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► **To cite this version:**

Rosa Ana Lopez Rodriguez, Markus Nolf, Remko A. Duursma, Eric Badel, Richard J. Flavel, et al.. Mitigating the open vessel artefact in centrifuge-based measurement of embolism resistance. *Tree Physiology*, 2019, 39 (1), pp.143-155. 10.1093/treephys/tpy083 . hal-01854603

**HAL Id: hal-01854603**

**<https://hal.science/hal-01854603>**

Submitted on 6 Aug 2018

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## Mitigating the open vessel artefact in centrifuge based measurement of embolism resistance

Journal:	<i>Tree Physiology</i>
Manuscript ID	TP-2018-172.R1
Manuscript Type:	Methods paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Rosana, Lopez; Universidad Politecnica de Madrid, Sistemas y Recursos Naturales; Université Clermont Auvergne, INRA, PIAF Nolf, Markus ; western sydney university, Hawkesbury Institute for the Environment Duursma, Remko; Western Sydney University Hawkesbury Institute for the Environment Badel, Eric; Université Clermont Auvergne, INRA, PIAF Flavel, Richard; University of New England, School of Environmental and Rural Science Cochard, Hervé; Université Clermont Auvergne, INRA, PIAF Choat, Brendan; Western Sydney University Hawkesbury Institute for the Environment
Keywords:	Xylem Embolism, Drought Resistance, vulnerability to cavitation, cavitron, centrifuge technique, x-ray micro CT


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 Manuscripts

1 **Title:** Mitigating the open vessel artefact in centrifuge based measurement of embolism resistance

2 **Running head:** Mitigating artefacts in vulnerability curves

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4

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1  
2  
3 20 **Abstract** (300 words max)  
4

5  
6 21 Centrifuge-based techniques to assess xylem vulnerability to embolism are increasingly being used,  
7  
8 22 although we are yet to reach a consensus on the nature and extent of artefactual embolism  
9  
10 23 observed in some angiosperm species. In particular, there is disagreement over whether these  
11  
12 24 artefacts influence both the spin (Cavitron) and static versions of the centrifuge technique equally.  
13

14  
15 25 We tested two methods for inducing embolism: bench dehydration and centrifugation. We used  
16  
17 26 three methods to measure the resulting loss of conductivity: gravimetric flow measured in bench-  
18  
19 27 dehydrated and centrifuged samples (static centrifuge), in situ flow measured under tension during  
20  
21 28 spinning in the centrifuge (Cavitron), and direct imaging using X-ray microCT observations in stems  
22  
23 29 of two species of *Hakea* that differ in vessel length.  
24

25  
26 30 Both centrifuge techniques were prone to artefactual embolism in samples with maximum vessel  
27  
28 31 length longer, or similar, to the centrifuge rotor diameter. Observations with microCT indicated that  
29  
30 32 this artefactual embolism occurred in the outer most portions of samples. The artefact was largely  
31  
32 33 eliminated if flow was measured in an excised central part of the segment in the static centrifuge or  
33  
34 34 starting measurements with the Cavitron at pressures lower than the threshold of embolism  
35  
36 35 formation in open vessels. The simulations of loss of conductivity in centrifuged samples with a new  
37  
38 36 model, CAVITOPEN, confirmed that the impact of open vessels on the vulnerability to embolism  
39  
40 37 curve was higher when vessels were long, samples short and when embolism is formed in open  
41  
42 38 vessels at less negative pressures. This model also offers a robust and quantitative tool to test and  
43  
44 39 correct for artefactual embolism at low xylem tensions.  
45  
46

47  
48 40 **Keywords**  
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50  
51 41 Vulnerability to embolism, xylem embolism, drought, centrifuge technique, Cavitron, X-Ray microCT,  
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53 42 CAVITOPEN.  
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## 44 Introduction

45 Xylem water transport is dependent upon water held in a metastable state of water; evaporation of  
46 water from the leaf cell walls generates tension, which is transmitted through the water column to  
47 the roots. Water under tension is prone to cavitation, i.e. the abrupt transition from a metastable  
48 liquid to a gas, resulting in the formation of gas emboli that block the xylem conduits and impairs  
49 water transport (Tyree and Sperry 1988). As tension in the xylem sap increases, for example during  
50 drought, so does the probability of embolism formation. During severe or prolonged droughts,  
51 hydraulic failure can result in the complete loss of hydraulic conductance in the xylem and  
52 subsequent canopy dieback, or whole plant death (Brodribb and Cochard 2009; Nardini et al. 2013;  
53 Rodríguez-Calcerrada et al. 2017; Urli et al. 2013; Venturas et al. 2016). Hydraulic failure is now  
54 considered a principal cause of drought-induced plant mortality and forest die off (Choat et al. 2012;  
55 Sala et al. 2010). The projected rise in global mean temperature and frequency of extreme climate  
56 events over the next century will impact forest ecosystems and shift species distribution ranges. In  
57 this sense, resistance to embolism has emerged as a crucial parameter to understanding species  
58 ecology, differences in water use strategies, and for predicting future mortality events (Brodribb  
59 2017).

60 Xylem resistance to embolism is usually characterized with a vulnerability curve, showing the  
61 decrease in hydraulic conductivity as a function of the xylem tension. Since the publication of the  
62 first vulnerability curves for woody plants were published in 1985 (Sperry 1985) and 1986 (Tyree and  
63 Dixon 1986), a number of techniques that allow for more rapid measurement of vulnerability have  
64 been introduced (see Cochard et al. (2013) for a detailed review). However, although the time  
65 required for construction of a vulnerability curve has been dramatically reduced, recent work  
66 suggests that some of these methods are prone to experimental artefact (Choat et al. 2010; Cochard  
67 et al. 2010; Sperry et al. 2012; Torres-Ruiz et al. 2014). This has led to re-examination of  
68 methodology used to measure vulnerability to embolism (Jansen et al. 2015).

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2  
3 69 The most straightforward technique for inducing embolism is bench dehydration, wherein whole  
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5 70 plants or long branches are gradually dehydrated to various xylem tensions and hydraulic  
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7 71 conductivity of excised segments is measured gravimetrically before and after removing air from  
8  
9 72 embolised conduits (Sperry and Tyree 1988; Tyree and Zimmermann 2002). Bench dehydration relies  
10  
11 73 on natural desiccation of plant tissues and is therefore considered as the best reference method  
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13 74 with which to validate other techniques (Cochard et al. 2013; Ennajeh et al. 2011; Sperry et al. 2012).  
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15 75 This method is not completely free of artefacts and issues associated with disequilibrium in water  
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17 76 potential within a stem, blockage of flow by resin/mucilage (Cobb et al. 2007), and excision of  
18  
19 77 samples under tension can all alter the vulnerability curve significantly (Wheeler et al., 2013).  
20  
21 78 Although most of these issues can be minimised by adoption of suitable protocols (eg. Torres-Ruiz et  
22  
23 79 al., 2015), the bench dehydration technique requires several days and a substantial amount of plant  
24  
25 80 material to obtain a vulnerability curve for one species. As such, Holbrook et al. (1995) and Pockman  
26  
27 81 et al. (1995) proposed the use of a centrifugal force to create a defined negative pressure in the  
28  
29 82 xylem sap of excised plant stems, allowing for rapid and consistent generation of vulnerability  
30  
31 83 curves. Pockman et al. (1995) constructed vulnerability curves for several species by comparing the  
32  
33 84 hydraulic conductivity before and after spinning branches with their ends exposed to air, removing  
34  
35 85 segments at both ends before measuring conductivity in the remaining, middle section of the  
36  
37 86 sample. Alder et al. (1997) modified this technique with a centrifuge rotor designed to keep the  
38  
39 87 segment ends immersed in water during spinning, allowing the conductivity of a single segment to  
40  
41 88 be remeasured at different tensions to create an entire vulnerability curve for a single sample. This  
42  
43 89 important innovation allowed repeated measurements to be made on the same plant material,  
44  
45 90 reducing the number of samples required for construction of a curve and strengthening the results  
46  
47 91 statistically. Finally, Cochard (2002), Cochard et al. (2005) and Li et al. (2008) further modified the  
48  
49 92 centrifuge method and designed new rotors which allowed measuring the conductivity of the  
50  
51 93 segment while it is spinning and under tension. This further increased the efficiency of measurement  
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53 94 and allowed for flow measurements to be made under tension.  
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3 95 Although centrifuge based techniques induce embolism by increasing tension in sample xylem, the  
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5 96 patterns of embolism spread through the sample may differ from a naturally dehydrated sample (Cai  
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7 97 et al. 2010). The tension profile in the centrifuged segment is highest in the axis of rotation (i.e. in  
8  
9 98 the middle section of the segment) and declines towards the segment ends (Cochard et al. 2005),  
10  
11 99 while during natural dehydration the tension profile across the segment is expected to remain  
12  
13 100 approximately constant (Cai et al. 2010). Nevertheless, the vulnerability curves generated by  
14  
15 101 centrifugation agree well with the bench-top method in conifers and short-vesseled angiosperm  
16  
17 102 species (Alder et al. 1997; Cochard et al. 2005; Cochard et al. 2010; Li et al. 2008). In contrast,  
18  
19 103 inconsistent results have been obtained for species with long vessels, specifically those in which a  
20  
21 104 significant number of vessels in the sample are longer than the centrifuge rotor (Choat et al. 2010;  
22  
23 105 Jacobsen and Pratt 2012; Sperry et al. 2012; Torres-Ruiz et al. 2014).

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25  
26 106 Since 2005 the number of vulnerability curves constructed by centrifugation has increased  
27  
28 107 exponentially (see Fig. 3 in Cochard et al. (2013)). Accordingly, considerable effort has been devoted  
29  
30 108 to testing and validation of centrifuge techniques, whether measuring the flow gravimetrically after  
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32 109 spinning (static centrifuge method), or while centrifuging (Cavitron method). However, we are yet to  
33  
34 110 reach a consensus on the nature and extent of artefactual embolism observed with centrifuge  
35  
36 111 techniques. In particular, there is disagreement over whether these artefacts influence both spin  
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38 112 (Cavitron rotor) and static versions of the centrifuge technique equally (Hacke et al. 2015; Sperry et  
39  
40 113 al. 2012). In recent years, the application of x-ray computed microtomography (microCT) to the  
41  
42 114 study of plant hydraulics has emerged as a potentially powerful tool to validate hydraulic  
43  
44 115 techniques. In addition to providing a non-invasive assay of xylem function, it allows for analyses of  
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46 116 spatial and temporal patterns of embolism formation (Brodersen et al. 2013; Choat et al. 2016;  
47  
48 117 Dalla-Salda et al. 2014; Torres-Ruiz et al. 2016).

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53 118 In this study we evaluated the performance of both centrifuge techniques against bench  
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55 119 dehydration in order to examine possible discrepancies associated with each technique. First, we

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3 120 tested two methods for inducing embolism: bench dehydration and centrifugation. We then tested  
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5 121 three ways of measuring the resulting loss of conductivity: gravimetric flow measured in bench-  
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7 122 dehydrated and centrifuged samples (static centrifuge), *in situ* flow measured under tension during  
8  
9 123 spinning in the centrifuge (Cavitron), and direct imaging using X-ray microCT observation. All  
10  
11 124 experiments were carried out with two species of the genus *Hakea* that differ in vessel length. *H.*  
12  
13 125 *dactyloides* is a short vesseled species with maximum vessel length shorter than 14 cm, whereas *H.*  
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15 126 *leucoptera* has longer vessels and maximum vessel length is ca. 25 cm. Additionally, we compared  
16  
17 127 results obtained using two rotor diameters (14 and 27 cm) to assess the effect of sample length, and  
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19 128 measured hydraulic flow both in the whole, spun segments and excised middle sections. Spatial  
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21 129 patterns of embolism within samples were visualized with X-ray microCT after centrifugation in  
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23 130 order to provide further insight into potential discrepancies. Finally, a new model, CAVITOPEN was  
24  
25 131 developed to simulate the effect of vessel and sample lengths on centrifuge estimates of embolism  
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27 132 resistance. We hypothesized that i) both centrifuge techniques, the static centrifuge and the  
28  
29 133 cavitron, are prone to similar artefacts when constructing vulnerability curves of long-vesseled  
30  
31 134 species; ii) the shape of the vulnerability curve of centrifuged samples will depend on the amount of  
32  
33 135 cut open vessels; iii) image techniques and standard flow measurements will produce similar  
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35 136 vulnerability curves.  
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## 39 137 **Material and methods**

### 40 138 *Plant Material*

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45 139 Experiments were carried out on branch material of two diffuse-porous species of the same genus  
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47 140 exhibiting different vessel lengths, *Hakea dactyloides* (Gaertn.) Cav. and *Hakea leucoptera* R. Br.  
48  
49 141 Branches were sampled from natural populations of *H. dactyloides* at Mount Banks (33° 34' 46" S,  
50  
51 142 150° 21' 56" E; NSW, Australia) and *H. leucoptera* at Binya State Forest (34° 11' 16" S, 146° 16' 13"  
52  
53 143 E; NSW, Australia) from May to September 2016 (late autumn-winter in the South Hemisphere). Sun  
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55 144 exposed branches of 1.5-2.0 m length were collected in the field in the early morning and  
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3 145 immediately placed in black plastic bags with moistened paper towels to prevent transpiration with  
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5 146 their cut ends covered with Parafilm. In the laboratory they were kept at 4 °C until measured.  
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8 147 *Midday xylem water potential in the field and Native embolism*  
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11 148 Midday xylem water potential was measured in the field in November 2015, February 2016 and June  
12  
13 149 2016. Two leaves of five plants per species were covered with aluminium foil and sealed with a  
14  
15 150 plastic bag 1 hour before excision and measurement with a pressure chamber (PMS Instrument Co.,  
16  
17 151 Albany, OR, USA).  
18

19  
20 152 Native embolism was determined in current-year, one-year and two-year old segments of 5  
21  
22 153 branches per species to ensure that the effects of previous natural water stress were minimised.  
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24 154 Note that segments containing 1-year and 2-year-old growth were necessary to fit in the 27 cm rotor  
25

26 155 of the centrifuge. Measuring native embolism we also wanted to control for sample collection date  
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28 156 because branches were cut at different times during late autumn-winter 2016 to avoid long storage.  
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30 157 Branch proximal end was cut underwater to release tension for 30 min (Torres-Ruiz et al. 2015;  
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32 158 Wheeler et al. 2013) and then the branch was progressively recut under water to segments 50 mm  
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34 159 long. Note that at least twice the maximum vessel length was removed from the cut end after  
35

36 160 tension relaxation. Thereafter, the edges of these segments were trimmed using a razor blade. Initial  
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38 161 conductivity ( $K_h$ ) was measured in 50 mm long segments with filtered, degassed 2 mmol KCl solution  
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40 162 at low pressure ( $\leq 4$  kPa) with a liquid flowmeter (LiquiFlow L13-AAD-11-K-10S; Bronkhorst High-  
41

42 163 Tech B.V., Ruurlo, the Netherlands). The segments were then flushed with the same solution at a  
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44 164 minimum of 0.20 MPa for 15 min to remove embolism and subsequently determine maximum  
45

46 165 hydraulic conductivity ( $K_{max}$ ). The native percentage loss of conductivity (PLC) was calculated for  
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48 166 each segment as:  
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51  
52 167  $PLC = 100 \times (1 - K_h / K_{max})$  (equation 1)  
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3 168 Specific hydraulic conductivity ( $K_S$ ) was calculated dividing  $K_{max}$  by the xylem cross-sectional area  
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5 169 (average distal and proximal xylem area measured with a calliper).  
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8 170 *Maximum vessel length and vessel length distribution*  
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10  
11 171 Ten branches per species were sampled from the same plants as used for hydraulic measurements  
12  
13 172 to determine maximum vessel length with the air perfusion technique (Ewers and Fisher 1989). Once  
14  
15 173 in the lab, 60 cm long segments were flushed for 1 h with degassed, filtered 2 mmol KCl solution at  
16  
17 174 0.18-0.20 MPa to remove any embolism. Then each segment was infiltrated with compressed air at  
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19 175 0.05 MPa at its distal end with an aquarium air pump while the basal end was repeatedly shortened  
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21 176 by 2 cm under water until air bubbles emerged. The remaining sample length was assumed as  
22  
23 177 maximum vessel length.  
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25  
26 178 An estimate of the amount of vessels longer than the centrifuge rotor diameter and longer than half  
27  
28 179 the rotor diameter (open to centre vessels) was assessed in four branches of *H. dactyloides* and five  
29  
30 180 branches of *H. leucoptera* by measuring the decrease in PLC after air injection (Cochard et al. 1994;  
31  
32 181 Torres-Ruiz et al. 2014). Briefly, 35 cm long segments were flushed as described above to remove  
33  
34 182 embolism. Then, tubing was attached to the distal end of these segments and compressed air was  
35  
36 183 injected into the samples at 0.1 MPa for 10 min using a pressure chamber. This pressure was  
37  
38 184 sufficient to empty the open vessels but not high enough to move water through wet pit membranes  
39  
40 185 between adjacent vessels (Ewers and Fisher, 1989). PLC was determined in 3 cm long segments  
41  
42 186 across the sample as described for native embolism. At the injection point, PLC is close to 100%  
43  
44 187 because all the vessels are air filled and progressively decrease to 0 for a length longer than the  
45  
46 188 longest vessel in the sample. The PLC at each distance from the injection point corresponds to the  
47  
48 189 percentage of contribution to flow from vessels longer than this distance. If all the vessels were of  
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50 190 equal diameter, this percentage would correspond to the number of vessels longer than the distance  
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52 191 from the injection point. In this case of the two *Hakea* species used are diffuse porous and vessel  
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54 192 diameters within the same sample did not vary greatly. Thus the curves in Fig. 1 represent a proxy of  
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3 193 vessel distribution of the two species, although not as accurate as anatomy, and allow to estimate  
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5 194 the amount of open vessels from a certain cut point.

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8 195 *Bench dehydration technique*

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10 196 Branches were dehydrated gradually in the laboratory at ca. 23 °C. Xylem water potential ( $\Psi_x$ ) was  
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12 197 measured with a pressure chamber (PMS Instrument Co., Albany, OR, USA) in bagged leaves  
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14 198 (wrapped with aluminium foil and a plastic bag at least 1 h before sampling). When the target  $\Psi_x$  to  
15  
16 199 construct the VC was reached, branches were sealed into a plastic bag with moistened paper towels  
17  
18 200 for 1 h to equilibrate  $\Psi_x$ . Water potential was measured again in two bagged leaves of the same  
19  
20 201 branchlet to confirm homogeneous  $\Psi_x$  in the sample. The  $\Psi_x$  of the sample was considered  
21  
22 202 equilibrated if the difference between the three  $\Psi_x$  (one measured before sealing the branch and  
23  
24 203 two measured after equilibration) was not higher than 0.1 MPa. Afterwards tension was released for  
25  
26 204 30 minutes by cutting the branch proximal end under water and PLC was determined in one-year-old  
27  
28 205 segments as for native embolism. Vulnerability curves were generated by plotting PLC against  $\Psi_x$ .  
29  
30 206 For *H. leucoptera* 7 branches were dehydrated and 4 different branchlets per branch were measured  
31  
32 207 at different  $\Psi_x$  to construct the vulnerability curve and for *H. dactyloides* we used 12 branches and  
33  
34 208 two branchlets per branch. All branchlets were far apart (at least four branch orders) and after  
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36 209 collection the cutting surface was covered with parafilm to avoid air entry in the rest of the sample.  
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41 210 *Centrifuge techniques*

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44 211 We compared two centrifuge techniques: i) the static centrifuge method described by Alder et al.  
45  
46 212 (1997) and ii) the *in situ* flow technique (Cavitron (Cochard 2002; Cochard et al. 2005)). In the static  
47  
48 213 centrifuge two different sizes of custom-built rotors, 14 cm and 27 cm, were used to test the effect  
49  
50 214 of segment length and fraction of open vessels. All hydraulic conductivity measurements were  
51  
52 215 performed using filtered, degassed 2 mmol KCl solution and a flow meter (see Native embolism  
53  
54 216 section).

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3 217 Static centrifuge measurements were carried out on 20 branches per species. Branches were  
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5 218 trimmed under water and both ends were shaved to a final length of 14 or 27 cm. The initial  
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7 219 hydraulic conductivity was measured as described above (see Native embolism section) with a  
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9 220 pressure head of 7.5 kPa. Subsequently, 14-cm long branches were spun in the centrifuge (Sorvall RC  
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11 221 5C Plus) for 5 minutes at increasing pressure steps. Foam pads saturated with the solution used for  
12  
13 222 measurements were placed in the reservoirs of the rotor to maintain sample ends in contact with  
14  
15 223 the solution even when the rotor was stopped (Tobin et al. 2013). After each step, samples were  
16  
17 224 removed and  $K_h$  was measured on the whole segment as described for native embolism. In the  
18  
19 225 27cm-long branches we modified the single spin method (Hacke et al. 2015) so that two  
20  
21 226 measurements were made in each centrifuged segment. The initial  $K_h$  was measured before spinning  
22  
23 227 in the 27-cm long sample. After spinning,  $K_h$  was measured on the whole segment and the first PLC  
24  
25 228 was calculated. Subsequently, a 4 cm-long segment was cut from the middle section and its  $K_h$  was  
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27 229 measured. The second PLC was determined in this 4 cm-long segment after flushing to obtained the  
28  
29 230 maximum  $K_h$  ( $K_{max}$ ) as described for native embolism.

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33 231 *In situ* flow centrifuge measurements (Cavitron technique) were carried out on six branches per  
34  
35 232 species using a modified bench top centrifuge (H2100R, Cence Xiangyi, Hunan, China). For the static  
36  
37 233 centrifuge, samples were trimmed under water to a length of 27 cm to fit in the rotor. Initial  
38  
39 234 conductivity,  $K_i$ , was determined at a xylem pressure of  $-0.5$  MPa in *H. dactyloides* and 1.5 MPa in *H.*  
40  
41 235 *leucoptera*. The xylem pressure was then lowered stepwise by increasing the rotational velocity, and  
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43 236  $K_h$  was again determined while the sample was spinning. The PLC at each pressure step was  
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45 237 quantified as

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48 238 
$$PLC = 100 \times (1 - K_h / K_i).$$
 (equation 2)  
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51 239 *X-ray microCT imaging*  
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3 240 A subset of branches of *H. leucoptera* was transported to the University of New England in Armidale  
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5 241 (NSW, Australia). They were gradually dehydrated to five different xylem water potentials ranging  
6  
7 242 from -4.8 MPa to -9 MPa as for the bench dehydration method. After measuring  $\Psi_x$ , tension was  
8  
9 243 relaxed by cutting the proximal end of the branch under water leaving it submerged for 30 minutes.  
10  
11 244 Then the branch was sequentially cut back under water and finally 10-mm-long segments were  
12  
13 245 excised under water from current-year shoots, wrapped in Parafilm, inserted into a plexiglass tube  
14  
15 246 and then placed in an X-ray microtomography system (GE-Phoenix V|tome|xs, GE Sensing &  
16  
17 247 Inspection Technologies, Wunstorf, Germany) to visualize embolized vessels. Another subset of  
18  
19 248 branches of *H. leucoptera* was centrifuged to five (-5, -6, -7, -8, -9 MPa) and three (-5, -6, -7 MPa)  
20  
21 249 different water potentials in the static centrifuge using 27 cm and 14 cm long segments,  
22  
23 250 respectively. They were immediately submerged in liquid paraffin wax and preserved at 4 °C for  
24  
25 251 three days until measured in the same facility (Cochard et al. 2015). Seven branches of *H. dactyloides*  
26  
27 252 were also centrifuged at four (-3, -4, -5, -6 MPa) and three (-3, -4, -5 MPa) water potentials with the  
28  
29 253 27 and 14 cm rotors, respectively, following the same protocol. One branch of *H. leucoptera* was  
30  
31 254 prepared as the centrifuged samples but was not spun in the centrifuge to detect any possible  
32  
33 255 artefact due to sample preparation. All samples were scanned at the middle of the sample.  
34  
35 256 Additionally, in three 27 cm long samples we scanned at 6 cm and 12 cm from the axis of rotation to  
36  
37 257 examine embolism profiles across a sample.  
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40  
41 258 X-ray scan settings were 90 kV and 170 mA, and 1800 projections, 600 ms each, were acquired  
42  
43 259 during the 360° rotation of the sample. The resultant images covered the whole cross section of the  
44  
45 260 sample in 8.7 mm length with a spatial resolution of 8.7  $\mu\text{m}$  per voxel. At the end of the scan, the  
46  
47 261 sample was cut back to 30 mm length, injected with air at >1 MPa pressure and rescanned at the  
48  
49 262 same location as before to visualize all empty vessels in the fully embolized cross section. After  
50  
51 263 three-dimensional reconstruction with Phoenix datos|x2 Reconstruction Version 2.2.1-RTM (GE  
52  
53 264 Sensing & Inspection Technologies, Wunstorf, Germany), volumes were imported into ImageJ 1.49k  
54  
55 265 (Schneider et al. 2012). A median Z projection of c. 100  $\mu\text{m}$  along the sample axis was extracted from  
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the middle of the scan volumes following the protocol in Nolf et al. (2017). PLC of each sample was estimated calculating the theoretical hydraulic conductance based on the conduit dimensions of embolized and functional vessels (Choat et al. 2016). To measure conduit dimensions, a radial sector of the transverse section was selected in the same microCT scan and all their embolized vessels were measured manually. The image of this sector was then binarized so the dimensions of the selected embolized vessels matched with the manually drawn vessels. This threshold value was then used for binarizing the image of the whole cross section and all the embolized vessels were measured using the Analyse Particles function in Image J. Theoretical specific hydraulic conductivity ( $K_{sth}$ ) was calculated as:

$$K_{sth} = \frac{\sum \left( \frac{D^4 \pi}{128 \eta} \cdot \frac{\Delta p}{\Delta x} \right)}{A} \quad (\text{equation 3})$$

Where  $D$  is the equivalent circular vessel diameter based on vessel area,  $\eta$  viscosity of water,  $\Delta p/\Delta x$  pressure gradient per xylem length,  $A$  xylem cross-sectional area.

The current theoretical specific hydraulic conductivity ( $K_{sth}$ ) for each sample was calculated by subtracting the summed specific hydraulic conductivity of embolized vessels from the  $K_{sth(max)}$  of that sample, calculated as the  $K_{sth}$  of the sample after air injection. The pressure gradient used for calculations of  $K_{sth}$  was similar to the pressure gradient used in the hydraulic measurements, 0.06 MPa m<sup>-1</sup>.

#### *Vulnerability curve fitting and statistical analysis*

Vulnerability curves were fitted using a Weibull function (Ogle et al. 2009) in R 3.2.0 (R Core Team, 2015) using the fitplc package (Duursma and Choat 2017). Confidence intervals of  $P_{12}$ ,  $P_{50}$  and  $P_{88}$  ( $\Psi_x$  at 12, 50 and 88 % loss of conductivity, respectively) and the slope of the curve at 50% loss of conductivity ( $S_{50}$ ) were used to compare between methods. Confidence intervals (CI) for the bench

290 dehydration and the static centrifuge techniques were obtained using bootstrap resampling (999  
 291 replicates). Methods were considered to be statistically different if the 95% CIs did not overlap.

292 Differences in native embolism and specific initial conductivity between sampling dates were tested  
 293 with a one-way ANOVA. Means were compared using a Tukey test at 95% confidence. Vulnerability  
 294 curve parameters across methods were compared at the  $\Psi_x$  corresponding with three levels of loss  
 295 of conductivity: 12%, 50% and 88% ( $P_{12}$ ,  $P_{50}$  and  $P_{88}$ , respectively) and the slope of the VC at 50% loss  
 296 of conductivity ( $S_{50}$ ).

### 297 *CAVITOPEN- simulation of the effect of open vessels in a centrifuged sample*

298 To disentangle the effects of centrifugation on 'true' vessel embolism at the centre of the samples,  
 299 where more vessels are closed at both ends and tension is maximum, from draining of open vessels  
 300 at both sample ends a new model, CAVITOPEN, was developed. In a centrifuged sample, the  
 301 variation of xylem pressure ( $P$ ) with distance from the axis of rotation ( $r$ ) is given by the following  
 302 equation (Alder et al. 1997):

$$303 \quad dP/dr = \rho\omega^2 r \quad \text{(equation 4)}$$

304 where  $\rho$  is the density of water, and  $\omega$  the angular velocity.

305 Integrating this equation from  $R$  (distance from the axis of rotation to the water reservoir) we can  
 306 obtain the pressure at  $r$  ( $P_r$ ):

$$307 \quad P_r = 0.5 \rho\omega^2 (R^2 - r^2) \quad \text{(equation 5)}$$

308 The effect of vessel length on 'true' vessel embolism in a spun sample has already been modelled by  
 309 Cochard et al (2005). Briefly, if the vessels are infinitely long, the VC obtained by centrifugation  
 310 should yield the correct  $P_{50}$  value. When the vessels are infinitely short the  $P_{50}$  value is  
 311 underestimated due to the variation of xylem pressure inside the spun sample (eq. 4) and the  
 312 consequent gradient of embolism along the sample: xylem pressure is minimum in the middle of the

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3 313 sample and null at the extremities (eq. 5). Since the loss of conductivity is measured on the whole  
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5 314 sample, an underestimation of the degree of embolism in the middle of the sample is predicted. This  
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7 315 effect of vessel length was further tested with the CAVITOPEN model and found marginal, i.e. the  
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9 316 shift in the VC was negligible, compared to the draining effect. For sake of simplicity, this effect was  
10  
11 317 no longer considered in the simulations. To simulate the draining effect at both sample ends, we first  
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13 318 hypothesized that vessel ends follow a logarithmic distribution following the vessel length  
14  
15 319 probability density function proposed by Cohen et al. (2003) and assuming vessel ends uniformly  
16  
17 320 distributed across the length of the sample:

20 321 
$$N_x = N_0 \cdot \exp(-x/L_{max})$$
 (equation 6)

22  
23 322 where  $N_x$  is the number of open vessels at the distance  $x$  from sample ends,  $N_0$  the total number of  
24  
25 323 vessels and  $L_{max}$  the maximum vessel length.

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27  
28 324 The second assumption of the model is that open vessels drain when the minimum pressure in the  
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30 325 vessel exceeds a threshold value  $P_{open}$ . Because of the quadratic distribution of the pressure in the  
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32 326 sample, vessels having their end wall located closer to the sample ends, i. e. further from the centre  
33  
34 327 of rotation, will drain at a higher rotational velocity.

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36  
37 328 The branch segment was discretised in 0.1 mm thick sections arranged in serial. The xylem pressure  
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39 329 in the middle of the segment was set to a pressure varying from 0 to -12 MPa in 1 MPa steps. The  
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41 330 model then computes the pressure at steady state in each 0.1 mm section and determines the PLC  
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43 331 caused by 'true' embolism (non-open vessels) and by draining (open vessels). Finally, the PLC of the  
44  
45 332 whole segment is computed which enables the construction of the vulnerability curve. We tested  
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47 333 the model for different theoretical  $L_{max}$  values and the 4 rotors sizes used in our experiments. To  
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49 334 validate the model we used the values of PLC obtained for *H. leucoptera* in the static centrifuge with  
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51 335 the 27 cm rotor. The CAVITOPEN model was fit to the measurements using constrained numerical  
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336 optimization to estimate four parameters:  $P_{50}$ ,  $S_{50}$ ,  $L_{max}$  and  $P_{open}$ . All routines were implemented as  
337 an R package (available from (Duursma 2017)).

## 338 Results

### 339 *Native embolism and minimum xylem water potential in the field.*

340 Midday xylem water potential decreased from -1.02 to -1.51 MPa in *H. dactyloides* and from -1.35 to  
341 -2.62 MPa in *H. leucoptera* from November 2015 to February 2016. In June 2016, the water potential  
342 was -1.16 MPa in *H. dactyloides* and -1.42 MPa in *H. leucoptera*. Native embolism remained low in  
343 both species across the sampling dates. We measured higher PLC in two-year-old branch segments  
344 (< 13 %) than in current year growth (< 2 %) in *H. leucoptera* whereas in *H. dactyloides* native  
345 embolism was lower than 2% in all samples. Maximum xylem specific conductivity ( $K_{smax}$ ) was  $0.87 \pm$   
346  $0.10 \text{ kg m}^{-1} \text{ s}^{-1} \text{ MPa}^{-1}$  in *H. leucoptera* and  $1.29 \pm 0.09 \text{ kg m}^{-1} \text{ s}^{-1} \text{ MPa}^{-1}$  in *H. dactyloides* (mean  $\pm$  sd). No  
347 significant differences in native PLC or  $K_s$  ( $P > 0.05$ ; Table S1) were detected between sampling dates.

### 348 *Maximum vessel length and vessel length distribution*

349 Maximum vessel length as determined by air injection was 25 cm (standard deviation, sd = 5) in *H.*  
350 *leucoptera* and 10 cm (sd = 3) in *H. dactyloides*. Air injected branches of *H. dactyloides* showed 17%  
351 PLC at 7 cm from the injection point, 5% at 14 cm and less than 1% at 28 cm, whereas in *H.*  
352 *leucoptera* the PLC was always higher, 50%, 25%, and 5% at 7, 14 and 28 cm respectively (Fig. 1).  
353 Thus the number of open vessels at both ends when using the centrifuge technique differed  
354 between species.

### 355 *Vulnerability curves*

356 Vulnerability curves (VCs) obtained with the bench dehydration technique were s-shaped for both  
357 species, with significant embolism only occurring once a threshold water potential had been  
358 reached. This threshold was more negative in *H. leucoptera* (-6.3 MPa) than in *H. dactyloides* (-3.8

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3 359 MPa) (Fig. 2). VCs obtained with bench dehydration had the most negative  $P_{12}$  and the steepest  
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5 360 slopes of all methods (Table S2), meaning that embolism formation started at more negative  $\Psi_x$  and  
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7 361 conductivity was lost across a narrower range of  $\Psi_x$  compared with VCs generated by centrifugation.  
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10 362 When the centrifuge was used to induce embolism, results in the shorter-vesseled species, *H.*  
11  
12 363 *dactyloides*, were similar for the three techniques used to measure loss of conductivity, flowmeter,  
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14 364 Cavitron and microCT (average  $P_{50}$  with the 27 cm rotor in the static centrifuge and the Cavitron -4.8  
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16 365 MPa), and the CI at 95% overlapped with bench dehydration ( $P_{50} = -5.0$  MPa). The VC generated with  
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18 366 the 14 cm rotor for *H. dactyloides* yielded slightly less negative values ( $P_{50} = -4.3$  MPa; Table S2; Fig.  
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20 367 2). In contrast, VCs for *H. leucoptera* differed considerably depending on the method and the sample  
21  
22 368 length. Vulnerability parameters ( $P_{12}$ ,  $P_{50}$ ,  $P_{88}$ ) obtained with the Cavitron (-5.0, -7.1 and -9.0 MPa,  
23  
24 369 respectively) matched more closely with the bench dehydration VC (-6.3, -7.4 and -8.2 MPa). For  
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26 370 samples spun in the static centrifuge, we found a significant effect both of the rotor size and the  
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28 371 segment used to measure flow (whole, spun segment or excised middle section in the 27 cm rotor)  
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30 372 on apparent vulnerability to embolism: segments measured across their entire length exhibited  
31  
32 373 higher vulnerability to embolism compared to the bench-dehydration VC as shown by  $P_{12}$  (-1.2 and -  
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34 374 2.6 MPa for 14 and 27 cm rotors, respectively) and  $P_{50}$  (-5.3 and -6.0 MPa, respectively), but seemed  
35  
36 375 less vulnerable towards the dry end of the curve ( $P_{88}$  of -14.2 and -10.4 MPa, respectively; Table S2).  
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38 376 Both VCs were almost linear when flow was measured across the whole segment with a shift  
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40 377 towards more vulnerable values with the 14 cm rotor, but became s-shaped when only the middle  
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42 378 section of the 27 cm segment was measured (Fig. 2). Removing the segment ends resulted in a  
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44 379 steeper slope and significantly more negative values of  $P_{12}$  and  $P_{50}$ . The Cavitron and the middle  
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46 380 segment techniques yielded similar results and agreed well with the dehydration technique in  $P_{50}$   
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48 381 and  $P_{88}$  and with microCT image analysis (red triangles in Fig. 2).  
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53 382 *Patterns of embolism across a centrifuged sample*

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3 383 Within 27-cm-length centrifuged samples of *H. leucoptera*, microCT scans revealed that embolism  
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5 384 levels were consistently at their highest near the sample ends (at 12 cm from the axis of rotation)  
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7 385 when spun at equivalents of -5, -7 and -9 MPa in the static centrifuge (Fig. 3). At -5 and -7 MPa loss  
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9 386 of conductivity decreased from the basal end to the centre, contradicting theoretical expectations.  
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11 387 This trend was observed even at  $\Psi_x$  inducing less than 40% PLC based on the bench dehydration VC  
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13 388 (Fig. 3). Only at -9 MPa, that is, below  $P_{88}$  on bench dehydration, did levels of embolism converge  
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15 389 along the length of the sample at 80-90%.

### 18 390 *Influence of open vessels in the VC of a centrifuged sample*

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21 391 The simulations produced by the CAVITOPEN model confirmed that the shape of the VCs generated  
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23 392 by the centrifugation was largely dependent on vessel and sample lengths. As maximum vessel  
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25 393 length decreased, PLC of the whole sample decreased at a given  $\Psi_x$ , and the shape of the VC shifted  
26  
27 394 from exponential to sigmoidal (Fig. 4a). The same pattern was observed when the sample length  
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29 395 increased (Fig. 4b). For instance, with 14-cm-length centrifuged samples,  $P_{50}$  ranged from -0.6 MPa  
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31 396 to -7.7 MPa varying the maximum vessel length of the sample from 50 cm to 5 cm. Likewise, the  $P_{50}$   
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33 397 of a centrifuged sample with maximum vessel length of 15 cm ranged from -2.1 MPa in the 14-cm  
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35 398 rotor to -7.7 MPa using a 40-cm rotor. Embolism of vessels open from the cut surface (Fig. S1)  
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37 399 influenced values of PLC at high negative pressures, even in short-veesled samples, resulting in  
38  
39 400 rapid loss of conductivity followed by a plateau. The more open vessels and the less negative the  
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41 401 threshold of embolism of open vessels (Fig. 4c), the higher is this plateau and stronger the impact on  
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43 402 the VC (Fig. 4). VCs can be corrected if the first inflection point of the curve is considered the starting  
44  
45 403 point for initial conductivity ( $K_i$ ), i.e. 0% loss of conductivity. This is shown in Fig. 4d with actual  
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47 404 measurements of PLC obtained in 27-cm centrifuged samples of *H. leucoptera*. When the  
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49 405 CAVITOPEN model was fit (black circles and grey solid line, respectively) and we used the inflection  
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51 406 point as starting point for  $K_i$ , the corrected curve matched the reference VC obtained with bench  
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53 407 dehydration (Fig. 4d black solid line and orange dashed line, respectively). Alternatively, by fitting  
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3 408 the model using numerical optimization we estimated values of  $P_{50} = -6.9$  MPa,  $S_{50} = 49.7$ ,  $L_{max} =$   
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5 409 15.21 and  $P_{open} = -0.75$ .

## 6 7 8 410 **Discussion**

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10 411 We evaluated the reliability of two centrifuge based techniques commonly used to measure  
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12 412 vulnerability to embolism in angiosperm species and present a protocol that mitigates experimental  
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14 413 artefacts associated with open xylem vessels. Both the static centrifuge method and the *in-situ* flow  
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16 414 centrifuge method (Cavitron) were prone to artefactual embolism caused by open vessels, although  
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18 415 the errors were significantly greater in the static centrifuge method. In a species with maximum  
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20 416 vessel length longer or similar to the centrifuge rotor diameter, the static centrifuge significantly  
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22 417 overestimated xylem vulnerability to embolism if the whole spun segment was used to measure  
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24 418 flow. Observations with microCT indicated that artefactual embolism caused by centrifugation of  
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26 419 samples occurred in the outer most portions of samples. However, we demonstrated that  
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28 420 artefactual embolism was largely eliminated from static centrifuge if flow was measured in an  
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30 421 excised central part of the segment. This altered protocol yielded VCs similar to those obtained on  
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32 422 the same species with bench dehydration thus allowing these centrifuge techniques to accurately  
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34 423 measure vulnerability to embolism in longer vesseled species. We also present a new model  
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36 424 (CAVITOPEN) that simulates the impact of vessel draining at the cut end on the whole VC curve and  
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38 425 showed that errors were largely dependent on vessel length and rotor diameter. This model allows  
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40 426 researchers to quantitative test and avoid errors associated with the artefactual embolism. The  
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42 427 bench dehydration technique indicated that significant embolism was only initiated in both species  
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44 428 after water potential dropped below a threshold value, -3.8 MPa in *H. dactyloides* and -6.3 MPa in *H.*  
45  
46 429 *leucoptera*. PLC then increased rapidly and hydraulic conductivity was lost almost completely within  
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48 430 a span of 1 MPa (Fig. 2). These vulnerability curves have been classified as sigmoidal or s-shaped as  
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50 431 opposed to exponential or r-shaped curves, characterized by rapid conductivity losses as soon as the  
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52 432 water potential declines below zero (Cochard et al. 2013; Sperry et al. 2012). A third type of VC,

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3 433 intermediate between these two, exhibits a linear response, and is mainly found in diffuse porous  
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5 434 species when using centrifugation to induce embolism (Cochard et al. 2013).  
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8 435 Our results showed that VCs obtained with the static centrifuge technique and the Cavitron are  
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10 436 similar to bench dehydration in a short-vesseled species, i.e. a species with no through vessels (open  
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12 437 at both ends) in the segment and with few vessels open from the cut surface to the middle of the  
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14 438 segment. All centrifuge generated VCs for *H. dactyloides* were sigmoidal and similar to bench  
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16 439 dehydration VCs, with a slight shift towards more vulnerable values when using the 14 cm rotor (Fig.  
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18 440 2, Table S2) as recently found by Pengxian et al. (2018) in *Acer mono* when comparing in the static  
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20 441 centrifuge the 14 cm and 27 cm rotors. VCs of other short vesseled angiosperms such as *Betula*  
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22 442 *pendula* (Cochard et al. 2010), *Fagus sylvatica* (Aranda et al. 2014), *Populus tremuloides* (Schreiber et  
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24 443 al. 2011) or *Acer negundo* (Christman et al. 2009) were also sigmoidal when the static centrifuge or  
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26 444 the Cavitron were used. In contrast, the VC shape obtained for *H. leucoptera* samples differed  
27  
28 445 significantly depending on methodology resulting in a shift of  $P_{50}$  of 2 MPa in samples from the same  
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30 446 population (Fig. 2, Table S2). This dramatic change was observed previously in peach (*Prunus persica*)  
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32 447 when the length of the centrifuged samples was varied in a Cavitron; shorter samples were more  
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34 448 vulnerable to embolism ( $P_{50}$  shifted from -4.5 to -1 MPa) and VCs became r-shaped (Cochard et al.  
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36 449 2010). However, when using the static centrifuge to measure the same population, Sperry et al.  
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38 450 (Sperry et al. 2012) found that VCs were linear and relatively insensitive to the number of open  
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40 451 vessels with  $P_{50}$  less negative than -2 MPa using 14 cm and 27 cm samples. This difference in  
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42 452 sensitivity to the proportion of open vessels in the centrifuged samples has led some to conclude  
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44 453 that the original centrifuge method and rotor design are not subject to the open vessel artefact  
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46 454 (Hacke et al. 2015; Sperry et al. 2012). However, Torres-Ruiz et al. (2017) demonstrated that if the  
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48 455 amount of open vessels is relatively high in both rotors, 14 and 27 cm, VCs could be equally biased  
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50 456 and would appear statistically indistinguishable.  
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3 457 Recent publications have addressed this controversy, showing that long-vesseled species such as  
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5 458 grape vine, oaks, robinia or olive, with a high proportion of open vessels, produce similarly biased  
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7 459 results with both the static centrifuge and the Cavitron when compared with reference curves  
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9 460 generated by dehydration or non-invasive imaging (Choat et al. 2016; Choat et al. 2010; Pengxian et  
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11 461 al. ; Torres-Ruiz et al. 2014). Li et al. (2008) and Pengxian et al. (2018) tested the two centrifuge  
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13 462 methods head to head and found close correspondence in VCs across species with different xylem  
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15 463 anatomy. An extended literature survey of methods to measure vulnerability to embolism showed  
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17 464 that when using the centrifuge, VCs were sigmoidal in conifers and in long vessel species  
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19 465 exponential, whereas in diffuse porous species VCs varied from sigmoidal to linear or exponential  
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21 466 (Cochard et al. 2013). Our measurements and simulations made with the CAVITOPEN model explain  
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23 467 the different shapes of VCs and some disagreements between the static centrifuge and the Cavitron.  
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25 468 In short-vesseled angiosperms, we have shown that VCs by centrifugation agreed with each other  
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27 469 and closely matched the curves based on bench dehydration and microCT (Choat et al. 2016;  
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29 470 Cochard et al. 2010). In angiosperms with a proportion of vessels open to the middle but not the  
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31 471 whole way through, the standard protocol in the static centrifuge produces linear VCs (Sperry et al.  
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33 472 2012). Here the initial conductivity is measured before spinning, thus if the native embolism is low,  
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35 473 all the vessels are conductive, regardless of their length. As soon as the sample is spun, the  
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37 474 conductivity would be artificially reduced relative to the native state in proportion to the amount of  
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39 475 vessels open to centre. Sample with open vessels thus become artificially vulnerable to embolism at  
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41 476 the beginning of the VC (i.e. at less negative water potentials). For *H. leucoptera*, this translated into  
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43 477 less negative values of  $P_{12}$  in all centrifuged samples compared with those measured with the bench  
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45 478 dehydration technique creating a linear response or a plateau at high water potentials. Higher  
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47 479 differences in  $P_{12}$  were observed in *H. leucoptera* than in *H. dactyloides* according with a higher  
48  
49 480 proportion of vessels open to centre in the former species (Fig. 1). In the Cavitron, the initial  
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51 481 measurement was made while spinning at low tension and many open to centre vessels would  
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53 482 already be embolised in the initial measurement of conductivity, resulting in a lower artefactual loss  
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3 483 of conductivity in the subsequent water potentials of the VC. This may bias the curves slightly  
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5 484 pushing them to more negative values but it did not appear to be significant effect here as the  
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7 485 Cavitron curves for *H. leucoptera* were similar to bench dehydration curves.  
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10 486 The simulations of PLC with the CAVITOPEN model confirmed that the impact of open vessels on the  
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12 487 VC was higher when vessels were long, samples short and when open vessels cavitated at less  
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14 488 negative pressures (Fig. 4). If the samples were much shorter than the maximum vessel length of the  
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16 489 branch (see the results in Fig. 4 for the 14 cm rotor with  $L_{max}$  50cm), the resulting VC was exponential  
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18 490 (r-shaped), as observed in long-vessel angiosperms, and shifted to more linear or s-shaped when  
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20 491  $L_{max}$  was decreased or the sample length increased. One of the assumptions in the model is that  
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22 492 vessels open at the cut surface cavitate when they reach a threshold value; that is far less negative  
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24 493 than intact vessels whose two ends are included within the spun segment. This influences the shape  
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26 494 the VC at higher pressures creating a “bump” in the VC followed by a plateau. This effect can be  
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28 495 corrected to some extent if the first inflexion point of the VC is considered to be the 0% point for loss  
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30 496 of conductivity. In this case the initial conductivity ( $K_i$ ) value is shifted to a lower value corresponding  
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32 497 to the hydraulic conductivity of the plateau (Fig. 4d). The estimated values of  $P_{50}$  and  $S_{50}$  when the  
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34 498 CAVITOPEN model was fit to actual measurements agreed quite well with those obtained with  
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36 499 reference techniques and confirmed that this model can be used to correct open vessel artefacts for  
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38 500 centrifuge based VCs. The estimated  $L_{max}$  was however significantly shorter than  $L_{max}$  measured with  
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40 501 the air injection technique. The air injection technique has shown to produce higher  $L_{max}$  than the  
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42 502 rubber injection method (Pan et al. 2015), thus our values could be overestimated. On the other  
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44 503 hand, the model assumed that vessel lengths in a sample follow the density function proposed by  
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46 504 Cohen et al. (2003) which can be sensitive to the clustering of vessel lengths (Cai and Tyree 2014). It  
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48 505 is clear that the actual distribution of vessel lengths, network topology and connectivity are crucial  
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50 506 for the sensitivity to an open vessel artefact.  
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#### 55 **Origin of the open-vessel artefact**

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3 508 The physical mechanisms underlying this open-vessel artefact are yet to be fully elucidated. Some  
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5 509 studies suggest that microbubbles and particles can act as nucleation sites when they flow through  
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7 510 the sample as it spins in the Cavitron, causing premature embolism (Cochard et al. 2010; Sperry et al.  
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9 511 2012; Wang et al. 2014). In the static centrifuge, bubbles might be drawn into vessels while starting  
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11 512 the spin or while mounting or dismounting the stems to measure flow (Wang et al. 2014). In both  
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13 513 centrifuge techniques bubbles in open vessels can move by buoyancy while spinning toward the  
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15 514 region of lowest pressure at the center of rotation (Rockwell et al., 2014). Draining from open  
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17 515 vessels as a consequence of artefactual embolism when the centrifuge starts spinning appears to be  
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19 516 a common phenomenon in both rotors. Our microCT images showed that after spinning in the  
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21 517 centrifuge, most of the vessels were empty near the ends even though tension ought to be zero  
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23 518 (Cochard et al. 2005). The use of water saturated foam pads to avoid desiccation did not prevent this  
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25 519 (Hacke et al. 2015; Tobin et al. 2013). We discarded the possibility that sample manipulation before  
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27 520 spinning or during wax embedding had triggered vessel draining because we scanned control  
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29 521 samples that were not spun. These samples showed no embolism (Fig. 3). Furthermore, patterns of  
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31 522 embolism did not follow theoretical expectations based on the distribution of tension within the  
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33 523 spun sample. The embolism levels decreased from the ends to the center in a fashion consistent  
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35 524 with the amount of vessels open to center, opposite to that expected from profile in tension and in  
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37 525 agreement with the assumption of the CAVITOPEN model than open vessels artificially cavitate  
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39 526 when they reach a threshold pressure that is much less negative than in intact vessels. This pattern  
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41 527 was observed at water potentials inducing less than 40% loss of conductivity based on the VC  
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43 528 obtained using the middle segment of the centrifuged sample (Fig. 3), even though the centre of the  
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45 529 sample experienced the highest tensions. Embolism levels converged within the sample at -9 MPa at  
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47 530 80-90%. These results confirm that centrifugation drains open vessels and only reliably measure the  
48  
49 531 vulnerability of intact xylem vessels within the sample (Fig. S1). This is consistent with observations  
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51 532 made previously by Cochard et al. (2010) using the Cavitron; they reported that embolism was  
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53 533 higher in the basal and upstream ends relative to the centre of samples from species with vessels  
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3 534 that are predominately at least half as long as the spun segment. Cai et al. (2010) and Pengxian et al.  
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5 535 (2018) also reported higher PLC values than predicted by theory at both ends after spinning samples  
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7 536 in a Cavitron. Given that our results were obtained with the static centrifuge it is clear that the  
8  
9 537 overestimation of vulnerability for open to centre vessels occurs in both versions of the centrifuge  
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11 538 technique.

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14 539 The hydraulic continuity between vessels cut open at each end of the sample and vessels with their  
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16 540 terminal ends in this portion of the sample is probably re-established by refilling of vessels immersed  
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18 541 under water at both ends (Fig. 2 in Cochard et al. (2010)). This refilling would occur by capillarity  
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20 542 either while spinning in the Cavitron or while flow is measured gravimetrically (Fig. S2). Since the  
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22 543 middle of the centrifuged sample contains the majority of intact vessels, VCs constructed with the  
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24 544 static centrifuge technique of angiosperm species using only the central segment are more reliable  
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26 545 and in closer agreement with PLC generated by natural dehydration (Fig. 2). This modification is  
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28 546 technically easy to achieve and mitigates the open vessel artefact; however, it carries the  
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30 547 disadvantage that samples cannot be spun repeatedly to construct replicate curves for each sample  
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32 548 and thus more plant material is needed to construct each curve.

## 33 34 35 36 549 **Conclusion**

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39 550 We confirmed the validity of vulnerability curves constructed with both centrifuge methods for short  
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41 551 conduit angiosperm species, those with most conduits shorter than half the length of the centrifuge  
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43 552 rotor. A new model, CAVITOPEN was developed to simulate the effect of vessel length, rotor size and  
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45 553 vulnerability of open vessels in loss of conductivity of centrifuged samples. In species with maximum  
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47 554 vessel length similar to the centrifuge rotor, we recommend constructing vulnerability curves with  
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49 555 the Cavitron or measuring flow exclusively in the central part of the spun segment when using the  
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51 556 static centrifuge. Alternatively, artefactual embolism at low xylem tensions can be corrected if the  
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53 557 first inflexion point of the VC is considered to be the starting point for  $K_{max}$  (0 % loss of conductivity)  
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55 558 or by fitting the CAVITOPEN model to the measurements to estimate  $P_{50}$  and  $S_{50}$ . When samples

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3 559 contained a high proportion of open to centre vessels, the centrifuge technique is prone to error and  
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5 560 overestimates vulnerability to embolism. Determining the proportion of open to centre vessels or  
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7 561 performing the simple test recently proposed by Torres-Ruiz et al. (2017), which compares changes  
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9 562 in  $K_s$  before and after spinning in the centrifuge at low tensions, are highly advisable before using  
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11 563 any of the centrifuge techniques.

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14 564 The shape of the vulnerability curves obtained with bench dehydration were always sigmoidal while  
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16 565 in centrifuged samples the shape was determined by the presence of open vessels. While previous  
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18 566 studies have demonstrated that species with the longest vessel classes (eg. lianas, ring porous trees)  
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20 567 open vessels tend to exhibit exponential curves when measured in the centrifuge. Here we showed  
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22 568 that VCs with a linear shape are symptomatic of species with intermediate vessel lengths in which a  
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24 569 higher proportion of vessels open to centre of the test segment. The occurrence of this incipient  
25  
26 570 open vessel artefact can be mitigated by measurement of the excised central portion of the  
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28 571 segment.

### 31 572 **Acknowledgments**

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33  
34 573 This research was supported by a Marie Curie Fellowship to R.L. (FP7PEOPLE-2013-IOF-624473) and  
35  
36 574 an ARC Future Fellowship to B.C. (FT130101115). We thank Dr. Javier Cano, Adrián Cano, Teresa  
37  
38 575 Rosas and Jennifer Peters for field assistance, Dr. Iain M Young for X-ray microCT advice and  
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40 576 comments on the manuscript, Gavin McKenzie for lab support and Dr. Stephanie Stuart for her ideas  
41  
42 577 and writing assistance. No conflict of interests declared.

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3 715 **Figure legends**  
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6 716 **Figure 1.** Distribution of PLC in air-injected branches of *H. dactyloides* (black circles) and *H.*  
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8 717 *leucoptera* (open circles) at different positions from the injected end. Vertical bars represent the  
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10 718 standard error. Dashed lines indicate the two sample lengths used for the centrifuge methods, 14 cm  
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12 719 and 27 cm and dot lines indicate their respective half sample length.

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15 720 **Figure 2.** Xylem vulnerability to embolism curves and 95% confidence intervals (grey shaded areas)  
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17 721 of *Hakea dactyloides* (left panels) and *Hakea leucoptera* (right panels) obtained with two methods to  
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19 722 induce cavitation in the xylem, bench dehydration and centrifuge force and three methods to  
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21 723 measure the loss of conductivity, flowmeter (close circles), in situ flow method (open circles) and X-  
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23 724 ray microCT visualisation (red triangles). Vertical solid lines indicate  $P_{50}$  and vertical dashed lines  
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25 725 indicate the 95% confidence interval for  $P_{50}$ . Horizontal dashed lines indicated native xylem  
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27 726 embolism measured in the field. Two rotor sizes, 14 cm and 27 cm, were used in the static  
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29 727 centrifuge, and water flow in the whole segment or only in the central part was measured (see  
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31 728 methods for details).

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35 729 **Figure 3.** Transverse slices from X-ray microtomography (X-ray micro-CT) scans of branches of *Hakea*  
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37 730 *leucoptera* (maximum vessel length =  $25 \pm 5$  cm) scanned at three positions before spinning (left  
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39 731 column) and after spinning in the centrifuge at 5, 7 and 9 MPa. Embolized vessels appear as black  
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41 732 and water-filled conduits appear as grey. The estimated percent loss of conductivity (PLC) is shown  
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43 733 in each picture. Scale bar, 1 mm.

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46 734 **Figure 4.** Simulations with the CAVITOPEN model of the effect of threshold of embolism formation  
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48 735 (MPa) of cut open vessels (A), maximum vessel length (cm) (B), and rotor size (cm) (C) on xylem  
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50 736 vulnerability to embolism curves generated with centrifugation. In red, vulnerability curve of close  
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52 737 vessels at both ends. (D) The CAVITOPEN model was fit to measurements in *H. leucoptera* using  
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54 738 numerical optimization to estimate all four parameters: water potential at 50% loss of conductivity

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3 739 ( $P_{50}$ ), slope of the vulnerability curve ( $S_{50}$ ), maximum vessel length ( $L_{max}$ ) and threshold of embolism  
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5 740 formation of cut open vessels ( $P_{open}$ ). Circles represent the values obtained in our study with the  
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7 741 static centrifuge, 27 cm rotor in *H. leucoptera* when flow was measured in the whole segment (see  
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9 742 Methods for details); grey solid line is the fitted curve with the CAVITOPEN model; black solid line  
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11 743 represent the curve after correction and orange dashed line is the reference curve obtained with  
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13 744 bench dehydration for the species.

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For Peer Review

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8 1 **Title:** Mitigating the open vessel artefact in centrifuge based measurement of embolism resistance

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10 2 **Running head:** Mitigating artefacts in vulnerability curves

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**Abstract** (300 words max)

Con formato: Español (España)

Centrifuge-based techniques to assess xylem vulnerability to embolism are increasingly being used, although we are yet to reach a consensus on the nature and extent of artefactual embolism observed in some angiosperm species. In particular, there is disagreement over whether these artefacts influence both the spin (Cavitron) and static versions of the centrifuge technique equally.

We tested two methods for inducing embolism: bench dehydration and centrifugation. We used three methods to measure the resulting loss of conductivity: gravimetric flow measured in bench-dehydrated and centrifuged samples (static centrifuge), in situ flow measured under tension during spinning in the centrifuge (Cavitron), and direct imaging using X-ray microCT observations in stems of two species of *Hakea* that differ in vessel length.

Con formato: Fuente: Cursiva

Both centrifuge techniques were prone to artefactual embolism in samples with maximum vessel length longer, or similar, to the centrifuge rotor diameter. Observations with microCT indicated that this artefactual embolism occurred in the outer most portions of samples. The artefact was largely eliminated if flow was measured in an excised central part of the segment in the static centrifuge or starting measurements with the Cavitron at pressures lower than the threshold of embolism formation in open vessels. The simulations of loss of conductivity in centrifuged samples with a new model, CAVITOPEN, confirmed that the impact of open vessels on the vulnerability to embolism curve was higher when vessels were long, samples short and when embolism is formed in open vessels at less negative pressures. This model also offers a robust and quantitative tool to test and correct for artefactual embolism at low xylem tensions.

**Keywords**

Vulnerability to embolism, xylem embolism, drought, centrifuge technique, Cavitron, X-Ray microCT, CAVITOPEN.

## 44 Introduction

45 Xylem water transport is dependent upon water held in a metastable state of water; evaporation of  
46 water from the leaf cell walls generates tension, which is transmitted through the water column to  
47 the roots. Water under tension is prone to cavitation, i.e. the abrupt transition from a metastable  
48 liquid to a gas, resulting in the formation of gas emboli that block the xylem conduits and impairs  
49 water transport (Tyree and Sperry 1988). As tension in the xylem sap increases, for example during  
50 drought, so does the probability of embolism formation. During severe or prolonged droughts,  
51 hydraulic failure can result in the complete loss of hydraulic conductance in the xylem and subsequent  
52 canopy dieback, or whole plant death (Brodribb and Cochard 2009; Nardini et al. 2013; Rodríguez-  
53 Calcerrada et al. 2017; Urli et al. 2013; Venturas et al. 2016). Hydraulic failure is now considered a  
54 principal cause of drought-induced plant mortality and forest die off (Choat et al. 2012; Sala et al.  
55 2010). The projected rise in global mean temperature and frequency of extreme climate events over  
56 the next century will impact forest ecosystems and shift species distribution ranges. In this sense,  
57 resistance to embolism has emerged as a crucial parameter to understanding species ecology,  
58 differences in water use strategies, and for predicting future mortality events (Brodribb 2017).

59 Xylem resistance to embolism is usually characterized with a vulnerability curve, showing the decrease  
60 in hydraulic conductivity as a function of the xylem tension. Since the publication of the first  
61 vulnerability curves for woody plants were published in 1985 (Sperry 1985) and 1986 (Tyree and Dixon  
62 1986), a number of techniques that allow for more rapid measurement of vulnerability have been  
63 introduced (see Cochard et al. (2013) for a detailed review). However, although the time required for  
64 construction of a vulnerability curve has been dramatically reduced, recent work suggests that some  
65 of these methods are prone to experimental artefact (Choat et al. 2010; Cochard et al. 2010; Sperry  
66 et al. 2012; Torres-Ruiz et al. 2014). This has led to re-examination of methodology used to measure  
67 vulnerability to embolism (Jansen et al. 2015).

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8 68 The most straightforward technique for inducing embolism is bench dehydration, wherein whole  
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10 69 plants or long branches are gradually dehydrated to various xylem tensions and hydraulic conductivity  
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12 70 of excised segments is measured gravimetrically before and after removing air from embolised  
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14 71 conduits (Sperry and Tyree 1988; Tyree and Zimmermann 2002). Bench dehydration relies on natural  
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16 72 desiccation of plant tissues and is therefore considered as the best reference method with which to  
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18 73 validate other techniques (Cochard et al. 2013; Ennajeh et al. 2011; Sperry et al. 2012). This method  
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20 74 is not completely free of artefacts and issues associated with disequilibrium in water potential within  
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22 75 a stem, blockage of flow by resin/mucilage (Cobb et al. 2007), and excision of samples under tension  
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24 76 can all alter the vulnerability curve significantly (Wheeler et al., 2013). Although most of these issues  
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26 77 can be minimised by adoption of suitable protocols (eg. Torres-Ruiz et al., 2015), the bench  
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28 78 dehydration technique requires several days and a substantial amount of plant material to obtain a  
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30 79 vulnerability curve for one species. As such, Holbrook et al. (1995) and Pockman et al. (1995) proposed  
31  
32 80 the use of a centrifugal force to create a defined negative pressure in the xylem sap of excised plant  
33  
34 81 stems, allowing for rapid and consistent generation of vulnerability curves. Pockman et al. (1995)  
35  
36 82 constructed vulnerability curves for several species by comparing the hydraulic conductivity before  
37  
38 83 and after spinning branches with their ends exposed to air, removing segments at both ends before  
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40 84 measuring conductivity in the remaining, middle section of the sample. Alder et al. (1997) modified  
41  
42 85 this technique with a centrifuge rotor designed to keep the segment ends immersed in water during  
43  
44 86 spinning, allowing the conductivity of a single segment to be remeasured at different tensions to  
45  
46 87 create an entire vulnerability curve for a single sample. This important innovation allowed repeated  
47  
48 88 measurements to be made on the same plant material, reducing the number of samples required for  
49  
50 89 construction of a curve and strengthening the results statistically. Finally, Cochard (2002), Cochard et  
51  
52 90 al. (2005) and Li et al. (2008) further modified the centrifuge method and designed new rotors which  
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54 91 allowed measuring the conductivity of the segment while it is spinning and under tension. This further  
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56 92 increased the efficiency of measurement and allowed for flow measurements to be made under  
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58 93 tension.

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8 94 Although centrifuge based techniques induce embolism by increasing tension in sample xylem, the  
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10 95 patterns of embolism spread through the sample may differ from a naturally dehydrated sample (Cai  
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12 96 et al. 2010). The tension profile in the centrifuged segment is highest in the axis of rotation (i.e. in the  
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14 97 middle section of the segment) and declines towards the segment ends (Cochard et al. 2005), while  
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16 98 during natural dehydration the tension profile across the segment is expected to remain  
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18 99 approximately constant (Cai et al. 2010). Nevertheless, the vulnerability curves generated by  
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20 100 centrifugation agree well with the bench-top method in conifers and short-vesseled angiosperm  
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22 101 species (Alder et al. 1997; Cochard et al. 2005; Cochard et al. 2010; Li et al. 2008). In contrast,  
23  
24 102 inconsistent results have been obtained for species with long vessels, specifically those in which a  
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26 103 significant number of vessels in the sample are longer than the centrifuge rotor (Choat et al. 2010;  
27  
28 104 Jacobsen and Pratt 2012; Sperry et al. 2012; Torres-Ruiz et al. 2014).

29 105 Since 2005 the number of vulnerability curves constructed by centrifugation has increased  
30  
31 106 exponentially (see Fig. 3 in Cochard et al. (2013)). Accordingly, considerable effort has been devoted  
32  
33 107 to testing and validation of centrifuge techniques, whether measuring the flow gravimetrically after  
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35 108 spinning (static centrifuge method), or while centrifuging (Cavitron method). However, we are yet to  
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37 109 reach a consensus on the nature and extent of artefactual embolism observed with centrifuge  
38  
39 110 techniques. In particular, there is disagreement over whether these artefacts influence both spin  
40  
41 111 (Cavitron rotor) and static versions of the centrifuge technique equally (Hacke et al. 2015; Sperry et  
42  
43 112 al. 2012). In recent years, the application of x-ray computed microtomography (microCT) to the study  
44  
45 113 of plant hydraulics has emerged as a potentially powerful tool to validate hydraulic techniques. In  
46  
47 114 addition to providing a non-invasive assay of xylem function, it allows for analyses of spatial and  
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49 115 temporal patterns of embolism formation (Brodersen et al. 2013; Choat et al. 2016; Dalla-Salda et al.  
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51 116 2014; Torres-Ruiz et al. 2016).

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60 117 In this study we evaluated the performance of both centrifuge techniques against bench dehydration  
118 in order to examine possible discrepancies associated with each technique. First, we tested two

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8 119 methods for inducing embolism: bench dehydration and centrifugation. We then tested three ways of  
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10 120 measuring the resulting loss of conductivity: gravimetric flow measured in bench-dehydrated and  
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12 121 centrifuged samples (static centrifuge), *in situ* flow measured under tension during spinning in the  
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14 122 centrifuge (Cavitron), and direct imaging using X-ray microCT observation. All experiments were  
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16 123 carried out with two species of the genus *Hakea* that differ in vessel length. *H. dactyloides* is a short  
17  
18 124 vesseled species with maximum vessel length shorter than 14 cm, whereas *H. leucoptera* has longer  
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20 125 vessels and maximum vessel length is ca. 25 cm. Additionally, we compared results obtained using  
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22 126 two rotor diameters (14 and 27 cm) to assess the effect of sample length, and measured hydraulic  
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24 127 flow both in the whole, spun segments and excised middle sections. Spatial patterns of embolism  
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26 128 within samples were visualized with X-ray microCT after centrifugation in order to provide further  
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28 129 insight into potential discrepancies. Finally, a new model, CAVITOPEN was developed to simulate the  
29  
30 130 effect of vessel and sample lengths on centrifuge estimates of embolism resistance. We hypothesized  
31  
32 131 that i) both centrifuge techniques, the static centrifuge and the cavitron, are prone to similar artefacts  
33  
34 132 when constructing vulnerability curves of long-vesseled species; ii) the shape of the vulnerability curve  
35  
36 133 of centrifuged samples will depend on the amount of cut open vessels; iii) image techniques and  
37  
38 134 standard flow measurements will produce similar vulnerability curves.

135

## 136 **Material and methods**

### 137 *Plant Material*

138 Experiments were carried out on branch material of two diffuse-porous species of the same genus  
139 exhibiting different vessel lengths, *Hakea dactyloides* (Gaertn.) Cav. and *Hakea leucoptera* R. Br.  
140 Branches were sampled from natural populations of *H. dactyloides* at Mount Banks (33° 34' 46'' S,  
141 150° 21' 56'' E; NSW, Australia) and *H. leucoptera* at Binya State Forest (34° 11' 16'' S, 146° 16' 13'' E;  
142 NSW, Australia) from May to September 2016 (late autumn-winter in the South Hemisphere). Sun

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8 143 exposed branches of 1.5-2.0 m length were collected in the field in the early morning and immediately  
9 144 placed in black plastic bags with moistened paper towels to prevent transpiration with their cut ends  
10  
11 145 covered with Parafilm. In the laboratory they were kept at 4 °C until measured.

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14 146 *Midday xylem water potential in the field and Native embolism*

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16 147 Midday xylem water potential was measured in the field in November 2015, February 2016 and June  
17 148 2016. Two leaves of five plants per species were covered with aluminium foil and sealed with a plastic  
18  
19 149 bag 1 hour before excision and measurement with a pressure chamber (PMS Instrument Co., Albany,  
20  
21 150 OR, USA).

22  
23 151 Native embolism was determined in current-year, one-year and two-year old segments of 5 branches  
24  
25 152 per species to ensure that the effects of previous natural water stress were minimised. ~~due to~~Note  
26  
27 153 that segments containing 1-year and 2-year-old growth were necessary to fit in the 27 cm rotor of the  
28  
29 154 centrifuge. ~~Measuring native embolism we also wanted and~~ to control for sample collection date  
30  
31 155 because branches were cut at different times during late autumn-winter 2016 to avoid long storage.

32 156 Branch proximal end was cut underwater to release tension for 30 min (Torres-Ruiz et al. 2015;  
33  
34 157 Wheeler et al. 2013) and then the branch was progressively recut under water to segments 50 mm  
35  
36 158 long. Note that at least twice the maximum vessel length was removed from the cut end after tension  
37  
38 159 relaxation. Thereafter, the edges of these segments were trimmed using a razor blade. Initial  
39  
40 160 conductivity ( $K_i$ ) was measured in 50 mm long segments with filtered, degassed 2 mmol KCl solution  
41  
42 161 at low pressure ( $\leq 4$  kPa) with a liquid flowmeter (LiquiFlow L13-AAD-11-K-10S; Bronkhorst High-Tech  
43  
44 162 B.V., Ruurlo, the Netherlands). The segments were then flushed with the same solution at a minimum  
45  
46 163 of 0.20 MPa for 15 min to remove embolism and subsequently determine maximum hydraulic  
47  
48 164 conductivity ( $K_{max}$ ). The native percentage loss of conductivity (PLC) was calculated for each segment  
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50 165 as:

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$$PLC = 100 \times (1 - K_i / K_{max})$$
 (equation 1)

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8 167 Specific hydraulic conductivity ( $K_s$ ) was calculated dividing  $K_{max}$  by the xylem cross-sectional area  
9 168 (average distal and proximal xylem area measured with a calliper).

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12 169 *Maximum vessel length and vessel length distribution*

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14 170 Ten branches per species were sampled from the same plants as used for hydraulic measurements to  
15  
16 171 determine maximum vessel length with the air perfusion technique (Ewers and Fisher 1989). Once in  
17  
18 172 the lab, 60 cm long segments were flushed for 1 h with degassed, filtered 2 mmol KCl solution at 0.18-  
19  
20 173 0.20 MPa to remove any embolism. Then each segment was infiltrated with compressed air at 0.05  
21  
22 174 MPa at its distal end with an aquarium air pump while the basal end was repeatedly shortened by 2  
23  
24 175 cm under water until air bubbles emerged. The remaining sample length was assumed as maximum  
25  
26 176 vessel length.

27 177 An estimate of the amount of vessels longer than the centrifuge rotor diameter and longer than half  
28  
29 178 the rotor diameter (open to centre vessels) was assessed in four branches of *H. dactyloides* and five  
30  
31 179 branches of *H. leuoptera* by measuring the decrease in PLC after air injection (Cochard et al. 1994;  
32  
33 180 Torres-Ruiz et al. 2014). Briefly, 35 cm long segments were flushed as described above to remove  
34  
35 181 embolism. Then, tubing was attached to the distal end of these segments and compressed air was  
36  
37 182 injected into the samples at 0.1 MPa for 10 min using a pressure chamber. This pressure was sufficient  
38  
39 183 to empty the open vessels but not high enough to move water through wet pit membranes between  
40  
41 184 adjacent vessels (Ewers and Fisher, 1989). PLC was determined in 3 cm long segments across the  
42  
43 185 sample as described for native embolism. At the injection point, PLC is close to 100% because all the  
44  
45 186 vessels are air filled and progressively decrease to 0 for a length longer than the longest vessel in the  
46  
47 187 sample. The PLC at each distance from the injection point corresponds to the percentage of  
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49 188 contribution to flow from vessels longer than this distance. If all the vessels were of equal diameter,  
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51 189 this percentage would correspond to the number of vessels longer than the distance from the injection  
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53 190 point. In this case of the two *Hakea* species used are diffuse porous and vessel diameters within the  
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55 191 same sample did not vary greatly. Thus the curves in Fig. 1 represent a good proxy of vessel distribution

Con formato: Fuente: Cursiva

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8 192 of the two species, although not as accurate as anatomy, and allow to estimate the amount of open  
9 193 vessels from a certain cut point.

#### 194 *Bench dehydration technique*

195 Branches were dehydrated gradually in the laboratory at ca. 23 °C. Xylem water potential ( $\Psi_x$ ) was  
196 measured with a pressure chamber (PMS Instrument Co., Albany, OR, USA) in bagged leaves (wrapped  
197 with aluminium foil and a plastic bag at least 1 h before sampling). When the target  $\Psi_x$  to construct  
198 the VC was reached, branches were sealed into a plastic bag with moistened paper towels for 1 h to  
199 equilibrate  $\Psi_x$ . Water potential was measured again in two bagged leaves of the same branchlet to  
200 confirm homogeneous  $\Psi_x$  in the sample. The  $\Psi_x$  of the sample was considered equilibrated if the  
201 difference between the three  $\Psi_x$  (one measured before sealing the branch and two measured after  
202 equilibration) was not higher than 0.1 MPa. Afterwards tension was released for 30 minutes by cutting  
203 the branch proximal end under water and PLC was determined in one-year-old segments as for native  
204 embolism. Vulnerability curves were generated by plotting PLC against  $\Psi_x$ . For *H. leucoptera* 7  
205 branches were dehydrated and 4 different branchlets per branch were measured at different  $\Psi_x$  to  
206 construct the vulnerability curve and for *H. dactyloides* we used 12 branches and two branchlets per  
207 branch. All branchlets were far apart (at least four branch orders) and after collection the cutting  
208 surface was covered with parafilm to avoid air entry in the rest of the sample.

#### 209 *Centrifuge techniques*

210 We compared two centrifuge techniques: i) the static centrifuge method described by Alder et al.  
211 (1997) and ii) the *in situ* flow technique (Cavitron (Cochard 2002; Cochard et al. 2005)). In the static  
212 centrifuge two different sizes of custom-built rotors, 14 cm and 27 cm, were used to test the effect of  
213 segment length and fraction of open vessels. All hydraulic conductivity measurements were  
214 performed using filtered, degassed 2 mmol KCl solution and a flow meter (see Native embolism  
215 section).



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8 216 Static centrifuge measurements were carried out on 20 branches per species. Branches were trimmed  
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10 217 under water and both ends were shaved to a final length of 14 or 27 cm. The initial hydraulic  
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12 218 conductivity was measured as described above (see Native embolism section) with a pressure head of  
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14 219 7.5 kPa. Subsequently, 14-cm long branches were spun in the centrifuge (Sorvall RC 5C Plus) for 5  
15  
16 220 minutes at increasing pressure steps. Foam pads saturated with the solution used for measurements  
17  
18 221 were placed in the reservoirs of the rotor to maintain sample ends in contact with the solution even  
19  
20 222 when the rotor was stopped (Tobin et al. 2013). After each step, samples were removed and  $K_h$  was  
21  
22 223 measured on the whole segment as described for native embolism. In the 27cm-long branches we  
23  
24 224 modified the single spin method (Hacke et al. 2015) so that two measurements were made in each  
25  
26 225 centrifuged segment. The initial  $K_h$  was measured before spinning in the 27-cm long sample. After  
27  
28 226 spinning,  $K_h$  was measured on the whole segment and the first PLC was calculated. Subsequently, a 4  
29  
30 227 cm-long segment was cut from the middle section and its  $K_h$  was measured. The second PLC was  
31  
32 228 determined in this 4 cm-long segment after flushing to obtained the maximum  $K_h$  ( $K_{max}$ ) as described  
33  
34 229 for native embolism.

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36 230 *In situ* flow centrifuge measurements (Cavitron technique) were carried out on six branches per  
37  
38 231 species using a modified bench top centrifuge (H2100R, Cence Xiangyi, Hunan, China). For the static  
39  
40 232 centrifuge, samples were trimmed under water to a length of 27 cm to fit in the rotor. Initial  
41  
42 233 conductivity,  $K_i$ , was determined at a xylem pressure of  $-0.5$  MPa in *H. dactyloides* and  $1.5$  MPa in *H.*  
43  
44 234 *leucoptera*. The xylem pressure was then lowered stepwise by increasing the rotational velocity, and  
45  
46 235  $K_h$  was again determined while the sample was spinning. The PLC at each pressure step was quantified  
47  
48 236 as

$$45 \quad 237 \quad PLC = 100 \times (1 - K_h / K_i). \quad (\text{equation 2})$$

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60 238 *X-ray microCT imaging*

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8 239 A subset of branches of *H. leucomelaena* was transported to the University of New England in Armidale  
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10 240 (NSW, Australia). They were gradually dehydrated to five different xylem water potentials ranging  
11  
12 241 from -4.8 MPa to -9 MPa as for the bench dehydration method. After measuring  $\Psi_x$ , tension was  
13  
14 242 relaxed by cutting the proximal end of the branch under water leaving it submerged for 30 minutes.  
15  
16 243 Then the branch was sequentially cut back under water and finally 10-mm-long segments were excised  
17  
18 244 under water from current-year shoots, wrapped in Parafilm, inserted into a plexiglass tube and then  
19  
20 245 placed in an X-ray microtomography system (GE-Phoenix V|tome|xS, GE Sensing & Inspection  
21  
22 246 Technologies, Wunstorf, Germany) to visualize embolized vessels. Another subset of branches of *H.*  
23  
24 247 *leucomelaena* was centrifuged to five (-5, -6, -7, -8, -9 MPa) and three (-5, -6, -7 MPa) different water  
25  
26 248 potentials in the static centrifuge using 27 cm and 14 cm long segments, respectively. They were  
27  
28 249 immediately submerged in liquid paraffin wax and preserved at 4 °C for three days until measured in  
29  
30 250 the same facility (Cochard et al. 2015). Seven branches of *H. dactyloides* were also centrifuged at four  
31  
32 251 (-3, -4, -5, -6 MPa) and three (-3, -4, -5 MPa) water potentials with the 27 and 14 cm rotors,  
33  
34 252 respectively, following the same protocol. One branch of *H. leucomelaena* was prepared as the  
35  
36 253 centrifuged samples but was not spun in the centrifuge to detect any possible artefact due to sample  
37  
38 254 preparation. All samples were scanned at the middle of the sample. Additionally, in three 27 cm long  
39  
40 255 samples we scanned at 6 cm and 12 cm from the axis of rotation to examine embolism profiles across  
41  
42 256 a sample.

39 257 X-ray scan settings were 90 kV and 170 mA, and 1800 projections, 600 ms each, were acquired during  
40  
41 258 the 360° rotation of the sample. The resultant images covered the whole cross section of the sample  
42  
43 259 in 8.7 mm length with a spatial resolution of 8.7  $\mu\text{m}$  per voxel. At the end of the scan, the sample was  
44  
45 260 cut back to 30 mm length, injected with air at >1 MPa pressure and rescanned at the same location as  
46  
47 261 before to visualize all empty vessels in the fully embolized cross section. After three-dimensional  
48  
49 262 reconstruction with Phoenix datos|x2 Reconstruction Version 2.2.1-RTM (GE Sensing & Inspection  
50  
51 263 Technologies, Wunstorf, Germany), volumes were imported into ImageJ 1.49k (Schneider et al. 2012).  
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53 264 A median Z projection of c. 100  $\mu\text{m}$  along the sample axis was extracted from the middle of the scan

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8 265 volumes following the protocol in Nolf et al. (2017). PLC of each sample was estimated calculating the  
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10 266 theoretical hydraulic conductance based on the conduit dimensions of embolized and functional  
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12 267 vessels (Choat et al. 2016). To measure conduit dimensions, a radial sector of the transverse section  
13  
14 268 was selected in the same microCT scan and all their embolized vessels were measured manually. The  
15  
16 269 image of this sector was then binarized so the dimensions of the selected embolized vessels matched  
17  
18 270 with the manually drawn vessels. This threshold value was then used for binarizing the image of the  
19  
20 271 whole cross section and all the embolized vessels were measured using the Analyse Particles function  
21  
22 272 in Image J. Theoretical specific hydraulic conductivity ( $K_{sth}$ ) was calculated as:

$$23 \quad K_{sth} = \frac{\sum \left( \frac{D^4 \pi}{128 \eta} \cdot \frac{\Delta p}{\Delta x} \right)}{A} \quad \text{(equation 3)}$$

24  
25 274  
26 275 Where  $D$  is the equivalent circular vessel diameter based on vessel area,  $\eta$  viscosity of water,  $\Delta p/\Delta x$   
27  
28 276 pressure gradient per xylem length,  $A$  xylem cross-sectional area.  
29

30 277  
31 278 The current theoretical specific hydraulic conductivity ( $K_{sth}$ ) for each sample was calculated by  
32  
33 279 subtracting the summed specific hydraulic conductivity of embolized vessels from the  $K_{sth(max)}$  of that  
34  
35 280 sample, calculated as the  $K_{sth}$  of the sample after air injection. The pressure gradient used for  
36  
37 281 calculations of  $K_{sth}$  was similar to the pressure gradient used in the hydraulic measurements, 0.06 MPa  
38  
39 282  $m^{-1}$ .

#### 40 283 41 42 284 *Vulnerability curve fitting and statistical analysis*

43  
44 285 Vulnerability curves were fitted using a Weibull function (Ogle et al. 2009) in R 3.2.0 (R Core Team,  
45  
46 286 2015) using the fitplc package (Duursma and Choat 2017). Confidence intervals of  $P_{12}$ ,  $P_{50}$  and  $P_{88}$  ( $\Psi_x$   
47  
48 287 at 12, 50 and 88 % loss of conductivity, respectively) and the slope of the curve at 50% loss of  
49  
50 288 conductivity ( $S_{50}$ ) were used to compare between methods. Confidence intervals (CI) for the bench

289 dehydration and the static centrifuge techniques were obtained using bootstrap resampling (999  
 290 replicates). Methods were considered to be statistically different if the 95% CIs did not overlap.

291 Differences in native embolism and specific initial conductivity between sampling dates were tested  
 292 with a one-way ANOVA. Means were compared using a Tukey test at 95% confidence. Vulnerability  
 293 curve parameters across methods were compared at the  $\Psi_x$  corresponding with three levels of loss  
 294 of conductivity: 12%, 50% and 88% ( $P_{12}$ ,  $P_{50}$  and  $P_{88}$ , respectively) and the slope of the VC at 50% loss  
 295 of conductivity ( $S_{50}$ ).

296 *CAVITOPEN- simulation of the effect of open vessels in a centrifuged sample*

297 To disentangle the ~~combined~~ effects of centrifugation on 'true' vessel embolism at the centre of the  
 298 samples, where more vessels are closed at both ends and tension is maximum, from draining of open  
 299 vessels at both sample ends a new model, CAVITOPEN, was developed. In a centrifuged sample, the  
 300 variation of xylem pressure ( $P$ ) with distance from the axis of rotation ( $r$ ) is given by the following  
 301 equation (Alder et al. 1997):

$$302 \quad dP/dr = \rho\omega^2r \quad (\text{equation 4})$$

303 where  $\rho$  is the density of water, and  $\omega$  the angular velocity.

304 Integrating this equation from  $R$  (distance from the axis of rotation to the water reservoir) we can  
 305 obtain the pressure at  $r$  ( $P_r$ ):

$$306 \quad P_r = 0.5 \rho\omega^2(R^2 - r^2) \quad (\text{equation 5})$$

307 The effect of vessel length on 'true' vessel embolism in a spun sample has already been modelled by  
 308 Cochard et al (2005). Briefly, if the vessels are infinitely long, the VC obtained by centrifugation should  
 309 yield the correct  $P_{50}$  value. When the vessels are infinitely short the  $P_{50}$  value is underestimated due  
 310 to the variation of xylem pressure inside the spun sample (eq. 4) and the consequent gradient of  
 311 embolism along the sample: xylem pressure is minimum in the middle of the sample and null at the

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8 312 extremities (eq. 5). Since the loss of conductivity is measured on the whole sample, an  
9  
10 313 underestimation of the degree of embolism in the middle of the sample is predicted. This effect of  
11  
12 314 vessel length was further tested with the CAVITOPEN model and found marginal, i.e. the shift in the  
13  
14 315 VC was negligible, compared to the draining effect. For sake of simplicity, this effect was no longer  
15  
16 316 considered in the simulations. To simulate the draining effect at both sample ends, we first  
17  
18 317 hypothesized that vessel ends follow a logarithmic distribution following the vessel length probability  
19  
20 318 density function proposed by Cohen et al. (2003) and assuming vessel ends uniformly distributed  
21  
22 319 across the length of the sample:

$$22 \quad N_x = N_0 \cdot \exp(-x/L_{max}) \quad \text{(equation 6)}$$

23  
24 321 where  $N_x$  is the number of open vessels at the distance  $x$  from sample ends,  $N_0$  the total number of  
25  
26 322 vessels and  $L_{max}$  the maximum vessel length.

27  
28 323 The second assumption of the model is that open vessels drain when the minimum pressure in the  
29  
30 324 vessel exceeds a threshold value  $P_{open}$ . Because of the quadratic distribution of the pressure in the  
31  
32 325 sample, vessels having their end wall located closer to the sample ends, i. e. further from the centre  
33  
34 326 of rotation, will drain at a higher rotational velocity.

35  
36 327 The branch segment was discretised in 0.1 mm thick sections arranged in serial. The xylem pressure  
37  
38 328 in the middle of the segment was set to a pressure varying from 0 to -12 MPa in 1 MPa steps. The  
39  
40 329 model then computes the pressure at steady state in each 0.1 mm section and determines the PLC  
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42 330 caused by 'true' embolism (non-open vessels) and by draining (open vessels). Finally, the PLC of the  
43  
44 331 whole segment is computed which enables the construction of the vulnerability curve. We tested the  
45  
46 332 model for different theoretical  $L_{max}$  values and the 4 rotors sizes used in our experiments. To validate  
47  
48 333 the model we used the values of PLC obtained for *H. leucoptera* in the static centrifuge with the 27 cm  
49  
50 334 rotor. The CAVITOPEN model was fit to the measurements using constrained numerical optimization

335 to estimate four parameters:  $P_{50}$ ,  $S_{50}$ ,  $L_{max}$  and  $P_{open}$ . All routines were implemented as an R package  
336 (available from (Duursma 2017)).

## 337 Results

### 338 *Native embolism and minimum xylem water potential in the field.*

339 Midday xylem water potential decreased from -1.02 to -1.51 MPa in *H. dactyloides* and from -1.35 to  
340 -2.62 MPa in *H. leucoptera* from November 2015 to February 2016. In June 2016, the water potential  
341 was -1.16 MPa in *H. dactyloides* and -1.42 MPa in *H. leucoptera*. Native embolism remained low in  
342 both species across the sampling dates. We measured higher PLC in two-year-old branch segments (<  
343 13 %) than in current year growth (< 2 %) in *H. leucoptera* whereas in *H. dactyloides* native embolism  
344 was lower than 2% in all samples. Maximum xylem specific conductivity ( $K_{smax}$ ) was  $0.87 \pm 0.10 \text{ kg m}^{-1}$   
345  $\text{s}^{-1} \text{ MPa}^{-1}$  in *H. leucoptera* and  $1.29 \pm 0.09 \text{ kg m}^{-1} \text{ s}^{-1} \text{ MPa}^{-1}$  in *H. dactyloides* (mean  $\pm$  sd). No significant  
346 differences in native PLC or  $K_s$  ( $P > 0.05$ ; Table S1) were detected between sampling dates.

### 347 *Maximum vessel length and vessel length distribution*

348 Maximum vessel length as determined by air injection was 25 cm (standard deviation, sd = 5) in *H.*  
349 *leucoptera* and 10 cm (sd = 3) in *H. dactyloides*. Air injected branches of *H. dactyloides* showed 17%  
350 PLC at 7 cm from the injection point, 5% at 14 cm and less than 1% at 28 cm, whereas in *H. leucoptera*  
351 the PLC was always higher, 50%, 25%, and 5% at 7, 14 and 28 cm respectively (Fig. 1). Thus the number  
352 of open vessels at both ends when using the centrifuge technique differed between species.

### 353 *Vulnerability curves*

354 Vulnerability curves (VCs) obtained with the bench dehydration technique were s-shaped for both  
355 species, with significant embolism only occurring once a threshold water potential had been reached.  
356 This threshold was more negative in *H. leucoptera* (-6.3 MPa) than in *H. dactyloides* (-3.8 MPa) (Fig.  
357 2). VCs obtained with bench dehydration had the most negative  $P_{12}$  and the steepest slopes of all

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8 358 methods (Table S2), meaning that embolism formation started at more negative  $\Psi_x$  and conductivity  
9 359 was lost across a narrower range of  $\Psi_x$  compared with VCs generated by centrifugation.

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12 360 When the centrifuge was used to induce embolism, results in the shorter-vesseled species, *H.*  
13 361 *dactyloides*, were similar for the three techniques used to measure loss of conductivity, flowmeter,  
14 362 Cavitron and microCT (average  $P_{50}$  with the 27 cm rotor in the static centrifuge and the Cavitron -4.8  
15 363 MPa), and the CI at 95% overlapped with bench dehydration ( $P_{50} = -5.0$  MPa). The VC generated with  
16 364 the 14 cm rotor for *H. dactyloides* yielded slightly less negative values ( $P_{50} = -4.3$  MPa; Table S2; Fig.  
17 365 2). In contrast, VCs for *H. leucoptera* differed considerably depending on the method and the sample  
18 366 length. Vulnerability parameters ( $P_{12}$ ,  $P_{50}$ ,  $P_{88}$ ) obtained with the Cavitron (-5.0, -7.1 and -9.0 MPa,  
19 367 respectively) matched more closely with the bench dehydration VC (-6.3, -7.4 and -8.2 MPa). For  
20 368 samples spun in the static centrifuge, we found a significant effect both of the rotor size and the  
21 369 segment used to measure flow (whole, spun segment or excised middle section in the 27 cm rotor) on  
22 370 apparent vulnerability to embolism: segments measured across their entire length exhibited higher  
23 371 vulnerability to embolism compared to the bench-dehydration VC as shown by  $P_{12}$  (-1.2 and -2.6 MPa  
24 372 for 14 and 27 cm rotors, respectively) and  $P_{50}$  (-5.3 and -6.0 MPa, respectively), but seemed less  
25 373 vulnerable towards the dry end of the curve ( $P_{88}$  of -14.2 and -10.4 MPa, respectively; Table S2). Both  
26 374 VCs were almost linear when flow was measured across the whole segment with a shift towards more  
27 375 vulnerable values with the 14 cm rotor, but became s-shaped when only the middle section of the 27  
28 376 cm segment was measured (Fig. 2). Removing the segment ends resulted in a steeper slope and  
29 377 significantly more negative values of  $P_{12}$  and  $P_{50}$ . The Cavitron and the middle segment techniques  
30 378 yielded similar results and agreed well with the dehydration technique in  $P_{50}$  and  $P_{88}$  and with microCT  
31 379 image analysis (red triangles in Fig. 2).

#### 380 *Patterns of embolism across a centrifuged sample*

381 Within 27-cm-length centrifuged samples of *H. leucoptera*, microCT scans revealed that embolism  
382 levels were consistently at their highest near the sample ends (at 12 cm from the axis of rotation)

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8 383 when spun at equivalents of -5, -7 and -9 MPa in the static centrifuge (Fig. 3). At -5 and -7 MPa loss of  
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10 384 conductivity decreased from the basal end to the centre, contradicting theoretical expectations. This  
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12 385 trend was observed even at  $\Psi_x$  inducing less than 40% PLC based on the bench dehydration VC (Fig.  
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14 386 3). Only at -9 MPa, that is, below  $P_{88}$  on bench dehydration, did levels of embolism converge along the  
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16 387 length of the sample at 80-90%.

#### 17 388 *Influence of open vessels in the VC of a centrifuged sample*

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19 389 The simulations produced by the CAVITOPEN model confirmed that the shape of the VCs generated  
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21 390 by the centrifugation was largely dependent on vessel and sample lengths. As maximum vessel length  
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23 391 decreased, PLC of the whole sample decreased at a given  $\Psi_x$ , and the shape of the VC shifted from  
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25 392 exponential to sigmoidal (Fig. 4a). The same pattern was observed when the sample length increased  
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27 393 (Fig. 4b). For instance, with 14-cm-length centrifuged samples,  $P_{50}$  ranged from -0.6 MPa to -7.7 MPa  
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29 394 varying the maximum vessel length of the sample from 50 cm to 5 cm. Likewise, the  $P_{50}$  of a centrifuged  
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31 395 sample with maximum vessel length of 15 cm ranged from -2.1 MPa in the 14-cm rotor to -7.7 MPa  
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33 396 using a 40-cm rotor. Embolism of vessels open from the cut surface (Fig. S1) influenced values of PLC  
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35 397 at high negative pressures, even in short-vesseled samples, resulting in rapid loss of conductivity  
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37 398 followed by a plateau. The more open vessels and the less negative the threshold of embolism of open  
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39 399 vessels (Fig. 4c), the higher is this plateau and stronger the impact on the VC (Fig. 4). VCs can be  
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41 400 corrected if the first inflection point of the curve is considered the starting point for initial conductivity  
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43 401 ( $K_i$ ), i.e. 0% loss of conductivity. This is shown in Fig. 4d with actual measurements of PLC obtained in  
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45 402 27-cm centrifuged samples of *H. leucoptera*. When the CAVITOPEN model was fit (black circles and  
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47 403 grey solid line, respectively) and we used the inflection point as starting point for  $K_i$ , the corrected  
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49 404 curve matched the reference VC obtained with bench dehydration (Fig. 4d black solid line and orange  
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51 405 dashed line, respectively). Alternatively, by fitting the model using numerical optimization we  
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53 406 estimated values of  $P_{50} = -6.9$  MPa,  $S_{50} = 49.7$ ,  $L_{max} = 15.21$  and  $P_{open} = -0.75$ .

#### 51 407 **Discussion**



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8 408 We evaluated the reliability of two centrifuge based techniques commonly used to measure  
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10 409 vulnerability to embolism in angiosperm species and present a protocol that mitigates experimental  
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12 410 artefacts associated with open xylem vessels. Both the static centrifuge method and the *in-situ* flow  
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14 411 centrifuge method (Cavitron) were prone to artefactual embolism caused by open vessels, although  
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16 412 the errors were significantly greater in the static centrifuge method. In a species with maximum vessel  
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18 413 length longer or similar to the centrifuge rotor diameter, the static centrifuge significantly  
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20 414 overestimated xylem vulnerability to embolism if the whole spun segment was used to measure flow.  
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22 415 Observations with microCT indicated that artefactual embolism caused by centrifugation of samples  
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24 416 occurred in the outer most portions of samples. However, we demonstrated that artefactual  
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26 417 embolism was largely eliminated from static centrifuge if flow was measured in an excised central part  
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28 418 of the segment. This altered protocol yielded VCs similar to those obtained on the same species with  
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30 419 bench dehydration thus allowing these centrifuge techniques to accurately measure vulnerability to  
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32 420 embolism in longer vesseled species. We also present a new model (CAVITOPEN) that simulates the  
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34 421 impact of vessel draining at the cut end on the whole VC curve and showed that errors were largely  
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36 422 dependent on vessel length and rotor diameter. This model allows researchers to quantitative test  
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38 423 and avoid errors associated with the artefactual embolism. The bench dehydration technique  
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40 424 indicated that significant embolism was only initiated in both species after water potential dropped  
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42 425 below a threshold value, -3.8 MPa in *H. dactyloides* and -6.3 MPa in *H. leucoptera*. PLC then increased  
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44 426 rapidly and hydraulic conductivity was lost almost completely within a span of 1 MPa (Fig. 2). These  
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46 427 vulnerability curves have been classified as sigmoidal or s-shaped as opposed to exponential or r-  
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48 428 shaped curves, characterized by rapid conductivity losses as soon as the water potential declines  
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50 429 below zero (Cochard et al. 2013; Sperry et al. 2012). A third type of VC, intermediate between these  
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52 430 two, exhibits a linear response, and is mainly found in diffuse porous species when using  
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54 431 centrifugation to induce embolism (Cochard et al. 2013).

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60 432 Our results showed that VCs obtained with the static centrifuge technique and the Cavitron are similar  
433 to bench dehydration in a short-vesseled species, i.e. a species with no through vessels (open at both

ends) in the segment and with few vessels open from the cut surface to the middle of the segment.

All centrifuge generated VCs for *H. dactyloides* were sigmoidal and similar to bench dehydration VCs, with a slight shift towards more vulnerable values when using the 14 cm rotor (Fig. 2, Table S2) as recently found by Pengxian et al. (2018) in *Acer mono* when comparing in the static centrifuge the 14 cm and 27 cm rotors. VCs of other short vesseled angiosperms such as *Betula pendula* (Cochard et al. 2010), *Fagus sylvatica* (Aranda et al. 2014), *Populus tremuloides* (Schreiber et al. 2011) or *Acer negundo* (Christman et al. 2009) were also sigmoidal when the static centrifuge or the Cavitron were used. In contrast, the VC shape obtained for *H. leucoptera* samples differed significantly depending on methodology resulting in a shift of  $P_{50}$  of 2 MPa in samples from the same population (Fig. 2, Table S2). This dramatic change was observed previously in peach (*Prunus persica*) when the length of the centrifuged samples was varied in a Cavitron; shorter samples were more vulnerable to embolism ( $P_{50}$  shifted from -4.5 to -1 MPa) and VCs became r-shaped (Cochard et al. 2010). However, when using the static centrifuge to measure the same population, Sperry et al. (Sperry et al. 2012) found that VCs were linear and relatively insensitive to the number of open vessels with  $P_{50}$  less negative than -2 MPa using 14 cm and 27 cm samples. This difference in sensitivity to the proportion of open vessels in the centrifuged samples has led some to conclude that the original centrifuge method and rotor design are not subject to the open vessel artefact (Hacke et al. 2015; Sperry et al. 2012). However, Torres-Ruiz et al. (2017) demonstrated that if the amount of open vessels is relatively high in both rotors, 14 and 27 cm, VCs could be equally biased and would appear statistically indistinguishable.

Recent publications have addressed this controversy, showing that long-vesseled species such as grape vine, oaks, robinia or olive, with a high proportion of open vessels, produce similarly biased results with both the static centrifuge and the Cavitron when compared with reference curves generated by dehydration or non-invasive imaging (Choat et al. 2016; Choat et al. 2010; Pengxian et al. ; Torres-Ruiz et al. 2014). Li et al. (2008) and Pengxian et al. (2018) tested the two centrifuge methods head to head and found close correspondence in VCs across species with different xylem anatomy. An extended literature survey of methods to measure vulnerability to embolism showed

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8 460 that when using the centrifuge, VCs were sigmoidal in conifers and in long vessel species exponential,  
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10 461 whereas in diffuse porous species VCs varied from sigmoidal to linear or exponential (Cochard et al.  
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12 462 2013). Our measurements and simulations made with the CAVITOPEN model explain the different  
13 463 shapes of VCs and some disagreements between the static centrifuge and the Cavitron. In short-  
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15 464 vesseled angiosperms, we have shown that VCs by centrifugation agreed with each other and closely  
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17 465 matched the curves based on bench dehydration and microCT (Choat et al. 2016; Cochard et al. 2010).  
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19 466 In angiosperms with a proportion of vessels open to the middle but not the whole way through, the  
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21 467 standard protocol in the static centrifuge produces linear VCs (Sperry et al. 2012). Here the initial  
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23 468 conductivity is measured before spinning, thus if the native embolism is low, all the vessels are  
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25 469 conductive, regardless of their length. As soon as the sample is spun, the conductivity would be  
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27 470 artificially reduced relative to the native state in proportion to the amount of vessels open to centre.  
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29 471 Sample with open vessels thus become artificially vulnerable to embolism at the beginning of the VC  
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31 472 (i.e. at less negative water potentials). For *H. leucomptera*, this translated into less negative values of  
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33 473  $P_{12}$  in all centrifuged samples compared with those measured with the bench dehydration technique  
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35 474 creating a linear response or a plateau at high water potentials. Higher differences in  $P_{12}$  were  
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37 475 observed in *H. leucomptera* than in *H. dactyloides* according with a higher proportion of vessels open to  
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39 476 centre in the former species (Fig. 1). In the Cavitron, the initial measurement was made while spinning  
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41 477 at low tension and many open to centre vessels would already be embolised in the initial  
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43 478 measurement of conductivity, resulting in a lower artefactual loss of conductivity in the subsequent  
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45 479 water potentials of the VC. This may bias the curves slightly pushing them to more negative values but  
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47 480 it did not appear to be significant effect here as the Cavitron curves for *H. leucomptera* were similar to  
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49 481 bench dehydration curves.

46 482 The simulations of PLC with the CAVITOPEN model confirmed that the impact of open vessels on the  
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48 483 VC was higher when vessels were long, samples short and when open vessels cavitated at less negative  
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50 484 pressures (Fig. 4). If the samples were much shorter than the maximum vessel length of the branch  
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52 485 (see the results in Fig. 4 for the 14 cm rotor with  $L_{max}$  50cm), the resulting VC was exponential (r-

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8 486 shaped), as observed in long-vesseled angiosperms, and shifted to more linear or s-shaped when  $L_{max}$   
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10 487 was decreased or the sample length increased. One of the assumptions in the model is that vessels  
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12 488 open at the cut surface cavitate when they reach a threshold value; that is far less negative than intact  
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14 489 vessels whose two ends are included within the spun segment. This influences the shape the VC at  
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16 490 higher pressures creating a “bump” in the VC followed by a plateau. This effect can be corrected to  
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18 491 some extent if the first inflexion point of the VC is considered to be the 0% point for loss of  
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20 492 conductivity. In this case the initial conductivity ( $K_i$ ) value is shifted to a lower value corresponding to  
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22 493 the hydraulic conductivity of the plateau (Fig. 4d). The estimated values of  $P_{50}$  and  $S_{50}$  when the  
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24 494 CAVITOPEN model was fit to actual measurements agreed quite well with those obtained with  
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26 495 reference techniques and confirmed that this model can be used to correct open vessel artefacts for  
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28 496 centrifuge based VCs. The estimated  $L_{max}$  was however significantly shorter than  $L_{max}$  measured with  
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30 497 the air injection technique. The air injection technique has shown to produce higher  $L_{max}$  than the  
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32 498 rubber injection method (Pan et al. 2015), thus our values could be overestimated. On the other hand,  
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34 499 the model assumed that vessel lengths in a sample follow the density function proposed by Cohen et  
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36 500 al. (2003) which can be sensitive to the clustering of vessel lengths (Cai and Tyree 2014). It is clear that  
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38 501 the actual distribution of vessel lengths, network topology and connectivity are crucial for the  
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40 502 sensitivity to an open vessel artefact.

### 503 **Origin of the open-vessel artefact**

40 504 The physical mechanisms underlying this open-vessel artefact are yet to be fully elucidated. Some  
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42 505 studies suggest that microbubbles and particles can act as nucleation sites when they flow through  
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44 506 the sample as it spins in the Cavitron, causing premature embolism (Cochard et al. 2010; Sperry et al.  
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46 507 2012; Wang et al. 2014). In the static centrifuge, bubbles might be drawn into vessels while starting  
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48 508 the spin or while mounting or dismounting the stems to measure flow (Wang et al. 2014). In both  
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50 509 centrifuge techniques bubbles in open vessels can move by buoyancy while spinning toward the region  
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52 510 of lowest pressure at the center of rotation (Rockwell et al., 2014). Draining from open vessels as a

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8 511 consequence of artefactual embolism when the centrifuge starts spinning appears to be a common  
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10 512 phenomenon in both rotors. Our microCT images showed that after spinning in the centrifuge, most  
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12 513 of the vessels were empty near the ends even though tension ought to be zero (Cochard et al. 2005).  
13 514 The use of water saturated foam pads to avoid desiccation did not prevent this (Hacke et al. 2015;  
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15 515 Tobin et al. 2013). We discarded the possibility that sample manipulation before spinning or during  
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17 516 wax embedding had triggered vessel draining because we scanned control samples that were not  
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19 517 spun. These samples showed no embolism (Fig. 3). Furthermore, patterns of embolism did not follow  
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21 518 theoretical expectations based on the distribution of tension within the spun sample. The embolism  
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23 519 levels decreased from the ends to the center in a fashion consistent with the amount of vessels open  
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25 520 to center, opposite to that expected from profile in tension and in agreement with the assumption of  
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27 521 the CAVITOPEN model than open vessels artificially cavitate when they reach a threshold pressure  
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29 522 that is much less negative than in intact vessels. This pattern was observed at water potentials  
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31 523 inducing less than 40% loss of conductivity based on the VC obtained using the middle segment of the  
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33 524 centrifuged sample (Fig. 3), even though the centre of the sample experienced the highest tensions.  
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35 525 Embolism levels converged within the sample at -9 MPa at 80-90%. These results confirm that  
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37 526 centrifugation drains open vessels and only reliably measure the vulnerability of intact xylem vessels  
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39 527 within the sample (Fig. S1). This is consistent with observations made previously by Cochard et al.  
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41 528 (2010) using the Cavitron; they reported that embolism was higher in the basal and upstream ends  
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43 529 relative to the centre of samples from species with vessels that are predominately at least half as long  
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45 530 as the spun segment. Cai et al. (2010) and Pengxian et al. (2018) also reported higher PLC values than  
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47 531 predicted by theory at both ends after spinning samples in a Cavitron. Given that our results were  
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49 532 obtained with the static centrifuge it is clear that the overestimation of vulnerability for open to centre  
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51 533 vessels occurs in both versions of the centrifuge technique.

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60 534 The hydraulic continuity between vessels cut open at each end of the sample and vessels with their  
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62 535 terminal ends in this portion of the sample is probably re-established by refilling of vessels immersed  
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64 536 under water at both ends (Fig. 2 in Cochard et al. (2010)). This refilling would occur by capillarity either

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8 537 while spinning in the Cavitron or while flow is measured gravimetrically (Fig. S2). Since the middle of  
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10 538 the centrifuged sample contains the majority of intact vessels, VCs constructed with the static  
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12 539 centrifuge technique of angiosperm species using only the central segment are more reliable and in  
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14 540 closer agreement with PLC generated by natural dehydration (Fig. 2). This modification is technically  
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16 541 easy to achieve and mitigates the open vessel artefact; however, it carries the disadvantage that  
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18 542 samples cannot be spun repeatedly to construct replicate curves for each sample and thus more plant  
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20 543 material is needed to construct each curve.

#### 20 544 **Conclusion**

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23 545 We confirmed the validity of vulnerability curves constructed with both centrifuge methods for short  
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25 546 conduit angiosperm species, those with most conduits shorter than half the length of the centrifuge  
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27 547 rotor. A new model, CAVITOPEN was developed to simulate the effect of vessel length, rotor size and  
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29 548 vulnerability of open vessels in loss of conductivity of centrifuged samples. In species with maximum  
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31 549 vessel length similar to the centrifuge rotor, we recommend constructing vulnerability curves with the  
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33 550 Cavitron or measuring flow exclusively in the central part of the spun segment when using the static  
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35 551 centrifuge. Alternatively, artefactual embolism at low xylem tensions can be corrected if the first  
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37 552 inflexion point of the VC is considered to be the starting point for  $K_{max}$  (0 % loss of conductivity) or by  
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39 553 fitting the CAVITOPEN model to the measurements to estimate  $P_{50}$  and  $S_{50}$ . When samples contained  
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41 554 a high proportion of open to centre vessels, the centrifuge technique is prone to error and  
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43 555 overestimates vulnerability to embolism. Determining the proportion of open to centre vessels or  
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45 556 performing the simple test recently proposed by Torres-Ruiz et al. (2017), which compares changes in  
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47 557  $K_s$  before and after spinning in the centrifuge at low tensions, are highly advisable before using any of  
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49 558 the centrifuge techniques.

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51 559 The shape of the vulnerability curves obtained with bench dehydration were always sigmoidal while  
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53 560 in centrifuged samples the shape was determined by the presence of open vessels. While previous  
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55 561 studies have demonstrated that species with the longest vessel classes (eg. lianas, ring porous trees)

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8 562 open vessels tend to exhibit exponential curves when measured in the centrifuge. Here we showed  
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10 563 that VCs with a linear shape are symptomatic of species with intermediate vessel lengths in which a  
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12 564 higher proportion of vessels open to centre of the test segment. The occurrence of this incipient open  
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14 565 vessel artefact can be mitigated by measurement of the excised central portion of the segment.

15 566 **Acknowledgments**

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18 567 This research was supported by a Marie Curie Fellowship to R.L. (FP7PEOPLE-2013-IOF-624473) and  
19  
20 568 an ARC Future Fellowship to B.C. (FT130101115). We thank Dr. Javier Cano, Adrián Cano, Teresa Rosas  
21  
22 569 and Jennifer Peters for field assistance, Dr. Iain M Young for X-ray microCT advice and comments on  
23  
24 570 the manuscript, Gavin McKenzie for lab support and Dr. Stephanie Stuart for her ideas and writing  
25  
26 571 assistance. No conflict of interests declared.

27 572

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709 **Figure legends**

710 **Figure 1.** Distribution of PLC in air-injected branches of *H. dactyloides* (black circles) and *H.*  
 711 *leucoptera* (open circles) at different positions from the injected end. Vertical bars represent the  
 712 standard error. Dashed lines indicate the two sample lengths used for the centrifuge methods, 14 cm  
 713 and 27 cm and dot lines indicate their respective half sample length.

714 **Figure 2.** Xylem vulnerability to embolism curves and 95% confidence intervals (grey shaded areas)  
 715 of *Hakea dactyloides* (left panels) and *Hakea leucoptera* (right panels) obtained with two methods to  
 716 induce cavitation in the xylem, bench dehydration and centrifuge force and three methods to  
 717 measure the loss of conductivity, flowmeter (close circles), in situ flow method (open circles) and X-  
 718 ray microCT visualisation (red triangles). Vertical solid lines indicate  $P_{50}$  and vertical dashed lines  
 719 indicate the 95% confidence interval for  $P_{50}$ . [Horizontal dashed lines indicated native xylem](#)  
 720 [embolism measured in the field.](#) Two rotor sizes, 14 cm and 27 cm, were used in the static  
 721 centrifuge, and water flow in the whole segment or only in the central part was measured (see  
 722 methods for details).

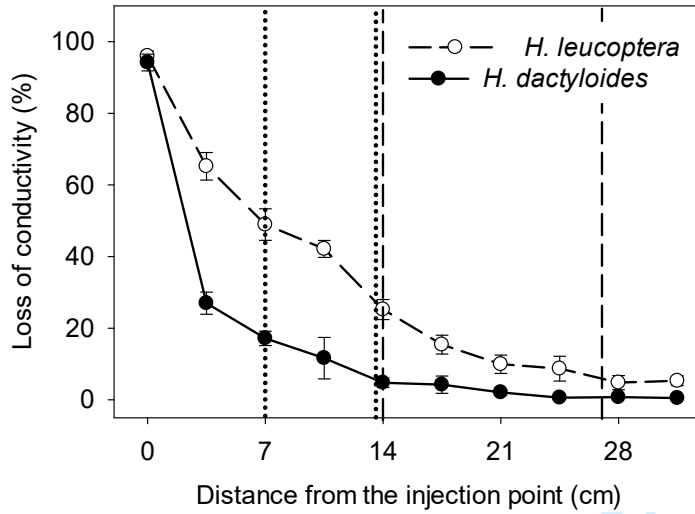
723 **Figure 3.** Transverse slices from X-ray microtomography (X-ray micro-CT) scans of branches of *Hakea*  
 724 *leucoptera* (maximum vessel length =  $25 \pm 5$  cm) scanned at three positions before spinning (left  
 725 column) and after spinning in the centrifuge at 5, 7 and 9 MPa. Embolized vessels appear as black  
 726 and water-filled conduits appear as grey. The estimated percent loss of conductivity (PLC) is shown  
 727 in each picture. Scale bar, 1 mm.

728 **Figure 4.** Simulations with the CAVITOPEN model of the effect of threshold of embolism formation  
 729 (MPa) of cut open vessels (A), maximum vessel length (cm) (B), and rotor size (cm) (C) on xylem  
 730 vulnerability to embolism curves generated with centrifugation. In red, vulnerability curve of close  
 731 vessels at both ends. (D) The CAVITOPEN model was fit to measurements in *H. leucoptera* using  
 732 numerical optimization to estimate all four parameters: water potential at 50% loss of conductivity

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8 733 ( $P_{50}$ ), slope of the vulnerability curve ( $S_{50}$ ), maximum vessel length ( $L_{max}$ ) and threshold of embolism  
9 734 formation of cut open vessels ( $P_{open}$ ). Circles represent the values obtained in our study with the static  
10 735 centrifuge, 27 cm rotor in *H. leucoptera* when flow was measured in the whole segment (see Methods  
11 736 for details); grey solid line is the fitted curve with the CAVITOPEN model; black solid line represent the  
12 737 curve after correction and orange dashed line is the reference curve obtained with bench dehydration  
13 738 for the species.  
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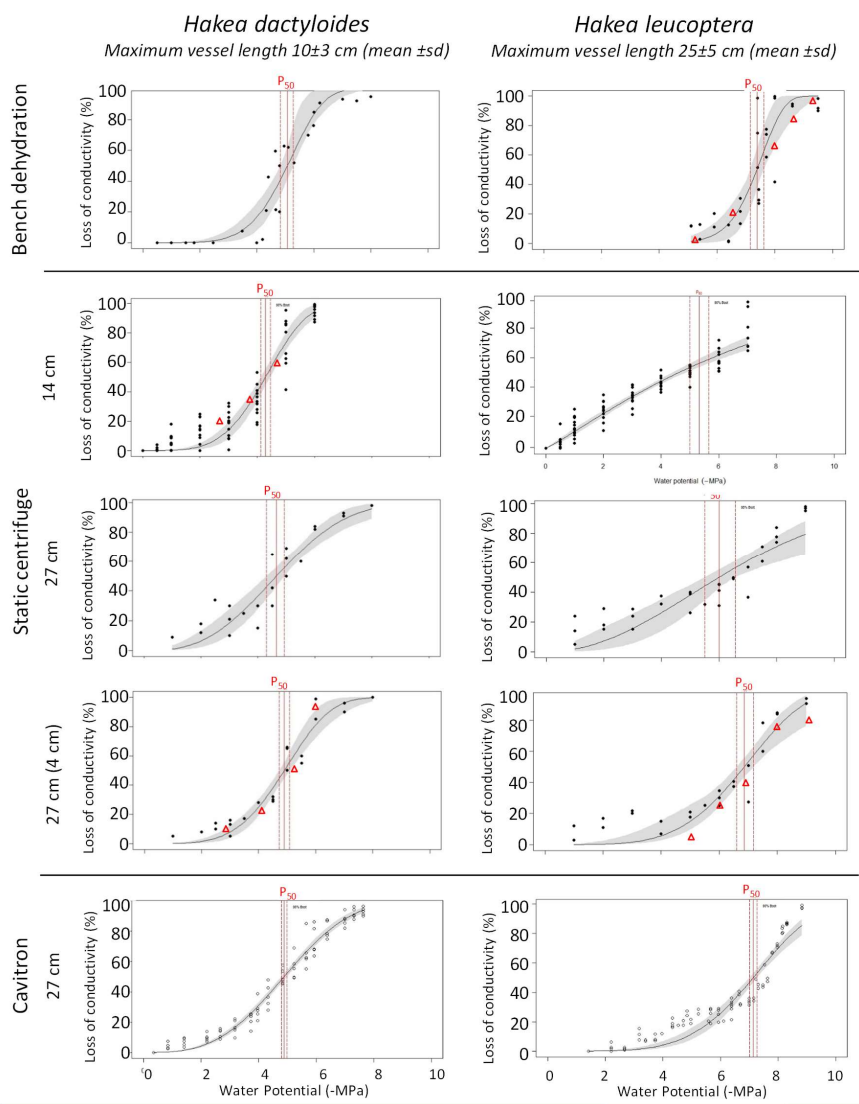
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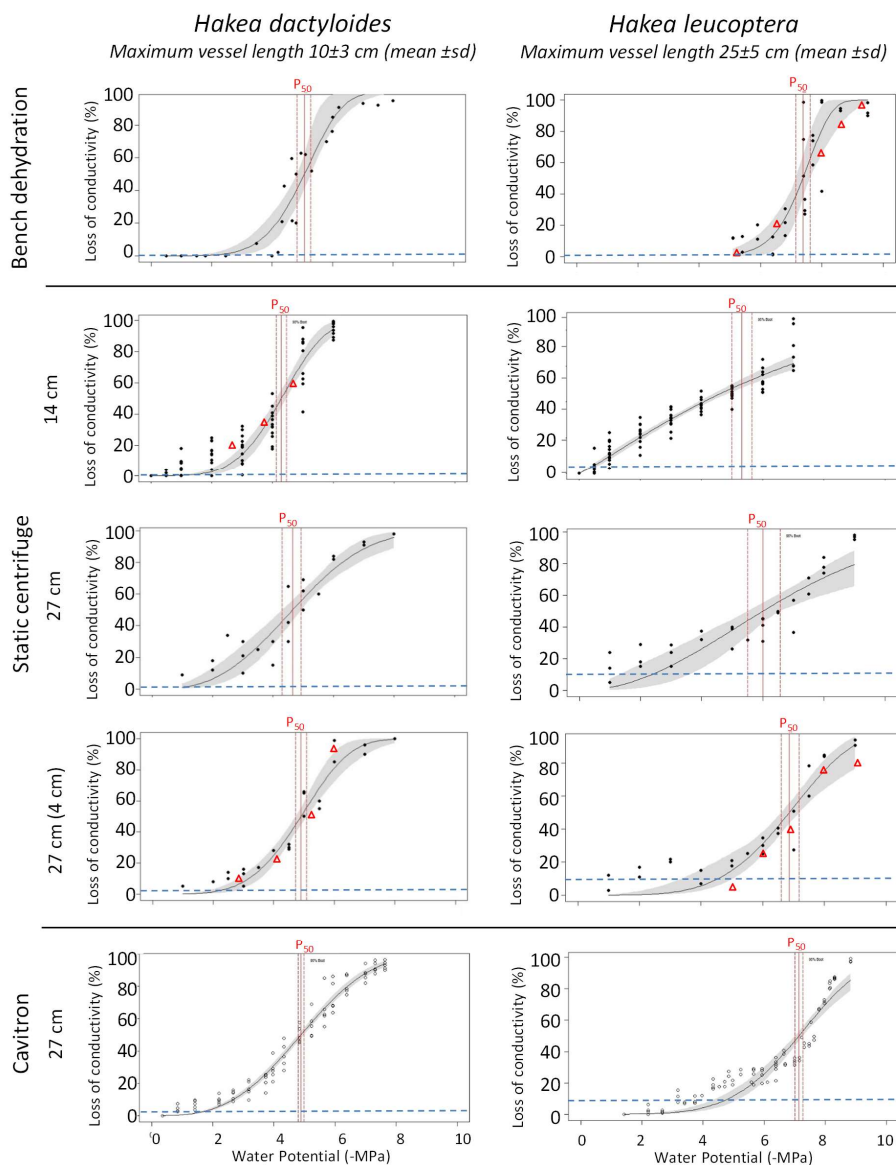
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742 **Figure 1.** Distribution of PLC in air-injected branches of *H. dactyloides* (black circles) and *H.*  
 743 *leuoptera* (open circles) at different positions from the injected end. Vertical bars represent the  
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 745 and 27 cm and dot lines indicate their respective half sample length.

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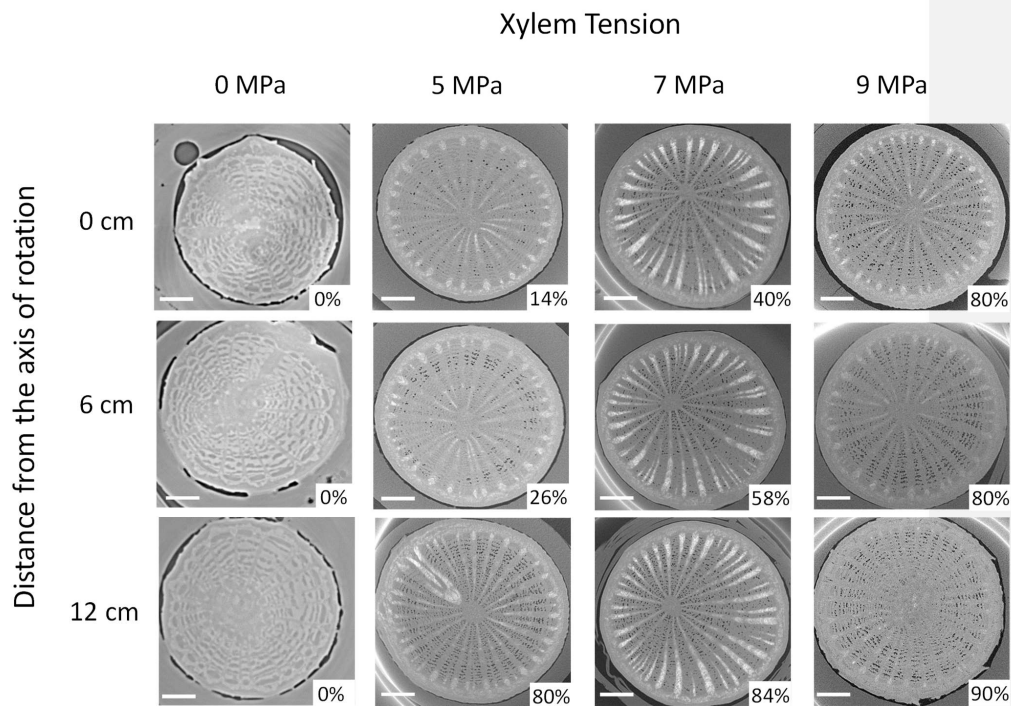


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749 **Figure 2.** Xylem vulnerability to embolism curves and 95% confidence intervals (grey shaded areas)  
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 752 measure the loss of conductivity, flowmeter (close circles), in situ flow method (open circles) and X-  
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 754 indicate the 95% confidence interval for  $P_{50}$ . Two rotor sizes, 14 cm and 27 cm, were used in the

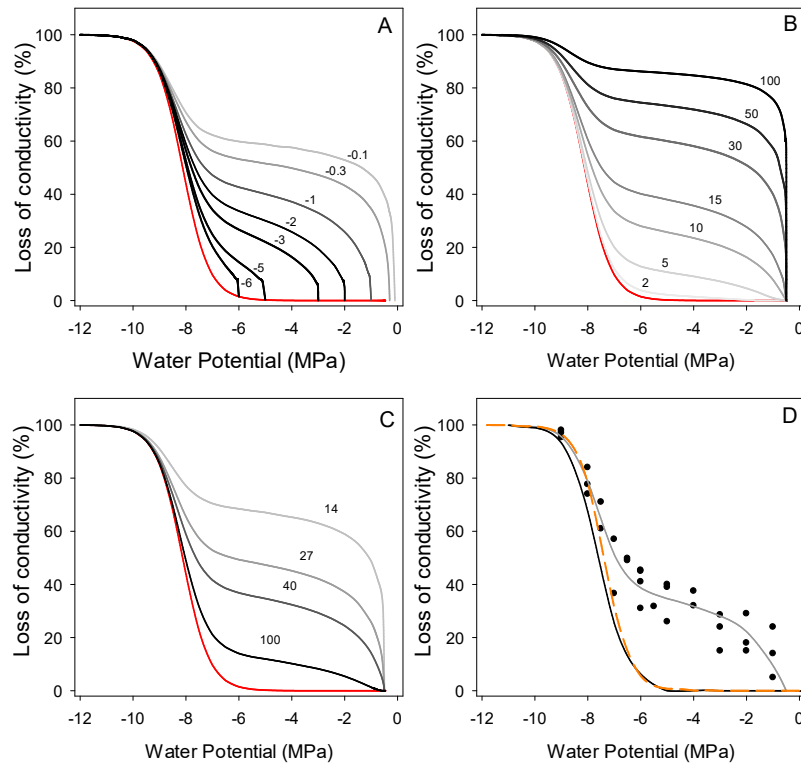
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755 static centrifuge, and water flow in the whole segment or only in the central part was measured (see  
 756 methods for details).



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 758 **Figure 3.** Transverse slices from X-ray microtomography (X-ray micro-CT) scans of branches of *Hakea*  
 759 *leucoptera* (maximum vessel length =  $25 \pm 5$  cm) scanned at three positions before spinning (left  
 760 column) and after spinning in the centrifuge at 5, 7 and 9 MPa. Embolized vessels appear as black  
 761 and water-filled conduits appear as grey. The estimated percent loss of conductivity (PLC) is shown  
 762 in each picture. Scale bar, 1 mm.





**Figure 4.** Simulations with the CAVITOPEN model of the effect of threshold of embolism formation (MPa) of cut open vessels (A), maximum vessel length (cm) (B), and rotor size (cm) (C) on xylem vulnerability to embolism curves generated with centrifugation. In red, vulnerability curve of close vessels at both ends. (D) The CAVITOPEN model was fit to measurements in *H. leucoptera* using numerical optimization to estimate all four parameters: water potential at 50% loss of conductivity ( $P_{50}$ ), slope of the vulnerability curve ( $S_{50}$ ), maximum vessel length ( $L_{max}$ ) and threshold of embolism formation of cut open vessels ( $P_{open}$ ). Circles represent the values obtained in our study with the static centrifuge, 27 cm rotor in *H. leucoptera* when flow was measured in the whole segment (see Methods for details); grey solid line is the fitted curve with the CAVITOPEN model; black solid line represent the curve after correction and orange dashed line is the reference curve obtained with bench dehydration for the species.

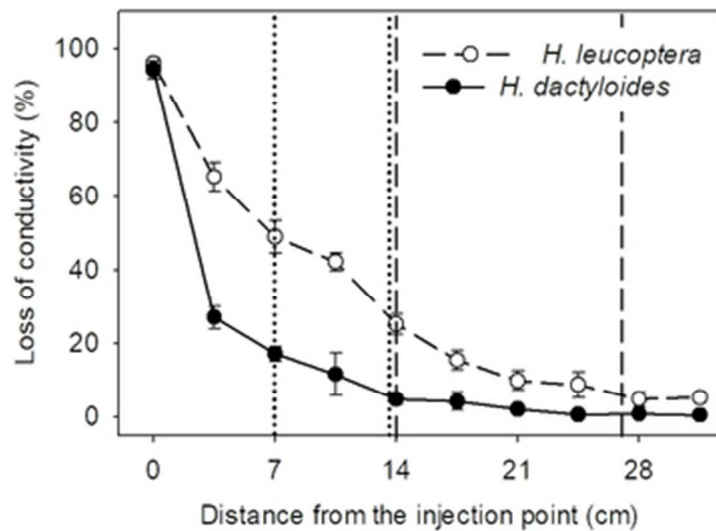


Figure 1. Distribution of PLC in air-injected branches of *H. dactyloides* (black circles) and *H. leucoptera* (open circles) at different positions from the injected end. Vertical bars represent the standard error. Dashed lines indicate the two sample lengths used for the centrifuge methods, 14 cm and 27 cm and dot lines indicate their respective half sample length.

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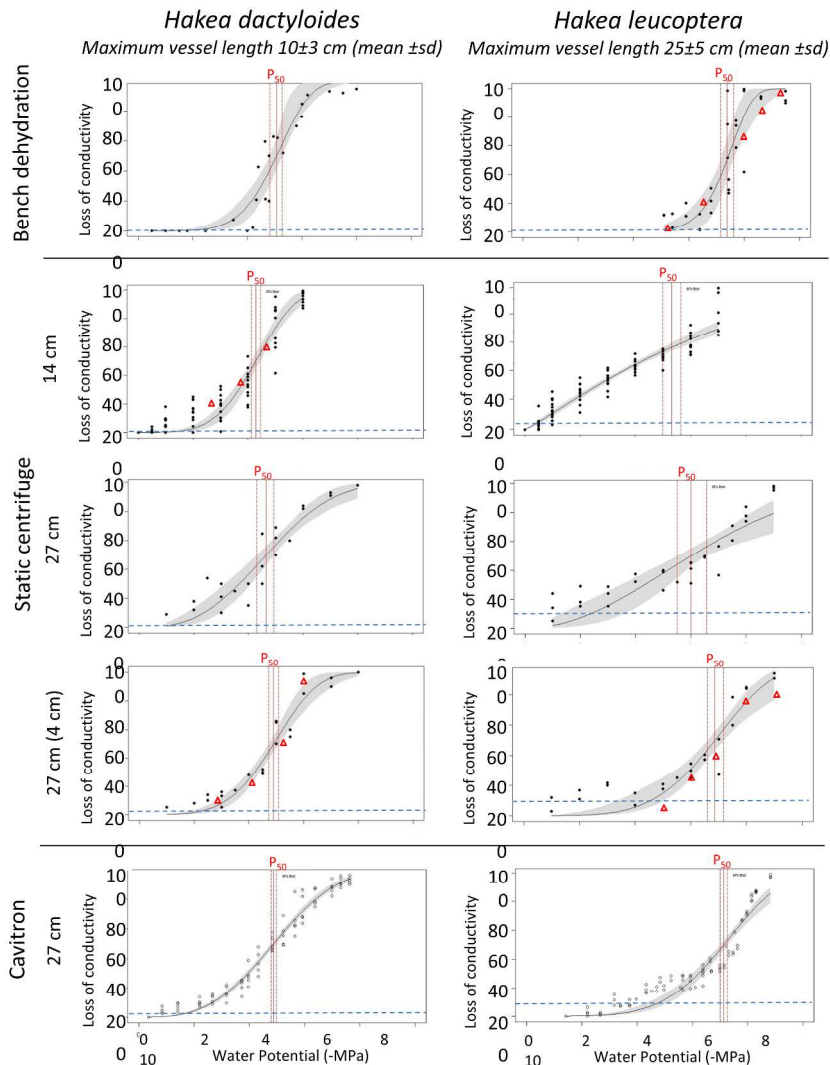


Figure 2. Xylem vulnerability to embolism curves and 95% confidence intervals (grey shaded areas) of *Hakea dactyloides* (left panels) and *Hakea leucoptera* (right panels) obtained with two methods to induce cavitation in the xylem, bench dehydration and centrifuge force and three methods to measure the loss of conductivity, flowmeter (close circles), in situ flow method (open circles) and X-ray microCT visualisation (red triangles). Vertical solid lines indicate P50 and vertical dashed lines indicate the 95% confidence interval for P50. Horizontal dashed lines indicated native xylem embolism measured in the field. Two rotor sizes, 14 cm and 27 cm, were used in the static centrifuge, and water flow in the whole segment or only in the central part was measured (see methods for details).

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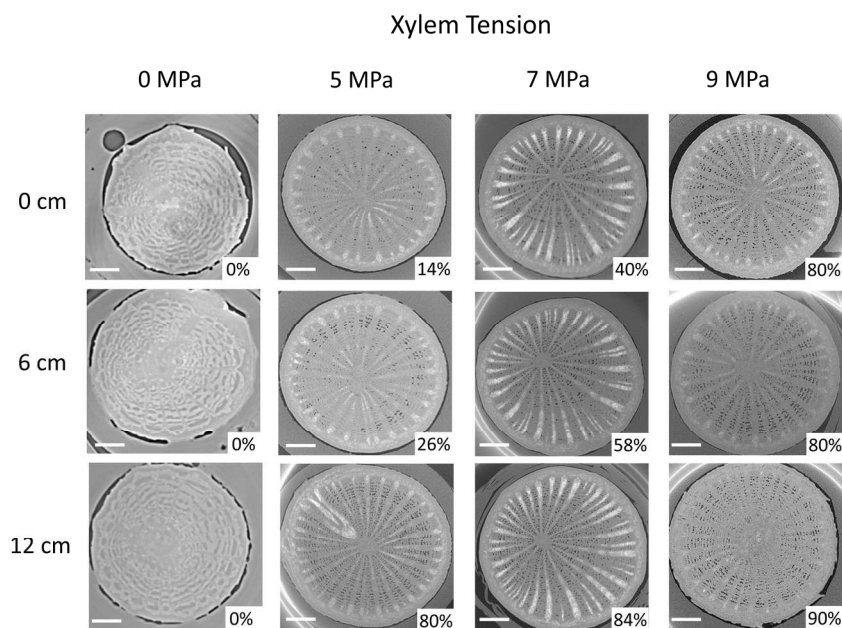


Figure 3. Transverse slices from X-ray microtomography (X-ray micro-CT) scans of branches of *Hakea leucoptera* (maximum vessel length =  $25 \pm 5$  cm) scanned at three positions before spinning (left column) and after spinning in the centrifuge at 5, 7 and 9 MPa. Embolized vessels appear as black and water-filled conduits appear as grey. The estimated percent loss of conductivity (PLC) is shown in each picture. Scale bar, 1 mm.

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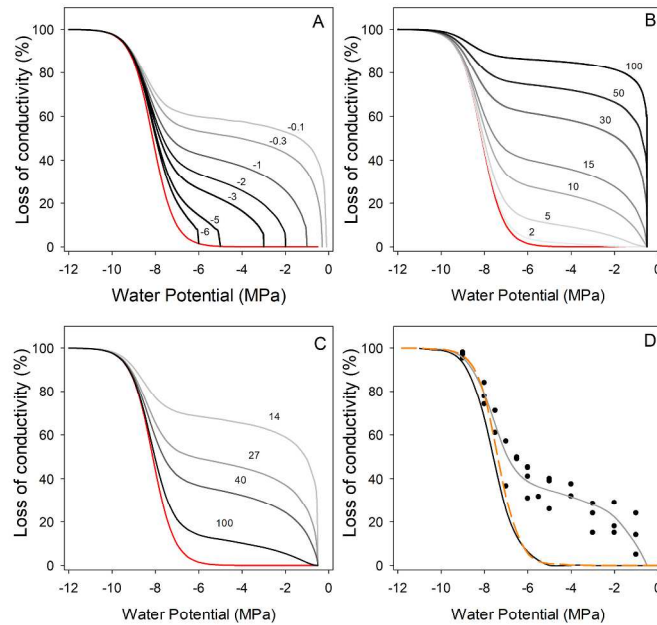


Figure 4. Simulations with the CAVITOPEN model of the effect of threshold of embolism formation (MPa) of cut open vessels (A), maximum vessel length (cm) (B), and rotor size (cm) (C) on xylem vulnerability to embolism curves generated with centrifugation. In red, vulnerability curve of close vessels at both ends. (D) The CAVITOPEN model was fit to measurements in *H. leucoptera* using numerical optimization to estimate all four parameters: water potential at 50% loss of conductivity ( $P_{50}$ ), slope of the vulnerability curve ( $S_{50}$ ), maximum vessel length ( $L_{max}$ ) and threshold of embolism formation of cut open vessels ( $P_{open}$ ). Circles represent the values obtained in our study with the static centrifuge, 27 cm rotor in *H. leucoptera* when flow was measured in the whole segment (see Methods for details); grey solid line is the fitted curve with the CAVITOPEN model; black solid line represent the curve after correction and orange dashed line is the reference curve obtained with bench dehydration for the species.

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