment levels. *Eucalyptus wandoo* showed similar patterns, but only showed an initial increase in stomatal conductance followed by a sharp decrease to levels before rewatering. This may have been due to an accumulation and subsequent translocation of
ABA from roots to shoots. Overall, seedlings of both species responded similarly to drought and subsequent rewatering. As anticipated, the leaf water potential reached lower levels in *E. wandoo* than in *E. marginata*, which potentially allowed *E. wandoo* to extract more water from the soil, but resulted in higher PLC.
References


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Drought stress does not deplete non-structural carbohydrate pools in two *Eucalyptus* species with contrasting water-use strategies

**Summary**

- Seedlings of *Eucalyptus marginata* Sm. and *E. wandoo* Blakely were exposed to a 63-day drought to test if contrasting water use strategies would lead to differences in the dynamics of carbohydrate reserves and their role in embolism repair.

- Several harvests were carried out to measure predawn, xylem, and leaf water potentials, loss of stem hydraulic conductivity (PLC), and non-structural carbohydrates in leaves, stems and roots. In addition, I monitored soil water content and stomatal conductance.

- The relatively isohydric species *Eucalyptus marginata* reduced its stomatal conductance and thus maintained high leaf water potentials ($\Psi_L$) of approx. -2 MPa and displayed a PLC of about 50%. In addition, *E. marginata* showed complete recovery of stem hydraulic conductivity within 18 hours after rewatering while the xylem was still under substantial tension. The relatively anisohydric species *Eucalyptus wandoo* only reduced its stomatal conductance when $\Psi_L$ were below -3 MPa, and upon rewatering recovered its water status but did not restore stem conductivity and transpiration completely: its PLC was still close to 60%.

- Despite contrasting water-use strategies, total non-structural carbohydrate concentrations were not depleted in droughted seedlings of either *Eucalyptus* species. Starch concentrations were only reduced in leaves of droughted *E. marginata* as well as droughted and rewatered *E. wandoo* seedlings but not significantly altered by drought or rewatering in sapwood, bark and cambium, or roots. High soluble sugar concentrations in the sapwood could be significant for osmoregulation and embolism repair in *Eucalyptus* seedlings during drought.
Introduction

The occurrence of climate-related tree mortality in forests across the world (Allen et al., 2010) highlights the importance of understanding the processes leading to tree death, especially if we aim to predict responses of forests to a future drier and hotter climate (McDowell et al., 2011). Currently, the physiological mechanisms leading to drought-induced tree mortality and recovery from drought are not entirely understood. It is clear, however, that plant water and carbon relations are inevitably linked through stomatal regulation, and two main mechanisms have been proposed to explain tree death during drought. Firstly, plants could die from hydraulic failure if the water loss was not sufficiently regulated and the accumulation of xylem embolisms impeded water transport. Secondly, avoiding water loss through stomatal closure could lead to reduced carbon assimilation and result in carbon starvation. McDowell et al. (2008) proposed that the mechanism leading to tree death could depend on water-use strategies (i.e. isohydric versus anisohydric) in combination with the duration and intensity of the drought. They suggested that carbon starvation only occurs when the drought intensity is low but the duration long, whereas a short but intense period of drought would lead to hydraulic failure.

The evidence for carbon starvation as a mechanism for drought-induced tree mortality is limited and it has been debated whether or not carbon starvation occurs under natural conditions, since plants often accumulate sugars under drought conditions (Hartmann et al., 2013). This is thought to be a result of a sustained reduction in sink demand as plant growth ceases before photosynthesis is affected (Hsiao, 1973; Muller et al., 2011). In addition, carbon demands for respiration can be down-regulated, which could delay the moment that carbon reserves would reach critical levels (Hartmann et al., 2013; McDowell & Sevanto, 2010). Despite the fact that carbon pools have been shown to decrease in some studies (e.g. Quirk et al., 2013, Hartmann et al., 2013), it remains difficult to link reductions in carbon pools to tree death, mainly because tree death occurred when carbohydrate reserves were not completely depleted but substantially reduced (e.g. >80%; Galiano et al., 2011). In addition, an accumulation of starch but a depletion of soluble sugars may indicate that the use of stored starch was impaired during drought. Currently, it is unknown if carbohydrates can even be utilised until all pools are exhausted (McDowell & Sevanto, 2010).
While it remains unclear if carbon starvation is a common cause of tree death under natural conditions, the experimental manipulation of carbon pools has given us insight into carbon metabolism during drought. O’Brien et al. (2014), for instance, altered carbohydrate pools but not growth in seedlings of ten tropical tree species by applying different sequences of low and high light intensities. They showed that increased carbon pools extended the period that plants survived during drought and suggested that this was due to a greater capacity to maintain hydraulic function. Sevanto et al. (2014) suggested that hydraulic failure could be associated with a reduced ability for osmoregulation when carbon pools are low. At the same time, Klein et al. (2014) pointed out that the ability to repair embolisms might be more important than just maintaining water potentials ($\Psi$) above a certain threshold, and there is increasing evidence that carbohydrates have an important role during the refilling of embolised vessels. Salleo et al. (2009), for example, observed that the starch concentration in wood parenchyma cells decreased during xylem refilling and increased again after refilling was completed. They suggested that starch depolymerisation in wood parenchyma cells and subsequent transport of sugars to the xylem drives the inward flow of water and lead to refilling of vessels. The increased sink activity could then result in enhanced unloading of sugars and water from the phloem (Nardini et al., 2011). Zwieniecki & Holbrook (2009) proposed that xylem-parenchyma cells could continuously secrete sugars into vessels and that the sugars accumulate upon cavitation and assist in osmotically-driven refilling. Secchi & Zwieniecki (2011) later suggested that sucrose could be secreted into vessels and trigger refilling once its concentration in embolised vessels reached a certain threshold. In summary, carbohydrate reserves might not often get depleted enough during drought to cause tree mortality, but non-structural carbohydrates certainly are likely important during drought and the recovery from drought.

Since it has been suggested that water-use strategies could determine how trees die, I used two Eucalyptus species with contrasting water-use strategies to study aspects of carbon and water relations during drought and the recovery from drought. Both species, Eucalyptus marginata Sm. and E. wandoo Blakely, are endemic to south-western Australia and are common forest and woodland species. The two species have distinct distribution ranges and largely grow on contrasting soil types, however, their habitat shows some overlap (Verboom & Pate, 2015). Eucalyptus marginata is dominant in
areas of comparatively higher rainfall (600 - 1250 mm annual precipitation; Boland et al., 2006) and reduces its water use in response to dry conditions and thus maintains high leaf water potentials (Poot & Veneklaas, 2013), unless it can access water resources with its deep root system (Dell et al., 1983). *Eucalyptus wandoo* predominantly grows in drier areas (400 - 700 mm annual precipitation; Boland et al., 2006) and develops considerably low \( \Psi_L \) before substantially reducing its stomatal conductance (Colquhoun et al., 1984). Poot & Veneklaas (2013) studied water relations of *E. marginata* and *E. wandoo* in the field, where they naturally co-occurred, and found that *E. wandoo* displays lower predawn \( (\Psi_{PD}) \) and midday \( (\Psi_L) \) water potentials as well higher stomatal conductance throughout most of the study period (i.e. over one year for most of the measured parameters). In addition, loss of 50% conductivity occurs at much lower xylem water potentials in *E. wandoo* than in *E. marginata*. *Eucalyptus marginata* is considered to be a relatively isohydric species, while *E. wandoo* is known to be a species with a relatively anisohydric water-use strategy.

The present experiment was designed to study carbon and water relations over a long period of progressive drought. I aimed at investigating the relationship between carbohydrate concentrations and the ability of *Eucalyptus* seedlings to repair embolisms upon rewatering. Based on previously observed differences in water-use strategies, I predicted that drought would affect carbon and water relations differently in the two *Eucalyptus* species. I hypothesised that *E. marginata* would show an early reduction in stomatal conductance in response to drought and thus maintain high \( \Psi_L \). As a result of reduced stomatal conductance, carbohydrate pools would likely decline over the course of the experiment. This could then reduce the ability of *E. marginata* seedlings to repair embolisms. In contrast, I hypothesised that *E. wandoo* would maintain higher stomatal conductance throughout most of the experiment and consequently display lower \( \Psi_L \) and relatively high levels of carbohydrate reserves. The increased tension in the xylem would result in an accumulation of embolisms, which could lead to a loss in hydraulic conductivity despite high carbohydrate concentrations. Assuming that embolism repair is related to high carbohydrate concentrations, I expected better embolism repair in *E. wandoo* than in *E. marginata*. 
**Materials and Methods**

*Plant material and experimental setup*

One and a half year old *E. marginata* Sm. and *E. wandoo* Blakely seedlings were obtained from Muchea Tree Farm (Muchea, WA, Australia). Ninety seedlings per species were transplanted into free-draining pots made out of stormwater pipes (840 mm long, 150 mm diameter). The bottom 5 cm of the pots was covered with gravel to ensure good drainage. The pots were then filled with 20.0 kg of a soil mix consisting of one part sand and two parts of a loamy sand (w/w) collected at the University of Western Australia farm (Ridgefield: S 32° 30’ 23’’ – E 116° 59’ 31’’, West Pingelly, Australia). Both soils were dried and sieved (4 mm mesh size) before they were mixed. After transplanting in December 2013, plants were kept well-watered by hand-watering them daily. At the same time, a slow-release irrigation system (2 L/h) was set up to facilitate establishment of the seedlings. The seedlings were watered six times a day with c. 33 mL using this irrigation system. The pots were arranged in four blocks (3 x 50 and 1 x 35 pots) in a glasshouse at the University of Western Australia (Crawley, WA, Australia) and the treatments were allocated in a Latin square design within the block. The irrigation system was removed six weeks before the start of the experiment and pots were watered to the pot weight corresponding with a soil water content (SWC) of 13% until the experiment was started. Throughout the course of the experiment, all pots were weighed twice a week to calculate SWC and transpiration and to adjust pot weights of well-watered control pots to a SWC of 13%. Pots with no seedling were used to measure evaporation from well-watered and droughted (i.e. non-rewatered) pots. The initial number of such pots was five and four, respectively, but after every harvest, additional pots from which the seedling had been removed, were added to measure water loss through soil evaporation. The water content and water distribution in these pots was assumed to best represent that of the pots that did still have plants.

*Treatments and harvests*

Almost six months after transplanting, 66 *E. marginata* and 75 *E. wandoo* seedlings of similar size were selected for the experiment and allocated to the treatment groups and harvest dates. From day 0, pots were either well-watered (control) or not watered. Plants in the drought treatment were never watered again, whereas plants in the rewatering treatment only received water on the day before their harvest. Since I expected less
variability over time in the well-watered control plants, only five replicates were allocated to this treatment for each of the harvests on days 0, 35, and 63 of the experiment. For the drought and rewatering treatment, seven replicates were allocated to each harvest carried out on day 0, 28, 35, 42, and 63 for *E. marginata* and day 0, 28, 35, 42, 49, and 63 for *E. wandoo*. The last harvest was conducted before the soil water content reached critical levels for plant survival to avoid mortality. A set of spare seedlings, which experienced the same drought treatment but were excluded from the experiment because they were larger than most other seedlings, were monitored during the drydown. The critical soil water content was determined based on the SWC that was reached when the spare seedlings started to show signs of complete tissue desiccation in leaves, which was later followed by leaf discolouration and leaf shedding. Rereowering of rewatered seedlings was conducted in the evening before each harvest by adding 1 L of water to each pot. Assuming that the SWC was below 4% in the upper part of the soil towards the end of the drought period due to root water uptake and evaporation, 1 L of water would have been enough to increase the SWC to more than 15% in the top third of the pot without risking loss by drainage. Due to the large number of samples, each harvest was carried out over a period of one to three days. Replicates from each of the three treatments as well as the two species were spread evenly over the harvest days, to reduce potential effects of confounding factors (e.g. cloud cover) on physiological measurements. Temperature and relative humidity within the glasshouse were measured every 15 minutes using a SHD100 temperature and humidity sensor (Schneider Electric, Rueil-Malmaison, France). The light intensity was recorded every 15 minutes directly above the glasshouse using a LI-190SA quantum sensor (LI-COR® Biosciences, Lincoln, Nebraska, USA). The temperature ranged between 10 and 27°C over the course of the experiment and was on average 17°C. During the experiment, the relative humidity reached a maximum of 80% during the night and a minimum of 40% during the day. The light intensity was higher at the start of the experiment and reached daily maxima of more than 1200 µmol m⁻² s⁻¹ but decreased to daily maxima of approx. 600 µmol m⁻² s⁻¹.

**Physiological measurements**

In the evening before each harvest, seedlings were wrapped in opaque plastic bags. Predawn water potentials were measured at 7:30 h after which the bags were removed. Predawn, midday (Ψₒ) and stem xylem water potentials were measured on one leaf per
seedling using a Scholander-type pressure bomb (Model 1000, PMS instrument company, Albany, OR, USA). Leaves previously used for $\Psi_L$ measurements were snap-frozen in liquid nitrogen for later non-structural carbohydrate analyses. At midday, one leaf per seedling was wrapped more than one hour before seedlings were harvested to let the leaf equilibrate with the stem xylem and determine $\Psi_X$. Thereafter, $g_s$ was measured using a leaf porometer (SC-1 Leaf porometer, Decagon Devices, Pullman, WA, USA). In contrast to *E. marginata*, *E. wandoo* has stomata on both sides of the leaves, I thus measured adaxial and abaxial $g_s$ and combined the two obtained values. At 13:00 h, seedlings were removed from the pot by gently pulling them out of the soil. They were immediately wrapped in opaque plastic bags and transported to the nearby laboratory for hydraulic conductivity measurements. Leaves were removed from the shoot and tissue samples of sapwood, bark including cambium and the upper tap root (removed from approximately 40 mm below the shoot-root interface) were snap-frozen in liquid nitrogen and stored at -80°C for later non-structural carbohydrate analyses. The time lag of 18 h between rewatering and harvesting was chosen as physiological parameters responded quickly to rewatering in Chapter 2. In addition, physiological measurements such as water potentials and stomatal conductance had to be completed before the destructive harvest. A subsample of leaves was weighed and scanned to determine leaf area (LA). Leaves and stems were weighed and then dried in an oven (80°C, 96 h). Whole-plant stomatal conductance ($g_{sp}$) was calculated to give a measure of water use by multiplying the leaf-area based stomatal conductance with leaf area values ($g_{sp} = g_s \times LA$).

*Hydraulic conductivity measurements*

Hydraulic conductivity ($K_h$ in kg s$^{-1}$ m MPa$^{-1}$) is a measure of the bulk flow rate ($F$ in kg s$^{-1}$) of a liquid relative to its driving force (i.e. pressure in MPa) and adjusted for the length of the flow path (i.e. length of the measured stem segment in m). Sapwood-specific conductivity ($K_s$ in kg s$^{-1}$ m$^{-1}$ MPa$^{-1}$) represents $K_h$ normalised for the sapwood area of the measured stem segment. The bulk flow rate was measured as described by Sperry *et al.* (1988) using a flow meter (Liqui-Flow™, Bronkhorst High-tech B.V., Ruurlo, The Netherlands) instead of a balance. The harvested stem (including upper tap root) was cut under water just above the root-shoot interface. The stem was then cut again about 5 cm above the first and was also done under water. The second cut was made to avoid the ‘tension-cutting artefact’ (Venturas *et al.*, 2015). An approximately 4
A 40 cm piece was cut off and 5 mm of bark and cambium were removed at either end of the stem segment and wrapped in Parafilm M (Bemis NA, Neenah, WI, USA) before it was connected to tubing at the down-stream end of the flow meter. The other end of the flow meter was connected to a pressure head (400 mm, 3.915 kPa). The flow meter was connected to a computer that allowed plotting and monitoring of the flow of a 20 mM potassium chloride (KCl) solution through the stem segment. After recording the native hydraulic conductivity, the stem segment was flushed several times to remove gas bubbles using a syringe that was filled with KCl solution and placed in a caulking gun. The caulking gun was connected to a pressure gauge in order to achieve a high (100 kPa) and relatively stable pressure. After flushing, the flow through the stem segment was measured for a second time to obtain a value of maximum hydraulic conductivity. The diameter and length of the stem segments were measured using callipers. The diameter was measured twice on each end of the stem segment and values were averaged to calculate sapwood area. The native sapwood-specific conductivity (K_{sn}) represents K_{s} calculated from the first measurement of the bulk flow rate, whereas maximum sapwood-specific conductivity (K_{smax}) represents K_{s} calculated from the second measurement of the bulk flow rate (after flushing of the stem segment to remove gas bubbles).

**Non-structural carbohydrate measurements**

The tissue samples were kept at -20°C for four weeks before they were dried under vacuum in a freeze-dryer (Benchtop K, VirTis, SP Industries Inc., Warminster, PA, USA). Samples were then ground into a fine powder using a ball mill grinder (Retsch, Haan, Germany). Fifty mg of ground sample material was extracted with 500 µL of 80% (v/v) ethanol in a hot water bath (30 min, 80°C). Samples were centrifuged (30 min at 6450 x g; Biofuge 13, Heraeus Instruments, Hanau, Germany) before the supernatant was transferred into Eppendorf tubes. The pellet was then resuspended in 500 µL of 80% (v/v) ethanol and the second extraction was done in the same way as the first one. Supernatants of both extractions were combined and 400 µL were transferred into HPLC tubes. The extracts were analysed for glucose, fructose and sucrose contents using HPLC (Chow & Landhäusser, 2004). The pellet was dried in a fume hood before the starch was solubilised with 350 µL of 0.2 M NaOH in a hot water bath (95°C, 30 min). The solution was neutralised with 140 µL of HCl/acetate buffer (0.5 M HCl and 0.1 M acetate). A subsample of 80 µL was taken from the solution and added to 600 µL
of enzyme mix. The enzyme mix consisted of 2000 µL of 10% amyloglucosidase solution and 20 µL of 10% α-amylase (Roche Diagnostics, Indianapolis, USA) in 50 mL of acetate buffer (50 mM). Samples were then incubated for 15 hours at 37°C (Hoch et al., 2002) and subsequently centrifuged at 6450 x g for 20 min. The supernatant was transferred into HPLC tubes for analysis. The starch concentration (g g⁻¹) of the samples was calculated by multiplying the glucose concentration with 0.9. A Waters 717 autosampler and 600E dual head pump (Waters Corp., Milford, USA) were used in combination with an evaporative light scattering detector (Grace, Columbia, USA). Separation was performed on a Grace Prevail™ ES Carbohydrate column (250 mm x 4.6 mm) at 30°C with 5 µm particle size. The mobile phase consisted of 75% acetonitrile in milli-Q water (at a flow rate of 1 mL min⁻¹).

**Statistical analyses**

Statistical analyses and graphing were performed using the software R (R Development Core Team, 2010). Linear models were generally used to analyse the data. The suitability of linear models was then confirmed by testing for violation of independence, homogeneity of variances, and normality of residuals. A generalised additive model was used to plot and analyse the relationship between ΨPD and SWC as well as the relationship between soluble sugars and ΨPD.

**Results**

In both species, the SWC in the drought treatment was much lower than that of well-watered controls, but reached lower levels of 4.1% in *E. wandoo* compared with 6.5% in *E. marginata* after water was withheld for 63 days (Fig. 1a, c). Along with decreasing SWC, ΨPD declined in both species over the course of the experiment (*P* < 0.001; Fig. 1b, d) but the relationship between ΨPD and SWC was different between the two species (*P* < 0.01), mainly because relatively anisohydric species *Eucalyptus wandoo* reached a lower minimum ΨPD of -5.2 MPa compared with a minimum ΨPD of -3.5 MPa in the relatively isohydric species *E. marginata* (*P* < 0.05). Mean ΨL in well-watered seedlings of both species stayed above -1.3 and -1.4 MPa in *E. marginata* and *E. wandoo*, respectively, throughout the experiment (Fig. 1e and g). The ΨL started to decrease 42 days after the start of the drought treatment in both species and mean ΨL
Figure 1 M e a n  ±  S E  o f  t h e  s o i l  w a t e r  c o n t e n t  ( S W C )  o v e r  t h e  c o u r s e  o f  t h e  e x p e r i m e n t  f o r  w e l l 
-watered (open symbols) and droughted (closed symbols) Eucalyptus marginata (a; n = 5-35) and E. wandoo (c; n = 5-42) seedlings, respectively. The relationship between predawn water potential (Ψ\(_{PD}\)) and SWC using values from well-watered and droughted but not rewatered seedlings of the relatively isohydric species E. marginata (b; \(r^2 = 0.73; n = 40\)) and the relatively anisohydric species E. wandoo (d; \(r^2 = 0.54; n = 44\)). Mean ± SE of midday water potentials (\(\Psi_{L}\)) on harvest days through the course of the experiment for well-watered (open symbols, n = 5), droughted (closed symbols, n = 7), and rewatered (crossed symbols, n = 7) seedlings of E. marginata (e) and E. wandoo (g), respectively. Lines in (e) and (g) represent mean \(\Psi_{L}\) for well-watered seedlings during the experiment and were fitted using linear regression. The relationship between \(\Psi_{L}\) and \(\Psi_{PD}\) for E. marginata (f; \(n = 65, r^2 = 0.69\)) and E. wandoo (h; \(n = 73, r^2 = 0.84\)). Lines were fitted using linear regression. Measurements were conducted about 18 hours after rewatering.
values were up to c. 0.9 MPa and 3.3 MPa lower in droughted seedlings of *E. marginata* and *E. wandoo*, respectively, compared with well-watered controls (*P* < 0.01). In *E. marginata*, the Ψₗ was higher in rewatered seedlings compared with well-watered seedlings at the end of the experiment (*P* < 0.001). In *E. wandoo*, on the other hand, Ψₗ in rewatered seedlings were generally lower than in well-watered seedlings but higher than in droughted seedlings (*P* < 0.001). The relationship between Ψₗ and Ψₚₐ was strong in both species (*P* < 0.001, *r*² = 0.86; Fig. 1f and h) but not affected by treatments.

Mean PLC was relatively constant over the course of the experiment in well-watered seedlings of both species and ranged between 21 and 33% in *E. marginata* and 28 and 34% in *E. wandoo*. However, the overall response of the two species to the treatments was different (*P* < 0.05) and there was a significant interaction between species, treatment, and time (*P* < 0.01, Fig. 2a and c). The two species were therefore analysed separately. In the relatively isohydric species *E. marginata*, PLC increased in the droughted seedlings (*P* < 0.05) and only 42 days after the start of the drought treatment (*P* < 0.01), whereas rewatered seedlings recovered to control levels throughout the entire experiment. At the end of the experiment, droughted seedlings of *E. marginata* had a PLC of about 51 ± 12% while well-watered and rewatered seedlings had a lower PLC of 21 ± 5% and 18 ± 4%, respectively. In *E. wandoo*, PLC was the same in droughted compared with well-watered seedlings during most of the experiment but it increased towards the end of the experiment (*P* < 0.05). Surprisingly, rewarered *E. wandoo* seedlings showed a higher PLC of 57 ± 7% compared with 32 ± 8% in well-watered and 40 ± 9% in droughted seedlings at the end of the experiment (*P* < 0.05). The relationship between PLC and Ψₓ was weak in both species but well-watered and rewatered *E. marginata* seedlings generally showed lower PLC (under 40%) and higher Ψₓ (above -2 MPa) compared with droughted seedlings. In *E. wandoo*, Ψₓ and PLC of rewatered seedlings were similar to observations from well-watered seedlings but droughted seedlings showed unexpectedly low PLC of ~40% at low Ψₓ of about -4 MPa. The low PLC observed in droughted *E. wandoo* during the last harvest was due to ~20% lower maximum sapwood-specific conductivity (Kₛₚₐ; i.e. Kₛ after flushing of the stem segment) of ~80 kg s⁻¹ m⁻¹ MPa⁻¹ in droughted seedlings compared with ~100 kg s⁻¹ m⁻¹ MPa⁻¹ in well-watered seedlings, while Kₛₚₐ was 20% higher in rewatered *E. wandoo* seedlings than in well-watered controls. Therefore, PLC was lower in
Figure 2: Progression of the percentage loss in hydraulic conductivity (PLC; mean ± SE) over the course of the experiment for well-watered (open symbols), droughted (closed symbols), and rewatered (crossed symbols) seedlings of the relatively isohydric species *Eucalyptus marginata* (a; n = 5-7) and the relatively anisohydric species *E. wandoo* (b; n = 6-9). Measurements were conducted 15 hours after rewatering had occurred. Grey dashed lines represent well-watered averages over time. Vertical lines, where present, denote standard errors. Black lines in (j) and (k) were fitted using linear regression.

Hydraulic conductivity (K) and PLC against xylem water potential (Ψ) for *E. marginata* (a, c) and *E. wandoo* (e, g). PLC against stomatal conductance (gsp) over the course of the experiment for well-watered (f) and rewatered (h) seedlings of the relatively isohydric species *E. marginata* (a, c) and *E. wandoo* (e, g). Stomatal conductance (gs) over the course of the experiment for well-watered (f) and rewatered (h) seedlings of the relatively anisohydric species *E. marginata* (a, c) and *E. wandoo* (e, g). Measurements were conducted 18 hours after rewatering had occurred. Grey dashed lines represent well-watered averages over time. Vertical lines, where present, denote standard errors. Black lines in (f) and (h) were fitted using linear regression.
droughted than in rewatered *E. wandoo* seedlings, despite the same K\textsubscript{sn} (i.e. native sapwood-specific conductivity) of ~50 kg s\textsuperscript{-1} m\textsuperscript{-1} MPa\textsuperscript{-1}. The opposite was the case in *E. marginata*, where rewatered seedlings showed an almost 30% reduction in K\textsubscript{smax} compared with well-watered controls. Consequently, a K\textsubscript{sn} of 55 kg s\textsuperscript{-1} m\textsuperscript{-1} MPa\textsuperscript{-1} relative to a K\textsubscript{smax} of ~70 kg s\textsuperscript{-1} m\textsuperscript{-1} MPa\textsuperscript{-1} resulted in 20% PLC and gave the impression of highly successful embolism refilling. In droughted *E. marginata* seedlings, K\textsubscript{smax} was reduced by ~20% to c. 80 kg s\textsuperscript{-1} m\textsuperscript{-1} MPa\textsuperscript{-1} but K\textsubscript{sn} was much lower (~30 kg s\textsuperscript{-1} m\textsuperscript{-1} MPa\textsuperscript{-1}) and thus resulted in a higher PLC of about 60%. The reduction in K\textsubscript{smax} of 20 and 30% in droughted and rewatered seedlings, respectively, was not statistically significant.

In both species, g\textsubscript{s} decreased somewhat over the course of the experiment in well-watered seedlings (Fig. 2e and g; P < 0.05) but ranged between c. 300 and over 500 mmol m\textsuperscript{2} s\textsuperscript{-1} in *E. marginata* and between c. 400 and 560 mmol m\textsuperscript{2} s\textsuperscript{-1} in *E. wandoo* over the course of the experiment. In the relatively isohydric species *E. marginata*, g\textsubscript{s} was the same in the droughted and rewatered seedlings throughout the experiment but declined with time compared with that in well-watered seedlings (P < 0.001). On the last day, g\textsubscript{s} was reduced by more than 50% in droughted and about 20% in rewatered compared with well-watered seedlings. In the relatively anisohydric species *E. wandoo*, droughted and rewatered seedlings had a high g\textsubscript{s} that reached a maximum of ~800 mmol m\textsuperscript{2} s\textsuperscript{-1} during the first 35 days of the experiment. Thereafter, g\textsubscript{s} decreased rapidly and was reduced by over 60% to c. 140 ± 40 mmol m\textsuperscript{2} s\textsuperscript{-1} in the droughted and rewatered compared with the well-watered seedlings at the end of the experiment (P < 0.001). In *E. marginata*, the relationship between whole-plant stomatal conductance (g\textsubscript{sp}) and hydraulic conductivity (K\textsubscript{h}) was relatively weak (P < 0.05, r\textsuperscript{2} = 0.28) and not affected by treatments. Also in *E. wandoo*, the relationship between g\textsubscript{sp} and K\textsubscript{h} (P < 0.001, r\textsuperscript{2} = 0.52) was not affected by treatments, but well-watered seedlings had a higher g\textsubscript{sp} and K\textsubscript{h} than droughted and rewatered seedlings (P < 0.05).

Seedlings of both species showed an accumulation of soluble sugars with decreasing Ψ\textsubscript{PD} in leaves as well as in sapwood over the course of the experiment (P < 0.001; Fig. 3a to d). In *E. marginata*, soluble sugar concentrations were about twice as high in leaves when Ψ\textsubscript{PD} values were below -3.0 MPa compared with Ψ\textsubscript{PD} values of about -0.5 MPa. The same soluble sugar concentration of ~40 mg g\textsuperscript{-1} dry weight was observed at
Ψ_{PD} above and below -0.5 MPa in rewatered and droughted *E. marginata* seedlings, respectively. Soluble sugars in rewatered seedlings showed a similar range of concentrations as in droughted seedlings but at lower Ψ_{PD} (P < 0.05). Also in *E. wandoo*, a twofold increase in the concentration of soluble sugars in leaves was observed, however, rewatering did not affect the relationship between the concentration of soluble sugars and Ψ_{PD}, as there were both small increases in Ψ_{PD} and small decreases in sugar concentrations. The accumulation of soluble sugars in sapwood in response to decreasing Ψ_{PD} was similar to that observed in leaves (P < 0.001). Both species showed a more than twofold increase in soluble sugar concentrations when Ψ_{PD} decreased from around -0.5 MPa to less than -3.0 MPa. Again, rewatering affected the relationship between soluble sugars and Ψ_{PD} only in *E. marginata* (P < 0.001) but not in *E. wandoo*, due to lower observed Ψ_{PD} at the same soluble sugar concentrations in rewatered compared with droughted *E. marginata* seedlings.

Towards the end of the drought treatment, total NSC concentrations in leaves were the same in all treatments and species, being around 66 mg g^{-1} dry weight (Fig. 4a). In *E. marginata*, glucose concentrations were higher in droughted seedlings than in well-watered and rewatered seedlings (P < 0.001). In contrast, glucose concentrations were the same in all treatments in *E. wandoo* seedlings. In *E. marginata*, fructose concentrations were almost twice as high in droughted compared with well-watered and rewatered seedlings (P < 0.01). In *E. wandoo*, on the other hand, fructose concentrations were the same in all treatments. Sucrose concentrations were the same in all treatment groups and species. Starch concentrations were slightly lower in droughted seedlings of *E. marginata* compared with well-watered (P = 0.05) but not rewatered (P = 0.08) seedlings. In *E. wandoo*, starch concentration was higher in well-watered than in droughted and rewatered seedlings (P < 0.05). In sapwood, total NSC concentrations were the same in both species but highest in droughted seedlings (P < 0.01; Fig. 4b). This was because concentrations of glucose and fructose but not of sucrose were greater in droughted *E. marginata* seedlings than in well-watered and rewatered seedlings (P < 0.01 for fructose and glucose). In *E. wandoo*, higher sucrose concentrations in droughted seedlings led to higher total NSC concentrations in droughted seedlings (P < 0.05). Sapwood starch concentrations were the same across species and treatments. Similar to sapwood, the highest total NSC concentrations of the bark including cambium were observed in droughted seedlings (P < 0.05; Fig. 4c). In *E. marginata,*
Figure 3 Soluble sugar concentration in leaves (top panels) and sapwood (bottom panels) of well-watered (open symbols), droughted (closed symbols), and rewatered (crossed symbols) *Eucalyptus marginata* (left panels) and *E. wandoo* (right panels) seedlings plotted against predawn water potentials ($\Psi_{PD}$). Rewatering occurred in the evening prior to the harvest day. Predawn water potentials were measured c. 13.5 hours after rewetting and the leaf and sapwood samples were taken c. 18 hours after rewetting. Lines represent the relationship between soluble sugars and $\Psi_{PD}$ and were fitted using generalised additive models ($n = 64\text{-}72$). Dashed lines represent 95% confidence intervals. Grey lines, where present, represent a different fit for rewatered seedlings where this relationship was significantly different to that of well-watered and droughted seedlings combined (black lines).

Glucose as well as fructose concentrations were almost three times greater in droughted than in well-watered and rewatered seedlings ($P < 0.01$). The sucrose concentration was higher in droughted and rewatered seedlings compared with well-watered seedlings in *E. marginata* ($P < 0.05$) but starch concentrations were the same across treatments. In *E. wandoo*, glucose concentrations were about twice as high in droughted compared with well-watered and rewatered seedlings ($P < 0.05$). Despite an about twice as high fructose concentrations in droughted seedlings, the fructose concentration in droughted seedlings was not statistically different from that of well-watered and rewatered
seedlings ($P = 0.11$). Also the sucrose and starch concentrations were the same across treatments in *E. wando*. Total NSC concentrations were more than 40% higher in roots of *E. wando* seedlings compared with *E. marginata* seedlings (Fig. 4d; $P < 0.01$) but not affected by the treatments. The higher total NSC concentrations in *E. wando* compared with *E. marginata* were due to ~70% higher concentrations in glucose and fructose ($P < 0.001$), respectively, as well as about four times higher starch concentrations ($P < 0.01$). Sucrose concentrations were the same in both species. In

![Graphs showing non-structural carbohydrate concentrations in tissue samples of well-watered (W; $n = 5$-14), droughted (D; $n = 7$-21), and rewated (R; $n = 7$-21) *Eucalyptus marginata* (right) and *E. wando* (left) seedlings. Tissue samples from leaves (a), sapwood (b), were collected 42 and 63 days after the start of the experiment for *E. marginata* and 42, 49 and 63 days after the start of the experiment for *E. wando*. Tissue samples of bark and cambium (c), as well as the upper tap root (d) were only collected 63 days after the start of the experiment. Rewatering occurred c. 18 hours before samples were collected. Vertical lines represent standard errors.](image)

**Figure 4** Average non-structural carbohydrate concentrations (i.e. glucose, fructose, sucrose, and starch) in tissue samples of well-watered (W; $n = 5$-14), droughted (D; $n = 7$-21), and rewatered (R; $n = 7$-21) *Eucalyptus marginata* (right) and *E. wando* (left) seedlings. Tissue samples from leaves (a), sapwood (b), were collected 42 and 63 days after the start of the experiment for *E. marginata* and 42, 49 and 63 days after the start of the experiment for *E. wando*. Tissue samples of bark and cambium (c), as well as the upper tap root (d) were only collected 63 days after the start of the experiment. Rewatering occurred c. 18 hours before samples were collected. Vertical lines represent standard errors.
roots of both species, concentrations of individual sugars were the same across treatments.

**Discussion**

I studied effects of drought and subsequent rewatering on carbon and water relations in seedlings of two *Eucalyptus* species with contrasting water-use strategies. The aim of the experiment was to understand to what extent water-use strategies determine a species’ ability to recover from drought and to get a better understanding of the response mechanisms involved in severe stress potentially leading to mortality. In support of my hypothesis, seedlings of the relatively isohydric species *E. marginata* maintained a high $\Psi_L$, which was only 0.9 MPa lower in droughted than in well-watered seedlings. In *E. wandoo*, on the other hand, $\Psi_L$ was up to 3.3 MPa lower in droughted compared with well-watered seedlings towards the end of the experiment confirming that *E. wandoo* displays a more anisohydric water-use strategy than *E. marginata*, and is in agreement with what has been found by Poot & Veneklaas (2013). The relatively conservative water-use strategy in *E. marginata* was also evident from a 10-25% lower $g_s$ compared with that of *E. wandoo* even under well-watered conditions. The reduced $g_s$ in well-watered plants at the end of the experiment is likely to be a result of lower light conditions towards the end of the experiment. An early reduction in $g_s$ in droughted *E. marginata* seedlings was associated with a slower decrease in SWC and a higher minimum SWC of 6.5% compared with 4.1% in *E. wandoo*. As a consequence, $\Psi_{PD}$ stayed above -2 MPa throughout the experiment in all but two *E. marginata* seedlings, while they decreased to below -5 MPa in *E. wandoo*.

In response to rewatering, $\Psi_L$ was higher in rewatered than in well-watered *E. marginata* seedlings. In *E. wandoo*, $\Psi_L$ of rewatered seedlings was almost as high as in well-watered seedlings, however, $\Psi_L$ did not always recover to levels observed in well-watered seedlings within 18 hours of rewatering. The generally full and fast recovery of $\Psi_L$ upon rewatering, even in severely-stressed plants, shows that water redistribution is very efficient, despite an incomplete recovery of the water transport system. The relationship between $\Psi_{PD}$ and $\Psi_L$ was not affected by drought or rewatering, which indicates that hydraulic gradients were maintained at relatively constant levels in both species. However, an accumulation of embolisms in response to decreasing $\Psi_X$ led to a loss of hydraulic conductivity in droughted seedlings of both species towards the end of
the experiment. PLC reached over 50% in *E. marginata*, but rewatered seedlings were able to repair embolisms and showed a PLC of less than 20% within 18 hours of rewatering, which was the same as in well-watered seedlings. This shows that embolism reversal occurs while the xylem is under substantial tension and corroborates earlier work by Martorell *et al.* (2014), who found that *Eucalyptus pauciflora* seedlings are able to repair embolisms to levels of well-watered control plants within only ten hours of rewatering. In contrast, well-watered *E. wandoo* showed more than 30% PLC throughout the entire experiment and PLC of ~40% in droughted seedlings was only slightly but not consistently higher than values from well-watered seedlings.

Surprisingly, rewatered *E. wandoo* seedlings showed the highest PLC of 60% at the end of the experiment. This was not the result of reduced embolism repair, since rewatered seedlings showed the same $K_{sn}$ as droughted seedlings but 50% higher $K_{smax}$ than droughted seedlings. The lower $K_{smax}$ observed in droughted and rewatered *E. marginata* seedlings could have been a result of tyloses permanently sealing off embolised vessels (Hacke & Sperry, 2001; McElrone *et al.*, 2010). Tyloses have been found in *E. marginata* in response to waterlogging (Davison & Tay, 1985) and in *E. wandoo* in response to pathogen infections (Hooper & Sivasithamparam, 2005), and are likely a common response to stress. However, since they are thought to permanently seal vessels, the higher $K_{smax}$ observed in rewatered *E. wandoo* seedlings suggests that tyloses were not responsible for the lower $K_{smax}$ in droughted *E. wandoo* seedlings. I cannot exclude the possibility that gas bubbles were not completely removed from narrow vessels when stem segments were flushed before $K_{smax}$ measurements (Sperry *et al.*, 1988) and that this would have affected the $K_{smax}$ values observed in droughted seedlings, while embolisms in such narrow vessels could have been repaired in rewatered *E. wandoo* seedlings. If the repair of narrow vessels had led to the 50% higher $K_{smax}$ in rewatered seedlings, rewatered seedlings would have likely shown higher $K_{sn}$ than droughted seedlings, which was not the case. Zwieniecki *et al.* (2001) showed a reduction in hydraulic resistance and increased conductivity when the xylem sap of 19 angiosperm species was experimentally enriched with KCl and proposed that the hydrogels associated with bordered pit membranes would shrink and allow a higher water flow. This hypothesis was supported by recent finding by Lee *et al.* (2012), who showed that hydrogels associated with bordered pit membranes shrink when exposed to a 50 mM KCl solution instead of de-ionised water and suggested that the hydraulic
permeability of the pit membrane could change in response to altered ionic concentration of the xylem sap. I thus suggest that observed changes in $K_{\text{smax}}$ in the present study could be associated with an increased hydraulic permeability due to changes in pit membranes in response to rewatering. If we assume that $E. \ marginata$ seedlings in all treatments had the same $K_{\text{smax}}$, adjusted PLC values would be almost 70% in the droughted and almost 50% in the rewatered seedlings. In $E. \ wandoo$, $K_{\text{smax}}$ was highest in rewatered seedlings, adjusted PLC would thus have been ~60% in droughted and ~40% in well-watered seedlings. This would mean that droughted $E. \ wandoo$ seedlings displayed the same loss in conductivity (i.e. ~60%) as rewatered seedlings, and thus points towards limited repair near the end of the drought treatment, while $E. \ marginata$ showed successful embolism repair. In summary, these findings highlight the importance of critical examination of $K_{\text{smax}}$ measurements for the estimation of PLC.

I expected that $E. \ marginata$ would reduce its stomatal conductance and thus avoid water loss and, consequently, deplete its carbohydrate reserves while $E. \ wandoo$ was expected to allow low water potentials and risk greater losses of conductivity. Interestingly, I found no indication of a depletion of carbon pools in either of the species. The opposite was the case, as both species accumulated soluble sugars in response to decreasing $\Psi_{PD}$, which correlated well with soil water availability. An accumulation of carbohydrates in response to drought has also been found for $Eucalyptus \ globulus$ and was an effect of attenuated growth during drought (Mitchell et al., 2014). An earlier reduction of growth rate in $E. \ marginata$ may be the reason for the observed high levels of NSC, despite an early reduction in $g_s$. I observed a c. twofold increase in soluble sugar concentrations in leaves and sapwood, respectively, in $E. \ marginata$ and $E. \ wandoo$ seedlings in response to a reduction in $\Psi_{PD}$ from c. -0.5 to about -3 MPa over the course of the experiment. In $E. \ marginata$, the same soluble sugar concentrations were observed at lower $\Psi_{PD}$ after rewatering and this may be a result of increased $\Psi_{PD}$ while concentrations of soluble sugars remained similar. In $E. \ wandoo$, on the other hand, soluble sugar concentrations decreased with increasing $\Psi_{PD}$. I surmise that at least some of the sugars found in sapwood were sugars secreted into vessels, in particular into embolised vessels in plants with low $\Psi_{PD}$. This would be consistent with the hypothesis of Zwieniecki & Holbrook (2009), who proposed a continuous secretion of sugars into vessels and that sugars would accumulate in
embolised but not water-filled vessels, despite the fact that starch pools were not significantly affected in the present study.

During the last 21 days of the experiment (i.e. when the drought treatment started to affect $\Psi_X$ and PLC), droughted seedlings of both species accumulated soluble sugars in the sapwood as well as in the bark and cambium. The increases in soluble sugar concentrations were due to increased glucose and fructose concentrations in *E. marginata* and mainly due to increased sucrose concentrations in *E. wandoo*. The high soluble sugar levels in stems and leaves, could help maintain hydraulic function (Sevanto *et al.*, 2014) but also contribute to embolism repair. In the present study, the starch concentrations were the same in both species and across treatments. This is in contrast to findings in *Laurus nobilis*, where starch depolymerisation in parenchyma cells was thought to be involved in embolism repair (Salleo *et al.*, 2009). Zwieniecki & Holbrook (2009) suggested that the complete depolymerisation of 1 mg of starch per gram of stem (fresh weight) of *Populus trichocarpa* would be sufficient to develop an osmotic force that is equivalent to -1 MPa throughout the xylem, which could drive refilling. I found no evidence for depolymerisation of starch into glucose in the sapwood of the two *Eucalyptus* species, but an increase in soluble sugars from around 15 to c. 40 mg g$^{-1}$ dry weight in response to a decrease in $\Psi_{PD}$ from about -0.5 MPa to c. -4 MPa. The vessel lumina account for about 13 and 22% of the cross-sectional area in *E. marginata* and *E. wandoo*, respectively (Ridge *et al.*, 1984), and are thus similar to what has been found for other species. I therefore believe that the secretion of at least 10 mg of soluble sugars per gram dry stem into vessels would have been possible and could have led to an osmotic force of about -10 MPa in embolised vessels, which could have driven refilling. In addition, the high NSC concentrations in bark including cambium could provide sugars for this process, as has been proposed by Nardini *et al.* (2011), who suggested that embolism refilling could be ‘a particular case of phloem unloading’. However, I did not measure the transfer of carbohydrates between different tissues, precluding further interpretation. In addition, it remains unknown what percentage of soluble sugars could get secreted into vessels and what concentrations were present in the xylem sap in response to the observed water potential range.

I show that carbohydrate pools in leaves, sapwood, and bark including cambium generally decline after rewatering. The fate of soluble sugars after rewatering, however,
is unclear. After rewatering and repair of embolised vessels, soluble sugars might be translocated in the transpiration stream to leaves, where they might be loaded into the phloem and transported to sink tissues. Alternatively, sugars might be polymerised and stored as starch. However, I found no significant increases in soluble sugar or starch concentrations in response to rewatering in any of the tissues, but roots had slightly higher NSC concentrations after rewatering. I suggest that the differences were not detected due to small increases in various tissues or because sugars were used in respiration or embolism repair, which would have been stimulated in response to rewatering (Saveyn et al., 2007). Since I found no depletion of NSC pools as a result of drought in any of the analysed tissues, I also suggest that carbohydrate transport was not affected in these seedlings during the drought (O’Brien et al., 2014).

Conclusions

I found that the contrasting water-use strategies of *E. marginata* and *E. wandoo* did not lead to differences in carbon pools. Both species reduced their water use in response to drought but the more anisohydric species *E. wandoo* allowed more negative leaf water potentials. The lower PLC in droughted compared with rewatered seedlings may have been caused by increases in the hydraulic permeability of hydrogels associated with bordered pit membranes in response to rewatering. Despite the lack of carbon depletion, I speculate that carbon metabolism would appear important during drought, as carbohydrates may be vital for osmoregulation to maintain hydraulic function and also to create an osmotic force to drive refilling. Future research should investigate the timing of embolism formation and repair along with the dynamics and distribution of NSC in xylem and associated tissues in more detail.
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Chapter 4

_Eucalyptus_ seedlings can display higher xylem vulnerability when grown in non-native soils

**Summary**

- Current global change scenarios predict a warmer and drier climate for south-western Australia. Changes in climatic conditions could alter the suitability of a species’ current habitat and lead to a shift in its distribution range. Future habitats with optimal climatic conditions may however not have optimal soil conditions. I aimed to study how two different soil types affect water relations and in particular the hydraulic system in two _Eucalyptus_ species with contrasting water-use strategies and soil preferences.

- Seedlings of _Eucalyptus marginata_ Sm. and _Eucalyptus wandoo_ Blakely were grown in a sandy and loamy soil, respectively. Once established, the pots were allowed to dry down to obtain a range of soil water contents and in order to study water relation parameters such as predawn and midday water potentials, stomatal conductance, and xylem vulnerability in response to plant water status.

- Seedlings of both species accumulated embolisms in response to reduced soil water availability. In _E. wandoo_, 50% loss of conductivity occurred at -1 MPa when grown in sandy soil compared with -2.5 MPa in loamy soil, despite lower leaf area to sapwood area ratios. In _E. marginata_, on the other hand, the xylem vulnerability was the same in both soil types.

- The survival of tree species in areas with favourable future climates could be threatened if the capacity of their hydraulic system is affected by the soil substrate.

**Introduction**

Recent events of large-scale tree dieback indicate that many forest ecosystems worldwide are threatened by changes in climate, with drought and heat being the strongest drivers for forest mortality (Allen *et al.*, 2010). In south-western Australia, a
recent event of extensive canopy collapse in the dominant tree species has been attributed to extreme drought and heat in 2010 and 2011 (Matusick et al., 2013). Since Mediterranean-type ecosystems have evolved to cope with summers of no or minimal rainfall, they were generally assumed to be more resilient to drought stress. However, a recent study by Choat et al. (2012) argues that trees across various ecosystems operate with similarly narrow hydraulic safety margins and are thus all at risk at locations with a drier future climate.

Most climate forecasting models predict that the climate of Mediterranean-type ecosystems is most likely to undergo severe changes (IPCC, 2007). Since 1920, the temperature in Australia has risen by 0.9°C and this trend is predicted to continue (BOM, 2014). During this period, south-western Australia became drier (BOM, 2014). Depending on the scenario (i.e. low or high carbon dioxide emissions in the future), temperatures in the southwest of Western Australia will increase by 1-5°C and annual precipitation will decrease by 0-30% by 2070 in comparison to averages from the period between 1980 and 1999 (BOM, 2014). If the climate predictions for south-western Australia become reality, there will be serious consequences for the flora and fauna of all ecosystems in this region, as drought events will become more frequent and severe (IPCC, 2014).

In response to changes in average temperature and annual rainfall, tree species will be exposed to more challenging climatic conditions, unless they migrate to areas that will have a suitable climate in the future. Such new areas do not necessarily have the same soils as those in the original distribution range. Since water availability is determined by the interaction between climate, soils and vegetation (Schymanski et al., 2009), it is important to understand the response of trees to drought in different soil types. In this study, I use two widespread Eucalyptus species that are endemic to the south-western Australia: Eucalyptus marginata Sm. and Eucalyptus wandoo Blakely. Even though their distribution ranges show some overlap, the two species tend to occur in areas with different soil types and different annual precipitation ranges (Verboom & Pate, 2015). Eucalyptus marginata generally inhabits areas with higher annual rainfall (600 - 1250 mm annual precipitation) compared with E. wandoo (400 - 700 mm annual precipitation; Boland et al., 2006). This species grows on a variety of soil types but is most abundant on deep sands or loamy sands, often gravelly, and often with clay at
several meters depth; its deep root system allows it to access the groundwater in certain areas. *Eucalyptus wandoo*, on the other hand, grows on heavier soils, mostly in drier areas (Boland et al., 2006). It can lower its leaf water potential substantially to extract water from dry soils (Colquhoun et al., 1984; Poot & Veneklaas, 2013).

Poot & Veneklaas (2013) studied water relations in *E. marginata* and *E. wandoo* as well as two other species in an area where all species co-occurred and areas that represented a typical habitat for each of the study species. When co-occuring, *Eucalyptus wandoo* exhibited higher $g_s$ and lower midday water potentials compared with *E. marginata*. *Eucalyptus wandoo* also showed a smaller reduction in sapwood velocity than *E. marginata* during dry summer months. Interestingly, the observed differences were less pronounced or disappeared when each species was measured in its typical habitat. Poot & Veneklaas (2013) concluded that the water availability to plants is not only determined by climate but also by soil properties and that *E. wandoo* might only be superior at extracting water from the soil when the soil properties are suitable. In addition, it has been shown that the vulnerability to embolism formation can be influenced by the soil texture, with higher critical water potential in desert shrubs grown in sandy soils compared to plants of the same species grown in loamy soil (Hacke et al., 2000; Sperry & Hacke, 2002). This would mean, that the survival of species migrating into climatically favourable future habitats could be threatened if the soil conditions are not suitable.

This study aims at testing the ability of the two *Eucalyptus* species to tolerate soil drought in two different soil types by adjusting their water use (or adapting their water use strategies). I hypothesise that both species will grow better in soils that are more similar to the soils found in their native habitat (i.e. sandy soil for *E. marginata* and loamy soil for *E. wandoo*). They will respond to decreasing soil water availability by reducing stomatal conductance and lowering leaf water potentials. I also predict that both species will accumulate embolisms when the soil dries and show a higher loss of conductivity in the sandy soil compared with the loamy soil at a given soil water potential.
**Materials and Methods**

**Plant material and experimental setup**

One and a half year old *Eucalyptus marginata* Sm. and *Eucalyptus wandoo* Blakely seedlings were obtained from Muchea Tree Farm (Muchea, WA, Australia). The provenance of seed used at the nursery is unknown. Twenty-four seedlings of similar size from each of the two species were selected and transplanted into free-draining pots made out of storm-water pipes (840 mm long, 150 mm diameter). Gravel was added to the bottom of each pot to ensure proper drainage. Pots were then filled with 20.5 or 21 kg of one of the two soil mixes to achieve similar soil volumes. The first soil mix consisted of one part (w/w) river sand and two parts of a loamy sand collected at the University of Western Australia farm (Ridgefield: S 32° 30’ 23” – E 116° 59’ 31”, West Pingelly, Australia). The second soil mix contained four parts of river sand and one part of the loamy sand. Both the river sand and the loamy sand were dried and sieved (4 mm mesh size) before they were mixed. Both soil mixes (i.e. the loamy and the sandy soil) were analysed to determine texture, nutrient concentrations, and pH. Soil analysis was performed by CSBP (Kwinana, WA, Australia). The loamy soil consisted of 80% sand, 13% clay, and 6% silt while the sandy soil consisted of 90% sand, ~7% clay, and ~2.5% silt. The pH was the same in both soils (~6.3) but nutrient levels were generally higher in the loamy soil, especially nitrogen (c. 4 and 1.4 mg kg⁻¹) and potassium (c. 120 and 50 mg kg⁻¹) concentrations, which were more than twice as high in the loamy compared with the sandy soil, and phosphorus concentrations, which were approximately 15% higher in the loamy (~14 mg kg⁻¹) compared with the sandy soil (~12 mg kg⁻¹). For the soil water retention curves of the two soil types, various amounts of dry and wet soil samples of each soil were mixed to obtain a range of SWC. The soil samples were then transferred into plastic bags and left overnight to equilibrate. The soil water potentials ($\Psi_{\text{Soil}}$) of the soil samples were then measured using a dewpoint water potential meter (WP4-C, Decagon Devices, Pullman, WA, USA). The samples were subsequently weighed and oven-dried (80°C, 96 h) before they were weighed again and % SWC (g H₂O g⁻¹ soil) calculated. After transplanting, pots were kept at field capacity by hand-watering them daily. At the same time, a slow-release drip irrigation system (2 L h⁻¹) was set up to facilitate establishment of the seedlings. The seedlings were watered six times a day with c. 33 mL for the first month using this irrigation system. The 48 pots were arranged in a Latin square design in a glasshouse at the University of Western Australia in Crawley, Western Australia.
Treatments and harvests

About six months after transplanting, the drought treatment was initiated and water was withheld from all the pots. The pots were then allowed to dry down to different target weights, weighing the pots twice a week. In addition, stomatal conductance (gs) was measured regularly using a leaf porometer (SC-1 leaf porometer, Decagon Devices, Pullman, WA, USA). Further physiological measurements were initiated when the soil water content reached below 5% or gs values were reduced by more than 50% in some of the seedlings of each treatment. To confirm that seedlings across all treatments (i.e. species and soil type) showed a similar range of ΨPD (i.e. a measure of water availability to the seedlings), one leaf per seedling was removed for ΨPD measurements. This was needed to get a range of xylem water potential (ΨX) values for xylem vulnerability curves. Seedlings were harvested over the course of four days after water had been withheld for 40 days. In the evenings before each harvest, seedlings were wrapped in plastic bags, allowing ΨPD measurements to be measured at 7:30 am. ΨPD, ΨX, and midday water potential (ΨL) were measured using a Scholander-type pressure bomb (Model 1000, PMS instrument company, Albany, OR, USA). One leaf per seedling was wrapped more than one hour before seedlings were harvested to let the leaf equilibrate with the xylem and determine ΨX. Stomatal conductance was measured one more time at midday before seedlings were harvested. During harvest, the shoot of the seedling was cut above the soil and transferred into opaque plastic bags. Immediately after that, seedlings were transported to the nearby laboratory where hydraulic conductivity was measured as outlined below. Thereafter, the shoots were separated into leaves and stems. A subsample of leaves was weighed and scanned to determine leaf area. All leaves and stems were then dried in an oven (80°C, 72 h). Whole-plant stomatal conductance was estimated by multiplying leaf-level gs with total plant leaf area (gsp = gs x LA). Harvests were carried out over four days to ensure that hydraulic conductivity measurements could be conducted within a short time after shoots were removed from the pots.

Hydraulic conductivity measurements

Hydraulic conductivity (Kh in kg s⁻¹ m MPa⁻¹) is a measure of the bulk flow rate (F in kg s⁻¹) of a liquid relative to its driving force (i.e. pressure drop due to pressure head in MPa) and adjusted for the length of the flow path (i.e. length of the measured stem segment in m). Kh was measured as described by Sperry et al. (1988) using a flow meter.
(Liqui-Flow™, Bronkhorst High-tech B.V., Ruurlo, The Netherlands) instead of a balance. The harvested stem was recut under water and a 4 cm piece was cut off c. 5 cm above the first cut. The second cut was made to avoid the ‘tension-cutting artefact’ (Venturas et al., 2015). Five mm of bark and phloem were removed on either end of the stem segment before it was connected to tubing at the down-stream end of the flow meter. The other end of the flow meter was connected to a pressure head (400 mm, 3.915 kPa). The flow meter was connected to a computer that allowed plotting and monitoring of the flow of a 20 mM potassium chloride (KCl) solution through the stem segment. After recording the initial conductivity, the stem segment was flushed several times to remove gas bubbles using a syringe that was filled with KCl solution and placed in a chalking gun. The chalking gun was connected to a pressure gauge in order to achieve a high (100 kPa) and relatively stable pressure. After flushing, the flow through the stem segment was measured for a second time to obtain a value of maximum conductivity. The diameter and length of the stem segments were measured using callipers. The diameter was measured twice on each end of the stem segment.

Leaf area to sapwood area ratio
A representative subsample of leaves from each seedling was scanned and the total leaf area (LA) was determined based on fresh weights of all leaves relative to the fresh weight of the leaf subsample. Sapwood area was calculated based on the sapwood diameters of the stem segment that was used for hydraulic conductivity measurements. Leaf area and sapwood area values in mm² were used to calculate the leaf area to sapwood area ratio (Pérez-Harguindeguy et al., 2013).

Statistical analysis
Statistical analyses and plotting were performed using the software R (R Development Core Team, 2010). Xylem vulnerability curves were analysed and plotted using generalised additive models. The relationships between gₛ and SWC, whole plant stomatal conductance (gₛₚ) and Kₘ as well as gₛₚ and hydraulic gradient for the different species and soil types were analysed with generalised least square models due to uneven variance between the two species or soil types. The variance structure was accounted for using a weights term in the model. Soil water retention curves for each soil type were plotted and analysed using a linear model with a second-order polynomial function.
**Results**

There was a strong correlation between PLC and $\Psi_X$ ($P < 0.001$) when the data from both species and soil types were analysed together but no effect of species or soil type alone. In the same analysis, the interaction term between species and soil type was significant ($P = 0.024$), indicating that the two species responded differently to the soils. The data set was then split up to analyse the effect of soil type separately for each of the *Eucalyptus* species. In *E. marginata* (Fig. 1a), no difference between soil types was detected but PLC correlated well with $\Psi_X$ ($P < 0.001$). The soil type had, however, a significant effect on PLC in *E. wandoo* ($P < 0.01$, Fig. 1b) and PLC was between ~20-40% higher in the sandy soil throughout the observed range of $\Psi_X$. The correlation between PLC and $\Psi_X$ remained strong ($P = 0.011$). *Eucalyptus wandoo* reached a 50% loss of conductivity ($\Psi_{50}$) at c. -1 MPa in the sandy soil and almost -2.5 MPa in the loamy soil. In *E. marginata*, on the other hand, $\Psi_{50}$ was around -1.5 MPa in both soil types. The SWC reached low levels in all the treatments (i.e. soil type and species), but small differences in SWC led to large differences in the $\Psi_X$ that were measured. *Eucalyptus marginata* seedlings reached a minimum $\Psi_X$ of about -2 MPa in loamy soil and less than -3 MPa in sandy soil. In *E. wandoo* seedlings, the $\Psi_X$ was about 1 MPa lower in the respective soils.

![Figure 1](image-url)

**Figure 1** Xylem vulnerability curves based on percentage loss of conductivity (PLC) and xylem water potentials ($\Psi_X$) in response to a dry-down of soil for *Eucalyptus marginata* (a) and *E. wandoo* (b) grown in a sandy soil (open circles and squares) or a loamy soil (closed circles and squares). Each data point represents an individual pot-grown seedling. Solid and dashed lines represent means fitted with generalised linear models in loamy and sandy soils, respectively, and dashed lines represent corresponding 95% confidence intervals ($n = 11-12$).
Stomatal regulation did not differ in the two soil types because the relationship between $g_s$ and $\Psi_{PD}$ was not affected by the soil type, however differences existed between the two *Eucalyptus* species (Fig. 2a and b). Overall, stomatal conductance was higher in *E. wandoo* compared with *E. marginata* ($P < 0.001; r^2 = 0.46$) but declined with lower $\Psi_{PD}$ in both species. The generalised additive model’s explained deviance (i.e. $r^2$) was low for both species (*E. marginata*: $P = 0.029$, $r^2 = 0.37$; *E. wandoo*: $P = 0.023$, $r^2 = 0.25$). The variability in $g_s$ was substantial even at high $\Psi_{PD}$, especially in *E. wandoo*. When
Ψ_{PD} was above -1.2 MPa in *E. wandoo*, g_s ranged between about 300 and almost 800 mmol m^{-2} s^{-1} in sandy soil and between 100 and c. 680 mmol m^{-2} s^{-1} in loamy soil. Ψ_{PD} declined with decreasing SWC in both species and soil types (Fig. 2c and d; *P* < 0.001; \( r^2 = 0.70 \)), but neither the species nor the soil type alone had an effect on the relationship between Ψ_{PD} and the SWC. However, the relationships between Ψ_{PD} and SWC were generally tighter in loamy soil \( (r^2 > 0.80) \) compared with sandy soil \( (r^2 < 0.20) \). The variability of Ψ_{PD} in *E. marginata* was large (-0.2 to -2.1 MPa) in sandy soil when the SWC was between 4 and 6%, possibly because not all seedlings accessed water in the deeper layers of the soil. In *E. wandoo*, on the other hand, the observations of the lowest Ψ_{PD} were clearly associated with low SWC in the loamy soil. Over most of the SWC range in this experiment, the Ψ_{Soil} was more negative in the loamy soil compared with the sandy soil \( (P < 0.05, r^2 = 0.93) \) but particularly when the SWC was between 2 and 4% (Fig. 3).

![Figure 3](image.png)

**Figure 3** Logarithm of negative soil water potential (log(-Ψ_{Soil})) derived from soil water retention curves against soil water contents (SWC). Lines were fitted using second-order polynomial linear regression \( (n = 9-11) \).

The two different soils affected allometric relationships that influenced plant water relationships. Plants of both species had a 25-30% higher leaf area to sapwood area ratio (A_L:A_S) in loamy compared with sandy soil (Fig. 4). To examine if this may have caused a greater demand for water by the leaves relative to the ability of stems to supply that water, Fig. 5 plots whole-plant stomatal conductance \( (g_{sp} = g_s \times LA) \) against stem hydraulic conductivity \( (K_h) \). Overall, the relationship between \( g_{sp} \) and \( K_h \) was influenced
by species (P < 0.001) and soil type (P < 0.01; Fig. 5a). In *E. wandoo*, $g_{sp}$ correlated with $K_h$ but was the same in the two soil types. In *E. marginata*, on the other hand, $g_{sp}$ was higher in loamy soil compared with sandy soil but the correlation with $K_h$ was only significant in the loamy soil (P < 0.001, $r^2 = 0.82$). The hydraulic gradient ($\Psi_{PD} - \Psi_L$) did not correlate well with $g_s$ but $g_s$ was higher in *E. wandoo* compared with *E. marginata* in both soil types (P < 0.01; Fig. 5b) and coincided with a slightly greater hydraulic gradient. The interaction between species and soil type indicated that the relationship between $g_s$ and hydraulic gradient was affected differently by soil type in the two species (P < 0.05), as *E. marginata* had lower $g_s$ in sandy soil while *E. wandoo* had lower $g_s$ in loamy soil. Overall, the soil type alone had no effect on the relationship between $g_s$ and the hydraulic gradient.

**Figure 4** Leaf area to sapwood area ratio for *Eucalyptus marginata* and *E. wandoo* seedlings grown in sandy and loamy soil, respectively. Bars represent means and vertical lines represent standard errors ($n = 11-12$).

**Discussion**

The results of this study show that the hydraulic system of seedlings of two *Eucalyptus* species can respond differently to soil water deficit depending on the soil type. While *E. marginata* seedlings had very similar xylem vulnerability curves in the sandy and the loamy soil, *E. wandoo* seedlings accumulated significantly more embolisms when grown in the sandy compared with the loamy soil. PLC was about 20-40% higher over the range of observed $\Psi_X$ in *E. wandoo* when grown in sandy soil, and it is worth noting that this was despite a ~25% higher $A_L:A_S$ ratio in loamy soil. A higher $A_L:A_S$ ratio
would be expected to cause an increased demand for water and consequently a higher xylem tension, which would cause the formation of xylem embolisms, unless the water demand was reduced through the reduction of stomatal conductance. However, the stomatal response to decreasing $\Psi_{pd}$ was the same in both soils, and the soil type did not have an effect on the relationship between $g_s$ and the hydraulic gradient in *E. wandoo*. When taking into account that the leaf area was c. 25% higher in the loamy soil in *E. wandoo*, $g_s$ was reduced relative to $K_h$ but the relationship between $g_{sp}$ and $K_h$ was the same overall. This is interesting because it suggests that the water loss and water supply are tightly regulated in both soil types in *E. wandoo* and that the leaf area and $g_s$ are potentially adjusted to allow maximum use of the hydraulic system.

![Figure 5](image_url)

**Figure 5** Whole plant stomatal conductance (i.e. stomatal conductance adjusted with leaf area values for each seedling) against hydraulic conductivity (a) for *Eucalyptus marginata* and *E. wandoo* in sandy and loamy soil, respectively. Lines represent linear regressions for each combination of species and soil type ($n = 11-12$). Whole plant stomatal conductance against hydraulic gradient (i.e. difference between predawn and midday water potential; b) for *E. marginata* and *E. wandoo* in sandy and loamy soil, respectively. Crosses represent means and standard deviations calculated for x and y variables for each combination of species and soil type ($n = 11-12$).

The evidence suggests that an accumulation of embolisms in *E. wandoo* grown in sandy soil was not a result of higher xylem tension since a 50% loss of conductivity ($\Psi_{50}$) occurred at a 1.5 MPa lower $\Psi_X$ in the loamy soil compared with the sandy soil. A $\Psi_{50}$ value of -2.5 MPa is much closer to what has been found under natural conditions (e.g. -3.3 MPa; Poot & Veneklaas, 2013). In a similar study, Sperry & Hacke (2002) tested the effects of soil characteristics (i.e. sandy soil versus loamy soil) on water relations of eight shrub species in a desert landscape. They found that $\Psi_L$ were generally lower in the loamy soil and that lower water potentials coincided with higher PLC but, at the
same time, they observed higher vulnerability to root embolisms in the sandy soil as expressed by a 0.9 MPa lower $\Psi_{75}$ in the loamy soil. *Eucalyptus wandoo* is known to operate at very low midday leaf water potentials of below -4 MPa in the field (Poot & Veneklaas, 2013) and I therefore suggest that the increased vulnerability to cavitation in *E. wandoo* is caused by the unfavourable soil texture in sandy soil. Low water potentials help extract tightly bound water from the soil (Sperry & Hacke, 2002) and I propose that a more gradual decrease in soil water potential in fine-textured loamy soils might allow *E. wandoo* to remain in ‘hydraulic contact’ when the soil dries. In sandy soils, on the other hand, water in soil pores can become discontinuous as the $\Psi_{\text{soil}}$ decreases rapidly when roots take up water (Passioura, 1988). *Eucalyptus wandoo* might not be able to respond to rapid decreases in $\Psi_{\text{soil}}$, which could lead to hydraulic failure in parts of the root system, particularly in the drier upper soil layer. Embolisms may have propagated from the roots to the stems. $\Psi_{\text{pd}}$ stayed above -1.15 MPa in *E. wandoo* when grown in sandy soil but decreased to -3.5 MPa in loamy soil. Hydraulic failure in the roots in the upper soil layer likely led to a shift towards greater water use from deeper soil layers (Hacke et al., 2000), where soil water was readily accessible. This would mean that the leaves stayed hydrated but that plants rapidly depleted water in the upper layers of the soil while transpiring, and could explain the unexpectedly high PLC at moderate $\Psi_{\text{x}}$ and $\Psi_{\text{pd}}$.

In contrast to *E. wandoo*, PLC was the same in the sandy and loamy soil in *E. marginata* despite an even higher stimulation of leaf growth, resulting in a 30% higher $A_L:A_S$ ratio in the loamy soil compared with the sandy soil. Again, the relationship between $g_s$ and $\Psi_{\text{pd}}$ was the same in both soil types, but $g_{sp}$ was much higher in *E. marginata* in the loamy soil and correlated well with $K_h$. In the sandy soil, *E. marginata* had lower $g_{sp}$ even though the hydraulic gradient was the same as in the loamy soil. In addition, there was no correlation between $g_{sp}$ and $K_h$. I therefore suggest that *E. marginata* ‘sensed’ when $\Psi_{\text{soil}}$ decreased (in the upper part of the pot) and adjusted its water use before the $\Psi_{\text{soil}}$ dropped below a certain threshold when water uptake is prevented due to discontinuity of water in the soil pores (Passioura, 1988). Interestingly, the $\Psi_{\text{pd}}$ stayed above -1.5 MPa in *E. marginata* throughout the observed range in SWC (~2-10.5%) in both soil types, apart from two outliers that possibly did not have access to the deeper soil water resources in the pots (which was supported by low $g_s$ values in these plants). The high $\Psi_{\text{pd}}$ could indicate that root water uptake was tightly regulated
to avoid a localised depletion of soil water and subsequent consequences for root integrity. I suggest that localised fast decreases in $\Psi_{\text{Soil}}$ in the upper layers of the soil led to the synthesis of abscisic acid (ABA) in the roots of *E. marginata* (Cahill *et al.*, 1986). If roots ‘sensed’ decreases in $\Psi_{\text{Soil}}$ and the translocation of ABA reduced water use in the shoot (Davies *et al.*, 1990; Chaves *et al.*, 2003), a loss of hydraulic conductivity due to steep $\Psi_{\text{Soil}}$ decreases could be avoided. Certainly, compared to *E. wandoo*, *E. marginata* had smaller hydraulic gradients. In conclusion, I believe that the similarities in xylem vulnerability in sandy and loamy soil in *E. marginata* are a result of an early adjustment of water use in response to root signalling. Since *E. wandoo* only showed a distinct reduction in $g_s$ once the $\Psi_{\text{PD}}$ dropped below -2 MPa, I suggest that the response to decreasing soil water availability might occur at lower $\Psi_{\text{Soil}}$. This makes sense for a species adapted to fine-textured soils, as the soil water availability decreases more slowly in loamy soils. It is therefore advantageous to lower $\Psi_L$ in order to extract more water from the soil, as has been seen for *E. wandoo* when grown in its native habitat (Poot & Veneklaas, 2013).

The higher $A_L:A_S$ ratios observed in the loamy soil, compared to the sandy soil, in both species may be due to differences in soil fertility as concentrations of nitrogen, phosphorus, and potassium were higher in the loamy soil compared with the sandy soil. Bucci *et al.* (2006) also observed higher $A_L:A_S$ ratios in five woody savannah species at increased nutrient availability. Interestingly, in these species, the increased leaf areas coincided with higher embolism resistance (i.e. lower $\Psi_{50}$). In contrast, Atwell *et al.* (2009) found that nitrogen fertilisation predisposed *Eucalyptus pauciflora* seedlings to a higher risk of desiccation due to increased xylem vessel diameters and a mismatch between supply and demand. Since I did not measure root biomass, I do not know if differences in nutrient availability stimulated root growth along with leaf area increases, and how this could have affected the results. However, nutrient addition generally stimulates aboveground growth more than belowground growth (Lambers *et al.*, 2008). In field-grown *Eucalyptus saligna*, for example, nutrient addition only stimulated aboveground but not belowground biomass increases (Giardina *et al.*, 2003). Increased $A_L:A_S$ ratios can be also be a result of higher water availability or soil texture. Access to shallow groundwater led to higher leaf area to sapwood area ratios in *Eucalyptus kochii ssp. borealis* trees compared with trees without access to shallow groundwater (Carter & White, 2009). Since seedlings in this experiment grew under well-watered conditions
for over six months before the water was withheld, I do not believe that differences in AL:AS ratios were a direct effect of water availability. It has also been shown that shifts in biomass allocation patterns can be a direct effect of soil texture in plants that grown under well-watered conditions and the same nutrient concentrations (Xie et al., 2012). In this study, cotton plants (Gossypium herbaceum) had higher leaf biomass in loamy soil but higher root biomass in sandy soil. They concluded that the presence of air-filled soil pores makes roots less effective at taking up water and nutrients and that this caused a shift in biomass allocation, even under well-watered conditions. Whilst I do not know how nutritional effects influenced AL:AS ratios and xylem vulnerability, I propose that the observed patterns in PLC, gs, and Ψ in E. marginata and E. wandoo are best explained through direct and species-specific effects of the soil texture on water relations.

Conclusions
This experiment shows that adaptations to characteristics of the native habitat, especially the soil texture, can govern water relations and the regulation of water use in two Eucalyptus species. The migration of Eucalyptus wandoo into areas closer to the coast of south-western Australia, where the climate is predicted to be more favourable in the future, could be threatened, as this species might not be able to adjust its water use patterns to the predominantly sandy soils in the new environment. The results of this study highlight the importance of understanding how plants interact with the soil and suggest that the climate alone is not a reliable predictor of plant performance in new environments. These results could have implications for species distribution models and assisted migration programs. I propose that risk assessments for climate change impacts and potential translocations of tree species should take plant-soil interactions into consideration, to ensure the survival of trees species in a future warmer and drier climate.
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General discussion and conclusions

The understanding of processes that lead to drought-induced mortality and the recovery from drought are of utmost importance in the face of a changing global climate. If we aim to predict responses of forests to higher temperatures and reduced rainfall, we need to understand (1) the physiological mechanisms involved in drought-induced mortality and the recovery from drought; (2) species-specific responses to drought; (3) how drought affects entire ecosystems; and (4) feedback of drought-induced mortality on atmospheric CO₂ concentrations and associated climate-forcing. The understanding of physiological processes during drought could also be used to make informed decisions with regards to forest management and species translocations in order to grow forests that will be more resilient to future climatic conditions.

This thesis aims to contribute to the current knowledge of the mechanisms leading to drought-induced mortality and the recovery from drought through the study of carbon and water relations during drought and in response to rewatering. Also, I tested effects of contrasting soil types on plant water relations, in particular the hydraulic system, to understand how plant water relations and water-use strategies determine a species’ success in a given soil substrate. Two common and widespread Eucalyptus species, E. marginata Sm. and E. wandoo Blakely, were used in all experiments, because they differ in their water-use strategies. The specific aims of this thesis were (1) to determine the timing of embolism repair and its temporal coordination with the recovery of stomatal conductance, leaf water potential, and leaf water content in response to rewatering after a drought treatment; (2) to understand how carbon and water relations are affected throughout the course of a 63-day drought treatment and in response to rewatering; (3) to assess effects of contrasting water-use strategies on carbon and water relations during drought and the recovery from drought; (4) to understand the relationship between stem carbon pools and embolism repair; and (5) to test how the soil water availability in contrasting soil substrates affects water relations and the
hydraulic system in the two Eucalyptus species, and if the two species can adjust their water use and water-use strategies to soils that are not typical for their native habitat.

Here, I summarise the findings from each experimental chapter and discuss them in relation to findings from the other chapters and the wider literature. I also highlight the relevance of my research, as well as findings from the literature, and suggest further research.

**Water-use strategies in response to drought and growth in contrasting soil substrates**

The two species were chosen because of known differences with regards to their water-use strategies. Overall, the water use was greater in E. wandoo compared with *E. marginata*, as evidenced by higher *g*ₘ and transpiration rates over most of the observed range in soil water content. *Eucalyptus marginata*, the more isohydric species, showed an earlier reduction in *g*ₘ in response to decreasing soil water availability and thus higher midday leaf water potentials (Ψₑ) than those in *E. wandoo*. In contrast, *g*ₘ was reduced at lower soil water contents (SWC) in *E. wandoo*, causing lower predawn and midday water potentials. In its natural habitat, *E. wandoo* operates at very negative Ψₑ of below -4 MPa (Poot & Veneklaas, 2013), which may help extract tightly bound water from the soil and thus enhance the water uptake from dry soils (Colquhoun *et al.*, 1984; Sperry & Hacke, 2002). This may give *E. wandoo* a competitive advantage, because the growing period in a Mediterranean-type environment may be prolonged if more soil water can be extracted and photosynthesis can be maintained at lower Ψₑ. Since *E. wandoo* displayed greater xylem vulnerability in the sandy soil than in the loamy soil (Chapter 4), the strategy to reduce leaf water potentials to extract more soil water may only be successful in soils that show gradual decreases in Ψₑ as the soil dries, which is characteristic for fine-textured soils such as loamy soils. Furthermore, the adaptation to soil properties and rainfall patterns in their natural habitat could be associated with differences in stomatal regulation in the two *Eucalyptus* species. Stomatal conductance decreased in both species in response to decreasing Ψₚ, but this occurred at lower Ψₚ and thus lower Ψₑ in *E. wandoo*. Davies *et al.* (1990) suggested that plants need to have an ability to ‘judge’ how much soil water is available and proposed that ABA is involved in the signalling mechanism in plants. Decreases in Ψₑ can lead to the synthesis of ABA in roots, which is then translocated to leaves where it reduces *g*ₑ (Davies *et al.*, 1990; Chaves *et al.*, 2003). A reduced water loss caused by an ABA-
mediated reduction in $g_s$ in response to low $\Psi_{\text{Soil}}$ helps avoid critical $\Psi_{\text{Soil}}$ that potentially lead to embolisms in water-conducting tissues, and could eventually result in hydraulic failure. Loamy soils have a greater capacity to hold water, and their soil water availability decreases more gradually (Gupta & Larson, 1979). A potential production of ABA could therefore occur more slowly and at lower $\Psi_{\text{Soil}}$ in loamy soils. Since the soil type did not change the relationship between stomatal conductance and $\Psi_{\text{PD}}$ (Chapter 4), *E. wandoo* may not be able to reduce its water loss before critical $\Psi_{\text{Soil}}$ are reached in sandy soils, because of its adaptation to loamy soils. An early reduction of $g_s$ at relatively high $\Psi_{\text{PD}}$ in *E. marginata* ensures that critical $\Psi_{\text{Soil}}$ are avoided in both soil types. As expected, *E. marginata* was overall more conservative in its water use than *E. wandoo*.

According to Sperry *et al.* (1998), the hydraulic continuum from soil to leaf has two weak links where the continuum can break and cause plant death, because of interrupted water transport. Firstly, steep water potential gradients in soils can impede water uptake by roots. When soils dry, water in soils is not only held at lower $\Psi_{\text{Soil}}$, but can also become discontinuous in soil pores (Passioura, 1988) which prevents water movement towards roots and water uptake by roots. Secondly, xylem embolisms can impede water transport from roots to leaves and progressive embolisms can result in hydraulic failure. In sandy soils, decreases in $\Psi_{\text{Soil}}$ occur more rapidly and are associated with hydraulic discontinuity (Passioura, 1988). Sperry *et al.* (1998) found that the water transport in plants with a high resistance to embolism formation or in plants adapted to coarse-textured soils would be limited by insufficient water supply in the rhizosphere, and not due to impaired xylem water transport. However, a loss of water uptake leads to embolisms in roots and potentially reduces whole-plant hydraulic conductance (Domec *et al.*, 2004). It is thus likely that rhizosphere constraints were responsible for the 20-40% higher PLC over the observed range in xylem water potentials in *E. wandoo* in sandy soil compared with those in loamy soil. A slow decrease in $\Psi_{\text{Soil}}$ in loamy soils, on the other hand, may allow *E. wandoo* to remain in ‘hydraulic contact’ until lower critical $\Psi_{\text{Soil}}$ are reached, at which point stomata would start to close. Furthermore, morphological differences in roots may contribute to the ability to take up water from either soil type. However, root morphological traits were not studied in this thesis. I suggest that in *E. marginata*, a species that mainly grows on sandy soils, relatively high water potentials are maintained to avoid critical $\Psi_{\text{Soil}}$ that would have consequences for
root integrity, especially in sandy soils. My findings thus suggest that the two *Eucalyptus* species have a limited ability to adjust their water-use strategies to soil that are not typical to their native habitat.

Loss of hydraulic conductivity in response to drought

As a consequence of lower soil water availability and associated $\Psi_{PD}$, the tension in the xylem in the two *Eucalyptus* species led to a reduction in hydraulic conductivity, due to an accumulation of embolisms. When soil moisture was depleted to a SWC of ~5% in the small pots used in Chapter 2, *E. wandoo* displayed a PLC of ~90%. Despite similarly low SWC of less than 5%, PLC was only about 40% in *E. wandoo* seedlings grown in large pots (Chapter 3). In *E. marginata*, PLC was ~75% in small pots at a SWC of about 5%, and around 50% at a SWC of ~6.5% in the large pots. I suggest that some of the discrepancy found in the two experiments is a result of differences in pot size, since the same loamy soil was used for the experiments in Chapter 2 and 3, but the pots were much larger in Chapter 3, holding approximately five times more water. This could have led to a slower onset of drought conditions in the larger pots, and have given the seedlings more time to adjust their water use. Furthermore, the size and especially depth of the pots would have allowed for a more natural distribution of roots and soil water. Lobet *et al.* (2014) pointed out that the hydraulic architecture of root systems is important when soil water availability decreases and soil hydraulic conductivity becomes limiting. During dry periods, the soil water availability decreases in the upper part of the soil first, due to soil evaporation and preferential water uptake from upper layers of the soil, because of high root densities and short distances for water transport (Lobet *et al.*, 2014). *Eucalyptus marginata* showed high $\Psi_L$ of around -2 MPa when grown in the large pots, despite very low soil water availability in the upper layers of the soil towards the end of the drought treatment. I suggest that root water uptake changed to uptake from soil layers with higher soil water availability deeper in the soil profile in *E. marginata* when grown in large pots. Farrington *et al.* (1996) showed that *E. marginata* took up water from deeper soil layers during summer months when the water availability was low in upper soil layers. Due to the limited soil volume, this was likely not possible in the small pots and thus led to $\Psi_L$ lower than -2 MPa in *E. marginata* in Chapter 2. In contrast, $\Psi_L$ gradually decreased to similarly low levels of below -4 MPa in the loamy soil in *E. wandoo*, despite differences in soil volume in the experiments of Chapters 2 and 3. When *E. wandoo* seedlings were grown in the sandy
soil in Chapter 4, however, $\Psi_L$ were higher and associated with higher $\Psi_{PD}$ and SWC. The unexpectedly high xylem vulnerability at high $\Psi_{PD}$ in $E. \ wandoo$ grown in sandy soil suggests that a depletion of soil water in the upper layers of the pot was compensated for by taking up water from deeper soil layers, where water was readily available, but may not have been sufficient to prevent the formation of embolisms or repair embolisms.

There is increasing evidence that roots redistribute soil water within the soil profile (Meinzer et al., 2001). The redistribution of water occurs because roots transfer water from wetter to drier parts of the soil in response to water potential gradients within the soil and between roots and the surrounding soil (Domec et al., 2004). This hydraulic redistribution is thought to occur overnight, when the transpirational water demand is minimal, and consequently allows for an equilibration of the plant water status with the soil. If water was moved to the upper layers of the soil overnight, this water could then be taken up by roots and transported to transpiring leaves during the day. Hydraulic redistribution would thus ensure a better water supply to leaves and may help avoid the formation of embolisms. The redistribution of soil water, however, could have been more successful in the loamy soil where hydraulic continuity between roots and the soil remains intact over a greater $\Psi_{Soil}$ range, since a loss of hydraulic continuity between roots and soil water is likely to occur at higher $\Psi_{Soil}$ in the sandy soil. If the redistribution and subsequent uptake of soil water was limited in dry soils, this could exacerbate rhizosphere constraints in the hydraulic continuum necessary for plant water uptake and transport. Avoiding the depletion of soil water beyond a certain threshold could give $E. \ marginata$ the ability to take advantage of hydraulic redistribution in either soil type, while a reduction of leaf water potentials to extract more water from the soil in $E. \ wandoo$ could have led to a loss of hydraulic continuity in the sandy soil, and had consequences for root integrity and water uptake.

The idea of vulnerability segmentation suggests that the water-conducting tissues of plants have different vulnerabilities to embolism formation, with more expendable organs such as petioles and small branches being more likely to embolise than stems and roots (Tyree & Ewers, 1991). Vulnerability segmentation protects water-conducting tissues in roots and stems against hydraulic failure, while the water transport in the xylem of more short-lived tissues such as small branches and leaves is impeded earlier
during drought. With a loss of leaf area, some of the demand for water would be alleviated, enhancing the water supply to the remaining tissues. Tsuda & Tyree (1997) found that a 50% loss of hydraulic conductivity was reached at much higher $\Psi_X$ in petioles than in roots of *Acer saccharinum*. However, Meinzer *et al.* (2001) suggest in their review that the root xylem is usually more vulnerable to embolism formation than the shoot xylem. Since I did not measure root conductivity, I am unable to judge to what extent root hydraulic function was impeded in response to soil water deficit. Furthermore, it is not clear if embolisms could propagate from roots into the stem xylem through air-seeding, or if impaired water transport in roots would lead to increased tension in the stem that then caused xylem embolisms. Higher conductivity losses in stems of seedlings grown in small pots in Chapter 2 compared with that in large pots in Chapter 3 could partially be caused by embolism-induced conductivity losses in roots that propagated into the stem xylem. In the large pots, on the other hand, hydraulic redistribution of water could have contributed to the capacity of roots to maintain water uptake (Domec *et al.*, 2004), even from the upper and drier layers of the soil. Hydraulic redistribution could thus have helped protect the stem xylem from embolisms that were not tension-induced but a result of hydraulic limitations in parts of the root system and potentially a propagation of embolisms from the root system.

Recovery from embolisms upon rewatering

In Chapter 2, which assessed the short-term dynamics of embolism repair after rewatering of droughted seedlings, the reduction of PLC was used as a measure of embolism repair. Seedlings of both *Eucalyptus* species showed a fast recovery of PLC upon rewatering in Chapter 2, indicating that the repair of xylem embolism started rapidly after soil water was available and while the bulk of the xylem was under tension. Furthermore, embolism repair occurred while seedlings of the two *Eucalyptus* species were (at least intermittently) transpiring and while the light in the controlled-environment room was on. My findings provide thus another example of successful embolism repair under conditions that contradict the old paradigm, which states that xylem pressure has to be above atmospheric pressure for embolism reversal (Tyree *et al.*, 1999).

In *E. marginata*, the recovery from embolisms was continuous and PLC decreased from almost 75% to about 50% within eight hours after rewatering. In *E. wandoo*, a reduction
in PLC from 90% to less than 60% occurred within only two hours after rewatering, but PLC did not decrease any further within the following six hours. Despite a rapid decrease in PLC and a recovery of about 30% in both species, a complete reversal of embolisms did not occur within eight hours. In contrast, Martorell et al. (2014) found that Eucalyptus pauciflora seedlings recovered from a PLC of almost 90% to control levels of about 30% within only six hours. Plotting PLC against $\Psi_X$ to obtain a ‘xylem-recovery curve’ supported the idea that the recovery from embolisms was more gradual and complete in E. marginata compared with that in E. wandoo, as evidenced by a wide range of PLC at low $\Psi_X$ (i.e. < -1.5 MPa) in E. wandoo, but a relatively gradual reduction in PLC in response to increasing $\Psi_X$ in E. marginata. When seedlings were measured 18 hours after rewatering, in Chapter 3, $\Psi_L$ recovered to greater than -1 MPa in E. marginata seedlings and PLC was as low as in well-watered controls (i.e. ~20%). In E. wandoo, $\Psi_L$ also recovered to higher than -2 MPa but a recovery of PLC was not evident towards the end of the 63-day drought treatment. The recovery of $\Psi_L$ indicates that water was not limiting for refilling in E. wandoo. Since E. wandoo seedlings recovered from 90 to 60% PLC in less time in Chapter 2, I suggest that the long-term exposure to dry conditions in the upper part of the soil could have impeded root recovery and that this was the reason why $\Psi_L$, but not PLC recovered to control levels. In E. marginata, the higher soil water potentials, possibly in combination with hydraulic redistribution, could have contributed to the survival of roots in the upper layers of the soil (Domec et al., 2004). Upon rewatering, those roots could then have contributed to the fast recovery of E. marginata seedlings in Chapter 3.

Recovery of stomatal conductance, leaf water potentials, and relative water content
The recovery of leaf water potentials occurred fast in response to rewatering. Leaf water potentials in both E. marginata and E. wandoo increased from about -3 MPa to c. -1 MPa within only 4 hours (Chapter 2). In E. marginata, $\Psi_L$ were higher in rewatered than in well-watered seedlings within c. 18 hours after rewatering (Chapter 3). In E. wandoo, on the other hand, $\Psi_L$ did not always recover to control levels within 18 hours of rewatering, indicating that recovery was impaired in plants that experienced very low $\Psi_L$. Also, $g_s$ increased quickly and continuously in E. marginata, and they reached pre-drought levels four to five hours after rewatering. In E. wandoo, however, the initial increase in $g_s$ came to a halt after only three hours, and $g_s$ decreased rapidly and reached levels close to pre-rewatering at the end of the experiment. Furthermore, $g_s$ did not
recover within 18 hours after rewatering (Chapter 3) in *E. wandoo*. Martorell *et al.* (2014) found that *g*ₜ had not recovered ten days after rewatering in *E. pauciflora* seedlings, despite a complete recovery of PLC within only six hours after rewatering. This was attributed to stomatal closure in response to a residual abscisic acid (ABA) signal in leaves (Lovisolo *et al.*, 2008).

In a study by Burgess & Bleby (2006), *E. wandoo* trees in a natural forest showed almost no increase in transpiration after a summer rainfall event; however, these trees had been transpiring at reasonably fast rates before the rainfall and the lack of response to rainfall was tentatively attributed to hydraulic limitations. My findings do not suggest that hydraulic limitations would be responsible for the observed delay in the recovery of *g*ₜ, because of the fast recovery of leaf water potentials. Brodribb & McAdam (2013) studied stomatal regulation in two conifer species with contrasting water-use strategies in response to drought conditions. They found that foliar ABA led to stomatal closure in both species; however, ABA concentrations decreased in the anisohydric species during the course of the drought treatment, but stomata remained closed, possibly due to high xylem tension (i.e. low water potentials). Upon rewatering, stomatal conductance increased immediately in the anisohydric species with low foliar ABA concentrations. In contrast, ABA concentrations remained high throughout the entire drought treatment in the more isohydric species, and resulted in a delayed stomatal opening after rewatering. Brodribb & McAdam (2013) concluded that the presence of foliar ABA prevented stomatal opening in the more isohydric species, while the anisohydric species could take advantage of the increased water availability. I did not measure ABA concentrations, but suggest that a delay in the recovery of *g*ₜ may be advantageous to avoid further loss of hydraulic conductivity in cases where the wetting of soils is short-lived. Small rainfall pulses occur irregularly during summer or at the start of winter in the Mediterranean-type climate of south-western Australia, but they can be followed by long periods without precipitation. If the production of a strong enough ABA signal leading to stomatal closure in *E. wandoo* only occurred in response to prolonged exposure to very negative Ψ<sub>Soil</sub>, a reversal of stomatal closure before soil water was replenished could increase the risk of hydraulic failure. In *E. marginata*, on the other hand, the water use is tightly regulated, and therefore increasing *g*ₜ to take advantage of small rainfall pulses would likely not pose a risk to the hydraulic system, because *g*ₜ can be rapidly reduced when water becomes scarce. I thus suggest that not only the water-
strategy but also commonly experienced rainfall patterns led to differences in the regulation of stomatal conductance in the two *Eucalyptus* species.

When discussing the effects of drought on the hydraulic system in *E. marginata* and *E. wandoo*, it is important to evaluate the implications of the observed reduction in $K_{s\text{max}}$ in response to drought in both species (Chapter 3). A reduction of 20-30% in $K_{s\text{max}}$ was apparent in droughted seedlings of both species: $K_{s\text{max}}$ was around 100 kg s$^{-1}$ m$^{-1}$ MPa$^{-1}$ in well-watered and ~80 kg s$^{-1}$ m$^{-1}$ MPa$^{-1}$ in droughted seedlings. This reduction could be due to blockage in vessels, potentially caused by tyloses that are produced by parenchyma cells to occlude embolised vessels. The production of tyloses is a common response to stress, such as drought (Hacke & Sperry, 2001; McElrone *et al.*, 2010), waterlogging (Davison & Tay, 1985), or pathogen attacks (Hooper & Sivasithamparam, 2005). The occlusion of xylem vessels by tyloses (or other substances) could reduce $K_{s\text{max}}$, and the comparison of $K_{s\text{max}}$ values for well-watered and droughted plants suggests that tyloses or other irreversible damage were responsible for 20-30% of the loss in conductivity in droughted plants. The loss in conductivity estimated through PLC is thought to be associated with reversible embolisms. Unexpectedly, the data in Chapter 3 suggest that rewatered *E. wandoo* plants had 50% higher $K_{s\text{max}}$ than droughted plants which would suggest that 50% more irreversible loss of conductivity had taken place in 18 hours in the droughted plants. This is very unlikely; instead, this result may be partly due to incomplete refilling of embolised vessels by the external pressure of 100 kPa applied before $K_{s\text{max}}$ was carried out. For this explanation to be true, refilling would have had to be more successful in rewatered than droughted plants. A possible explanation is that the natural embolism repair mechanism is particularly effective in narrow vessels, especially if this occurs through exudation of sugars into the vessels, as narrower vessels have a greater surface to volume ratio. In contrast, refilling through external pressure, a standard procedure in PLC estimates, may refill wider vessels first. If this procedure was not 100% successful for narrow vessels in droughted plants, but narrow vessels in rewatered plants were already refilled through the natural mechanism, this would lead to a higher $K_{s\text{max}}$ value in rewatered than droughted plants. *Eucalyptus wandoo* is known to have very dense wood with an abundance of small-diameter xylem vessels (Ridge *et al.*, 1984), which may explain the unexpected contrast between rewatered and droughted plants in this species, but not in *E. marginata*. However, the 50% higher $K_{s\text{max}}$ in rewatered compared with droughted *E. wandoo* was
not associated with higher $K_{sn}$. If refilling of narrow vessels was enough to stimulate $K_{smax}$ by 50%, it should have translated into a higher $K_{sn}$ to some extent. This was not the case, because $K_{sn}$ was the same in droughted and rewatered seedlings. I thus surmise that rewatering changed the thickness of hydrogels associated with bordered pit membranes (Lee et al., 2012). Hydrogels can be involved in the regulation of hydraulic conductivity, due to changes in resistance to water flow associated with the thickness of the hydrogels in bordered pit membranes. Rewatering could have increased the permeability of hydrogels in *E. wandoo* and caused increased $K_{smax}$ after embolism removal during pressurisation. However, it is not clear if and to what extent hydrogels could contribute to the observed differences in $K_{smax}$ in droughted and rewatered *Eucalyptus* seedlings.

**Effect of drought and rewatering on non-structural carbohydrate pools**

Contrasting water-use strategies can have an influence on carbon pools during drought and may determine the causes of drought-related mortality (McDowell et al., 2008). Because isohydric species reduce their water loss through stomatal closure and consequently diminish photosynthetic carbon assimilation, they are likely to deplete carbon pools and may die from carbon starvation during prolonged periods of drought. Anisohydric species, in contrast, maintain relatively high $g_s$ to allow gas-exchange, but risk xylem embolisms due to high xylem tension, which could eventually lead to death as a result of hydraulic failure. I thus expected, that the relatively isohydric species, *E. marginata*, would deplete its carbon pools in response to drought, while *E. wandoo*, the relatively anisohydric species, would show a progressive reduction in hydraulic conductivity, but show high carbon pools during drought. However, I found no depletion, but rather an accumulation of soluble sugars in both *Eucalyptus* species in response to drought. Soluble sugar concentrations increased with decreasing $\Psi_{PD}$ in stems of both species (Chapters 2 and 3), and an accumulation of soluble sugars in response to a prolonged drought was also apparent in bark including cambium, but not in roots (Chapter 3). The increased sugar pools in droughted seedlings, despite reduced $g_s$, indicate that growth had decreased before photosynthesis was greatly affected (Hsiao et al., 1976). As both species show similar changes in NSC concentrations, growth could have been affected earlier in the more isohydric species *E. marginata* compared with that in the more anisohydric species *E. wandoo*.
The cessation of growth is a common early response to drought due to the sensitivity of turgor pressure to water deficit (Hsiao et al., 1976), unless turgor can be maintained through mechanisms such as osmotic adjustment (Sharp et al., 2004). Mitchell et al. (2014) proposed three phases during a plant’s response to water deficit. In the first phase, carbon uptake and assimilation exceeds carbon use for growth and respiration leading to a positive carbon balance. During the second phase, growth ceases due to a water deficit, but carbon assimilation is maintained to some extent, which results in an accumulation of carbon. The last phase is characterised by cessation of net carbon uptake, because of stomatal closure during severe water shortage; therefore, carbon pools decrease as carbon needs for respiration exceed carbon assimilation. My findings do not indicate that the third phase was reached in the experiments in Chapter 2 or 3, despite a substantial reduction in gs. Since soil water was almost completely depleted, I surmise that hydraulic failure would have occurred instead of carbon starvation. The last harvest in Chapter 3 was conducted when some of the large spare seedlings showed complete tissue desiccation in leaves, followed by leaf shedding. These are signs of hydraulic failure and not carbon starvation (Brodribb et al., 2010). Furthermore, the overall carbon pools in leaves were the same across all treatments in both species which would be unexpected if carbon was limiting while water was available. I thus conclude that hydraulic failure would have been a more likely reason for the symptoms observed in the spare seedlings.

It has been suggested that increased carbon pools are important for osmoregulation (Sevanto et al., 2014). During the last harvests in Chapter 3, there was a trend for higher starch concentration in leaves of well-watered and rewatered seedlings, while droughted seedlings had higher soluble sugar concentrations. The hydrolysis of starch may assist with osmotic adjustment and the maintenance of hydraulic function during drought (Hsiao et al., 1976). Within 18 hours of rewatering, a reversal of this trend had occurred and soluble sugar as well as starch pools were similar to those found in well-watered seedlings, indicating that it was not necessary to maintain large soluble sugar pools for osmotic adjustment after rewatering. O’Brien et al. (2014) found that large carbon pools extended the period that plants survive during drought. Furthermore, large carbon pools may allow a reduction of water loss through stomatal closure, which would then extend the period until hydraulic failure occurred. An early cessation of growth may have allowed a reduction of stomatal conductance and carbon assimilation to a level that was
adequate for maintenance respiration and osmotic adjustment in *Eucalyptus* seedlings, without depleting carbon pools.

Upon rewatering of droughted seedlings, carbon pools in the sapwood as well as the bark in both species were rapidly reduced and were similar to carbon pools found in well-watered seedlings. If soluble sugars were exported in the transpiration stream (Zwieniecki & Holbrook, 2009), increased carbon pools would have been found in roots or leaves, which was not observed. However, small increases in leaves or roots may not have been detectable. Furthermore, soluble sugars were not converted into starch as no significant increases in the starch concentration were detected. It is possible that soluble sugars were converted into compounds that were not analysed and thus not detected or that sugars were used in metabolic processes. Thomas & James (1999), for example, found a conversion of fructose into fructans in response to rewatering after drought in *Lolium perenne*. Since carbon assimilation started rapidly after rewatering in *L. perenne*, metabolic carbon requirements may have been met and thus allowed a conversion of simple sugars into storage sugars. When droughted *Quercus robur* trees were rewatered after a drought treatment, stem respiration increased substantially, and initially exceeded the stem respiration rates under well-watered conditions (Saveyn *et al.*, 2007). This could indicate that large amounts of sugars are metabolised during the recovery from drought and may offer an explanation for the decreases of soluble sugar concentrations in rewatered *Eucalyptus* seedlings.

*Potential significance of maintaining high carbon pools*

Many woody species in Mediterranean-type climates resprout after disturbances such as fire and drought. In *Eucalyptus* species, resprouting is common, and can occur from stems, lignotubers, and roots (Grant *et al.*, 1997). In order to be able to resprout, meristems have to survive the disturbances and reserves need to be present to support regrowth (Bond & Midgley, 2001). Depleting carbon pools during severe drought stress may thus reduce the ability of a species to resprout if drought is alleviated before tree death occurs. Matusick *et al*. (2013) reported extensive canopy collapse in *E. marginata* and other dominant tree species in response to extreme heat and drought in southwestern Australia in 2010/2011. Most of the trees in affected patches showed foliar browning in the entire canopy, or even a complete loss of foliage. In the following year, however, many of the trees that were thought to be dead showed resprouting along the
stem or from lignotubers (especially *E. marginata*; personal communication, G. Matusick). Mitchell *et al.* (2013) suggested that maintaining high carbohydrate levels during non-lethal drought could enhance the resilience of species, as carbohydrates could be used during the recovery process to produce new xylem and leaves. It would thus make sense for species with resprouting capacity to accumulate carbohydrates during drought for fast and successful resprouting when the climatic conditions are favourable.

**Future research**

Much further research is needed to improve our understanding of the mechanisms leading to drought-induced mortality and the recovery from drought.

The experiments conducted in this thesis focused on embolism formation and repair in xylem vessels of stems. Since I found greater xylem vulnerability in *E. wandoo* grown in sandy soil, even when leaf water potentials were relatively high, it is likely that the root water uptake was affected and may have contributed to an impaired water transport in the stem xylem. I thus suggest that further research should aim at understanding the causes of root embolisms and if embolisms in root xylem vessels can propagate into the stem xylem. Would conductivity losses in a part of the root system then affect the water transport in the stem? And what would be the significance of a higher or lower xylem vulnerability in roots compared with that in stems (Tyree & Ewers, 1991; Tsuda & Tyree, 1997; Meinzer *et al.*, 2001)? Would a greater vulnerability in stems protect roots from critical xylem tensions or vice versa? Could the stem xylem recover completely while much of the xylem of the root system is still embolised? These questions illustrate that a better understanding of the relationship between root and stem hydraulic systems is needed, as it will not be possible to predict the responses of trees to drought without an understanding of those basic processes within trees.

The findings from Chapters 2 and 3 show that carbohydrates accumulate in stems of *Eucalyptus* seedlings during drought; however, it is not clear where exactly the sugars are located. In order to provide a driving force for the refilling of embolised vessels, soluble sugars would have to be excreted into xylem vessels (Zwieniecki & Holbrook, 2009; Nardini *et al.*, 2011). It is thus necessary to measure sugar concentrations in the xylem sap of water-filled and embolised vessels. Furthermore, the source of water for
refilling is not clear, but it has been suggested that phloem unloading of sugars and water is involved in the process. However, Zwieniecki & Holbrook (2009) argue that the osmotic gradient between the phloem and embolised vessels would not be sufficient to enable fast refilling. I found that leaf water potentials recovered faster than PLC, showing that water redistribution in the stem is successful with limited hydraulic conductivity of the stem. However, if water for refilling was only sourced from the phloem, the speed of recovery would be reduced along with higher reductions in conductivity. My findings indicate that recovery from over 90 to about 60% PLC was possible within two hours. I thus suggest that alternative mechanisms might play a role in the recovery from embolisms, such as vapour-driven refilling proposed by Zwieniecki & Holbrook (2009). In summary, future research should focus on concentrations of soluble sugars in xylem vessels and the sources of water for refilling of embolised vessels.

Conclusions
My findings show that differences in water-use strategies in two Eucalyptus species contribute to the formation and reversal of embolisms. The relatively isohydric species, E. marginata, showed a conservative use of water through maintenance of relatively high water potentials and low stomatal conductance. As a consequence, an accumulation of embolisms was avoided until very low soil water contents were reached. In E. wandoo, on the other hand, the formation of embolisms was a result of high xylem tension due to low water potentials. When the two Eucalyptus species were grown in contrasting soil substrates, a sandy and a loamy soil, it became apparent that stomatal regulation may occur at lower soil water contents in E. wandoo which is possibly an adaptation to the heavier soils in its natural environment. Gradual decreases in soil water potentials in loamy soils may allow for low water potentials, while low water potentials could result in hydraulic discontinuity in sandy soils that would impede water uptake. The higher xylem vulnerability in E. wandoo in sandy soils was thus likely associated with impeded water uptake from dry parts within the soil profile in the pots, while high leaf water potentials observed in E. marginata may have contributed to the maintenance of hydraulic conductivity within the soil, thus avoiding increased xylem vulnerability in sandy soils.
The recovery from drought-induced xylem embolisms in the two *Eucalyptus* species started rapidly after rewatering, but was not always complete. Differences in the duration of the drought treatment as a consequence of differences in soil volume in the experiments in Chapters 2 and 3 likely led to contrasting patterns of recovery in the two species. *Eucalyptus marginata* showed a much greater loss in conductivity and an incomplete recovery of conductivity within eight hours after rewatering in the small pots, but a complete recovery of PLC within 18 hours of rewatering in the large pots. In *E. wandoo*, PLC was also greater in the small pots compared with that in the large pots, despite similarly low soil water contents towards the end of the drought treatment; however, the recovery from xylem embolisms was impaired in the large pots. I suggest that root integrity could play a role during the recovery from drought. Low soil water potentials in the small pots could have impaired root water uptake upon rewatering in *E. marginata* and thus reduced its ability to recover from drought-induced xylem embolisms. In the large pots, the water potentials remained relatively high throughout the drought treatment which would have protected roots against embolisms and thus contributed to the recovery in *E. marginata*. The opposite could have been the case for *E. wandoo*, where the duration of the drought associated with very low soil water contents in the upper layers of the large pots could have impaired root function and consequently root water uptake and transport upon rewatering.

Differences in water-use strategies between the two *Eucalyptus* species did not result in differences in carbon pools in response to drought. Diminished stomatal conductance did not lead to reductions in carbon pools in *E. marginata*, potentially because of an early reduction of growth. In both species, NSC concentrations were the same or higher in response to drought, suggesting that the more isohydric water-use strategy in *E. marginata* did not necessarily predispose this species to carbon starvation. Higher NSC concentrations as a result of drought were associated with higher levels of soluble sugars, while starch concentrations largely remained the same across treatments. Higher soluble sugar concentrations are likely important for osmoregulation and the maintenance of hydraulic function during drought (Hsiao *et al.*, 1976; Sevanto *et al.*, 2014; O’Brien *et al.*, 2014). In response to rewatering, NSC concentrations recovered to levels similar to those of well-watered controls, indicating that sugars were used during the recovery or removed in the transpiration stream. If large amounts of NSC were used during embolism repair, drying and rewetting cycles could deplete carbon pools.
However, carbon pools did not decrease when *Picea abies* trees subjected to drying-rewetting cycles (Hartmann *et al.*, 2013). In summary, my findings support the idea that sugars play an important role during drought and the recovery from drought (Zwieniecki & Holbrook, 2009; Nardini *et al.*, 2011) but the mechanisms remain unknown.
References


