



Measurements done on excised stems indicate that hydraulic recovery can be an important strategy used by *Eucalyptus* hybrids in response to drought

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Abstract

Key message A high-risk hydraulic strategy might be linked to embolism recovery in *Eucalyptus* hybrids, allowing plants to have high hydraulic conductivity regardless of safety.

Abstract Plants use a variety of strategies to mitigate or reduce drought-induced hydraulic failure. Recovery of hydraulic conductivity after drought stress can be an important mechanism used to avoid drought-induced mortality; however, the underlying mechanism of hydraulic recovery is still poorly understood. We examined the hydraulic recovery response between *E. grandis* × *camaldulensis* (*GC*) and *E. urophylla* × *grandis* (*GU*) after drought. We aimed to determine if there is a trade-off between xylem safety and hydraulic recovery and what the underlying mechanism might be. Destructive measurements together with X-ray microtomography measurements were used to determine the extent of hydraulic recovery at various time intervals. We found two distinct hydraulic strategies used by plants. *GC* was more resistant to embolism formation as compared to *UG*; however, *GC* showed lower levels of hydraulic recovery after rewatering. Larger vessel sizes were related to increases in drought vulnerability. Hydraulic recovery was also related to functional traits of cells surrounding vessels, highlighting the possible role that these cells play to increase hydraulic conductance in the xylem and increasing connectivity between vessels. Our study suggest that hydraulic recovery can be an important hydraulic strategy used by *Eucalyptus* hybrids in response to drought. A high-risk hydraulic strategy might be linked to embolism recovery, allowing plants to have high hydraulic resistance regardless of safety.

Keywords Embolism · Recovery · Refilling · Drought stress

Introduction

An increase in drought-induced tree mortality (Hammond et al. 2019), which can be expected to have a significant impact on plant survival in a changing climate, has been found in several studies in the last few years (Anderegg et al. 2013; Allen et al. 2015; McDowell et al. 2018). Major increases in tree mortality are likely to have an impact on global carbon and hydrological cycles (Bonan 2008). It is,

therefore, important to understand the strategies that trees use to avoid drought-driven mortality to improve production and vegetation models (Fatichi et al. 2019; Hammond et al. 2019).

During drought stress the continuous water column in the xylem can be disrupted by the formation of air bubbles or embolism which can reduce hydraulic conductivity leading to hydraulic failure (Tyree and Sperry 1988; Sperry et al. 2017; Xiong and Nadal 2020). Embolism formation restricts the water movement through the soil–plant–atmosphere–continuum (SPAC) and can lead to stomatal closure which helps prevent further water loss (Becker et al. 2000). However, this leads to a reduction in CO₂ uptake which is needed for photosynthesis (Kramer and Boyer 1995; Becker et al. 2000). The restriction of water movement through the SPAC also affects the transport of carbohydrates, ultimately limiting metabolism and growth (McDowell et al. 2008; Anderegg et al. 2015; Trugman et al. 2018).

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A wide range of strategies are used by plants to mitigate or reduce hydraulic failure, ranging from avoiding embolism formation, refilling embolism or repairing damaged hydraulic pathways through regrowth (Ogasa et al. 2013; Sperry and Love 2015; Klein et al. 2018). Many previous studies have focussed on how plants avoid hydraulic failure (Pittermann et al. 2006; Gleason et al. 2016; Venturas et al. 2017; Barotto et al. 2018), with focus not always given to recovery strategies deployed by plants (Klein et al. 2018). While it has been suggested by several researchers that plants can recover lost hydraulic conductance, there is still a debate about the underlying mechanisms and whether or not recovery can take place over short time periods (Cochard et al. 2001; Nardini et al. 2011; Klein et al. 2018).

It is well established that embolism refilling can take place when soil water potential increases (Brodersen and McElrone 2013; Niu et al. 2017), with evidence showing that some species refill embolism at night following increases in soil water potential (Ogasa et al. 2013; Brodersen and McElrone 2013; Klein et al. 2018; Zeppel et al. 2019). The use of nonstructural carbohydrates (NSC's) have also been suggested to play a role in aiding hydraulic recovery (Nardini et al. 2011; Tomasella et al. 2019a, b). Liu et al. (2019) found that cortical photosynthesis promoted accumulation of NSC's in the bark and was correlated with water uptake assisting in hydraulic recovery, while Secchi et al. (2021) showed that recovery is energy dependant and that the accumulation of sugars within the apoplast assisted in recovery. The physiological characteristics of plants related to embolism refilling and whether the strategies used by plants depend on their resistance to cavitation is, however, still largely unknown (Ogasa et al. 2013; Fernández et al. 2019).

Understanding these phenomena is of particular relevance to the genus *Eucalyptus*, a planted hardwood with a wide distribution (Myburg et al. 2014) and of great commercial significance. It has been shown that there is a wide variation in hydraulic responses to drought within the *Eucalyptus* genus (Bourne et al. 2017). Recent work by Choat et al. (2018) using CT imaging found that embolised vessels in various *Eucalyptus* spp. were not completely refilled even 72 h after rewatering, however evidence of possible partial refilling was seen. Complete recovery was however not seen in the experiment, with air bubbles persisting in all vessels to some degree (Choat et al. 2018). Work done by Zeppel et al. (2019), however, showed evidence of hydraulic recovery within *Eucalyptus* spp. after 12 h rewatering.

Measurements of hydraulic recovery using percentage loss of hydraulic conductance (Ogasa et al. 2013; Zeppel et al. 2019) have, however, been criticised for possible measurement artefacts caused by cutting xylem under tension, which allows the entry of air into vessels even when xylem is excised under water (Wheeler et al. 2013; Torres-Ruiz

et al. 2015). The significance of these artefacts has, however, also been questioned (Trifilò et al. 2014; Ogasa et al. 2016), with some authors arguing that rehydration to relax xylem tension might be the cause of discrepancies seen in measurements and should be accompanied by in vivo measurements as well (Nardini et al. 2011, 2017; Ogasa et al. 2016; Savi et al. 2016; Nolf et al. 2017).

Despite concerns regarding measurement artifacts leading to overestimating xylem vulnerability to embolism (Wheeler et al. 2013; Torres-Ruiz et al. 2015; Charrier et al. 2016; Lamarque et al. 2018), the number of studies showing recovery when there is a reduction in drought stress has been growing (Brodersen and McElrone 2013; Nardini et al. 2017; Brodersen et al. 2018; Liu et al. 2019; Tomasella et al. 2019a; Secchi et al. 2021). Studies combining in vivo measurements, such as X-ray microtomography (Kim and Lee 2010; Torres-Ruiz et al. 2015; Choat et al. 2015; Knipfer et al. 2016) and magnetic resonance imaging (Zwieniecki et al. 2013; Ogasa et al. 2016) with traditional measurements of embolism formation and recovery, can help validate recovery responses seen in the xylem (Nardini et al. 2017). The use of CT scans has created the opportunity to study embolism recovery in conjunction with traditional hydraulic measurement methods to avoid possible controversies regarding refilling. More studies are also needed where plants are not rewatered, to exclude refilling through increased root pressure.

In response to these questions, we looked at a variety of physiological variables related to the hydraulics of a plant to understand the drought recovery response, focussing on two physiologically contrasting commercial *Eucalyptus* hybrids. We focussed on using both hydraulic measurements and imaging methods (micro-CT) of hydraulic recovery. We aimed to (1) determine if the strategy of xylem embolism recovery and xylem embolism resistance is related to specific physiological or anatomical variables and (2) determine if there is a trade-off between xylem safety and a plants ability to recover from embolism after drought.

Methods

Plant material

All work was done using 42 potted rooted cuttings of two *Eucalyptus* hybrids. A 2-year-old *E. grandis* × *camaldulensis* (GC) ($n=21$) and *E. urophylla* × *grandis* (UG) ($n=21$) plants were grown under the same conditions under shade netting and were watered to field capacity twice a day prior to experimental work. Plants were grown in 2 L pots in a soil mixture with a ratio of 1:2 fine sand and composted pine bark. When plants were initially planted in pots, plants were fertilised once with a composted chicken manure.

Plants were then periodically fertilised with the controlled release Haifo Multicote 8 (N:15%, P:3%, K:12%, S:7%) fertiliser until the start of sampling. The height of plants varied between 0.7 and 1.2 m, with the canopy starting roughly halfway up the stem. No significant difference in height was seen between the two hybrids. The average stem diameter of all plants was 6.47 ± 0.94 mm as measured at 20 cm above the base of the stem. All measurements were done during May–December 2020.

Vulnerability curves

Vulnerability curves were constructed using the bench dehydration method and plotting the changes of hydraulic conductivity over xylem water potential (Ψ_x) (Tyree and Sperry 1988). The xylem tension at which stems lose 50% of their hydraulic conductance (P50) is used as a measure of how resistant plants are to embolism formation caused by drought (Martin-StPaul et al. 2017).

Plants were cut at the base of the stem before dawn, placed in water and covered with a black plastic bag before being transported to the laboratory where they were dried to different Ψ_x . Bench dehydration was used as it relies on desiccation and the introduction of artefactual embolism can be minimised as compared to methods like the centrifuge technique (López et al. 2018). Xylem water potential was measured from excised leaves using a pressure chamber (SKYE plant moisture system, SKPM 1400 series). Cut plants were placed in a sealed bag with a damp paper towel and allowed to equilibrate water potential between leaves and stem for 30 min before any measurements were made.

After Ψ_x measurements were made, hydraulic conductivity (K_h) was measured to obtain Ψ_x and K_h pairs. Stems were recut roughly 2–5 cm above the base of the plant while being submerged in water. Stem segments of 15–20 cm, which is more than half the stem length, were then cut while submerged and ends were shaved with a razor blade before being connected to tubes. Using gravity to create a hydraulic pressure head, samples were first perfused with distilled water and measurements were only made when the flow rate became stable after a few minutes (Torres-Ruiz et al. 2012). It is important to note that although steps have been taken to minimise cutting artifacts, possible cutting artifacts could however have occurred during sample preparation.

The K_h was calculated as the flowrate (kg s^{-1}) over the water potential difference (MPa^{-1}) along the stem segment. The hydraulic pressure head never exceeded 5.5 kPa to ensure that emboli were not artificially flushed during measurements. Flowrate was measured on an analytical balance, which can make measurements up to 3 decimal points, as change in water mass over time. Flowrate was measured at three different hydraulic pressure heads, including flow rate at no hydraulic pressure head to improve the accuracy of

K_h measurements of each stem segment (Torres-Ruiz et al. 2012).

Xylem recovery index

The Xylem Recovery Index (XRI) is based on the ratio of hydraulic conductivity under rewatered conditions and wet conditions (Ogasa et al. 2013). Zeppel et al. (2019) adjusted the XRI to account for the extent of drought experienced. The hydraulic method similar to the method used to construct vulnerability curves was used to make hydraulic measurements. Plants were dehydrated until more than 65% of hydraulic conductivity was lost based on Ψ_x (*UG*: mean = -2.86 ± -0.9 MPa; *GC*: mean = -3.26 ± -0.23 MPa). Cut stems with intact canopies that were dehydrated during the construction of vulnerability curves were rehydrated overnight (at least 15 h) by placing the cut base of stems in water before measuring the hydraulic conductivity of each species after rewatering. Hydraulic measurements were made in three stems of plants before dehydration, on three stems of plants that were dehydrated and then three stems of plants that were rehydrated overnight after dehydration.

The hydraulic conductivity of cut stems of plants were measured before drought, during drought and after rewatering using the method described above. XRI can be calculated as:

$$\text{XRI} = \frac{K_{hD} - K_{hRW}}{K_{hD} - K_{hW}}$$

where K_{hD} is the K_h measured during drought, K_{hRW} is the K_h measured after rewatering and K_{hW} is the K_h measured before drought is induced.

Micro-CT scans and Image analysis

Plants were cut at the base of the stem late afternoon before scanning and allowed to dehydrate overnight until >65% loss of hydraulic conductivity was achieved based on leaf water potential (Ψ_l) measurements. Dehydrated cut stems with plant canopies intact were scanned the following morning and then placed in water to allow rehydration after scanning for two hours. Cut plants with intact canopies were rehydrated for 2 h by placing cut base of stems in water. A single replication was also done on plants that were rehydrated for 24 h. Leaf water potential measurements were done on rehydrated plants to ensure that recovery took place. The rehydrated plants were then rescanned. The scanned area on the stem was marked to ensure that the same area was rescanned over time. Scans were taken in the central part of the stem below leaf formation, which was more than 16 cm away from the cut base. The scanned distance from the cut also minimised any recovery

artefacts seen as a result of capillary action. It is important to note that although steps were taken to minimise the introduction of artefactual embolism, it could not have been fully excluded from the study. The duration of scans was 30 min and to ensure that plants lost minimal amount of water during scans, the leaves of plants were wrapped in clingwrap and the cut stem was sealed with parafilm for the duration of the scan.

All scans were done at the CT facility situated at Stellenbosch University (du Plessis et al. 2016). To generate an image with 15 µm voxel size, 1800 projections were taken during a 360° rotation with 140 kV and 80 µA. Cross-sectional (2D) images were extracted from the middle of the 3D scan volume using Volume Graphics VGStudioMax 3.1. An adaptive Gauss filter was used to reduce noise in the image.

All cross-sectional images were further analysed using the image analysis software ImageJ (Schneider et al. 2012). A threshold was applied to all images to extract only black pixels. This ensured that only air-filled vessels were captured, since air appeared as black on images due to density differences. To determine which vessels refilled, an overlay was created with images taken at dehydration and after rewatering. Images were aligned based on feature selection and visually inspected to ensure alignment (Fig. 1). Within ImageJ, the *analyse particles* function was used to measure vessel dimensions and co-ordinates. Vessel co-ordinates were used to calculate the distance to the nearest vessel neighbour as well as the distance of vessels from the perimeter of the stem.

To determine which vessels were embolised during dehydration, cross sectional slices of stems were made after the CT scanning and stained with an alcian blue and safranin solution. Images of the cross sections and 2D images generated from the CT scans were aligned based on feature selection (Figs. 10 and 11 in supplementary material). To determine the accuracy of vessel area extraction during image processing, the percentage extracted vessel area was compared with the black/white pixel ratio of the original cross-sectional CT images.

In between scans, plants were removed from the CT scanner and placed in water. The plants had to be placed back in the CT scanner after rewatering. Even though the area that was scanned was marked on each stem, there was a small margin of error due to alignment differences.

The theoretical hydraulic conductivity ($\text{kg MPa}^{-1} \text{s}^{-1} \text{m}$) was calculated for all embolised and refilled vessels (Tombesi et al. 2010). The K_h of embolised and refilled vessels were calculated based on the Hagen–Poiseuille’s law (Tyree and Ewers 1991) modified by Tombesi et al. (2010):

$$K_h = \left(\frac{\pi \rho}{128 \eta} \right) \sum_{i=1}^n d_i^4$$

where η is viscosity of water set at $1 \times 10^{-9} \text{ MPa}\cdot\text{s}$; ρ is fluid density of water set at 1000 kg m^{-3} and d is the radius of a vessel (m) (Tombesi et al. 2010).

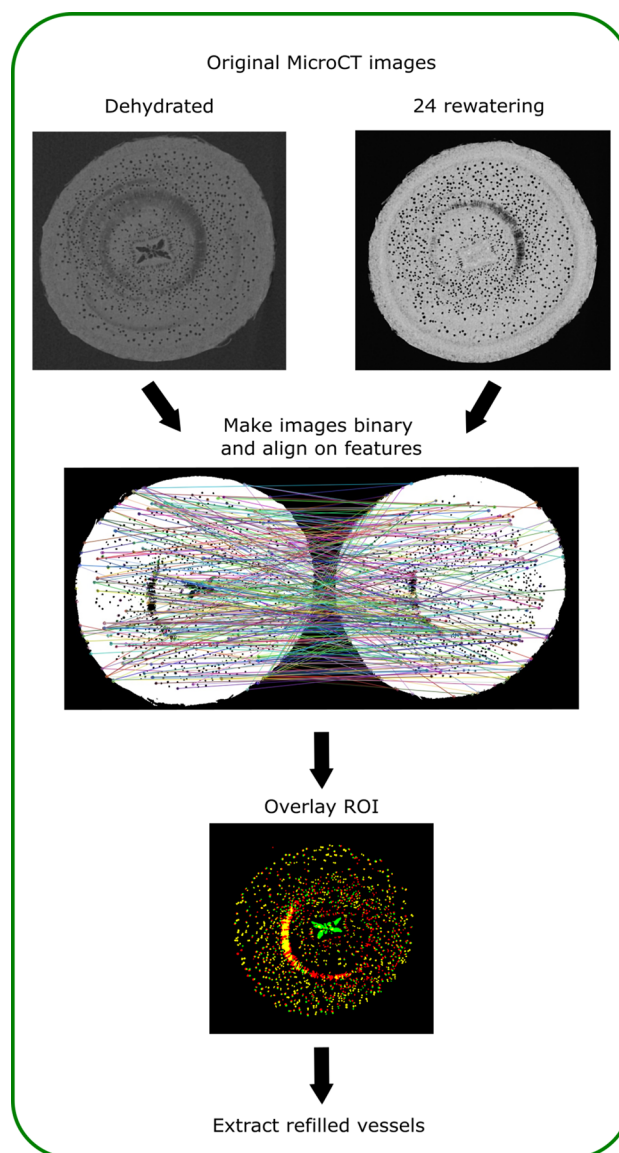


Fig. 1 Image processing pipeline to extract refilled vessels as captured from cross-sectional images from CT scans

Anatomical variables

In order to visually determine the extent to which water exits vessels and moves into adjacent tissue, a 1% safranin solution was allowed to flow through a 15 cm stem segment using a similar set-up as described in the vulnerability curve methods. This was done for three plants before dehydration for both hybrids. The hydraulic pressure head ranged between 5–7 kPa. The solution flowed through the stem segment until the safranin solution was seen at the distal end of the stem. Cross sections of the stem segments were manually made in the middle of each 15 cm stem segment.

The following measurements were made from photos of the cross sections using ImageJ software (Schneider et al.

2012): vessel area (mm^2), the stained areas (mm^2) (area that was stained by the safranin dye outside of the vessel), bridge length (μm) (the distance between two vessels if there is a continuous stained area between them) and number of connecting vessels (vessels that are connected by the stained area) (Fig. 2).

Cross-sections of stems after CT scanning was also taken and stained with an Alcian blue and safranin solution. This allowed for the pattern detection of nonlignified cells surrounding vessels (which can include parenchyma and vasicentric tracheids) (Vazquez-Cooz and Meyer 2002).

All images used for processing were obtained using a Motic BA310Pol polarizing microscope with the 40 \times objective.

Statistical analysis

All data was tested for normality before analysis. Homogeneity of variances was also tested before analysis. A single sample t-test was used to test for difference in the recovery strategies, which included the vulnerability curves and xylem recovery index, used between the two hybrids.

A Spearman's correlation matrix was used (since data did not show normality) to determine whether there was any significant relationship between anatomical features and functional hydraulic traits. The relationship between the following variables were tested: vessel area (mm^2), the stained area (mm^2), bridge length (μm) and number of connecting vessels. A non-parametric Kruskal–Wallis test was used to determine if there was a significant difference in anatomical features and hydraulic traits between hybrids. Distribution curves were used to compare vessel size distribution between the two hybrids.

From the CT-scanned images the percentage difference in vessel area and vessel k_h was used to determine if vessels refilled. Distribution curves were used to compare vessel size and the distance of the nearest vessel neighbour distribution between the refilled and embolised vessels for both hybrids.

Results

Functional hydraulic traits

The two hybrids exhibited different xylem morphological characteristics (Table 1). There was a significant difference seen in the distribution of vessel sizes between the two hybrids ($H = 14.139$, $p < 0.05$), with larger vessels found in *UG* but a higher count of vessels in *GC* (Table 1) (Fig. 3). *GC* showed high counts of smaller vessels with the highest frequency of vessels close to its mean. *UG* had the largest vessels with a maximum vessel area of 0.015 mm^2 as compared to the maximum vessel size of 0.008 mm^2 seen in *GC*.

Importantly, there was also a distinct difference seen in the hydraulic strategies used by the two hybrids. The vulnerability curves of the two hybrids were different, with *GC* losing halve of its hydraulic conductivity at a Ψ_x (P50) of -2.72 MPa as compared to *UG* which had a P50 value of -2.33 MPa ($t = -2.65$, $p = 0.008$) (Fig. 4). The hydraulic recovery response was also different between

Table 1 Kruskal–Wallis H value and significance levels of hydraulic characteristics between *E. urophylla* \times *grandis* (*UG*) and *E. grandis* \times *camaldulensis* (*GC*)

	Kruskal–Wallis test	<i>UG</i>	<i>GC</i>
Bridge length (μm)	$H = 5.109$	51.38 ± 38.17	34.933 ± 25.34
Vessel area (μm^2)	$H = 14.139^{***}$	2290 ± 1116	1933.8 ± 3000
Stained area (μm^2)	$H = 52.423^{***}$	9766 ± 5924	5333 ± 4553
Connecting vessels	$H = 3.837^*$	0.957 ± 1.141	1.385 ± 1.624

Mean and standard deviation of hydraulic characteristic of each hybrid ($n = 3$)

Significant values are indicated as follows: * indicates significance of $0.05 < p < 0.06$, ** indicates significance of $0.001 < p < 0.05$, *** indicates significance of $p < 0.001$

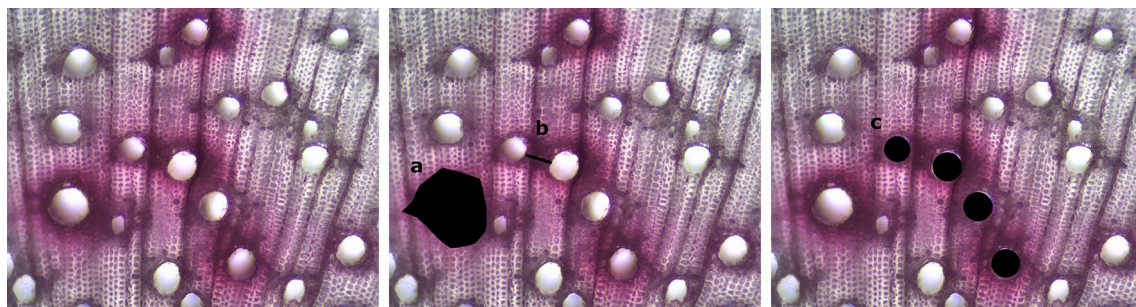


Fig. 2 Safranin stained zone surrounding vessels. The following measurements were made on the photos: Stained zone (a), Bridge length (b) and Connecting vessels (c). Photos were taken at a 40 \times magnification

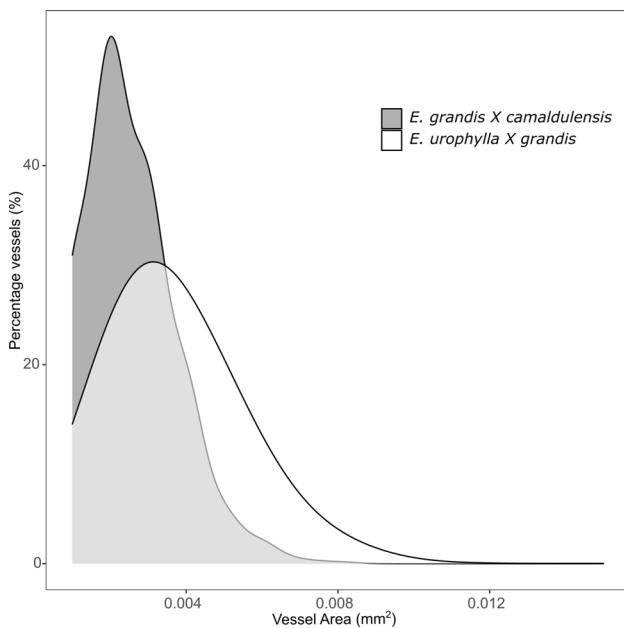


Fig. 3 Vessel area size distribution of *E. urophylla* × *grandis* and *E. grandis* × *camaldulensis* as percentage over vessel area size

the two hybrids. *UG* had a much higher XRI ($H=3.857$, $p<0.05$) as compared to *GC* (Fig. 5). It is important to note that both hybrids showed a level of hydraulic recovery, with the measured hydraulic conductivity increasing after rewatering.

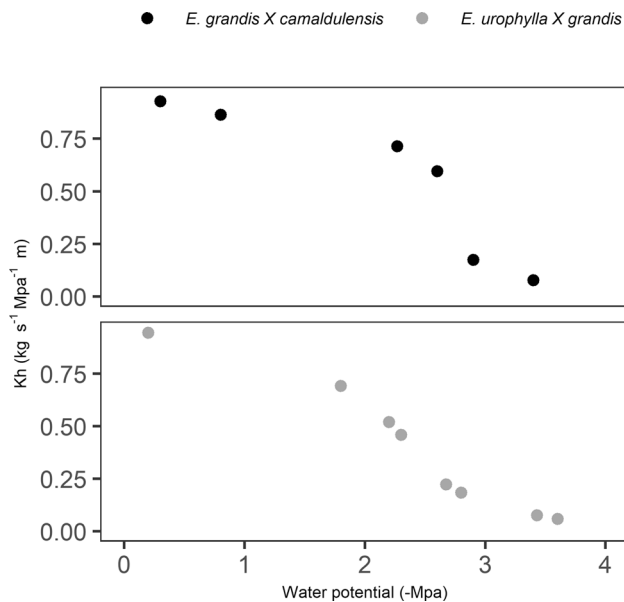


Fig. 4 Vulnerability curves indicating the hydraulic conductivity (K_h ; $\text{kg m MPa}^{-1} \text{s}^{-1}$) over decrease xylem water potential (Ψ_x). A significant difference was seen between the curves of the two hybrids ($t=-2.65$, $p=0.008$)

Functional traits of vessels and surrounding cells

There was a significant difference in the area of safranin staining around vessels between the two hybrids ($H=52.423$, $p<0.05$) (Table 1). *UG* showed larger areas of safranin staining when compared with *GC* (Table 1).

The bridge length (continuous stained distance between two vessels) was on average longer in *UG* (mean = $51.38 \mu\text{m}$) compared to *GC* (mean = $34.99 \mu\text{m}$). The connecting vessels (vessels that are connected by the stained area) were different between the two hybrids ($H=3.837$, $p=0.05$), with more connecting vessels seen in *GC*.

For both hybrids, there was a positive relationship between safranin-stained area and vessel size (*UG*: $r_s=0.312$, $p<0.05$; *GC*: $r_s=0.374$, $p<0.05$). In *GC* the bridge length was significantly correlated to the stained area ($r_s=0.374$, $p<0.05$), however this correlation was not seen in *UG* ($r_s=0.088$, $p>0.05$). The vessel area and bridge length were not significantly correlated in either hybrid (Tables 2 and 3 in Supplementary material).

Patterns of blue staining was also observed around vessels, when cross-sectional stem segments were stained with a safranin and Alcian blue double stain.

Recovery response

Plants were dehydrated until more than 65% of hydraulic conductivity was lost based on Ψ_1 (*UG*: mean = $-2.86 \pm -0.9 \text{ MPa}$; *GC*: mean = $-3.26 \pm -0.23 \text{ MPa}$) and estimated vulnerability

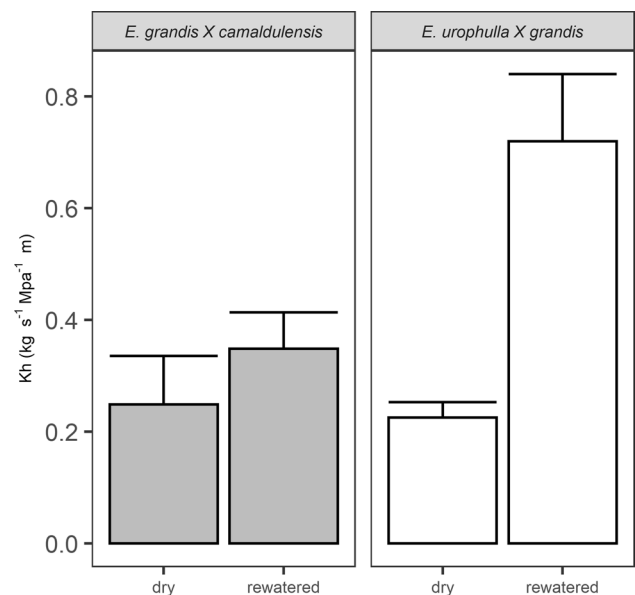


Fig. 5 Mean hydraulic conductivity of stems measured during drought and after rewatering. Error bars show 95% confidence interval

curves. Recovery of Ψ within both species was assessed after 2 h of rewatering (*UG*: mean = -1.07 ± -0.64 MPa; *GC*: mean = -1.13 ± -0.42 MPa) and 24 h of rewatering (*UG*: -0.425 ± -0.26 MPa; *GC*: -0.5 ± -0.20 MPa).

From the CT scan results, evidence of vessel refilling was seen for both hybrids (Figs. 6 and 7). When analysing the black/white pixel ratio there was a significant difference in percentage of vessels seen that refilled between the two hybrids ($H=3.857$, $p<0.05$), with a higher percentage of vessels refilling in *UG* after 2 h (Fig. 8). Similar trends were, however, clearly seen whether the black/white pixel ratio or the K_h calculated from extracted vessels was used during analysis and it was decided to report results of analysis done on the extracted vessel K_h only (Fig. 8). In both hybrids the K_h that recovered were however small (*UG*: mean = $2.99 \pm 3.31\%$, *GC*: mean = $0.35 \pm 0.39\%$). Vessels were; however, not analysed on a longitudinal axis during CT-scan analysis. No distinguishment was therefore made between complete and partial refilling.

When comparing the percentage extracted vessel K_h to the black/white pixel ratio of the original cross-sectional CT images refilled vessel area was overestimated when analysing the extracted vessel area. During the image analysis in both hybrids there was an overestimate of percentage of vessels that recovered during rewatering with an estimated mean error of 1.31% in measurements of *GC* and an estimated mean error of 2.15% in measurements of *UG* (Fig. 8).

The vessels that embolised in both hybrids were significantly closer to each other as compared to the vessels that refilled (*GC*: $H=150.58$, $p<0.05$, *UG*: $H=86.18$, $p<0.05$) (Fig. 9).

The vessels that refilled in *UG* were significantly closer to the stem perimeter ($H=719.79$, $p<0.5$), however this trend was not seen in *GC* (Fig. 9). The vessels that refilled in both hybrids had significantly smaller k_h than the vessels that stayed embolised (*GC*: $H=160.65$, $p<0.05$, *UG*: $H=343.49$, $p<0.05$).

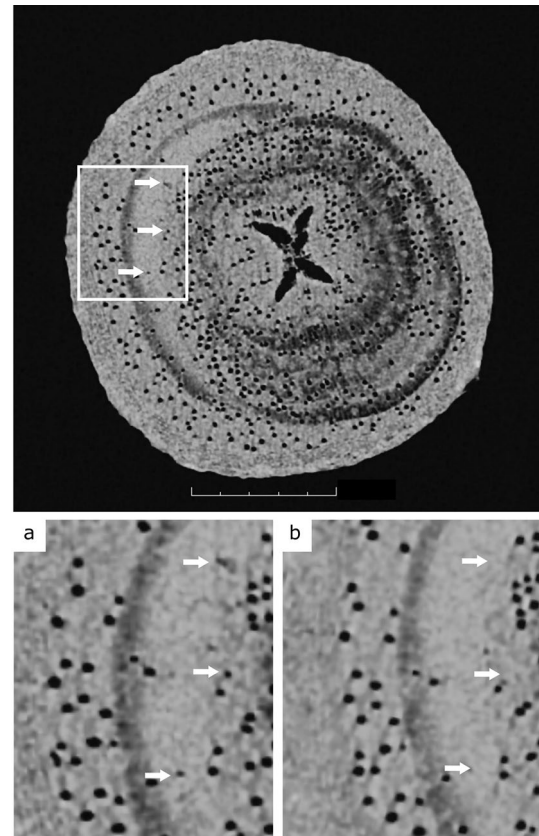


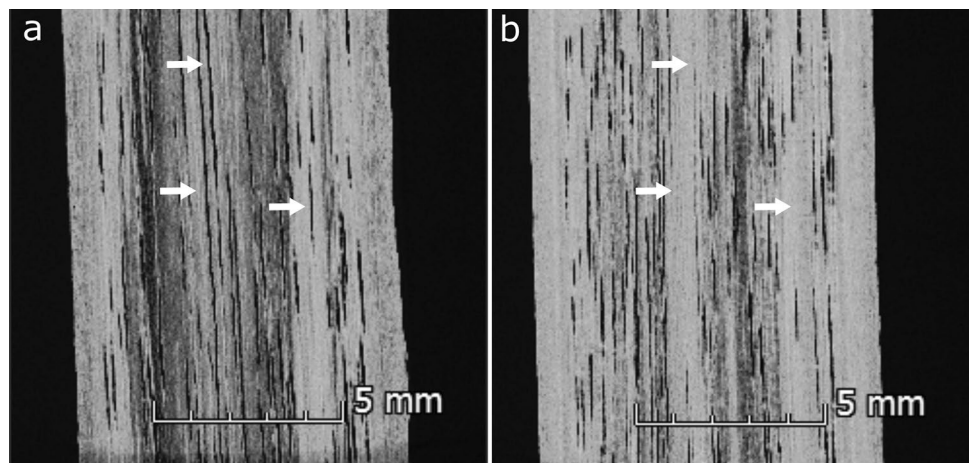
Fig. 7 Images showing refilled vessels during dehydration (a) and after 2 h of rewatering (b) in *E. grandis* × *camaldulensis*. White arrows indicate examples of refilled vessels. The white box shows area of focus

Discussion

Trade-off between safety, efficiency and recovery

Although the underlying mechanisms relating to embolism

Fig. 6 Longitudinal CT sections showing refilling of vessels in *E. urophylla* × *grandis*. CT scans show stem when dehydrated (a) and 24 h after rewatering (b). White arrows indicate areas of clear change seen after rewatering



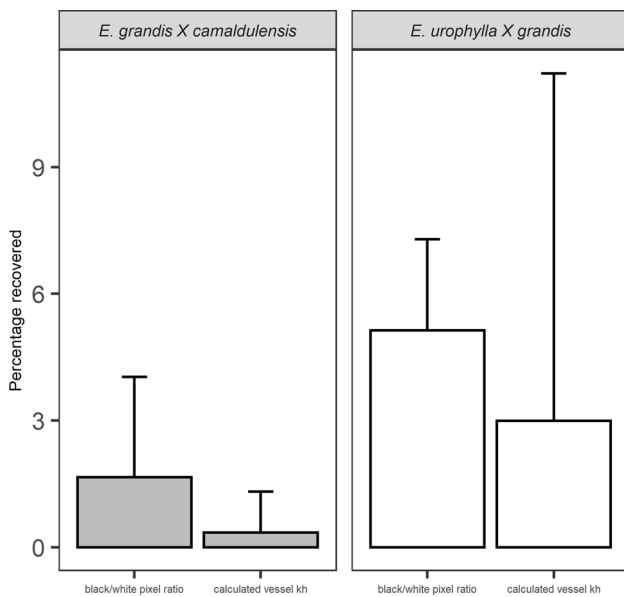


Fig. 8 Mean percentage vessels that refilled of each hybrid after 2 h of re-watering as calculated from black pixels of original CT scans and as calculated from extracted vessels after image processing. Error bars show 95% confidence interval. A significant difference was seen between two hybrids ($H=3.857$, $p<0.05$)

recovery are still not well understood, embolism recovery is increasingly considered an important hydraulic strategy used by plants in response to drought (Nardini et al. 2011). Plants often function at Ψ_x close to hydraulic failure thresholds (Ogasa et al. 2013). Drought levels in the field often exceed P50 values; however, certain plants can nonetheless survive post drought (Klein et al. 2011; Trifilò et al. 2015).

In this study, there was a trade-off seen between resistance to embolism and ability to recover hydraulic conductance following drought. Two distinct hydraulic strategies were observed between the two *Eucalyptus* hybrids, with the hybrid *UG* that is considered more drought-vulnerable (Van der Willigen and Pammenter 1998; Drew et al. 2009) having a higher rate of hydraulic recovery as compared to the embolism resistant hybrid. On the other hand, *GC* showed a greater resistance to embolism and hydraulic loss, an effect also seen by other authors (Van der Willigen and Pammenter 1998; Souden et al. 2020). It has been found that there is a correlation between P50 values and a plants ability to recover embolism (Klein et al. 2018), indicating that there might be a trade-off between hydraulic recovery and vulnerability.

Hydraulic measurements have been criticised for over-estimating drought vulnerability due to the introduction of artificial emboli during the cutting procedure (Wheeler et al. 2013; Torres-Ruiz et al. 2015). Although the possibility

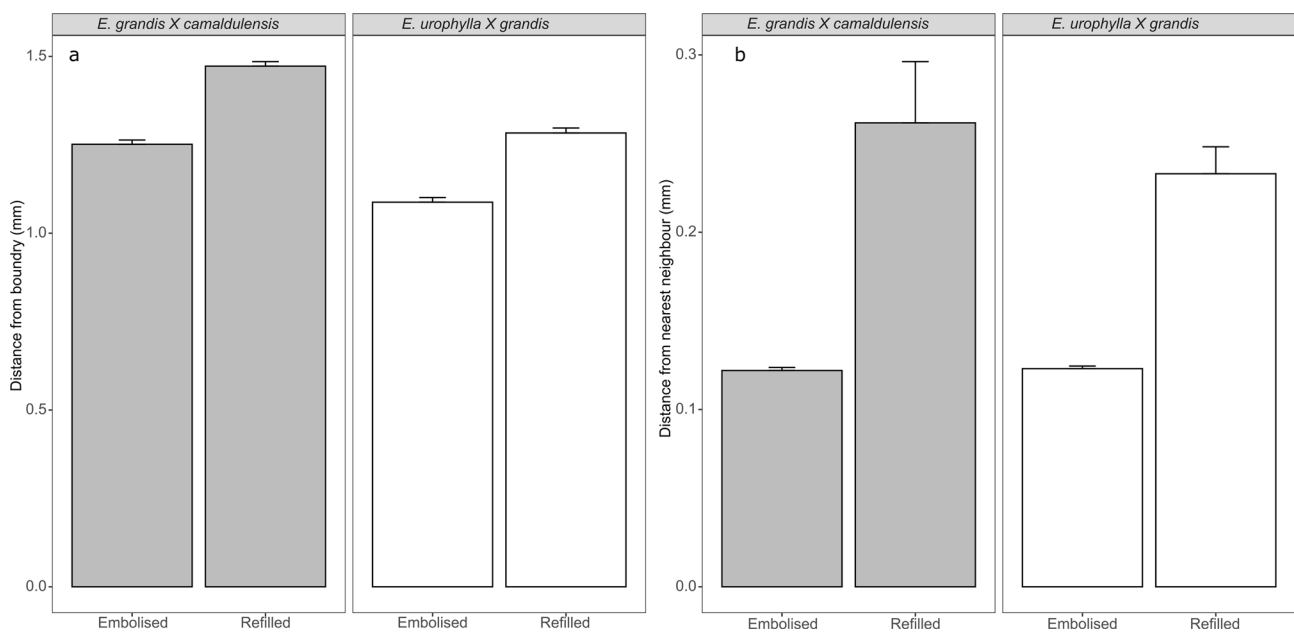


Fig. 9 Figure a show the mean distance of refilled and embolised vessels to stem perimeter; Figure b show the mean distance of embolised and refilled vessels to nearest other vessel. The vessels that embolised in both hybrids were significantly closer to each other as compared to the vessels that refilled (*GC*: $H=150.58$, $p<0.05$, *UG*: $H=86.18$,

$p<0.05$). The vessels that refilled in *UG* were significantly closer to the stem perimeter ($H=719.79$, $p<0.5$); however, this trend was not seen in *GC*. All information was extracted from CT scan images after two hours rewatering for both *E. grandis X camaldulensis* and *E. urophylla X grandis*. Error bars show 95% confidence intervals

of cutting artifacts could not be excluded from the study, both hybrids were subjected to the same conditions and measurement methods. Different responses could still be seen between the two hybrids further supporting that the responses seen was not due to measurement artifacts. The responses seen in *UG* and *GC* in our study; however, corresponds with the results seen in other studies where it was shown that *GC* is more resistant to embolism formation and drought as compared to *UG* (Van der Willigen and Pammenter 1998; Drew et al. 2009; Saadaoui et al. 2017; Souden et al. 2020), further highlighting the possible link to embolism resistance and recovery.

In our study, larger mean vessel area was seen in the *UG* hybrid that is putatively more vulnerable to drought. This correlation has been seen in other studies (Hacke et al. 2006; Lobo et al. 2018) with the previous studies noting the trade-off between xylem safety and efficiency, but related it to vessel length and not vessel area (Lens et al. 2011). Fernández et al. (2019) found that in several *Eucalyptus* species, vessel safety might be correlated to the area of non-vessel cells surrounding each vessel rather than vessel size.

In the previous studies a trade-off has been observed between efficiency and safety of vessels, with larger vessels compromised by decreases in safety (Gleason et al. 2016). The efficiency of vessels is dependent on their ability to transport water, while safety refers to vessels resisting formation and spread of embolism when soil water decreases (Sperry 2003). A plant's ability to recover hydraulic conductance can, however, also increase a plant's resilience against drought, allowing plants to have high efficiency and resilience regardless of safety (Ogasa et al. 2013).

The two hydraulic strategies seen within this study, highlighted that a high-risk hydraulic strategy might be coupled to a plant's ability to refill embolism. If plants cannot recover hydraulic conductance, they might be more conservative with the hydraulic strategy they use.

Embolism recovery

In our study, embolism recovery took place in both hybrids over short time periods (2–24 h) when plants were rehydrated. It has been previously showed that the recovery of embolism takes place over short time periods by measuring the percentage loss of hydraulic conductance over a drought–recovery cycle (Ogasa et al. 2013; Zeppel et al. 2019); however, in this study the use of successive CT scans on the same samples following rewatering gave further evidence that embolism recovery took place. Although the introduction of artefactual embolisms during sample preparation could not be excluded from the study, both hybrids were subjected to the same conditions and measurement methods. Different responses could still be seen between

the two hybrids further supporting that the responses seen was not due to measurement artifacts.

Earlier studies proposed that embolism refilling can occur within 10 min to a few hours indicating that novel refilling is rapid; however, these refilling times have been criticised as possible artifacts of destructive measurements and pressure-induced embolism (Cochard et al. 2000; Hacke and Sperry 2003; Nardini et al. 2011; Duursma et al. 2019). Hydraulic measurements can be used as evidence that hydraulic recovery takes place (Klein et al. 2018); however, the use of in vivo measurements in concert with this approach allows us to independently visually assess the extent of embolism recovery over time (Brodersen and McElrone 2013).

The use of hydraulic measurements in recovery studies have been criticised since stem segments are often cut while the xylem is under tension and it has been suggested that xylem tension should be relaxed before hydraulic measurements take place (Wheeler et al. 2013; Torres-Ruiz et al. 2015). It is suggested that cutting stem segments while xylem is under pressure can lead to the introduction of additional air bubbles into the xylem leading to overestimating xylem vulnerability to drought due to embolism formation (Wheeler et al. 2013). The significance of these artifacts has; however, been questioned (Trifilò et al. 2014; Ogasa et al. 2016). Ogasa et al. (2016) showed that cavitation and refilling is seen when xylem excision takes place while under tension; however, they found that the occurrence of this is not frequent and that artifacts can be minimised by increasing the distance of measurement from the excised end.

The use of micro-CT scans have also cast doubt on the results from studies that used hydraulic measurements in *Laurus nobilis* (Cochard et al. 2015); however, in the study done by Cochard et al. (2015), hydraulic measurements on the same plant material used during the micro-CT scans was not done. A direct comparison could therefore not have been made (Cochard et al. 2015; Nardini et al. 2017). It has also been argued that discrepancies in hydraulic measurements are due to the refilling of embolism during rehydration to relax xylem tension (Nardini et al. 2011; Savi et al. 2016) and should be accompanied by in vivo measurements for validation (Ogasa et al. 2016; Nardini et al. 2017; Nolf et al. 2017).

During this study only a small percentage of hydraulic recovery was seen after two hours using the micro-CT scans. During the hydraulic measurements, a larger recovery was seen after 15 h of rehydration. Concerns regarding cellular damage due to repetitive exposure to radiation during CT scans has been raised by recent studies (Petruzzellis et al. 2018), which could potentially hinder active refilling. During this study, it was therefore important to reduce the exposure of plants to only two CT scans to ensure that cellular damage was kept to a minimum. In the study done by Petruzzellis et al. (2018) they found

that damage was minimal if any was seen after one CT scan; however, cellular damage was seen after the second or third CT scan. During this study, plants were therefore only exposed to one CT scan, before recovery was evaluated during the second scan. During the second scan recovery was still seen in both hybrids; however, to different extents.

It is clear that more studies are needed to understand the source of discrepancies between direct and indirect measurements of hydraulic recovery (Nardini et al. 2017). Although changes in hydraulic measurements can be influenced by artefacts, it has been shown that these are minimal (Ogasa et al. 2016; Nardini et al. 2017) suggesting that large changes in hydraulic measurements after rehydration are due to hydraulic recovery. Hydraulic measurements should therefore also be validated in studies through the use of in vivo measurements, such as micro-CT scans (Brodersen et al. 2010; Choat et al. 2015).

Choat et al. (2018) have found that evidence of refilling seen in cross sectional CT scans in *Eucalyptus* species are often misleading, suggesting that refilled vessels are indications of either partial refilling or a vessel juncture appearing as two embolised vessels. Evidence of partial refilling was seen in *E. saligna* indicating the possibility of an underlying mechanism moving water into embolised vessel. Complete refilling was; however, not seen over a short time frame (Choat et al. 2016). Although the mechanism of refilling can vary between species, the formation of water droplets on the inside of vessels which grows to fill the vessel has been observed in grapevine and laurel (Tyree et al. 1999; Knipfer et al. 2016; Brodersen et al. 2018). In a study done by Knipfer et al. (2016) evidence of partial refilling was seen in grapevines in the absence of root pressure, with evidence that refilling was osmotically driven (Knipfer et al. 2016). A detailed analysis of the same sort in *Eucalyptus* may provide additional insight into the process. In our study no distinction was made between complete and partial refilling. Although there are indications of complete refilling in some vessels (Fig. 6), it is possible that evidence of refilling in other vessels indicate only partial refilling of the vessels.

Excluding the possible regrowth of new hydraulic pathways (Nardini et al. 2014), it has been proposed that embolism recovery can take place either through dissolution of the air bubble when xylem pressures reaches atmospheric pressures or through active or novel refilling (Brodersen and McElrone 2013; Klein et al. 2018), with the latter taking place while the majority of vessels are still under a negative potential (Nardini et al. 2011). There has been a debate on whether embolism refilling can take place under negative tensions (termed novel refilling) (Nardini et al. 2011), with novel refilling only being observed through use of non-invasive measurements in grapevines (Brodersen et al. 2018). We believe our study provides further evidence suggesting that

water movement into embolised vessels is a mechanism of hydraulic recovery.

Novel refilling implies that vessels can recover embolism while a large area of functional vessels are still under a negative water potential (Nardini et al. 2011). The physical limitations of refilling under negative tensions have been previously detailed (Vesala et al. 2003); however, it has been suggested that thermodynamic constraints would not allow for novel refilling (Vesala et al. 2003; Zwieniecki and Holbrook 2009; Rockwell et al. 2014). It is significant, in the light of this ongoing discussion, that in our study embolism recovery did apparently take place while a large portion of vessels were still under negative water potentials. This highlights that refilling under negative water potentials could be possible and shows the value of CT scan measurements of embolism refilling (Brodersen and McElrone 2013; Brodersen et al. 2018). Embolism recovery due to an increase in root pressure could be excluded from our study since negative Ψ were still seen in all plants after rewatering.

It has been hypothesized that the role of living cells surrounding vessels (parenchyma, vasicentric tracheids) play an active role in embolism refilling (Nardini et al. 2011; Barotto et al. 2016; Secchi et al. 2021). Nardini et al. (2011) suggests that embolism refilling is a result of sugars and ions being relocated to embolised vessels and changing osmotic gradients. This shift in osmotic gradients drives the flow of water from living cells or neighbouring vessels into embolised vessels (Salleo et al. 2004; Knipfer et al. 2016). The upregulation of certain genes relating to carbohydrate metabolism have also been noted in parenchyma surrounding vessels during drought (Secchi and Zwieniecki 2010). The movement of water into tissues immediately surrounding vessels was distinct in our study. This would be expected if the presence of living cells surrounding vessels increases and contributes to the hydraulic conductivity between vessels (Barotto et al. 2016), aiding embolism recovery. Previous studies have shown that the presence of tracheids and parenchyma surrounding vessels improves the hydraulic connectivity between vessels (Salleo et al. 2004; Loepfe et al. 2007; Sano et al. 2011; Barotto et al. 2016).

By increasing the interconnection between vessels, cells surrounding vessels can act as bridges allowing easier water movement between vessels and can contribute to embolism refilling (Barotto et al. 2016). The extent of staining seen was also connected to the percentage of recovery, with *UG* showing larger staining areas as well as increased hydraulic recovery. The staining patterns observed in our study highlights the conductive role of cells surrounding vessels, possibly increasing the connectivity between vessels. Based on the results, it seems likely that cells surrounding vessels will contribute to recovery through increasing hydraulic connectivity.

Conclusion

We observed two distinct hydraulic strategies within *Eucalyptus* hybrids. There was a trade-off between resistance to embolism formation and the ability to recover hydraulic conductivity following drought. A high-risk hydraulic strategy might be linked to embolism recovery, allowing plants to have high hydraulic resistance regardless of safety. While much focus has been given previously to the trade-off between hydraulic efficiency and safety, our results suggest that hydraulic recovery is also an important component to consider when assessing plant hydraulic strategies. Accordingly, it would be important to understand which taxonomic groups utilise refilling as a hydraulic strategy to improve vegetation models. The effect of drought stress on production is highly linked to changes in hydraulic conductance. It is important to understand the different mechanisms that govern the responses of plant species to drought stress in order to understand how plant production will be affected by changes in climate.

Previously focus has been given to understand how plant physiology is influenced by drought ultimately leading to tree mortality, with focus often not given to hydraulic recovery after drought. Understanding the hydraulic recovery response of plants, will give further insight into the impact of drought on trees. It is still unclear, whether or not there is a loss of hydraulic conductivity threshold where plants will not be able to recover hydraulic conductance or whether subsequent droughting events will lead to hydraulic recovery fatigue (Hammond et al. 2019).

Author contribution AS designed the experiment, performed measurements and analysis, and led the writing. DMD contributed to data interpretation, discussion and compiling the final version.

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